Original Article

Sodium nitrite therapy fails to improve tissue perfusion in a mouse model of hind limb ischemia: Slight differences in methodology may be responsible casting suspicion on the reliability and predictive value of this model

Jeff S. McKee, Benjamin D. Brooks

Department of Life Sciences, Baxter Healthcare Corporation, Illinois, USA

Abstract

Background: The nude mouse model of hind limb ischemia is used to evaluate human-derived, cell-based therapeutics intended to promote tissue perfusion. The criticism of the mouse model of hind limb ischemia is the absence of a well-characterized positive control. The suitability of sodium nitrite ($NaNO_2$) was evaluated. The rationale for doing so was based on a report that $NaNO_2$ induced unprecedented tissue perfusion in wild-type mice using a similar model. The objective was to evaluate $NaNO_2$ to improve tissue perfusion in nude mice as well as their wild-type counterparts. **Materials and Methods:** The mice underwent surgically induced, unilateral hind limb ischemia, and received either $NaNO_2$ or a vehicle intraperitoneally, twice daily, for seven days. Hind limb tissue perfusion was evaluated on days one, four, seven, and fourteen post-surgery. **Results:** No increase in tissue perfusion was observed in the nude or wild-type mice treated with $NaNO_2$ when compared with the vehicle. Nude mice exhibited significantly lower tissue perfusion compared to wild-type mice, irrespective of the treatment. **Conclusions:** $NaNO_2$ failed to increase tissue perfusion and, therefore, did not appear suitable for use as a positive control in this model. This is in stark contrast to a previous report indicating that $NaNO_2$ significantly increased tissue perfusion in wild-type mice using a similar model. The exact cause is not known, but is probably due to differences in methodology employed between laboratories. The lower tissue perfusion in nude mice is a novel finding, suggesting this strain may have less pre-existing collateral vessels and/or a reduced capacity to form new vessels as compared to wild-type mice.

Key words: Hind limb ischemia, nude mouse, peripheral arterial disease, peripheral vascular disease, sodium nitrite

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INTRODUCTION

The prevalence of peripheral vascular disease in people over 55 years is 10 – 25% and increases with age.[1] In the USA peripheral arterial disease (PAD) affects 12 – 20% of Americans of age 65 and older. Despite its prevalence and cardiovascular risk implications for heart attack and stroke, only 25% of PAD patients are currently

Address for correspondence:
Dr. Jeff McKee, Department of Life Sciences, 25212 W Illinois Route 120, WG2-1S, Round Lake, IL 60073, USA.
E-mail: jeff_mckee@baxter.com

undergoing treatment.[2] Peripheral arterial disease can lead to morbidity and eventually limb amputation if not adequately treated. Given the aging population, PAD will continue to be a challenge to the medical community and offer pharmaceutical companies an opportunity to develop and ultimately market effective therapies to treat the disease. In fact, many companies have currently invested in the clinical development of therapeutics to improve tissue perfusion and limb salvage. One such therapeutic area receiving a great deal of attention is the use of adult, human-derived stem cells.

The animal model typically employed in the early preclinical development of the evaluation of the efficacy of therapeutics targeting PAD, is the mouse model of hind limb ischemia. This model typically involves surgical intervention targeting the femoral artery and disrupting the circulation to the distal portion of one of the hind limbs. A major shortcoming of this model is the absence of a well-characterized positive control. Such a positive control would be defined as one that consistently produces statistically higher tissue perfusion as compared to a negative control, demonstrating that the model is responding as expected. This would increase confidence in the translation of the preclinical results to the clinical setting and would enable informed decisions about which therapies undergo further development. In recent times, it has been reported that sodium nitrite ($NaNO_2$) administered intraperitoneally, twice daily, for seven days, to wild-type mice, resulted in statistically higher tissue perfusion in the affected limb, as determined by laser Doppler imaging (LDI), three, five, and seven days after transection of the femoral artery.[3] The proposed action of sodium nitrite appears to be mediated by its metabolite, nitric oxide, which is known to regulate vascular smooth muscle tone and angiogenesis. The increase in perfusion was unprecedented in the literature with blood flow values in the affected hind limb reaching approximately 75% of the pre-surgery values as early as day 3 post-treatment.

