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Beneficial Effect of a Short-Acting NO Donor for the Prevention of Neointimal Hyperplasia

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Abstract

Nitric oxide (NO)-based therapies effectively inhibit neointimal hyperplasia in animal models of arterial injury and bypass grafting, but are not available clinically. We created a simple, effective, locally-applied NO-eluting therapy to prevent restenosis following vascular procedures. We investigated the efficacy of perivascular delivery of two different distinctly different diazeniumdiolate NO donors, 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO), (short half-life) and diazeniumdiolated poly(acrylonitrile) (PAN/NO), (long half-life), in powder or gel form (30% poloxamer 407), at inhibiting neointimal hyperplasia using the rat carotid artery injury model. Two weeks post-injury, all of the NO-eluting therapies successfully reduced neointimal hyperplasia. However, most dramatically, PROLI/NO powder reduced intimal area by 91.2% ($P<0.05$) versus injury alone. PROLI/NO powder was noted to reduce the medial area (40.2% vs. injury alone, $P<0.05$), while other groups showed no such effect. Three days post-injury, each NO treatment group significantly reduced cellular proliferation. However, inflammatory markers revealed a distinct pattern: PAN/NO groups displayed increased leukocyte infiltration ($P<0.05$) whereas PROLI/NO groups displayed less macrophage infiltration ($P<0.05$). In conclusion, perivascular delivery of diazeniumdiolate NO donors in powder or gel form effectively inhibits neointimal hyperplasia. Application of short-acting PROLI/NO powder most effectively inhibited neointimal hyperplasia and inflammation and may represent a simple, clinically applicable NO-eluting therapy to prevent neointimal hyperplasia and restenosis following open vascular interventions.

Keywords

nitric oxide; neointimal hyperplasia; arterial injury; perivascular; vascular smooth muscle cell proliferation

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INTRODUCTION

The preeminent cause of death in the United States and Europe remains atherosclerosis, accounting for an estimated 50% of all mortality and costing upwards of \$400 billion in the U.S. alone.[1] The long-term benefits of percutaneous angioplasty, surgical arterial bypass, or endarterectomy, which are the current therapeutic modalities for complications of atherosclerosis, are significantly limited by restenosis secondary to neointimal hyperplasia. Neointimal hyperplasia is the pathologic consequence of the vascular healing response, which involves platelet adherence and aggregation, leukocyte chemotaxis, vascular smooth muscle cell (VSMC) proliferation and migration, and collagen deposition.[2,3] Although the pathogenesis of neointimal hyperplasia is multifactorial, the common insult appears to be endothelial denudation following therapeutic intervention. The endothelium is the source of several vasoprotective molecules. One of these vasoprotective molecules that is produced constitutively from endothelial cells is nitric oxide (NO), which has been shown to relax VSMC and confer anti-platelet and anti-inflammatory properties to the vessel wall.[4–6] NO is also a potent inhibitor of VSMC proliferation and migration.[7–10] Hence, the loss of NO in combination with exposure of the underlying elastic lamina and media to circulating blood elements initiates a cascade of events that result in the formation of neointimal hyperplasia.

Given the vasoprotective properties of NO, replacement of NO at the site of arterial injury should inhibit the development of neointimal hyperplasia. This has been demonstrated in numerous animal models which have utilized varying NO delivery methods including inhalational NO, delivery of the substrate L-arginine, delivery of NO-donors systemically or locally, gene therapy with endothelial NO synthase (eNOS) or inducible NO synthase (iNOS), and polymer-based approaches of NO-donor delivery.[11–29] All of these therapies have demonstrated varying degrees of success, yet none have been used in the clinical arena due to significant side effects, safety concerns, complexity of administration and, sometimes, lack of significant and durable effect.

