Research in Veterinary Science 150 (2022) 79–82

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

# Nitric oxide synthase inhibition reveals differences in the nitric oxide pathway in previously laminitic ponies

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ARTICLE INFO

*Keywords:*
Blood pressure
Equine
Endothelial cells
Laminitis
Nitric oxide

ABSTRACT

Previously laminitic (PL) ponies are reported to have higher blood pressure than non laminitic (NL) ponies. This relative hypertension may be related to endothelial cell dysfunction, similar to humans with metabolic syndrome. To investigate the relationship between laminitis predisposition and endothelial dysfunction, the effect of nitric oxide synthase (NOS) inhibition on the change in circulating nitric oxide (NO) concentrations and systemic blood pressure (BP) was determined. An intravenous NO sensor was used to measure changes in blood NO concentration during and after intravenous infusion of the NOS inhibitor L-nitroarginine methyl ester (L-NAME; 4 mg/kg IV) in PL and NL ponies. NO concentrations decreased and BP increased in response to L-NAME infusion, with a significantly ($p = 0.02$) greater rate of increase in systolic BP in PL (14.62 ± 1.88 mmHg/h) compared to NL (8.54 ± 1.88 mmHg/h) ponies. This greater effect on BP in PL compared to NL ponies is consistent with higher basal NO production in PL ponies, suggesting that the relative hypertension previously seen in PL ponies results in increased basal NO production, rather than being caused by reduced NO production as hypothesised. Up-regulation of the NO system may be a compensatory mechanism stimulated by the higher resting BP in PL ponies. Further investigation of the mechanism underlying the relative hypertension seen in PL ponies is required.

The endothelium is a monolayer of cells lining every blood vessel, which acts as a barrier between the blood and other tissues. It regulates coagulation, vasomotor function and inflammation, and plays a major role in cardiovascular homeostasis (Esper et al., 2006). In health the vasculature is maintained in a quiescent, vasodilated state due to the predominance of nitric oxide (NO) in the balance between vasodilators and vasoconstrictors (Deanfield et al., 2007). NO causes vasodilation by activating the enzyme soluble guanylate cyclase in smooth muscle cells, in turn causing cyclic guanosine monophosphate (cGMP) mediated smooth muscle relaxation (Loscalzo and Welch, 1995). NO is generated from L-arginine by the action of NO synthase (NOS) throughout the body (Govers and Rabelink, 2001).

Endothelial dysfunction is a pathological state characterised by a switch from the quiescent NO dominated environment to one occupied by reactive oxygen species (Deanfield et al., 2007). This leads to a reduction in production of substances with vasodilatory, antimitogenic and antithrombogenic actions, such as NO, prostacyclin and endothelium derived hyperpolarising factor, and an increase in production of substances with vasoconstricting, proliferative and thrombogenic actions, such as endothelin-1 (ET-1) and thromboxane (Flammer et al., 2012).

The measurement of circulating NO, or the change in NO concentration in response to stimuli or inhibitors is indicative of endothelial function (Flammer and Lüschera, 2010). NO is unstable in biological fluids with an estimated half-life in blood of 1.8 ms (Liu et al., 1998), hence the biologically active chemical cannot be measured directly in blood samples. One alternative is a NO selective electrochemical sensor (Berkels et al., 2001) used as a catheter-type NO sensor to measure real time intravascular NO in vivo (Takarada et al., 2010).

Laminitis manifests as a painful condition of the equine hoof, secondary to certain systemic diseases, most commonly the endocrinopathies (equine metabolic syndrome (EMS) and pituitary pars intermedia dysfunction), or excessive weight bearing (Patterson-Kane et al., 2018). Endothelial dysfunction may play a role in laminitis

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https://doi.org/10.1016/j.rvsc.2022.05.012
Received 15 June 2021; Received in revised form 7 April 2022; Accepted 23 May 2022
Available online 1 July 2022

pathophysiology. Supporting evidence includes the identification of hypertension in previously laminitic ponies (Bailey et al., 2008), and myocardial hypertrophy in ponies with EMS (Heliczer et al., 2017). In addition, endothelial dysfunction ex vivo in laminar and facial vessels from chronic laminitic animals, indicates the presence of systemic and not just local dysfunction (Morgan et al., 2016). Underlying endothelial dysfunction may place the animal closer to the threshold of clinical disease, requiring a lesser insult, in the form of ingested pasture, to induce endocrinopathic laminitis (Katz and Bailey, 2012).

