

Evaluation of Efficacy and Safety of  
Pulsed Inhaled Nitric Oxide in the  
Anesthetized Horse:  
Preparing for Clinical Use

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## Evaluation of Efficacy and Safety of Pulsed Inhaled Nitric Oxide in the Anesthetized Horse: Preparing for Clinical Use

### Abstract

Anesthetized, recumbent horses commonly develop hypoxemia secondary to ventilation/perfusion ( $V_A/Q$ ) mismatch and shunting of pulmonary blood ( $Q_s/Q_t$ ) through atelectatic lung regions. Hypoxemia in the anesthetized horse is difficult to treat and ventilatory therapy is often ineffective. Nitric oxide (NO) can be inhaled (iNO) to provide selective dilatation of pulmonary blood vessels with no systemic effects. However, conventional continuously delivered iNO is ineffective in the anesthetized horse. Conversely, pulse-delivered iNO (PiNO) may be effective and the lower PiNO dose could reduce the risk of NO accumulation in the anesthetic circuit, alleviating adverse effects from NO accumulation. However, a rapid and profound decrease in oxygen ('rebound effect'), potentially mediated by endothelin-1 (ET-1), can occur with abrupt cessation of iNO at the end of anesthesia and this would limit the usefulness of PiNO in the horse since recovery is a critical period. PiNO decreases  $V_A/Q$  mismatch but the mechanism is unknown. The aims were to determine the 1) pulse dose, efficacy and safety of PiNO during prolonged inhalation anesthesia and during recovery from anesthesia, and 2) mechanism of improved pulmonary function.

PiNO increased oxygen and decreased  $Q_s/Q_t$  in both dorsally (Paper I) and laterally (Paper II) recumbent horses for an approximate surgical duration (2.5 hours) when PiNO was delivered at the most effective pulse duration (first 30-45% of the first part of inspiration; Paper I). The efficacy continued into recovery following PiNO cessation and  $PaO_2$  was higher and  $Q_s/Q_t$  lower in PiNO horses than in control horses for the entire recovery period. No rebound effect occurred in horses still anesthetized or those recovering from anesthesia. ET-1 concentrations were not increased by PiNO. No residual NO was present in the breathing circuit. The mechanism of PiNO as determined by multiple inert gas elimination technique (MIGET) was improved matching of  $V_A$  and  $Q$ . With scintigraphy, the improvement was determined to be due to a movement of blood against gravity from the dependent, atelectatic regions of the lung to the non-dependent aerated portions of the lung.

In conclusion, we have shown that PiNO is effective and safe in anesthetized horses and recovering horses. PiNO redistributes blood flow from dependent to non-dependent regions of the lung, thereby decreasing  $Q_s/Q_t$  and improving  $V_A/Q$  matching. We are confident that PiNO is ready for clinical use in anesthetized horses.

*Keywords:* nitric oxide, horse, anesthesia, oxygenation, ventilation/perfusion

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To Bill whose generosity of love, time, support and patience is more than I ever imagined that I would have in my life - and more than I deserve; to my family members in Texas who always think I am successful, even when I am not; and finally to Görel and Anneli and Peter and Gunilla for friendship.

*Whether you think you can, or you think you can't, you are probably right.*

Henry Ford

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Nyman G, **Grubb TL**, Heinonen E, Friendin J, Edner A, Malavasi LM, Frostell C, Högman M. (2012) Pulsed delivery of inhaled nitric oxide counteracts hypoxaemia during 2.5 hours of inhalation anaesthesia in dorsally recumbent horses. *Vet Anaesth Analg* 39(5).480-487.
- II **Grubb TL**, Friendin JHM, Edner E, Funkquist P, Hedenstierna G, Nyman G. (2012) The effects of pulse-delivered inhaled nitric oxide on arterial oxygenation, ventilation-perfusion distribution and plasma endothelin-1 concentration in laterally recumbent isoflurane-anaesthetized horses. *Vet Anaesth Analg* (Accepted for publication 10-2012).
- III **Grubb TL**, Högman M, Edner A, Friendin JH, Heinonen E, Malavasi LM, Frostell CG, Ryden A, Alving K, Nyman GC. (2008) Physiologic responses and plasma endothelin-1 concentrations associated with abrupt cessation of nitric oxide inhalation in isoflurane-anesthetized horses. *Am J Vet Res* 69(3),423-430.
- IV **Grubb TL**, Edner A, Friendin JH, Funkquist P, Rydén A, Nyman G. (2012) Oxygenation and plasma endothelin-1 concentrations in healthy horses recovering from isoflurane anaesthesia administered with or without pulse-delivered inhaled nitric oxide. *Vet Anaesth Analg* [Epub ahead of print July 16, 2012]