The ideal NO-based therapy should, therefore, be locally delivered to achieve a high concentration at the site of injury, have minimal to no side effects, and be simple to administer. Our ultimate goal is to develop a NO-based therapy that can be easily applied to the perivascular surface of arteries or vein grafts in the operating room. To that end, we have studied two different variables: the specific NO donor and the delivery vehicle. For the NO donor, we utilized a unique class of compounds called diazeniumdiolates. Advantages of diazeniumdiolates over other NO donors include spontaneous NO release upon exposure to an aqueous environment, predictable rates of NO release, and a wide range of NO generation.[30,31] More specifically, we identified two distinctly different NO donors, 1-[2-(carboxylato)pyrrolidin-1-yl]diazen-1-ium-1,2-diolate (PROLI/NO) and diazeniumdiolated poly (acrylonitrile) (PAN/NO), with short and long half-lives, respectively.[32,33]

The aims for this study were three-fold: first, to evaluate the efficacy of these two markedly different diazeniumdiolate NO donors to prevent the development of neointimal hyperplasia; second, to evaluate two different delivery approaches, powder versus gel; and third, to evaluate the inflammatory response following application of these NO-donors. Our hypothesis is that locally delivered NO-based therapies will inhibit the development of neointimal hyperplasia and will be a simple, clinically applicable therapy.

MATERIALS AND METHODS

Rat Carotid Artery Injury Model

All animal procedures were performed in accordance with principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH)

Publication 85–23, 1996) and approved by the Northwestern University Animal Care and Use Committee. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 350–400 grams were anesthetized with inhaled isofluorane (0.5–2%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. Following a sterile prep and midline neck incision, the left common, internal and external carotid arteries were dissected and the internal and common carotid arteries were occluded. A 2 French arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA) was inserted into the external carotid artery and advanced into the common carotid artery. Uniform injury was created by inflating the balloon to 5 atmospheres of pressure for 5 minutes. After removal of the balloon, the external carotid artery was ligated and blood flow was restored. Next, one of the NO-eluting therapies was applied evenly to the external surface of the injured common carotid artery. The neck incision was closed. Rats were sacrificed at 3-day and 2-week time points for immunohistochemical and morphometric analysis, respectively.

Preparation of the Nitric Oxide-Eluting Therapies

PROLI/NO is a small molecule made by treating the amino acid proline with NO to attach the diazeniumdiolate group [$-\text{N}(\text{O})=\text{N}-\text{O}^-$] to the nitrogen (Figure 1).[33] PAN/NO is a polymer made by reacting the widely-used industrial material poly(acrylonitrile) with NO to attach the diazeniumdiolate group to carbon atoms in the polymer chain.[32] From prior studies, PROLI/NO has been noted to release a large amount of NO over a relatively short duration while PAN/NO releases a lower steady state of NO over a prolonged duration.[32–34] For the delivery vehicle, we utilized both a powder and gel form. The powder form was the PROLI/NO or PAN/NO alone. The gel utilizes a polylactic-polyglycolic acid polymer matrix (poloxamer 407), a reverse phase gel which is liquid at 4°C but gels at 37°C. We theorized that a gel would allow even application of the NO donor to the external arterial surface. To create the gels, 5% by weight of either PAN/NO or PROLI/NO powder was mixed with 30% poloxamer 407 (Pluronic F127 Surfactant Prill; BASF, Florham Park, NJ) in phosphate buffered saline (PBS) at 4°C. Either 200 µl of the NO-eluting poloxamer gel or 20 mg of the NO donor powder were applied as described above. Control groups included no injury, injury alone, and poloxamer gel alone. Treatment groups included PAN/NO gel, PAN/NO powder, PROLI/NO gel, PROLI/NO powder, and the combined PAN/NO+PROLI/NO gel. For the combination therapy, 2.5% by weight of each NO donor was mixed with 30% poloxamer 407.

Tissue Processing

Carotid arteries were harvested at 3 days or 2 weeks following *in-situ* perfusion-fixation with 250 ml of PBS and 500 ml of cold 2% paraformaldehyde. Vessels were placed in paraformaldehyde at 4°C for 1 hour, then cryoprotected in 30% sucrose at 4°C overnight. Tissue was quick-frozen in Tissue-Tek O.C.T. Compound (Tissue Tek, Hatfield, PA) and 5-µm sections were cut throughout the entire injured segment of the common carotid artery.

Histology

Carotid arteries harvested at 2 weeks (n=6/group) were examined histologically for evidence of neointimal hyperplasia and vascular remodeling using routine hematoxylin and eosin (H&E). Images of H&E stained sections were collected with light microscopy using an Olympus BHT microscope (Melville, NY).

