# Transfection of Endothelial Nitric Oxide Synthase Gene Improves Angiogenic Efficacy of Endothelial Progenitor Cells in Rabbits with Hindlimb Ischemia

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## Abstract

**Background:** The present study explored the effect of endothelial nitric oxide synthase (eNOS) gene transfer on the angiogenic potential of *ex vivo* expanded endothelial progenitor cells (EPCs) in a rabbit model of hindlimb ischemia.

**Methods:** Rabbit peripheral blood EPCs were cultured and transfected with mammalian expression vector pcDNA3.1-eNOS containing full-length human eNOS gene. Ischemia was induced in the right hind limb of three groups of rabbits by ligation of the distal external iliac artery and excision of the common and superficial femoral arteries. In one group of animals, ten days after the surgery, autologous eNOS-EPCs were transplanted intramuscularly in the ischemic limb. Two other groups received an equivalent number of unmodified EPCs or phosphate buffered saline (PBS) respectively.

**Results:** Two weeks after cell transplantation, the *in vivo* expression of eNOS was detected in limb tissue sections of eNOS-EPCs treated animals. Animals treated with eNOS-EPCs had a significant reduction in ischemic muscle necrosis and inflammation, augmentation in the capillary density ($P< 0.05$) and angiographic scores demonstrating distal arterial reconstitution and enhanced angiogenesis in comparison to animals transplanted with EPCs or PBS ($P< 0.05$).

**Conclusion:** We conclude that modification of EPCs by eNOS constitutes an effective strategy to improve the efficacy of EPCs for therapeutic angiogenesis.

## Introduction

Therapeutic angiogenesis represents an innovative strategy to treat vascular insufficiency. It is aimed at improving the compensatory mechanisms of angiogenesis and arteriogenesis by stimulating the growth and development of collateral vessels in critical peripheral or myocardial ischemia. Bone marrow-derived endothelial progenitor cells (EPCs) harvested from peripheral blood have been recently identified for their contribution to angiogenesis and revascularization of ischemic tissues [1]. EPCs proliferate and migrate in response to angiogenic growth factors and differentiate into mature endothelial cells *in situ* for blood vessel formation [2]. In recent years, transplantation of *ex vivo* expanded autologous EPCs has emerged as a novel therapeutic strategy that could provide a robust source of viable endothelial cells to supplement the contribution of endothelial cells resident in the adult vasculature. The regenerative potential of *ex vivo* expanded EPCs has been well demonstrated in animal models of limb and myocardial ischemia [3-7].

Despite the promising applications for tissue regeneration, the limited endogenous pool, the possible functional impairment associated with a variety of physiological and pathological phenotypes of clinical patients, and the limited replicative potential of EPCs for *ex vivo* expansion, largely impede their use for autologous transplantation [8, 9]. Given these findings, genetic modification of EPCs to over express angiogenic growth factors, enhance signaling activity of the angiogenic response, rejuvenate the bioactivity, and/or extend the life span of EPCs constitute various potential strategies that might address the limitations of EPC transplantation and thereby optimize therapeutic angiogenesis [10]. Recent studies have employed genetic modification strategies of EPCs with vascular endothelial growth factor (VEGF), telomerase reverse transcriptase, fibroblast growth factor-1 and stromal-derived factor-1 alpha genes to enhance their vasculogenic function [11-14].

Endothelial nitric oxide synthase (eNOS) has been shown to elicit multiple beneficial effects within the cardiovascular system. In addition to its vaso-dilatory properties, eNOS-derived nitric oxide (NO) has been reported to play a critical role in angiogenesis and arteriogenesis in response to tissue ischemia [15-17]. In our previous *in vitro* study, we demonstrated enhanced migration, differentiation and angiogenic potential of EPCs over expressing eNOS [18]. Accordingly, in the present study, we further evaluated the efficacy of genetically modified autologous EPCs over expressing eNOS vis-à-vis unmodified EPCs in inducing angiogenesis in a rabbit model of experimentally induced hind limb ischemia. The study indicates that eNOS modified EPCs show

enhanced angiogenic properties than their unmodified counterparts in the ischemic animals.