V **Grubb TL**, Lord P, Berger M, Larsson C, Ryden A, Nyman G.  
Redistribution of pulmonary perfusion in anesthetized horses receiving  
pulse-delivered inhaled nitric oxide. (manuscript).

Papers I-IV are reproduced with the permission of the publishers.

The contribution of TG to the papers included in this thesis was as follows:

- I Data collection, data analysis, manuscript preparation
- II Data collection, data analysis, manuscript preparation
- III Research planning, data collection, data analysis, manuscript preparation
- IV Research planning, data collection, data analysis, manuscript preparation
- V Study design, research planning, data collection, data analysis, manuscript preparation

## Abbreviations

ABP	Arterial blood pressure (SAP,MAP,DAP)
C	Control
CaO <sub>2</sub>	Oxygen content in arterial blood
Cc'O <sub>2</sub>	Oxygen content in pulmonary capillaries
CiNO	Continuously delivered inhaled nitric oxide
CvO <sub>2</sub>	Oxygen content in mixed venous (pulmonary arterial) blood
DAP	Diastolic arterial pressure
DO <sub>2</sub>	Oxygen delivery
DPAP	Diastolic pulmonary artery pressure
ET-1	Endothelin-1
ETiso	End-tidal concentration of isoflurane
FiO <sub>2</sub>	Fraction inspired oxygen
Hb	Hemoglobin
HPV	Hypoxic pulmonary vasoconstriction
HR	Heart rate
iNO	Inhaled nitric oxide
IPPV	Intermittent positive pressure ventilation
MAP	Mean arterial pressure
MIGET	Multiple inert gas elimination technique
MPAP	Mean pulmonary arterial pressure
NO	Nitric oxide
NO <sub>2</sub>	Nitric dioxide
P(A-a)O <sub>2</sub>	Partial pressure difference of alveolar-arterial O <sub>2</sub>
PaCO <sub>2</sub>	Partial pressure of CO <sub>2</sub> in arterial blood
PAH	Pulmonary arterial hypertension
PAO <sub>2</sub>	Partial pressure of oxygen in the alveolar gas
PaO <sub>2</sub>	Partial pressure of oxygen in arterial blood

PEEP	Positive end-expiratory pressure
pHa	pH of arterial blood
pH $\bar{v}$	pH of mixed venous blood
PiO <sub>2</sub>	Partial pressure of inhaled oxygen
PiNO	Pulse-delivered inhaled nitric oxide
P $\bar{v}$ CO <sub>2</sub>	Partial pressure of CO <sub>2</sub> in mixed venous blood
P $\bar{v}$ O <sub>2</sub>	Partial pressure of oxygen in mixed venous blood
Qs/Qt	Ratio of shunted blood (Qs) to total blood flow
Qt	Cardiac output
RSS	Residual sum of squares
SaO <sub>2</sub>	Oxygen/hemoglobin saturation of arterial blood
SAP	Systolic arterial pressure
SDQ	Unit of dispersion of perfusion
SDV	Unit of dispersion of ventilation
SPAP	Systolic pulmonary arterial pressure
S $\bar{v}$ O <sub>2</sub>	Oxygen/hemoglobin saturation of mixed venous blood
TIVA	Total intravenous anesthesia
V <sub>A</sub> /Q	Ventilation/perfusion ratio
VD/VT	Dead space ventilation
VT	Tidal volume