The purpose of this study was to evaluate the potential of $NaNO_2$ to increase hind limb tissue perfusion in nude mice. The response of nude mice to $NaNO_2$ treatment was our primary interest as this strain is routinely used in the evaluation of human-derived, cell-based therapeutics intended to promote tissue perfusion. Wild-type mice were also included in an attempt to reproduce the findings of Kumar *et al*.[3]

MATERIALS AND METHODS

Test and control articles

The test article was sodium nitrite. Two different sources of sodium nitrite were required based on the surgery schedule [Table 1] and the timing of the receipt of the USP grade sodium nitrite. On the first day of dosing, five of the eight mice in group three, and five of the nine mice in group four received sodium nitrite from Sigma-Aldrich (catalog number 431605-50G). Sodium nitrite was reconstituted in Dulbecco's Phosphate buffered saline (PBS) to a final dosing concentration of 18 µg / mL. Sodium Nitrite Injection, USP (30 mg / mL, lot QB017E8, Hope Pharmaceuticals, Scottsdale, AZ) was used for all other dosing and was diluted in PBS to a final dosing concentration of 18 µg / mL. The negative control article was PBS. Aseptic technique was used for all steps in preparing the test and control articles.

Test system

All animal care and use procedures were approved by the institution's animal care and use committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.[4] Twenty-one male Hsd : Athymic Nude-*Foxn1*$^{nu/nu}$ mice (C57BL / 6N background) and 20 male C57BL / 6NHsd wild-type mice were received from Harlan Laboratories, Inc. The nude mice were approximately 11 weeks old at the time of surgery and the wild-type mice were approximately nine weeks old at the time of surgery. The animals were acclimated for seven days. All animals were housed individually in microisolator caging in a temperature-monitored room on a 12 hour / 12 hour light / dark cycle. All mice received autoclaved water *ad libitum*, and autoclaved sterilizable rodent diet (Harlan Teklad, #2018SC) *ad libitum*, except during surgery, LDI, and dosing with test or control articles.

Experimental design

The experimental design is described in Table 1. To accommodate the number of animals, a similar number of animals from each group underwent surgery on each

Table 1: Experimental design

Group	Article	Mouse strain	Target dose per injection	Surgery day 1, N	Surgery day 2, N	Total N
1	PBS	Hsd:Athymic Nude-Foxn1nu/nu	NA	5	5	10
2	PBS	C57BL/6NHsd	NA	4	4	8
3	$NaNO^2$	Hsd:Athymic Nude-Foxn1nu/nu	165 µg/kg	5	6	11
4	$NaNO^2$	C57BL/6NHsd	165 µg/kg	5	4	9

of the two consecutive days. Animals underwent LDI of both hind limbs and a clinical assessment of the ischemic hind limb was performed one day post-surgery. One day post-surgery, animals of the same strain were assigned to treatment based upon their clinical scores, which was done to ensure that the severity of the ischemic insult was similar in the $NaNO_2$ and PBS-treated groups. After assignment to the treatment groups, the average LDI scores were checked, to ensure that they were also similar across the treatment groups. Once assigned to the treatment groups, the animals were dosed intraperitoneally with the test or the control article twice daily, for seven days, post-surgery. Repeat clinical assessments and LDI measurements were obtained at various time points during the recovery period of 14 days, post-surgery. The animals were euthanized after the last LDI time point.

Surgery

On the day of the surgery, the animals were anesthetized with isoflurane and placed on a heating pad to maintain body temperature. The animals were given buprenorphine pre-emptively (approximately 0.1 mg/kg SC) for pain management and four to five additional doses (6 – 12 hours apart) post-surgery. Only one hind limb per mouse underwent surgery. An incision in the groin area was made and the superficial epigastric artery (SEA) was identified and used as a landmark. The femoral artery and vein was ligated using a 6.0 prolene suture, proximal to the SEA, to induce ischemia. A second ligation was made proximal to the bifurcation point of the femoral artery into the saphenous and popliteal arteries. A third ligation of the superficial epigastric artery and vein was made, followed by a fourth ligation of the profunda femoris artery and vein. After all ligations, the femoral artery and vein were transected and excised to ensure disruption of the circulation. After surgery, the skin was closed with a 6.0 prolene suture and the animals were recovered.