## Materials and Methods

### Rabbit hind limb ischemia model

In total, twenty-eight New Zealand White rabbits of either sex weighing 2.2-3.1 kg were studied. Food and water were provided ad libitum. Ten rabbits were used for the isolation and characterization of EPCs. In the remaining, unilateral hindlimb vascular insufficiency was induced surgically by previously described methods [19]. Briefly, rabbits were anesthetized with intramuscular (in) injections of 0.5mg/kg atropine, 5mg/kg xylazine and 50mg/kg ketamine. Under sterile surgical conditions, a longitudinal incision was made on the medial thigh of one hind limb, extending from the inguinal ligament to a point just proximal to the patella. After the skin incision, the entire femoral artery and all its major branches including the inferior epigastria, deep femoral, lateral circumflex and superficial epigastria arteries were dissected free. The external iliac artery and all of the arteries listed were ligated. Finally, the femoral artery was excised from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. The incision was closed in three layers with 2.0 braided silk. All rabbits were closely monitored by the veterinary staff and received in injections of ampicillin-cloxacillin [10mg/kg body weight twice daily] and paracetamol [10mg/kg body weight once daily for 5 days]. EPCs for treatment were given after 10 days of surgery. Two weeks after cell transplantation, the rabbits were taken for angiogram analysis and thereafter euthanized with a single intravenous overdose of 1% sodium thiopentone and muscle tissue was extracted from the ischemic limb. The mid-portion of the tissue section was placed in phosphate buffered saline (PBS), mounted in cross section in OCT compound (Leica) and snap-frozen in liquid nitkrogen. Cryostat sections (5μm) were prepared on microscope slides for histological analysis. The remaining tissue was snap-frozen in liquid nitrogen for RNA extraction. All animals have received humane care in compliance with the international standards on animal welfare and Guide for the Care and Use of Laboratory Animals of the Institutional Animal Ethics Committee.

### Assessment of limb necrosis

All the rabbits were clinically evaluated for incidence of muscle atrophy and distal limb necrosis/ulcers by macroscopic examination at day 10 post-surgery (before treatment) and at day 20 post-surgery (after treatment). Muscle atrophy and necrosis was also evaluated histologically by staining the ischemic muscle tissue sections by hematoxylin and eosin staining at day 20 post-surgery in all the animal groups.

### EPC isolation and characterization

Samples of peripheral blood (12-15 ml) from the central auricular artery of rabbits were harvested 1 day before surgery and anti-coagulated with heparin. The isolation and culture of EPCs was done as previously reported [5]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Histopaque 1077, Sigma-Aldrich, USA) and after purification with PBS and RBC lysis, 1 x 10⁶ cells/cm² were seeded on fibronectin-coated, 6-well plates (Nunc) in M-199 (Sigma-Aldrich, USA), 20% FBS (Sigma-Aldrich, USA), 10 ng/ml vascular endothelial growth factor (VEGF) (US Biologicals, USA) and antibiotics, 100 U/ml benzyl penicillin and 100 μg/ml streptomycin. Medium change was done every three days.

The identity of EPC-CFUs at day 7 was confirmed by immunocytochemistry with 1:50 dilution of monoclonal anti human von Willebrand factor (vWF, Sigma-aldrich, USA), and anti-human platelet endothelial cell adhesion molecule (PECAM/CD31,Dako,Germany) as previously described [20]. After incubation with the primary antibodies, cells were incubated with 1:500 dilution of anti-mouse IgG rhodamine conjugate (Molecular Probes, Netherlands) and were counterstained for 5 minutes with 10 μg of nuclear dye Hoechst 33342 (Sigma-Aldrich, USA). Functionality and viability of EPCs were assessed by 1,1'-dioctadecyl-3,3,3',3-tetramethyl-indocarbocyanine perchlorate labeled acetylated low-density lipoprotein (DiI-ac-LDL, Invitrogen, USA) uptake and lectin (UEA-1, Sigma-Aldrich, USA) staining after 7 days in culture [8]. The stained cells were visualized under an inverted fluorescent microscope.