# 1 Introduction

## 1.1 Hypoxemia in the anesthetized horse

For a myriad of reasons, horses, like other animals, often require surgery under general anesthesia with positioning in either dorsal or lateral recumbency. Unfortunately, general anesthesia and recumbent positioning can create physiologic changes that have a much greater adverse impact in the equine patient than in smaller mammalian patients. General anaesthesia in the horse routinely impairs pulmonary gas exchange with a significant decrease in the partial pressure of arterial oxygen ( $\text{PaO}_2$ ), with values commonly below 10 kPa (75 mm Hg) and even below 8 kPa (60 mmHg), in spite of the fact that most patients are breathing  $>0.95$  fraction of inspired oxygen ( $\text{FiO}_2$ ) (Hall, Gillespie and Tyler 1968, Gillespie, Tyler and Hall 1969, Mitchell and Littlejohn 1974, Nyman and Hedenstierna 1989, Nyman et al. 1990, Keegan et al. 1991, Day et al. 1995). In these patients, oxygen-hemoglobin saturation ( $\text{SaO}_2$ ) also decreases and the pulmonary shunt ( $\text{Qs}/\text{Qt}$ ) and partial pressure of alveolar-arterial oxygen difference  $\text{P(A-a)O}_2$  increase, indicating significant pulmonary dysfunction. Positioning in dorsal (or supine) recumbency, which is a common position for horses under anesthesia, can profoundly exacerbate the hypoxemia when compared to hypoxemia occurring in lateral recumbency (Swanson and Muir 1986, Stegmann and Littlejohn 1987, Nyman, Funkquist and Kvarn 1988, Nyman and Hedenstierna 1989, Steffey et al. 1990, Gasthuys, de Moor and Parmentier 1991, Day et al. 1995, Whitehair and Willits 1999).

Because oxygen is a vital substrate for normal tissue function, low  $\text{PaO}_2$ , or hypoxemia, can cause numerous problems depending on which cells, tissue or organs are affected by the decrease in oxygen delivery. In horses, hypoxemia associated with anesthesia has been directly implicated in postanesthetic cerebral necrosis (McKay et al. 2002), sudden cardiac arrest (McGoldrick, Bowen and Clarke 1998), hepatic insult (Whitehair et al. 1996), decreased skeletal muscle oxygenation (Steffey, Willits and Woliner 1992, Whitehair et

al. 1996, Portier et al. 2009), increased serum lactate (Portier et al. 2009) and lactic acidemia (Taylor 1999).

Horses have a fairly high rate of anesthesia-related morbidity and mortality (Young and Taylor 1993, Mee, Cripps and Jones 1998, Johnston et al. 2002, Proudman et al. 2006, Bidwell, Bramlage and Rood 2007, Senior et al. 2007, Wagner 2008a) especially when compared to anesthesia-related morbidity and mortality reported in other species. Recently reported rates of anesthesia-related mortality are 1.9% in the horse (with an increase in mortality in unhealthy horses) (Johnston et al. 2002), 0.17% in dogs (Brodbelt et al. 2008), 0.24% in cats (Brodbelt et al. 2008) and 0.001% in humans (Bainbridge et al. 2012). Hypoxemia could potentially contribute to morbidity and mortality, especially in unhealthy horses, which often have a greater oxygen deficit than healthy horses (Edner, Nyman and Essen-Gustavsson 2007, McCoy et al. 2011).

Of the four phases of anesthesia (premedication, induction, maintenance and recovery), the anesthetic recovery period is generally the most dangerous phase of anesthesia for the horse (Richey et al. 1990, Young and Taylor 1993, Johnston et al. 2002, Franci, Leece and Brearley 2006, Senior et al. 2007, Bidwell et al. 2007, Wagner 2008a) and Day et al. (Day et al. 1995) stated that the presence of hypoxemia, as defined as  $\text{PaO}_2 < 60$  mmHg, 'indicates higher morbidity and higher incidence of postanesthetic complications, including arrhythmias, pulmonary insufficiency, pneumonia, and postanesthetic myositis'. Poor muscle perfusion caused by hypotension is directly implicated in anesthesia-related myopathies and neuropathies (Grandy et al. 1987, Richey et al. 1990, Young and Taylor 1993, Duke et al. 2006), however, the damage is probably due to decreased oxygen delivery and ischemia rather than to the mere presence of hypotension. Finally, horses with high  $\text{PaO}_2$  at the end of anesthesia had recoveries that were faster and of equal quality compared to horses with low  $\text{PaO}_2$  (Hopster et al. 2011). Faster recoveries that are still good quality, could be beneficial since time in recumbency can increase the likelihood of some post-anesthetic complications (eg, myopathy, neuropathy).