Dose justification and administration

The dose of sodium nitrite and the dosing regimen were based on the study of Kumar *et al*.[3] Kumar *et al* examined a range of sodium nitrite doses given intraperitoneally, twice per day, for seven days, and the optimal efficacious dose reported was 165 μg/kg.[3]

Clinical observations

A clinical assessment of the ischemic hind limb of each animal was performed daily for seven days, post-surgery [Table 2]. This scoring system was based on the study of Zbinden *et al*, which combined appearance and functional assessment.[5] They concluded that to increase the likelihood of obtaining reliable conclusions using the mouse hind limb ischemia model, the animals should be randomized to treatment groups on day one post-surgery

Table 2: Clinical assessment of ischemic hind limb

Clinical score	Description
0	Normal
1	Plantar flexion, mild discoloration
2	No plantar flexion, mild discoloration
3	No plantar flexion, moderate to severe discoloration
4	Any necrosis of the foot and/or toes

(and prior to treatment) based on their clinical score, such that the starting clinical score was similar across all treatment groups. This was based on a demonstration that the clinical score on day one post-surgery was negatively and significantly correlated with the degree of perfusion of the affected hind limb measured post-surgery.[5] Post-surgical observations and body weights were performed and recorded daily for seven days. Cage-side observations were performed daily thereafter.

Laser Doppler imaging

The Moor Instruments Inc., moorLDI2 laser Doppler blood flow imaging system uses a low intensity laser beam that is scanned across a tissue surface in a raster fashion using a moving mirror. There is no direct contact with the tissue being evaluated. Due to the configuration of the laser Doppler system it was not possible to perform LDI in a sterile, laminar flow hood. As such, the aseptic technique was used for all animal manipulations whenever possible. Laser Doppler images of the plantar surface of the foot (region of interest = ROI) from both hind limbs were captured on day one (approximately 24 hours after surgery) and also four, seven, ten, and fourteen days after surgery. Images were captured in the dark to prevent possible interference by ambient light. Daily LDI measurements were taken before the $NaNO_2$ or PBS injection, to obtain the representative, steady-state changes in perfusion from the previous measurement. The animals were anesthetized by box induction and mask maintenance with isoflurane balanced with oxygen. The platform on which the animals were placed was covered with a sterile, black cloth. The cloth was placed on top of a heating pad to maintain body temperature. A rectal temperature probe was used to monitor body temperature. A clear, plastic, open box was inverted and placed over the animal to allow the ambient temperature of the immediate area surrounding the mouse to be controlled. Laser Doppler imaging was not initiated until the ambient temperature probe inside the box read 33 degrees Celsius and rectal temperature was 37.8 degrees Celsius. The isoflurane concentration and the rectal and ambient temperatures were recorded at the time the LDI images were captured. Three quality LDI images per animal, per

time point, were captured. Inadequate perfusion of the non-ischemic limb was a technical basis to discard and repeat images as needed to obtain three quality images per animal, per time point. Adequate perfusion was defined as predominately red pixels in the non-ischemic limb with LDI settings of 60 for background (633 nm wavelength, Red) and a palette range of 0 to 1200.[6] Images were analyzed using Software Research Version 5.1D, Moor Instruments Inc. All animals met the LDI requirement of presenting with an LDI ratio of less than 0.25 on day one post-surgery.

Any animal exhibiting partial necrosis of the foot of the affected hind limb was removed from the study. These animals were removed from the study because the ROI is the foot and with partial necrosis the ROI is reduced, which artificially decreases the LDI ratio (ischemic/non-ischemic foot). Data from animals removed from study were not submitted for statistical analysis and are not included herein.