### eNOS gene transfer in EPCs

Human full-length wild-type eNOS cDNA (3.6 kb) was isolated by RT-PCR from the total RNAs of human dermal microvascular endothelial cells with the use of specific primers (forward: 5'-CCCAAGCTTACCATGGGCAACTTGAAGAGCCTG-3'; reverse: 5'-GGAATTCAGGGGCTGTTGGTGTCTGA-3') [18]. The isolated cDNA was confirmed to be identical to the previously reported clone (NM_000603) by sequencing and was sub-cloned into pcDNA3.1 expression vector (Invitrogen, USA) (pcDNA3-eNOS). After characterization, rabbit EPCs after 7 days were transfected with 2 μg of plasmid DNA, pcDNA3.1-eNOS using lipofectamine and plus reagent in serum-free OptiMEM medium (Sigma-Aldrich, USA) as per the manufacturer's instructions for six hours (Sigma-Aldrich, USA). Six hours after transfection, medium containing M199 + 10% FBS + VEGF was added. EPCs were transplanted in the rabbits after three days of transfection.

### Treatment groups and EPC transplantation

After femoral artery excision, the rabbits were randomly assigned to one of the three groups: (a) control group (receiving PBS, $n= 6$); (b) EPC group (receiving cultured EPCs, $n= 6$); (c) eNOS-EPC group (receiving cultured eNOS-EPCs, $n= 6$). At day 10, post-surgery, EPCs or eNOS-EPCs in culture were trypsinized (0.05% trypsin-0.02% EDTA) and collected for transplantation. Cells in 600μL of PBS were injected in into 5-6 different sites of the ischemic thigh skeletal muscles with a 22-gauge needle. The control group received 600 μL PBS. Overall, a mean of $1 \times 10^5 \pm 0.35 \times 10^5$ EPC were injected per rabbit.

### Tissue procurement, histological section preparation, and mRNA extraction

Muscle samples were weighed and total RNA was extracted with TRIZOL reagent (Invitrogen, USA) as per the instructions. RNA concentration was determined by spectrophotometry at 260 nm and 1μg RNA was reverse transcribed into complementary DNA (cDNA) using MMLV Reverse transcriptase (Promega, USA). Of the transcribed cDNA, 500 ng was used for the PCR to detect the expression of human eNOS transgene. The RT-PCR primer sequence and annealing temperature used were as reported earlier: *endothelial nitric oxide synthase, forward*: 5'-AAGATCTCCGCCTCGCTCA-3', *reverse*: 5'-GCTGTTGAAGCGGATCTTA-3', product size: 336 bp and annealing temp: 58ºC [20].

### Immunohistochemistry

Immunohistochemical detection of human eNOS in the tissue sections was performed by incubating 5μm-thick frozen sections in

1:100 dilution of monoclonal anti-human eNOS antibody (Sigma-aldrich, USA). Sections were then incubated in 1:200 dilution of FITC-labeled anti-mouse secondary antibody (Molecular probes, Netherlands) for 30 min in dark. The slides were washed, mounted with 50% glycerol and examined under a fluorescence microscope. Some of the cryosections were also stained with hematoxylin and visualized under a phase contrast microscope.

## Measurement of nitrite levels

The NO production in the tissue homogenates was assessed by measuring the levels of nitrite by a one-step colorimetric assay using modified Griess reagent (Sigma-aldrich, USA). Nitrite concentration (nmoles/ml) was determined using a calibration curve with sodium nitrite standards.