## 1.2 Causes of hypoxemia

To correct the problem we must first understand the cause of the problem. Hypoxemia has five causes: 1) decreased  $\text{FiO}_2$ , 2) impaired oxygen diffusion across the alveolar-arterial membrane, 3) hypoventilation, 4) alveolar ventilation/perfusion ( $V_A/Q$ ) mismatch, and 5) shunting of deoxygenated blood directly from the venous to the arterial circulation ( $Q_s/Q_t$ ). Decreased  $\text{FiO}_2$

could be a concern in anesthetized patients if the inspired oxygen was mixed with air, nitrous oxide, or some other gas that dilutes the concentration of inspired oxygen. However, nitrous oxide is rarely, if ever, used in horses and, although air mixtures are used, hypoxemia commonly occurs in horses breathing a wide range of  $F_iO_2$ , with  $F_iO_2$  of 0.95-1 the most commonly administered. However, high  $F_iO_2$  can contribute to hypoxemia by causing absorption atelectasis but, in the horse, this is a slow developing source of hypoxemia (Nyman et al. 1988) which can generally be alleviated with initiation of positive pressure ventilation early in the anesthetic period (Day et al. 1995). Thus, decreased or increased  $F_iO_2$  can be ruled out as the major cause of hypoxemia in the anesthetized horse. Diffusion impairment can also be ruled out as a common cause of hypoxemia in healthy, anesthetized horses. Hemoglobin is completely saturated with oxygen in the first 1/3 of the red blood cell transit time through the pulmonary capillary. Thus, saturation in patients without pulmonary disease is limited by perfusion rather than by diffusion. There is ample time for oxygenation, even if mild diffusion impairment exists. Moderate to severe interstitial lung disease, such as profound pulmonary edema, would be required to impair diffusion to the extent that oxygenation would be impaired. Horses with that degree of respiratory disease are unlikely to undergo anesthesia for clinical procedures and horses in anesthesia research studies reporting hypoxemia were healthy and free from pulmonary disease or dysfunction. Thus, diffusion impairment is unlikely to be a routine cause of hypoxemia in the anesthetized horse.

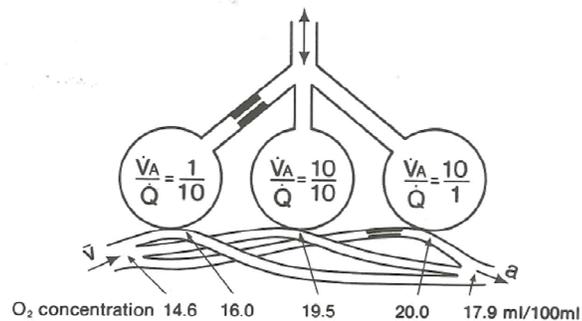
The three remaining causes of hypoxemia (hypoventilation,  $V_A/Q$  mismatch and  $Q_s/Q_t$ ) all contribute to hypoxemia in anesthetized horses. Hypoventilation, which is common in anesthetized horses, will cause an increase in the partial pressure of alveolar carbon dioxide ( $PACO_2$ ) and this contribution to hypoxemia is evidenced by the alveolar gas equation:

$$PAO_2 = PIO_2(PB-47) - (PACO_2)/0.8$$

In this equation,  $PAO_2$  is the partial pressure of alveolar oxygen,  $PIO_2$  is the partial pressure of inspired oxygen,  $PACO_2$  is the partial pressure of alveolar carbon dioxide (which is generally approximated by the partial pressure of arterial carbon dioxide [ $PaCO_2$ ]), and 0.8 is the respiratory exchange ratio that relates  $CO_2$  production to oxygen consumption (ie, normally 0.8 mole of  $CO_2$  is produced for every 1 mole of  $O_2$  consumed). Because arterial oxygen depends on a pressure gradient from alveolar oxygen, decreased  $PAO_2$  directly leads to decreased  $PaO_2$ . Fortunately, hypoventilation is easily diagnosed using arterial blood gas analysis and easily remedied using

mechanical ventilation (most commonly intermittent positive pressure ventilation; IPPV) to improve alveolar ventilation and eliminate excess  $\text{PaCO}_2$ . Furthermore,  $\text{PaO}_2$  in anesthetized horses can be as low as 8 kPa (60 mmHg), which, according to the alveolar gas equation, would require an extremely high  $\text{PaCO}_2$  if hypoventilation were the sole cause of hypoxemia in a horse. Thus, hypoventilation can be eliminated as the primary cause of hypoxemia in patients that are adequately ventilated. In addition, hypoxemia often persists even after hypercarbia has been remedied, emphasizing that other factors are causing the hypoxemia.