The moorLDI2 system allowed a retrospective analysis of the original images, to determine tissue perfusion from different ROIs. Select images were used to measure tissue perfusion from a secondary ROI, just above the ankle, including the calf, to capture the ROI used by Kumar *et al*.[3] The flux value from the new ROI of the ischemic limb was divided by the flux value from the new ROI of the non-ischemic limb and a new perfusion ratio was calculated. This retrospective analysis could only be performed on nude mice because the hair on the upper portion of the hind limbs of the wild-type mice prevented the laser from adequately scanning the skin surface. The images analyzed were limited to those obtained at the baseline (after surgery and prior to initiating treatment) and after three days of receiving $NaNO_2$ or PBS. The three days were selected based on a statistically significant increase in hind limb blood flow, which was as early as three days after initiating $NaNO_2$ therapy.[3]

Euthanasia

After the last LDI time point, the animals were euthanized by CO_2 inhalation.

Statistical analyses

Data submitted for statistical analysis included: Individual laser Doppler images from days one, four, seven, ten, and fourteen, and clinical scores from days one, four, and seven. The following were calculated: LDI ratio (ischemic / non-ischemic foot) for each image and the average LDI ratio per animal per time point. The following were statistically analyzed: Average LDI ratio and clinical score. The clinical score categorical response parameter was analyzed using an SAS procedure named CATMOD. The clinical score was analyzed on day one and the change in clinical score from day one was analyzed separately on days four and seven. The model included a factor for group effect. The main effect of the group was evaluated at $\alpha = 0.05$. If the main effect of the group was significant, then pairwise comparisons were performed and reported. The pairwise comparisons of Group 1 versus 2, 1 versus 3, 2 versus 4, and 3 versus 4 were performed at $\alpha = 0.05$ using the CONTRAST statement in CATMOD.

RESULTS

Unscheduled euthanasia

Three wild-type mice were euthanized due to inadvertent vessel rupture during surgery. Based on the presence of partial necrosis, five nude mice were removed from the study and euthanized (two from group 1 and three from group 3).

Clinical observations

The clinical scores for each group over time are summarized in Table 3. As designed, the animals of the same strain had very similar clinical scores on day one post-surgery, prior to the initiation of treatment with either PBS or $NaNO_2$. The day one post-surgery clinical scores were higher ($P < 0.0001$) for nude mice when compared with those of the wild-type mice, which was also observed on days four and seven, post-surgery. The post-surgery clinical scores on days four and seven, for all groups, were not significantly different from their respective scores on day one.

Tissue perfusion

The laser Doppler imaging ratios over time for each group, based on using the foot as the ROI, are depicted in Figure 1. As expected, animals of the same strain had the same LDI ratio on day one post-surgery, prior to the initiation of treatment with either PBS or $NaNO_2$. Day one post-surgery, however, the LDI ratios were lower ($P < 0.001$) for nude mice as compared to the wild-type mice, which was consistent with the strain difference

Table 3: Clinical scores (Mean ± Std. Err.)

Group	Days post-surgery		
	1	4	7
Hsd:Athymic Nude-*Foxn1*$^{nu/nu}$ with PBS (N=8)	3.25 ± 0.16*	3.38 ± 0.18*	3.75 ± 0.16*
C57BL/6NHsd with PBS (N=8)	1.13 ± 0.13†	1.13 ± 0.13†	1.13 ± 0.13†
Hsd:Athymic Nude-*Foxn1*$^{nu/nu}$ with $NaNO_2$ (N=8)	3.25 ± 0.16*	3.50 ± 0.19*	3.63 ± 0.26*
C57BL/6NHsd with $NaNO_2$ (N=9)	1.11 ± 0.11†	1.11 ± 0.11†	1.11 ± 0.11†

*,†denotes difference (P<0.0001)