Morphometric Analysis

Five evenly-spaced sections through each injured carotid artery were digitized using an Olympus BHT microscope and SPOT CCD software (Diagnostic Instruments, Sterling Heights, MI). Intimal area, medial area, and circumference were determined by measuring the

areas produced from the lumen, internal elastic lamina (IEE) and external elastic lamina (EEL) for each section using ImageJ software (National Institutes of Health).

Immunohistochemistry

Carotid arteries harvested at 3 days (n=2/group) were examined immunohistochemically for evidence of inflammation and proliferation. Sections were fixed using cold 2% paraformaldehyde and permeabilized with Triton-X100 in PBS and 0.5% bovine serum albumin (BSA; Vector, Burlington, CA). After sections were blocked with goat serum (Sigma, St Louis, MO), primary antibody was applied for 1 hour: anti-ED1 (macrophage, 1:500; Serotec, Raleigh, NC), anti-CD45 (lymphocyte, 1:500; BD Pharmingen, San Diego, CA), or anti-Ki67 (cellular proliferation, 1:500; BD Pharmingen, San Diego, CA). After rinsing, secondary antibody was applied for 1 hour (goat anti-mouse or goat anti-rabbit Alexa Fluor 555, 1:3000; Invitrogen, Carlsbad, CA). Sections were rinsed and coverslips were affixed with gelvatol. Three independent blinded observers qualitatively graded the amount of inflammation or cellular proliferation present in each treatment group on a scale of 0–3. Four equally spaced sections from each of two animals from each group were analyzed by all three blinded observers. Digital images were acquired using a Zeiss LSM-510 microscope (Hallbergmoos, Germany) with Zeiss LSM-510 software using a 40X objective.

Statistical Analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). Differences between multiple groups were analyzed using one-way analysis of variance with the Student-Newman-Keuls post hoc test for all pairwise comparisons. Differences between two groups were analyzed with the Student t-test (SigmaStat; SPSS, Chicago, IL). Statistical significance was assumed when $P < 0.05$.

RESULTS

Effects of Perivascular Delivery of NO-Eluting Therapies on Neointimal Hyperplasia and Vascular Remodeling Following Balloon Injury of the Carotid Artery

The aim of our study was to assess the effect of two very different diazeniumdiolate NO donors, PROLI/NO and PAN/NO, on neointimal hyperplasia. The NO donor was delivered directly to the external surface of the injured carotid artery in either powder or gel forms.

Analysis of the results revealed effective inhibition of neointimal hyperplasia with the different delivery methods and different NO donors. With respect to intimal area, PROLI/NO powder inhibited the neointimal formation to the greatest degree (12.6 ± 4.3 , 91.1% reduction) compared to arteries that underwent injury alone (142.4 ± 8.6 , $P < 0.05$, Table 1, Figure 2A, Figure 3). PROLI/NO in the poloxamer 407 gel produced the next most effective outcome, inhibiting intimal area by 77.5% (36.5 ± 6.9) compared to poloxamer 407 gel alone (162.8 ± 12.2 , $P < 0.05$). Both PAN/NO treatment groups were effective at reducing the intimal area, but were not as effective as PROLI/NO. Intimal area for PAN/NO powder was 47.6 ± 8.3 and for PAN/NO gel was 91.3 ± 11.5 , representing a 67% and 43.9% reduction relative to their respective controls, ($P < 0.05$). Combination therapy of PROLI/NO plus PAN/NO in the poloxamer 407 gel was nearly as effective as the PROLI/NO powder, inhibiting intimal area by 90.5% ($P < 0.05$). It should be noted that injured carotid arteries exposed to poloxamer 407 gel alone displayed greater intimal area compared to control injured arteries (162.8 ± 12.2 versus 142.4 ± 8.6 , $P = \text{NS}$), however, this did not reach statistical significance.