This pilot study aimed to determine if endothelial function differed between ponies which had not experienced episodes of laminitis (NL) and those which had had at least 2 previous episodes of laminitis (PL). NOS was inhibited by the systemic administration of the non-selective NOS inhibitor L-NAME and the effect on circulating NO and blood pressure monitored to identify potential differences in NO production in NL and PL ponies. This information indicates whether further studies of this pathway in the pathophysiology of laminitis predisposition are warranted.

The studies were approved by the Royal Veterinary College Ethics and Welfare Committee and conducted under Home Office Licence PPL number 70/6981.

The study was performed in 10 native breed pony mares (5 NL and 5 PL; see supplementary material). All PL ponies had experienced at least 2 episodes of naturally occurring endocrinopathic laminitis but no episodes within 12 months of the study commencing. NL ponies had never been diagnosed with laminitis in their lifetime within the research herd (at least 10 years). Episodes of laminitis were diagnosed based on clinical examination by an experienced equine veterinarian and confirmed with hoof radiography when required. All ponies were habituated to the techniques prior to the study and were also allowed to become accustomed to the examination room in which procedures were performed prior to each study session. Ponies were restrained in stocks, fed grass hay during data acquisition to decrease their movement and were encouraged to stand still, bearing weight evenly on all four limbs. The ambient room temperature was recorded.

The study was performed in autumn over 3 weeks. Both the jugular and cephalic veins were catheterised under local anaesthesia (mepivacaine hydrochloride; 20 mg per site). Heart rate (via a base-apex electrocardiogram; Cardiocap/5; Datex Ohmeda), respiratory rate, rectal temperature, systemic arterial blood pressure (non-invasive oscillometric method applied to the middle coccygeal artery; Cardiocap/5; Datex Ohmeda), capillary refill time and mucous membrane colour were recorded before and every 5 min during the study. Real-time NO concentrations were measured using an intravenous NO sensor (inNO-T Nitric Oxide Measuring System with amiNO-700 nitric oxide sensor; Innovative Instruments Inc. (Takarada et al., 2010)). The NO sensor was calibrated before each use by constructing a standard curve generated by adding varying concentrations of sodium nitrite to a reducing solution (sodium iodide in sulphuric acid) according to the manufacturer's instructions. The NO sensor was placed in the cephalic vein via the intravenous catheter and NO concentration was measured continuously throughout the study. The cephalic vein was chosen since it was being used for a separate simultaneous study. NOS was inhibited by intravenous infusion of L-NAME (4 mg/kg sterile filtered into 100 ml saline over 1 h (Sander et al., 1999); Sigma-Aldrich) via the jugular catheter. All measurements were performed throughout and for 1 h after the infusion. The sensor was cleaned using sterile saline immediately after use and disinfected between uses (Reprodis High Level Instrument Disinfectant).

Statistical analysis was performed using R version 4.0.3 (The R Project for Statistical Computing; https://www.r-project.org/). Normality and homogeneity of the data distribution was assessed by Shapiro-Wilk and Levene tests. The effect of predisposition to laminitis on baseline characteristics (heart rate, systolic, mean and diastolic blood pressure) was assessed using unpaired Student's *t*-tests. The rates of change of heart rate, blood pressure (systolic, diastolic and mean) and percentage change in NO concentration over the 2-h period during and after the L-NAME infusion were investigated using linear mixed effect models and ANOVA. The mean rates of change from baseline were calculated from the models and the effects of predisposition to laminitis and NOS inhibition on heart rate, blood pressure and NO concentration were assessed with laminitis as the explanatory variable and individual pony and time as the random effects. The data were displayed graphically by plotting best fit lines on scatter plots using the values for slope (rate of change) and intercept from the linear mixed effects models.

Statistical significance was accepted at $P \leq 0.05$.

There were no significant differences in baseline characteristics (age, weight, body condition score, heart rate, blood pressure) or room temperature between NL and PL ponies (Supplementary data). NOS inhibition significantly decreased NO concentration ($P = 0.04$), increased blood pressure ($P < 0.0005$) and decreased heart rate ($P = 0.0008$) over time (Table 1). The rate of change of systolic blood pressure was 1.7 times ($P = 0.02$) greater in PL than NL ponies (Table 1). There was no significant difference in the rate of change of NO concentration between the groups (Table 1).