## Analysis of capillary density

The collateral micro-vascular formation was evaluated by measuring the number of CD31 positive capillaries in two muscle tissue sections from each animal. Frozen sections were stained with antibody to CD31 followed by incubation with 1:500 dilution of biotinylated secondary antibody (Novocastra, UK). Sections were treated further with an avidin-biotin conjugated to horseradish peroxidase, developed with DAB (Novocastra, UK), and counterstained with hematoxylin. The number of capillaries was counted under a 20X objective lens using an image analysis system (Image Pro-plus 5.1, Media Cybernetics, Inc) in 5 randomly selected fields. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined.

## Angiographic analysis

The animals were pre-medicated with atropine [0.05mg/kg body weight (Bwt)] and xylazine [5mg/kg Bwt]. Ketamine [50 mg/kg Bwt] was used to induce the anesthesia. When the animals were under deep surgical anesthesia, under aseptic conditions, with a mid-ventral incision, laparotomy was performed and the abdominal aorta was exposed below the level of kidneys. After looping the aorta with umbilical tape, arterial cannulation was done using 20G i/v cannula with injection valve (Mediflon, Eastern Medikit Ltd., India) and the cannula was secured at site with braided silk sutures. Aorta was ligated anteriorly to prevent the back flow of the dye while the angiography was performed. The animal was positioned on its back (dorsoventral positioning) to obtain angiography of both the hind limbs simultaneously. Patency and blood flow to thigh region and shank regions were assessed using 7 ml of Iohexol (Non Ionic dye) "Omnipaque-350" (GE Healthcare, Ireland) for each rabbit, angiograhy was performed using Fluoroscopy "C" arm (Powermobil, Germany). Morphometric angiographic analysis of collateral vessel development was performed using a grid overlay that comprised 2.5 mm diameter circles arranged in rows spaced 5 mm apart as earlier reported [21]. This overlay was placed over the 4-second angiogram recorded at the level of the medial thigh. The number of contrast-opacified arteries crossing over circles and the total number of circles encompassing the medial thigh area were counted in a single

blind fashion. An angiographic score was calculated for each film as the number of circles crossed by opacified arteries divided by the total number of circles in the defined area of the ischemic thigh using Image Proplus 5.1, Media Cybernetics, Inc.

## Statistical analysis

All results are presented as mean ± SD. Comparisons between groups were made by 1-way ANOVA using SPSS software, version 11.5. A probability value of <0.05 (CI: 95%) was considered to indicate statistical significance.

## Results

All animals demonstrated weakness and limping of the right hind limb after the surgery. At day 10, about 67% of the animals showed significant muscle atrophy and 33% of the animals developed varying degrees of superficial tissue necrosis in their distal calves and toes. The macroscopic findings in different study groups before and after treatment are given in Table 1. The ischemic limbs of the eNOS-EPC treated groups showed complete absence of muscle atrophy and tissue necrosis at day 20 post-surgery. Histological studies of the ischemic muscle sections at day 20 post-surgery also revealed evidence of severe inflammation, necrosis and atrophy in the PBS treated animals in contrast to the EPC and eNOS-EPC treated animals which showed visible reduction in muscle necrosis and atrophy (Figure 1A-C).

## Characterization of EPCs

Under the culture conditions used in the present study, large round adherent cells appeared from the plated PBMCs at day 2 - day 3 (Supp Figure 1A), which then assumed a spindle-shaped, endothelial cell-like morphology at day 7 - day 8 (Supp Figure 1B). More than 90% of the adherent cells took up DiI-acetylated LDL (red fluorescence) and showed concurrent binding to FITC-UEA-1 lectin (green fluorescence) (Supp Figure 2A). After 7 days in culture, majority of the cells also expressed endothelial markers vWF (Supp Figure 2B) and CD31 (Supp Figure 2C).