Results of the intima/media (I/M) area ratio were similar to intimal area reductions (Figure 2C). Treatment with poloxamer 407 gel alone resulted in a slight increase in the I/M area ratio (1.08 ± 0.06) compared to injury alone (0.96 ± 0.04 , $P = \text{NS}$). Treatment with PAN/NO gel and

powder reduced the I/M area ratio by 46% and 67% compared to their controls (I/M 0.58 ± 0.06 and 0.32 ± 0.05 , respectively, $P < 0.05$). Treatment with PROLI/NO gel reduced the I/M area ratio by 69% (0.33 ± 0.05 , $P < 0.05$). Most dramatically, treatment with PROLI/NO powder reduced the I/M area ratio by 86% (0.13 ± 0.05 , $P < 0.05$). The combination therapy of PROLI/NO + PAN/NO powder resulted in an I/M area ratio reduction of 80% compared to injury alone (0.19 ± 0.07 , $P < 0.05$). Analysis of I/(I+M) revealed a pattern that was similar to I/M area ratio (Table 1).

The effects on medial remodeling and circumference were also interesting. PROLI/NO powder, which was most effective at inhibiting the development of the intima, was the only therapy to reduce medial area (40.2% versus injury alone, $P < 0.05$) (Figure 2B). The medial area for PROLI/NO gel (118.2 ± 8.0) was reduced compared to the poloxamer gel alone (149.2 ± 6.0) however, this was not statistically significant. PAN/NO appeared to have no effect on medial area following injury. With respect to circumference, PROLI/NO powder and the combination therapy of PROLI/NO and PAN/NO in the poloxamer gel did have an overall effect on circumference compared to their respective controls (Table 1, $P < 0.05$). Therefore, it appears that PROLI/NO powder not only inhibited the development of neointimal hyperplasia, but also effected vascular remodeling.

It should be noted that we observed no unusual toxic side effects from these therapies. There were no unusual deaths. We detected no hematomas, myocardial infarctions, unusual respiratory failure, or bleeding disorders. Furthermore, no foreign body responses were noted.

Effects of NO-Eluting Therapies on Inflammation Following Balloon Injury of the Carotid Artery

Next, we examined the effect of the different treatment groups on inflammation. In particular, carotid arteries were injured and exposed to the different treatment groups and harvested at 3 days, a time point suitable for evaluation of acute inflammation. Sections were stained with antibodies against ED1, an antigen specific for monocytes and tissue macrophages, and CD45, a cell surface marker specific for leukocytes. Interestingly, the control group that received the poloxamer 407 gel displayed more CD45 staining compared to injury alone ($P < 0.05$) (Figure 4). Similarly, both groups that were treated with PAN/NO displayed more CD45 staining compared to injury alone ($P < 0.05$). However, both groups that were treated with PROLI/NO displayed CD45 staining that was similar to injury alone.

With respect to ED1 staining, a slightly different pattern was observed. All treatment groups that included poloxamer 407 gel, including both PAN/NO and PROLI/NO, displayed ED1 staining similar to injury alone (Figure 5). However, PAN/NO powder and PROLI/NO powder exhibited less ED1-positive staining compared to injury alone ($P < 0.05$). Therefore, these data suggest that poloxamer 407 induces some vascular inflammation over injury alone and that this may not be an ideal delivery agent in the vasculature. However, PROLI/NO and PAN/NO powders appear to inhibit monocyte/macrophage infiltration, a property that is beneficial for vascular therapies directed toward inhibiting neointimal hyperplasia.

Effects of NO-Eluting Therapies on Proliferation Following Balloon Injury of the Carotid Artery

To further evaluate the inhibitory effect of NO on the development of neointimal hyperplasia, carotid arteries harvested at 3 days were evaluated for active proliferation using an antibody against Ki67, which detects actively proliferating cells. All groups that were treated with one of the NO-eluting therapies had fewer actively proliferating cells compared to injury alone or injury plus poloxamer 407 gel ($P < 0.05$; Figure 6). These data are consistent with the majority

of prior publications demonstrating an antiproliferative role for NO against both smooth muscle cells and adventitial fibroblasts.

DISCUSSION

The development of neointimal hyperplasia is the result of a localized activation of platelets, inflammatory cells, VSMC, and adventitial fibroblasts. This process is orchestrated by a complex interaction of mitogenic and chemotactic factors following endothelial cell injury that culminates in the formation of neointimal hyperplasia, vessel wall remodeling, and eventual luminal narrowing. We have demonstrated in this study that perivascular delivery of NO-eluting substances, PROLI/NO and PAN/NO, can dramatically inhibit neointimal hyperplasia in a rodent carotid artery balloon injury model. Application of PROLI/NO powder to the external surface of the injured artery produced a significantly greater amount of inhibition of the intima, I/M area ratio, and I/(I+M) compared to its counterpart, PAN/NO powder. While each donor had different effects on inflammation, PROLI/NO is the only treatment that affected vascular remodeling by reducing medial area and circumference and attenuated monocyte/macrophage infiltration.