The other two conditions,  $V_A/Q$  mismatch and  $Q_s/Q_t$ , represent a bigger problem for the horse as they are neither easily diagnosed nor easily treated.  $V_A/Q$  mismatch occurs in lung areas that are well-ventilated but poorly perfused or well-perfused but poorly ventilated, with a continuous spectrum of  $V_A/Q$  matching from low  $V_A/Q$  (minimal ventilation) to high  $V_A/Q$  (minimal perfusion) (*Figure 1*). Blood perfusing poorly ventilated alveoli (on left in *Figure 1*) is returned to the systemic circulation without having been fully oxygenated, thus contributing to hypoxemia. Blood perfusing high  $V_A/Q$  alveoli (on right in *Figure 1*) is well-oxygenated but is a small portion of total blood flow and thus does not balance the impact of hypoxemic blood returning from the low  $V_A/Q$  regions.

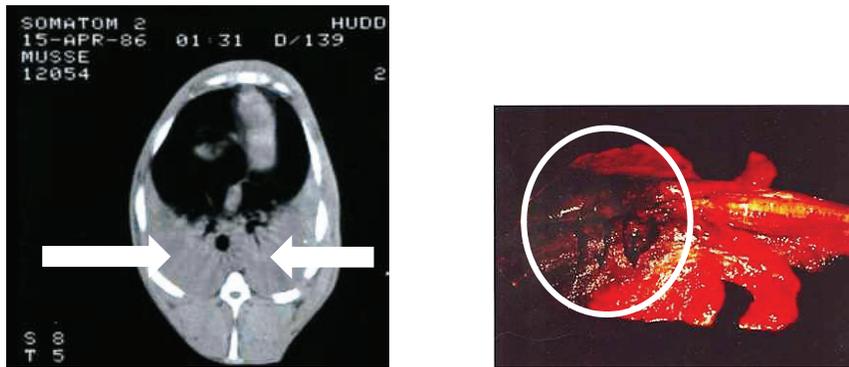


*Figure 1.* Diagram of ventilation ( $V_A$ ) and perfusion matching. The alveolus on the left is poorly ventilated but adequately perfused (low  $V_A/Q$ ), the alveolus in the middle has appropriate matching of  $V_A/Q$  and the alveolus on the right is adequately ventilated but poorly perfused (high  $V_A/Q$ ). Note the  $\text{PaO}_2$  impact of blood perfusing the alveolus on the left (large decrease in  $\text{PaO}_2$ ) versus the  $\text{PaO}_2$  impact of blood perfusing the alveolus on the right (no impact)(West 2011)

$V_A/Q$  mismatch was long thought to be the primary cause of hypoxemia in the anesthetized horse because anesthesia reduces functional residual capacity