## eNOS gene transfer and expression *In vivo*

Immunohistochemical staining of skeletal muscle sections with human specific eNOS antibody showed positive FITC-fluorescence in the tissues treated with eNOS-EPCs (Figure 2A, Figure 2B). No fluorescence was observed in the sections from EPC or PBS transplanted tissues (Supp Figure 3A-D). Human eNOS mRNA expression was detected in tissues homogenates of the eNOS-EPC treated rabbits whereas there was no mRNA expression in the limb tissue homogenates of EPC or PBS treated animals (Figure 2C).

## Tissue nitrite levels

As compared to the EPC (3.5 ± 0.35 nmoles/ml) or PBS (2.9 ± 0.28 nmoles/ml) treated animals, eNOS-EPCs treated rabbits showed about a 3-fold increase (9.4 ± 0.36 nmoles/ml, P< 0.01) in the local tissue nitrite levels (Figure 2D). There was however no significant difference between the nitrite levels in EPC treated and PBS treated animals.

| | Control n | | EPC-treated rabbits n | | eNOS-EPC treated rabbits n | |
|---|---|---|---|---|---|---|
| | Day 10 (Before Treatment) | Day 20 (After Treatment) | Day 10 (Before Treatment) | Day 20 (After Treatment) | Day 10 (Before Treatment) | Day 20 (After Treatment) |
| **Muscle atrophy** | 4 | 4 | 4 | 2 | 4 | 0 |
| **Tissue necrosis** | 2 | 2 | 2 | 1 | 2 | 0 |

n= no. of animals

**Table 1:** Macroscopic features observed in different groups of rabbits.
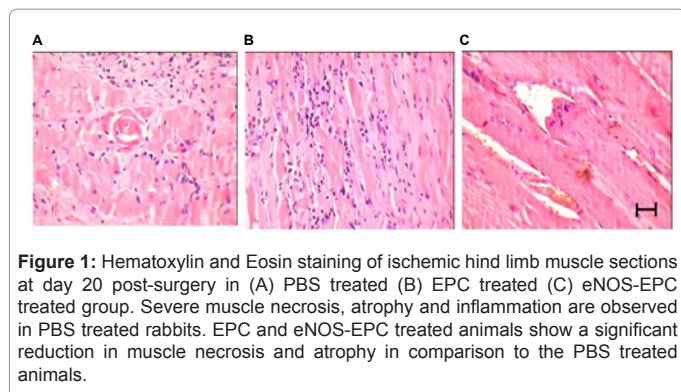
**Figure 1:** Hematoxylin and Eosin staining of ischemic hind limb muscle sections at day 20 post-surgery in (A) PBS treated (B) EPC treated (C) eNOS-EPC treated group. Severe muscle necrosis, atrophy and inflammation are observed in PBS treated rabbits. EPC and eNOS-EPC treated animals show a significant reduction in muscle necrosis and atrophy in comparison to the PBS treated animals.
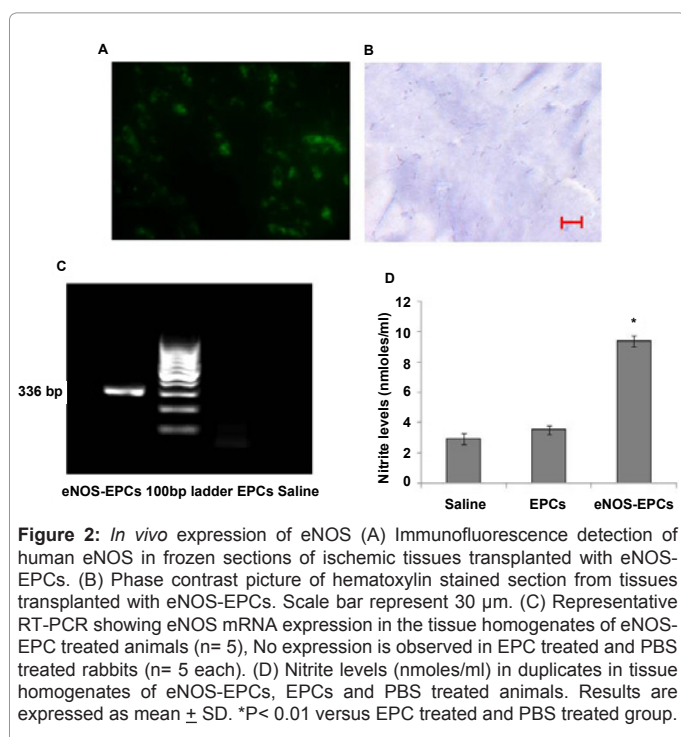