Our study validates the application of a perivascular NO donor as a means of supplementing exogenous NO production. Previous studies have augmented NO levels, however, these alternative means of NO supplementation have not made it to the clinical arena due to several limitations.[11–29,35] Systemic administration of NO is rapidly inactivated by hemoglobin in the circulating blood resulting in limited bioavailability. To overcome this limitation, larger doses of the NO donor must be administered systemically; however, these larger doses can produce adverse systemic hemodynamic and hemostatic effects, thereby precluding administration of biologically effective doses of NO.[25] Inhalational NO produced a 43% reduction in neointimal hyperplasia.[11] However, to achieve this level of inhibition, the rodents required continuous exposure to gaseous NO for 14 days. Later studies elucidated that inhalational NO bioavailability was nearly undetectable due to rapid inactivation by hemoglobin.[36] iNOS and eNOS gene therapy have demonstrated efficacy in the literature, [17–21] but the therapeutic use of gene therapy has been sidelined due to the safety concerns associated with viral-mediated gene delivery. It is possible that plasmid DNA delivery of one of the NOS isoforms may hold promise in the future; however, this remains to be determined. This said, the remaining therapeutically salient means of delivery, namely local perivascular application of NO, is highly promising.

Periadventitial delivery has been shown to be successful by several other investigators. Kaul et al. delivered SPER/NO in a polylactic-polyglycolic acid polymer matrix to the external surface of injured rat ileofemoral arteries and inhibited neointimal hyperplasia by 75% at 14 days.[12,25,27] Chaux et al. delivered SPER/NO in a biodegradable polymer to the external surface of vein grafts in hypercholesterolemic rabbits and found a 41% reduction in vein graft hyperplasia at 28 days.[23] Masters et al delivered a NO-releasing hydrogel covalently modified with a S-nitrocysteine and showed 75% inhibition of neointimal hyperplasia at 14 days in the rat carotid artery injury model.[35] Our periadventitial therapy resulted in greater than 90% inhibition of neointimal hyperplasia. The mechanism by which perivascular delivery of exogenous NO exerts its effect may involve additional facets that do not play a significant role with intraluminal delivery. In particular, local perivascular administration of NO appears to inhibit neointimal hyperplasia through two mechanisms: reduction of VSMC proliferation and prevention of macrophage and local fibroblast differentiation into VSMC. Adventitial fibroblasts have been shown to play an important role in formation of neointimal hyperplasia via the transformation of fibroblasts to myofibroblasts and subsequent differentiation into VSMC.[37] Additionally, Bayes-Genis et al. have suggested that macrophages may also differentiate into myofibroblasts, providing a progenitor population for VSMC.[38] Therefore,

external application of NO may affect both of these latter pathways by inhibiting VSMC, fibroblasts, and macrophages. In support of this hypothesis, in our study application of PROLI/NO powder, which resulted in the greatest inhibition of neointimal hyperplasia, also inhibited macrophage infiltration to the greatest extent. The next most effective therapy, PAN/NO powder, also inhibited macrophage infiltration.

An additional potential mechanism includes the downregulation of the inflammatory response to injury. Following injury, it has been well established that inflammatory infiltration occurs in both the injured media as well as the adventitia. Our study indirectly lends credence to the necessity of blocking the inflammatory response in addition to inhibiting VSMC and fibroblasts. Because PAN/NO-treated vessels had an increase in inflammation concurrent with a reduction in proliferation, it is possible that NO acted directly on the medial VSMC, and the adventitia was simply a convenient route of administration. However, based on the emerging data regarding the direct role of adventitial cells in neointimal formation,³⁵ it is likely that inhibition of these cells by periadventitial delivery of NO or other potent antiproliferative agents may offer a more effective therapeutic strategy.