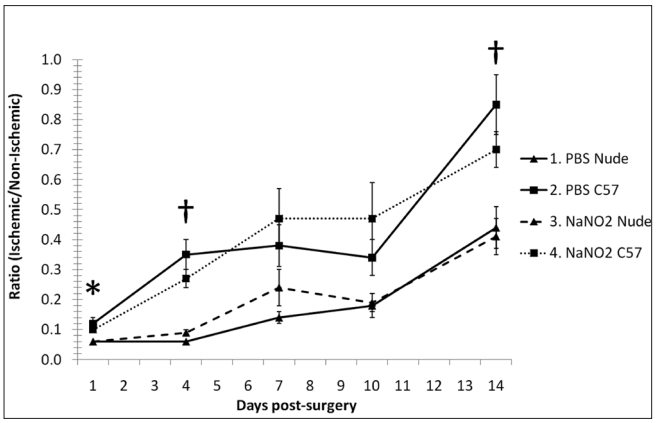

Figure 1: Laser Doppler image perfusion ratios (ischemic/non-ischemic foot) over time. Values represent the mean ± S.E.M. (n = 8 – 9). (*), significantly different PBS Nude versus PBS C57 ($P < 0.01$) and $NaNO_2$ Nude versus $NaNO_2$ C57 ($P < 0.05$); (†); significant difference in change from day one between PBS Nude versus PBS C57 at day four ($P < 0.0001$) and day 14 ($P < 0.05$); and $NaNO_2$ Nude versus $NaNO_2$ C57 at day four ($P < 0.001$) and day 14 ($P < 0.05$)

observed in the clinical scores on day one post-surgery [Table 3]. The lower LDI ratios in the nude mice on day one indicated lower perfusion of the ischemic foot as compared to the wild-type mice, and might indicate an inherently lower number of pre-existing collateral vessels in nude mice. The nude mice continued to exhibit lower LDI ratios at all post-treatment time points as compared to wild-type mice. This strain difference was significant at days four ($P < 0.001$) and 14 ($P < 0.05$) and could indicate a reduced capacity for vasculogenesis in nude mice as compared to wild-type mice. For both mouse strains, treatment with sodium nitrite had no effect on the LDI ratios, when compared to their respective PBS controls.

A representative image of the retrospective analysis performed on images from nude mice captured one day after surgery and prior to initiation of treatment (baseline), to determine the potential impact of using a different ROI on the calculated perfusion value, is depicted in Figure 2. The calculated average perfusion ratio derived from the area just above the ankle, including the calf, was higher (0.37) when compared to the average perfusion ratio derived from the foot (0.05). The average perfusion ratio of 0.37 for this new ROI was very similar to the average perfusion ratio (~0.35) for the equivalent ROI measured in wild-type mice reported by Kumar *et al.*[3] This data suggests that the use of different ROIs (foot vs. calf) could explain the difference in perfusion ratios observed in wild-type mice after surgery and prior to initiation of treatment between the present study and that reported by Kumar *et al.*[3]

A representative image of the retrospective analysis performed on images from nude mice captured three days after initiating treatment with $NaNO_2$ or PBS, to determine whether using the foot as the ROI in the present study

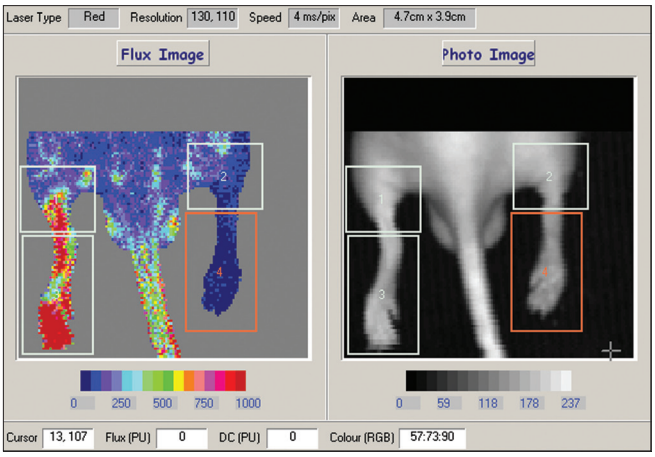

Figure 2: Representative image of the retrospective analysis performed on images from nude mice captured at baseline (one day after surgery and prior to initiating the treatment). The lower ROI boxes were used to calculate the LDI perfusion ratio of the foot and the upper ROI boxes were used to calculate a new LDI perfusion ratio just above the ankle, including the calf. Based on the mean blood flow statistics, the calculated average perfusion ratio for the calf was higher (0.37) as compared to the average perfusion ratio derived from the foot (0.05)