**Figure 2:** *In vivo* expression of eNOS (A) Immunofluorescence detection of human eNOS in frozen sections of ischemic tissues transplanted with eNOS-EPCs. (B) Phase contrast picture of hematoxylin stained section from tissues transplanted with eNOS-EPCs. Scale bar represent 30 μm. (C) Representative RT-PCR showing eNOS mRNA expression in the tissue homogenates of eNOS-EPC treated animals (n= 5), No expression is observed in EPC treated and PBS treated rabbits (n= 5 each). (D) Nitrite levels (nmoles/ml) in duplicates in tissue homogenates of eNOS-EPCs, EPCs and PBS treated animals. Results are expressed as mean ± SD. *P< 0.01 versus EPC treated and PBS treated group.

## Capillary/myocyte ratio

Representative anti CD31 immunostained sections of skeletal muscle tissue from various animal groups, two weeks after cell transplantation are shown in Figure 3A-C. Quantitative analysis revealed that capillary vessel numbers were significantly increased in rabbits receiving EPCs than those receiving PBS (2.7-fold, P< 0.05). Neo-capillary formation in rabbits receiving eNOS-EPCs was even more pronounced (4.5-fold relative to PBS treated group, P= 0.03 and 1.6-fold relative to EPC treated group, P= 0.05) (Figure 3D).

## Angiographic analysis

In the contrast angiography, there was restored perfusion in ischemic limbs in rabbits treated with eNOS-EPCs than those treated with EPCs or PBS (Figure 4A-C). Also, collateral vessel numbers and hence the angiographic score were significantly increased by 2.4 fold in eNOS-EPC treated group (0.7 ± 0.09) as compared to that observed in PBS (0.29 ± 0.03, P= 0.007) treated group and by 1.7 fold in comparison to EPC treated group (0.4 ± 0.04, P= 0.01) (Figure 4D). In eNOS-EPC treated group, a few of the growing collateral vessels were shown to

extend all the way from their original source to near-the ankle, where they seem to reconstitute the distal arterial tree.

## Discussion

The present study demonstrates that eNOS gene transfer in *ex vivo* expanded EPCs improves angiogenesis in a rabbit model of experimentally induced hind limb ischemia. In a hypoxic state brought about by tissue ischemia, angiogenesis is induced by an up regulation of VEGF. This is followed by changes in shear stress in preexisting collaterals that activate eNOS and induce its gene expression after chronic changes in blood flow [22]. Consequently, a certain amount of basal revascularization occurs in response to the ischemic conditions. In comparison with earlier studies which observed more than two-
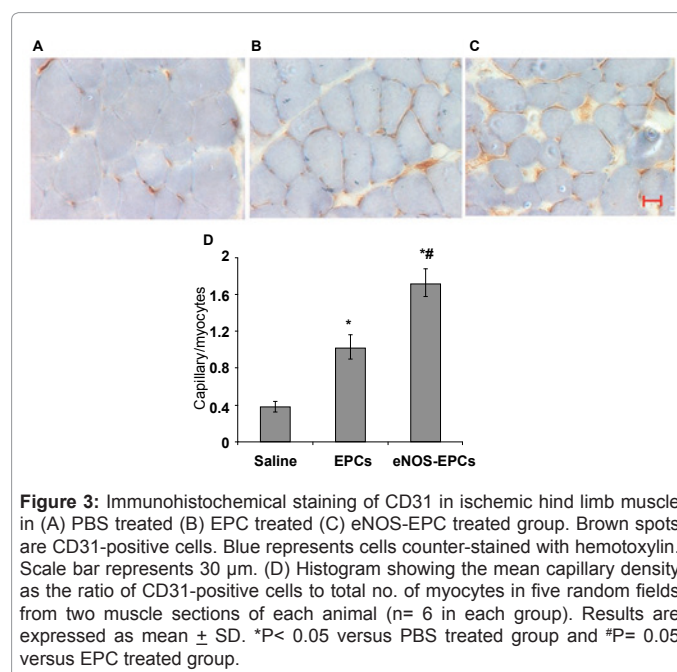