Several other findings of our study are novel in important and serendipitous respects. First, this is the only report of inhibiting neointimal hyperplasia following arterial injury using the diazeniumdiolates PROLI/NO and PAN/NO, the former having a very short half-life and the latter having a long half-life. It should be noted that the low molecular weight of the small molecule PROLI/NO when compared to the large polymer PAN/NO means that the total amount of NO released from any given quantity of PROLI/NO is considerably larger than the total amount of NO released from that same quantity of PAN/NO. Nevertheless, the finding that PROLI/NO was more effective compared to PAN/NO was surprising to us as we suspected the extended release of NO from PAN/NO would be more effective at attenuating neointimal hyperplasia than the short release of NO from PROLI/NO. Furthermore, the significant efficacy of the PROLI/NO powder over PAN/NO powder suggests an important role for early NO release following injury. An early reduction in platelet adhesion and inflammatory cell infiltration may be pivotal in halting the downstream mitogenic and chemotactic signaling cascade. Second, the non-polymer-based perivascular delivery of an NO donor (i.e., powder form) is novel in its simplicity, eliminating the need for a polymer vehicle. While the polymer was expected to improve localization and stability of NO, this was not the case in our study. Instead, the polymer, when applied without an NO donor, increased the degree of neointimal hyperplasia, and thus proved less effective, as compared to direct application of the NO donor. As an alternative interpretation to the above finding, the future use of an inert polymer may demonstrate a greater reduction in neointimal hyperplasia as compared to powder alone, and thereby, result in an even more effective use of the NO donors utilized in these studies. Interestingly, in a separate study, half as much PROLI/NO (10mg) was applied periadventitially following arterial injury in a nanofiber gel and resulted in a similar degree of inhibition of neointimal hyperplasia (unpublished data). It may be that the nanofiber gel increased the efficacy of the NO donor by prolonging the release of NO from the gel, extending the half-life, or exerting synergistic beneficial effects. Further study will be required, but these data suggest that there may be a threshold effect with the NO donor and its beneficial effects on the arterial injury response. We plan to study the optimal concentration once the therapies are evaluated in a large animal model. Third, while little is known about PAN/NO since this is a new diazeniumdiolate, PROLI/NO has been shown to have a very good safety profile with no identifiable carcinogenic or biological effects.[33] This is important if the therapy is targeted for human use. While Kaul et al. and Chaux et al. demonstrated efficacy with periadventitial delivery of SPER/NO, SPER/NO dissociates into NO and spermine, the latter of which has biologically active properties that may not be beneficial to the vasculature.[23,25,39]

Despite the significant efficacy of our therapies, some limitations exist. Most polymers, despite the rapid improvements in their safety profile, still carry some adverse reactions. For example, poloxamer 407 while having no known toxicity and a long history of safe use in a wide range of oral and topical pharmaceutical products, was shown to induce atherosclerosis in mice. [40] This appears to be secondary to lipid derangements and not secondary to direct effects on endothelial cells or macrophages. While little is known about PAN/NO, the parent polymer, namely polyacrylonitrile, it may have induced the inflammation we observed in the treatment groups that received PAN/NO. Further studies into the biologic effects of PAN/NO would be required before its use could be considered for clinical use. Lastly, the powder forms of PROLI/NO and PAN/NO yielded superior results, however there were two specific disadvantages. While delivery of the powder form of the NO donor is simple, and would seem easy to use, localized directed delivery can be challenging since the powder is fine, light-weight and difficult to control. Additionally, with direct use of the powder, it is difficult to titrate the amount of NO donor delivered to the adventitial surface, because a minimum is required in order to evenly coat the vessel.

In conclusion, these data demonstrate that direct application of the diazeniumdiolate NO donors PROLI/NO and PAN/NO to the external surface of an injured artery are highly efficacious at preventing the development of neointimal hyperplasia. However, delivery of PROLI/NO in the powder form was most effective and inhibited inflammation. This latter approach represents a very simple, easy to use therapy with no apparent side effects or safety concerns. Future studies include evaluating the efficacy of perivascular application of PROLI/NO in a more relevant model to humans. Our aim is to study the efficacy of this simple to use therapy in a porcine model of arterial injury and vein bypass grafting, as the true therapeutic potential of NO donors remains to be demonstrated in a milieu better approximating the human response to vascular injury.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
eNOS	endothelial nitric oxide synthase
H&E	hematoxylin and eosin
iNOS	inducible nitric oxide synthase
I/M	intima/media
NO	nitric oxide
PBS	phosphate buffered saline
SEM	standard error of the mean
VSMC	vascular smooth muscle cells

PROLI/NO

PAN/NO

Figure 1.
Chemical structures of PROLI/NO and PAN/NO.