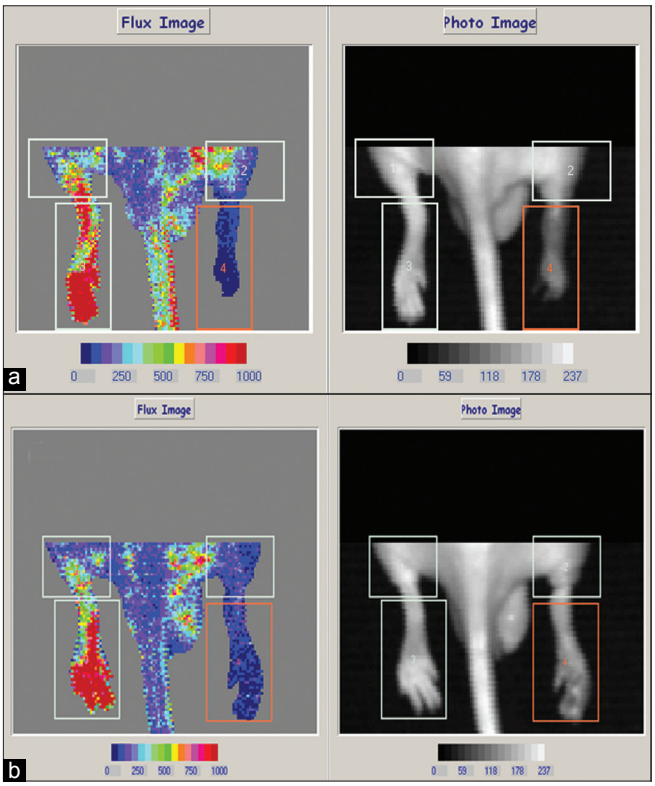

Figure 3: Representative image of the retrospective analysis performed on images from nude mice captured after four days of treatment with PBS (a) or $NaNO_2$ (b). Based on the mean blood flow statistics, the calculated perfusion ratio for the calf was higher (0.69) as compared to the original perfusion ratio derived from the foot (0.05) for PBS. Similarly, the calculated perfusion ratio for the calf was higher (0.54) as compared to the original perfusion ratio derived from the foot (0.08) for $NaNO_2$

prevented the detection of a therapeutic benefit with $NaNO_2$ therapy, is depicted in Figure 3. The calculated perfusion ratios derived from the area just above the

ankle, including the calf, were higher in both $NaNO_2$ and PBS-treated animals as compared to the perfusion ratios derived from the foot. For the PBS-treated animals, the new perfusion ratio was 0.50 (Mean, SE = 0.05, N = 8) as compared to 0.06 (Mean, SE = 0.00, N = 8) for the foot. For the $NaNO_2$-treated animals, the new perfusion ratio was 0.54 (Mean, SE = 0.05, N = 8) as compared to 0.09 (Mean, SE = 0.01, N = 8) for the foot. The salient observation is that the calculated perfusion ratios for both the original (foot) and new (calf area) ROI were similar between $NaNO_2$ and PBS-treated animals, providing further evidence to support the fact that there was no increase in tissue perfusion associated with $NaNO_2$ in the present study.

DISCUSSION

In the present investigation, we used a mouse model of hind limb ischemia to evaluate the potential of $NaNO_2$ therapy to increase tissue perfusion in nude mice. The rationale for doing so was the unprecedented ability of $NaNO_2$ therapy to improve tissue perfusion in wild-type mice using a similar model.[3] Kumar *et al* reported an approximate 75% recovery in blood flow to the ischemic hind limb, as determined by LDI, within as little as three days of transecting the femoral artery and initiating treatment with $NaNO_2$, relative to the PBS controls.[3] Under the conditions of the present study, the administration of $NaNO_2$ using the same treatment regimen failed to increase tissue perfusion of the ischemic hind limb in nude mice as well as wild-type mice. A noteworthy and novel finding in the present study was that nude mice exhibited significantly less tissue perfusion in the ischemic hind limb as compared to wild-type mice, which may suggest a lower capacity in the existence of the pre-existing collateral vessels and/or a decreased ability of nude mice to develop new vessels.