**Figure 3:** Immunohistochemical staining of CD31 in ischemic hind limb muscle in (A) PBS treated (B) EPC treated (C) eNOS-EPC treated group. Brown spots are CD31-positive cells. Blue represents cells counter-stained with hemotoxylin. Scale bar represents 30 μm. (D) Histogram showing the mean capillary density as the ratio of CD31-positive cells to total no. of myocytes in five random fields from two muscle sections of each animal (n= 6 in each group). Results are expressed as mean ± SD. *P< 0.05 versus PBS treated group and #P= 0.05 versus EPC treated group.
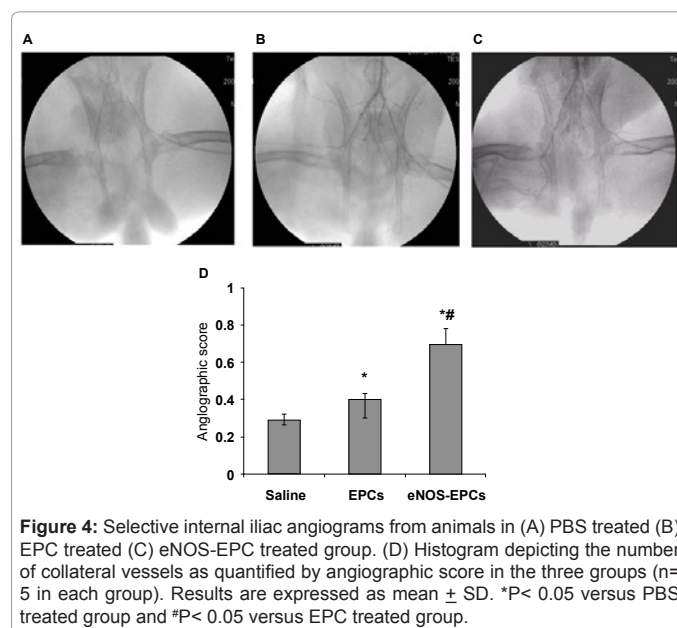


**Figure 4:** Selective internal iliac angiograms from animals in (A) PBS treated (B) EPC treated (C) eNOS-EPC treated group. (D) Histogram depicting the number of collateral vessels as quantified by angiographic score in the three groups (n= 5 in each group). Results are expressed as mean ± SD. *P< 0.05 versus PBS treated group and #P< 0.05 versus EPC treated group.