Fig. 1 shows the change in systolic blood pressure during and after NOS inhibition with the time series data from each pony plotted. The fitted lines correspond to the mean rates of change in Table 1.

No adverse side effects were noted during the study; the increase in blood pressure and decrease in heart rate were not to clinically detrimental values.

A NO sensor placed within the cephalic vein was used to directly measure the circulating NO concentration in real time, as previously reported in humans (Takarada et al., 2010). The NO concentration reduced over time during and after infusion of a NOS inhibitor (L-NAME). Blood pressure increased, as expected with reduced production of the main endothelial vasodilator (Rees et al., 1989). Heart rate decreased concurrently, presumably in response to the increased blood pressure due to the baroreceptor control mechanism (Robinson et al., 1966), thus confirming that this was a suitable means of measuring NO production.

Following NOS inhibition, overall, there was a greater rate of change of systolic blood pressure in PL ponies than NL ponies, suggesting a differential response to NOS inhibition in PL compared to NL ponies. The greater increase in blood pressure after NOS inhibition in PL ponies is the opposite to that predicted by the previous hypothesis, which suggested that NO production may be decreased in PL due to endothelial dysfunction (Katz and Bailey, 2012). One possible explanation is that basal NO production is in fact greater in PL ponies, since inhibition of its production had a larger effect on blood pressure over time. However, there was no significant difference in the rate of change of NO concentration between the groups ($p = 0.3$). This may be due to the small sample size in this pilot study. A sample size calculation performed using this pilot data indicated that 73 ponies would be required in each group to be sure of detecting a difference, should one exist. The lack of significant difference may be due to any increased basal production of NO acting locally rather than being released into the circulation (Vaughn et al., 1998). Alternatively, PL ponies may have a greater blood pressure response to NO due to increased sensitivity of their vasculature to NO. This, however, would be in contrast to studies on isolated blood vessels from experimentally induced laminitis, where receptor-mediated endothelial dependent relaxation is reduced (Schneider et al., 1999). It is possible that the hypertension seen previously in PL ponies is not due to decreased NO production but rather due to increased production of vasoconstrictors such as ET-1. The increased basal production of NO may be physiological compensation for the increase in vasoconstrictor tone.

The baseline absolute NO concentration could not be compared between individuals due to problems with calibrating the NO sensor in the standard solution. The sensor was reused for multiple experiments and despite careful cleaning, became less sensitive to the NO produced in the standard solution. Whilst calibration could still be performed, the

**Table 1**
Nitric oxide synthase (NOS) was inhibited with the administration of L-NAME (4 mg/kg IV over 1 h). NO concentration, heart rate (HR) and arterial blood pressure (mean [MABP], systolic [SABP] and diastolic [DABP]) responses to NOS inhibition in non laminitic (NL; $n = 5$) and previously laminitic (PL; $n = 5$) ponies following the infusion of the nitric oxide synthase inhibitor L-NAME (4 mg/kg IV) over 1 h (linear mixed effect model; ANOVA).

| Variable | Rate of change (all ponies; mean ± SEM) | Significance of NOS inhibition | Rate of change (individual groups; mean ± SEM) | | Significance of laminitis predisposition | |
|---|---|---|---|---|---|---|
| | | | NL | | PL | |
| NO (%/h) | −33.54 ± 13.93 | $P = 0.039$ | −18.7 ± 19.5 | | −48.3 ± 19.5 | $P = 0.3$ |
| HR (bpm/h) | −2.40 ± 0.50 | $P = 0.0008$ | −2.14 ± 0.74 | | −2.65 ± 0.74 | $P = 0.6$ |
| MABP (mmHg/h) | 8.88 ± 1.73 | $P = 0.0005$ | 6.06 ± 2.21 | | 11.68 ± 2.19 | $P = 0.1$ |
| SABP (mmHg/h) | 11.57 ± 1.34 | $P = 8 \times 10^{-6}$ | 8.54 ± 1.88 | | 14.62 ± 1.88 | $P = 0.02$ |
| DABP (mmHg/h) | 7.4 ± 0.97 | $P = 5 \times 10^{-13}$ | 5.75 ± 1.37 | | 8.99 ± 1.35 | $P = 0.09$ |