The failure of $NaNO_2$ therapy to increase hind limb tissue perfusion in wild-type mice in the present study, is in stark contrast to that reported by Kumar *et al*[3] and may be the result of differences in methodology between the two studies. The surgical intervention employed was one notable difference. The surgical interventions described to create the mouse model of hind limb ischemia vary considerably in the literature ranging from a single ligation and transection of the femoral artery to multiple ligations and transections including excision of the femoral artery and vein. Goto *et al* evaluated six different surgical interventions in mice including cutting and / or resecting the femoral vein, femoral artery or both the femoral artery and vein.[7] They concluded that all surgical interventions used, yielded similar blood flow values in the lower portion of the affected hind limb through two weeks post-surgery as measured by laser Doppler imaging (LDI). In contrast, Shireman and Quinones evaluated five different surgical interventions in mice; three protocols where surgical intervention was more proximal with one or two transections and two protocols where surgical intervention included multiple transections with or without excision of the femoral artery.[6] The latter two protocols resulted in significantly lower perfusion ratios as measured by LDI through 21 days post-surgery. According to the publication cited by Kumar *et al*[3] describing their surgical procedure, two ligatures were placed around the femoral artery proximal to the origin of the profunda femoris artery and then the femoral artery was transected between the two ligation sites.[8] In contrast, our surgical approach eliminated the possibility of any recanalization or short bridging-collateral formation, which could potentially bypass a single point of transection or occlusion and potentially restore blood flow to the affected limb. It could be argued that the more severe surgical intervention employed in the present study may have been too severe for a beneficial effect of sodium nitrite to be realized. Recent studies using the identical surgical intervention, however, have demonstrated statistically significant improvement in tissue perfusion in both C57BL/6 wild-type[9] and nude[10] mice following other therapeutic interventions. It is also worth noting that these authors used the same laser Doppler blood flow imaging system used in the present study as well as the same region of interest (foot) for determining changes in tissue perfusion.[9, 10] Furthermore, when comparing similar regions of interest Kumar *et al*[3] reported an average baseline (one day after surgery and prior to initiation of treatment) perfusion ratio of ~0.35 for wild-type mice compared to ~0.37 for nude mice in the present study. This suggested that blood flow in the lower portions of the affected hind limbs may have been more similar than dissimilar in the present study and Kumar *et al*[3].

Other notable differences in methodology between the present study and that of Kumar *et al*[3] were the imaging technology and the ROI used to assess tissue perfusion. Kumar *et al*[3] used the Vasamedics Laserflo BPM2 deep tissue penetrating laser Doppler device placing the tip of the laser beam over the medial calf muscle.[8] In contrast, we used the moorLDI2 laser Doppler blood flow imaging system. With the moorLDI2 system, the laser beam was scanned across in a raster fashion using a moving mirror enabling the user to scan the entire hind limb. The Vasamedics system, therefore, provides a focal, isolated measurement of perfusion; whereas, the moorLDI2 system provides a more global measurement of perfusion, providing a better picture of the total hind limb perfusion. To determine the potential impact of using a different ROI in the present study, images captured from nude mice at baseline (after surgery and prior to initiating the treatment)

and after three days of treatment were re-analyzed. A new ROI, just above the ankle, including the calf, was selected in order to capture the ROI Kumar *et al*[3] had analyzed. The calculated perfusion ratios for both the original (foot) and new (calf area) ROI were similar between the $NaNO_2$ and PBS-treated animals, providing further evidence to support our conclusion of no perceived therapeutic benefit associated with $NaNO_2$ therapy under the conditions of the present investigation.