2A.

2B.

2C.

Figure 2.

Graphical representation of the morphometric analysis of the rat carotid artery sections at 14 days: A) intimal area; B) medial area; C) intima/media area ratio. Units are arbitrary. (n = 6 per group). *P<0.05 versus injury alone. PAN-G: injury+PAN/NO gel; PAN-P: injury+PAN/NO powder; PRO-G: injury+PROLI/NO gel; PRO-P: injury+PROLI/NO powder; Combo: injury+PAN/NO+PROLI/NO gel.

Figure 3. Representative hematoxylin and eosin (H&E) stained rat carotid artery sections at 14 days. (n = 6 per group).

4B.

Figure 4.

Immunofluorescent staining for leukocytes (CD45) in the rat carotid artery sections 3 days following arterial injury. A) Graphical representation of scoring by three blinded observers (scale 0–3). B) Representative images of the immunofluorescent staining. Positive CD45 staining is indicated by red. Elastic lamina is green. * $P < 0.05$ versus injury alone. PAN-G: injury+PAN/NO gel; PAN-P: injury+PAN/NO powder; PRO-G: injury+PROLI/NO gel; PRO-P: injury+PROLI/NO powder.

5B.

Figure 5.

Immunofluorescent staining for macrophages (ED1) in the rat carotid artery sections 3 days following arterial injury. A) Graphical representation of scoring by three blinded observers (scale 0–3). B) Representative images of the immunofluorescent staining. Positive ED1 staining is indicated by red. Elastic lamina is green. * $P < 0.05$ versus injury alone. PAN-G: injury+PAN/NO gel; PAN-P: injury+PAN/NO powder; PRO-G: injury+PROLI/NO gel; PRO-P: injury+PROLI/NO powder.

Figure 6. Immunofluorescent staining for active cellular proliferation (Ki67) in the rat carotid artery sections 3 days following arterial injury. A) Graphical representation of scoring by three blinded observers (scale 0–3). B) Representative images of the immunofluorescent staining. Positive Ki67 staining is indicated by red. Elastic lamina is green. * $P < 0.05$ versus injury alone. PAN-G: injury+PAN/NO gel; PAN-P: injury+PAN/NO powder; PRO-G: injury+PROLI/NO gel; PRO-P: injury+PROLI/NO powder.

TABLE 1

Morphometric analysis of the carotid arteries 14 days after balloon injury.

Treatment Group	Outer Circumference	Lumen Area	Intimal Area	Medial Area	I/M Ratio	I/(I+M)
Injury Alone	101.1±2.16	531.9±19.8	142.4±8.6	152.9±9.5	0.96±0.04	0.48±0.01
Poloxamer Gel	101.6±2.08	517.1±23.9	162.8±12.2	149.2±6.0	1.08±0.06**	0.51±0.01**
PAN/NO Poloxamer Gel	94.6±2.79	482.2±45.6	91.3±11.5**	153.0±5.9	0.58±0.06*	0.34±0.03*
PAN/NO Powder	95.8±1.22	543.3±16.9	47.6±8.3*	143.6±5.5	0.32±0.05*	0.21±0.03*
PROL/NO Poloxamer Gel	96.5±1.68	592.9±23.4	36.5±6.9**	118.2±8.0	0.33±0.05**	0.22±0.03**
PROL/NO Powder	85.6±2.88*	485.3±37.6	12.6±4.3*	91.5±4.6*	0.13±0.05*	0.11±0.03*
PROL/NO + PAN/NO Poloxamer Gel	88.0±2.21**	496.8±25.9	15.3±5.3**	110.7±7.9	0.19±0.07**	0.12±0.04**

* P<0.05 versus Injury Alone

** P<0.05 versus Poloxamer Gel

Units are arbitrary.