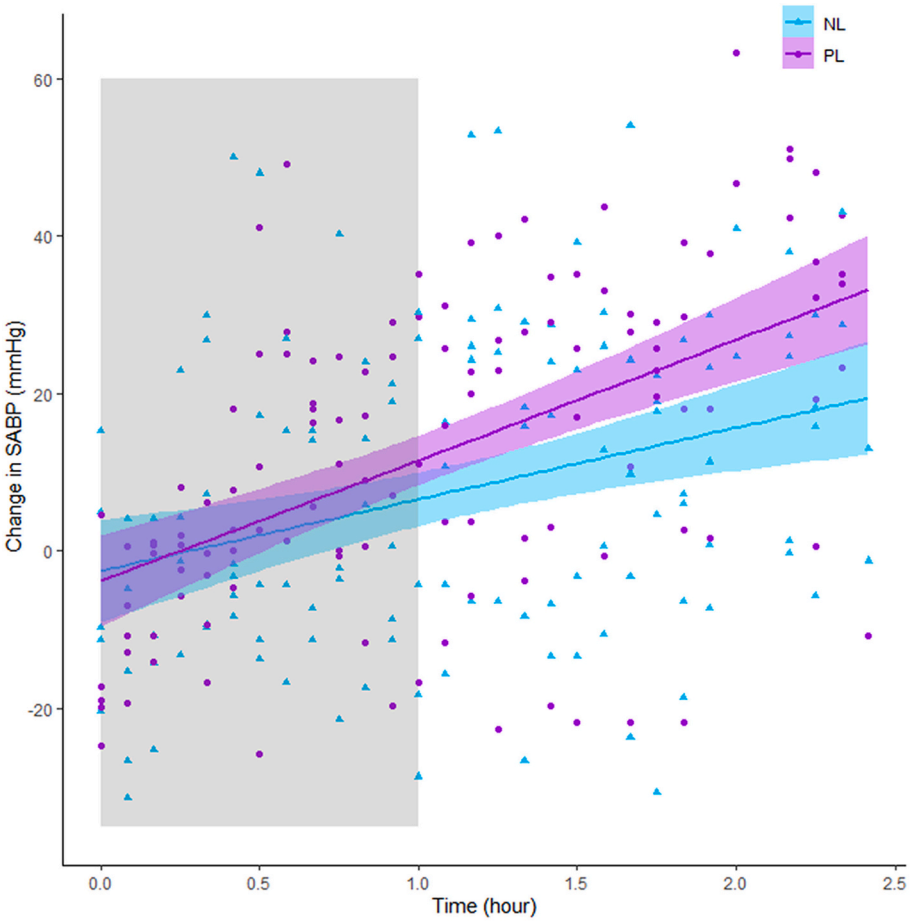


**Fig. 1.** Change in systolic arterial blood pressure (SABP) during (0−1*h*) and after (1–2.5 h) administration of the nitric oxide synthase inhibitor L-NAME (4 mg/kg IV over 1 h (shaded area)) in non laminitic (NL; $n = 5$) and previously laminitic (PL; n = 5) ponies. Each data point represents the value for an individual pony at that point in time. The mean rates of change of SABP for 5 NL ponies and 5 PL ponies are plotted as fitted lines with 95% confidence interval indicated ($p = 0.023$; linear mixed effect model; ANOVA).

baseline intravascular NO concentration between individual ponies was highly variable and calculations resulted in negative values in some instances so could not be considered accurate. Hence all values for NO concentration were calculated relative to baseline for that individual animal. These difficulties with the sensor may also have reduced the ability to detect a significant difference in change in NO concentration between the groups.

Further studies are required to investigate the theory of upregulated NO production in PL ponies, such as repeating this study following treatment with an ET receptor antagonist to assess the role of ET-1 vasoconstrictor activity on NO production.

## Declaration of Competing Interest

PA Harris is both a collaborating author and an employee of Mars Petcare UK which part funded this work.

## Acknowledgements

This work was performed as part of a PhD studentship joint funded by Mars Petcare UK and the Royal Veterinary College Melon Trust, grant ID 2785. We thank Dr. Ruby Chang for her assistance with statistical analysis.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2022.05.012.

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