Mouse strain differences in innate hind limb collateral vasculature are another variable that may have contributed to the failure of $NaNO_2$ therapy to increase hind limb perfusion in wild-type mice in the present study. Kumar *et al*[3] used C57BL / 6J mice (vendor information not provided), whereas, we used C57BL / 6NHsd mice from Harlan. Helisch *et al*[11] not only demonstrated significant differences in innate hind limb vasculature between different mouse strains, but even more surprising was that the differences observed were similar between the same strain of mouse, received from two different vendors. It was specifically noted that the C57BL / 6J mice received from Harlan exhibited a faster recovery in hind limb blood flow following surgery, as measured by LDI, compared to the C57BL / 6H mice received from Charles River.[11] Similarly, there has been considerable variability observed in the degree of necrosis following surgical intervention among different shipments of mice from the same vendor, which was attributed to the differences in innate hind limb collateral vasculature.[5]

A less obvious, but perhaps a very important difference between the present study and that of Kumar *et al*[3] is the source of sodium nitrite used for dosing. In the present study, five of the eight mice in group three and five of the nine mice in group four received sodium nitrite from Sigma-Aldrich only on the first day of dosing. According to the certificate of analysis the purity of the sodium nitrite was > 99.99% based on the trace metal basis. For the remaining animals in these two groups on the first day of dosing and for all the animals on all subsequent dosing days Sodium Nitrite Injection USP from Hope Pharmaceuticals was used. In contrast, Kumar *et al*[3] performed all dosing with sodium nitrite sourced from Sigma and did not include any product code information. Given that Sigma-Aldrich sells multiple forms of sodium nitrite with a range of purities it is not clear as to the purity of the sodium nitrite used by Kumar *et al*.[3] This does raise the question of whether the increase in tissue perfusion Kumar *et al*[3] observed was due to the sodium nitrite per se or to an impurity.

A novel finding in the present study was that nude mice exhibited, in general, significantly lower LDI ratios as compared to their wild-type counterparts. The lower LDI ratios in the nude animals correlated with the higher clinical scores observed in these animals. The decreased ability of nude mice to restore perfusion in the ischemic limb may be due to their lack of T lymphocytes, in particular CD4+ cells. One week after ischemia, $CD4^{-}$ mice demonstrated a reduced capacity to form collateral vessels, had lower macrophage numbers, and expressed lower vascular endothelial growth factor (VEGF) levels in the muscles from the ischemic hind limb compared to the wild-type mice.[12] They also reported a delay in the recovery of hind limb function in the $CD4^{-}$ mice relative to the wild-type mice. This is consistent with the higher clinical scores observed in nude mice in the present study compared to the wild-type mice. Furthermore, spleen-derived, purified CD4+ cells administered intravenously to $CD4^{-}$ mice, selectively localized to the ischemic limb, significantly increased perfusion as well as macrophage numbers and VEGF levels in the ischemic muscle.[12] These results indicate that CD4+ cells are important in orchestrating the arteriogenic response to acute hind limb ischemia. In contrast to the current study, Shireman and Quinones did not find any significant differences in the mean LDI ratios between nude mice (BALB / c background) and BALB / c heterozygous littermates through 28 days, post-hind limb ischemia.[6] To determine whether a different mouse background could change the susceptibility of nude animals to ischemia, C57BL / 6J nude mice were also evaluated and no significant differences in LDI ratios or extent of tissue necrosis was observed when compared with the heterozygous littermates.[6]

In conclusion, under the conditions of this study, sodium nitrite administered to nude or wild-type mice, twice daily, for seven consecutive days, by intraperitoneal injection, beginning approximately 24 hours after surgically-induced unilateral hind limb ischemia, failed to restore tissue perfusion of the ischemic hind limb as compared to treatment with PBS. This finding is in stark contrast to that reported by Kumar *et al*[3] and suggests that the outcome of efficacy studies using the mouse hind limb ischemia model may be too dependent on subtle differences in methodology employed between laboratories. This casts suspicion on the models' value to predict what may be observed clinically. It is important, therefore, for researchers to be aware of how slight methodological differences may affect the outcome of their efficacy studies and to incorporate this thinking into their risk-benefit decisions of moving a particular therapeutic into formal preclinical development.

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