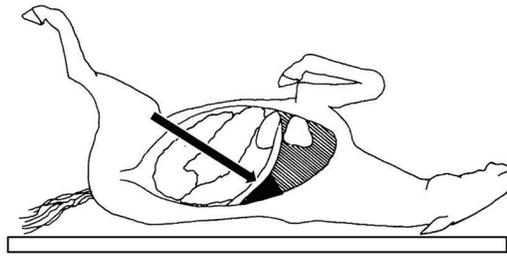
(FRC) to volumes at which airway closure could occur more easily (McDonell 1974, Sorenson and Robinson 1980) and reduces both ventilation (McDonell 1974) and blood flow (Stolk 1980) to the dependent lung regions. Thus, perfusion and ventilation are not always in the same place at the same time and it was theorized that anesthesia creates a low  $V_A/Q$  state leading to hypoxemia. However, it is actually shunt of blood through unventilated alveoli (designated  $Q_s/Q_t$  for ratio of shunted blood flow [ $Q_s$ ] to total blood flow [ $Q_t$ ]) that is the major cause of hypoxemia in the anesthetized horse. The deoxygenated blood from the shunt, termed 'venous admixture', is delivered directly into systemic circulation without being oxygenated and causes a decrease in  $PaO_2$ . In the standing conscious horse,  $Q_s/Q_t$  is approximately 1% as determined by the multiple inert gas elimination technique (MIGET)(Hedenstierna et al. 1987, Nyman, Bjork and Funkquist 1999), which is the gold standard for determination of the degree of shunt. This normal, low-level shunt is present in most (or perhaps all) mammalian species and is caused by deoxygenated blood from the bronchial veins (which transport blood from the pulmonary veins) and the Thebesian (or coronary) veins (which transport blood from the myocardium). Unlike other modalities used to determine venous admixture, MIGET can be used to differentiate  $Q_s/Q_t$  from areas of low  $V_A/Q$  and in the Nyman and Hedenstierna MIGET study (Nyman and Hedenstierna 1989) areas of low  $V_A/Q$  were minimal to absent in spontaneously breathing anesthetized horses in either dorsal or lateral recumbency, while  $Q_s/Q_t$  was increased to approximately 34% in dorsally recumbent and 20% in laterally recumbent horses. Thus, it would appear that  $Q_s/Q_t$  is the predominant problem.

This was further demonstrated when, using computed tomography (CT) and histologic analysis of lung tissue (*Figure 2*), large areas of complete atelectasis were identified in the dependent lung regions of ponies and horses anesthetized with halothane in oxygen and placed in dorsal recumbency (Nyman et al. 1990). In this study, the size of the atelectatic regions correlated to the degree of hypoxemia, emphasizing that the atelectatic regions were the direct cause of the hypoxemia. Clearly, areas that are completely atelectatic would be perfused but not ventilated and would cause  $Q_s/Q_t$  rather than low  $V_A/Q$ .



*Figure 2.* On the left is a CT scan from a pony showing the large area of consolidation in the dependent lung fields (indicated by arrows). On the right are the lungs from a horse euthanized during anesthesia. Note the large consolidated area (indicated by a circle) in the dependent lung fields (Nyman et al. 1990). Both images courtesy of Dr. Nyman.

Although both compression and absorption atelectasis can contribute to hypoxemia, the fact that these densities developed within 20 minutes of placing the horses in dorsal recumbency (Nyman et al. 1990) indicates that they were due to compression atelectasis. The rapid development of atelectasis was previously demonstrated by radiography in anesthetized horses in lateral recumbency (McDonnell, Hall and Jeffcott 1979). The shape of the equine diaphragm and the large size of the equine gastrointestinal tract are responsible for this compression (Sorenson and Robinson 1980). The diaphragm slopes dramatically under the caudal lung fields in the standing horse, allowing the liver and a large portion of the lung field to rest above the intestines, but also allowing the intestines to compress the caudal lung fields in a recumbent horse since recumbency places the intestines above the lung fields. (*Figure 3*) Absorption atelectasis can exacerbate the compression-induced hypoxemia but the effect is usually a slow decline in PaO<sub>2</sub> over time rather than an almost immediate decline in PaO<sub>2</sub> following recumbency (Nyman et al. 1988).



*Figure 3.* Schematic of a horse in dorsal recumbency. The arrow indicates the viscera resting on the caudal lung lobes causing atelectasis. Figure courtesy of Dr. Nyman.

Interestingly, collapsed lung regions identified by computed tomography (CT) scan are generally larger in proportion to body size and the impairment of oxygenation and percent of venous admixture are greater in anesthetized ponies (Nyman et al. 1990) than respective indices in anesthetized humans or sheep (Brismar et al. 1985, Strandberg et al. 1986, Hedenstierna et al. 1989). These differences emphasize the fact that anesthesia causes greater pulmonary compromise in the horse than in other species and the greater degree of compromise is most likely ‘related to different vertical lung heights and the effect of hydrostatic forces’ (Nyman et al. 1990) and to the visceral compression of the caudal lung fields as described above.