fold increase in angiogenic response after EPC therapy, we observed that the transplantation of *ex vivo* expanded autologous EPCs in the ischemic sites resulted in about two-fold increase in the angiogenic response over normal response to tissue ischemia after two weeks of EPC transplantation. This may be because in our study, EPC therapy was applied 10 days after the hind limb surgery in contrast to other studies that administered the cells one day after the surgery. Moreover, the dosage of EPCs used in the present study was about five to ten times less than that used in earlier studies [3-5]. The angiogenic effect was further enhanced by about four-fold over the normal response by an over expression of eNOS in culture-expanded EPCs, which could be robustly identified in the muscle tissues by RT-PCR, immunohistochemistry and increased nitrite levels in eNOS-EPC treated animals. This suggests that a combinatorial approach using eNOS and EPCs constitute an effective strategy to address the issue of quantitative and qualitative impairment of EPCs before *ex vivo* expansion and subsequent autologous readministration. The specific effects of eNOS transfection on EPCs are guarded by the fact that GFP transfection of EPCs did not result in any significant change/improvement in the functional properties of these cells under *in vitro* conditions [18]. It should also be noted that we didn't observe any macroscopic adverse effects of the EPC or eNOS-EPC therapy on other organs such as liver, spleen, lungs and contra-lateral limbs. The treatment effect was also observed in terms of significant improvement in the macroscopic and histological features of the ischemic muscle such as higher reduction in the muscle atrophy and necrosis in the animals transplanted with eNOS-EPCs as compared to that observed in animals treated with saline and unmodified EPCs. We preferred to deliver the cells locally in the ischemic region as the efficiency of recruitment is reduced if cells are introduced into the systemic venous circulation due to sequestration in the lung and other tissues.

The formation of sustainable and functional new blood vessels *in vivo* comprises of three important processes: vasculogenesis, angiogenesis, and arteriogenesis [23]. Capillary density of ischemic muscle reflects vasculogenesis and angiogenesis because they are processes of capillary formation from endothelial cells or EPCs. On the other hand, the angiographic score represents the number of arteries with a relatively large diameter because angiograms provide images of vessels of >50 µm in diameter. In our study, both histological and angiographic evidences demonstrate a significant improvement in the angiogenesis and arteriogenesis, respectively of eNOS-EPC treated animals as compared to the EPC or PBS treated animals, implicating that eNOS may be enhancing several reactions that involve both angiogenesis and arteriogenesis leading to the formation of collateral vessels.

A plethora of studies in recent years have reported the putative potential of eNOS in angiogenesis. In mouse hindlimb models, eNOS knockout mice show a severe form of critical limb ischemia [16]. Further, studies have demonstrated an enhancement in the ischemia-induced angiogenesis by a local overexpression of eNOS [17,24,25]. In addition, eNOS exerts a second messenger role in VEGF signaling and has been shown to be vital for VEGF-induced endothelial cell proliferation, migration and capillary formation both *in vitro* and *in vivo* [15,26]. Studies from our lab have also suggested that in presence of VEGF, eNOS over expression improves the angiogenic properties of EPCs including proliferation, migration and integration into tube-like structures *in vitro* [18]. Given a crucial role of eNOS-NO in the induction of angiogenesis and the fact that *ex vivo* expanded EPCs are preferentially recruited to the ischemic foci [4], it is logical to presume that EPCs transfected with eNOS may be leading to an increased local

overexpression of NO that in conjunction with other angiogenic factors such as VEGF may be directly or indirectly promoting angiogenic potential of the transplanted EPCs and/or differentiated ECs resident in the target ischemic tissue. The increased local bioavailability of NO may also augment the endogenous mobilization and functional activity of resident EPCs in these animals [27].

An added advantage of our therapy is that we have used a non-viral method of gene delivery that represents an important factor that may potentially enhance the safety profile of applications of gene therapy. Moreover, the need for long-term gene expression, which may be a prerequisite for certain types of inherited genetic defects, appears not to be a requirement for gene transfer strategies designed to promote neovascularization. Two to three weeks of gene expression appears to be sufficient to promote neovascularization and persistent blood flow.

In conclusion, the present study shows that autologous transplantation of eNOS modified *ex vivo* expanded EPCs enhances the angiogenic benefits of EPC therapy. As the viability of endogenous EPCs and also fully differentiated ECs is recurrently subjected to a variety of individual and environmental stress factors in peripheral and myocardial ischemic diseases, the concept of augmenting the regenerative and reparative potential of EPCs before *in vivo* transplantation constitutes a lucrative clinical approach for improving therapeutic angiogenesis in these diseases.

### Acknowledgements

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