### 1.3 Treating hypoxemia in anesthetized horses: Changing ventilation

Standard changes in ventilation (including common techniques like IPPV and/or positive end-expiratory pressure [PEEP]), and more advanced ventilatory techniques (including ventilation of selective lung fields or alveolar recruitment maneuvers) have been utilized in an attempt to improve oxygenation in anesthetized horses. Alterations in the composition of inspired gas, such as decreased  $F_{iO_2}$  or the addition of helium, have also been tried. IPPV does cause a decrease in  $P_{ACO_2}$  and can also improve oxygenation (Steffey et al. 1977, Gasthuys et al. 1991, Edner, Nyman and Essen-Gustavsson 2005). However,  $P_{aO_2}$  is not always improved (Shawley and Mandsager 1990, Nyman et al. 1990, Day et al. 1995) and IPPV may impair cardiovascular function, as evidenced by decreased  $Q$  and ABP secondary to IPPV-mediated positive intra-thoracic pressure (Hodgson et al. 1986, Shawley

and Mandsager 1990, Nyman et al. 1990, Gasthuys et al. 1991, Edner et al. 2005, Steffey et al. 2005a, Steffey et al. 2005b). In addition to the causes of hypoxemia that have been listed, hypoxemia can also occur secondary to decreased cardiac output ( $Q_t$ ) since inadequate pulmonary perfusion will decrease the amount of blood that reaches ventilated alveoli to be oxygenated. Thus, IPPV can make oxygenation worse rather than better (Day et al. 1995). Positive end expiratory pressure (PEEP) is often utilized to recruit low  $V_A/Q$  alveoli with the hopes of improving gas exchange. Although PEEP is more likely than IPPV alone to improve oxygenation, this technique does not always increase  $PaO_2$  and has an even greater negative effect on the cardiovascular system than IPPV used alone (Beadle, Robinson and Sorenson 1975, Swanson and Muir 1988, Wilson and Soma 1990, Nyman et al. 1990). PEEP may also have an adverse effect on the  $Q_s/Q_t$  as increased intrathoracic pressure, while expanding alveoli, may also redistribute blood into the atelectatic area (Nyman et al. 1990)

Because standard IPPV and PEEP do not always improve oxygenation in anesthetized horses, specialized ventilatory techniques have been investigated. In dorsally recumbent horses, the dependent lung regions have been selectively intubated and inflated using PEEP while the non-dependent lung regions have been intubated and ventilated by spontaneous breathing (Nyman et al. 1987, Nyman and Hedenstierna 1989). In laterally recumbent horses, each lung field (dependent and non-dependent) has been intubated and ventilated separately (Moens et al. 1994, Moens et al. 1998). With both of these techniques,  $PaO_2$  was improved and  $Q_s/Q_t$  was decreased. However, the techniques required specialized endotracheal tubes, intubation through a tracheotomy site and two anesthesia machines to ventilate each portion of the lung, thus these techniques are not practical for routine use. Also, recruitment maneuvers of 60-80  $cmH_2O$  peak inspiratory pressure (held for 10-12 seconds) have been used in an attempt to improve oxygenation in anesthetized horses (Hopster et al. 2011).  $PaO_2$  in horses receiving the recruitment maneuver was significantly higher than  $PaO_2$  in control horses at all times, but repeated recruitment was necessary to sustain the improvement. Furthermore, the recruitment maneuver did cause a cardiovascular impact and horses in the recruitment group required more dobutamine than control horses to maintain the same mean arterial blood pressure (MAP). Finally, the increased pressure techniques may not be benign for the aerated areas of the lung. Even in horses breathing spontaneously with no increase in inspiratory pressure, histologic exam of the aerated lung revealed evidence of hyperinflation and alveolar wall fragmentation (Nyman et al. 1990).

In addition to changing ventilatory techniques, improving oxygenation by changing the ventilatory gas has been attempted in anesthetized horses. Most horses under general anesthesia receive  $>0.95$   $\text{FiO}_2$  but decreasing the  $\text{FiO}_2$  to 0.21 in horses maintained on total intravenous anesthesia (TIVA) caused a decrease in  $\text{Qs/Qt}$  when compared to horses maintained on the traditional 0.95  $\text{FiO}_2$  (Marntell, Nyman and Hedenstierna 2005b). Horses maintained with inhalation anesthesia had a lower  $\text{P(A-a)O}_2$  when  $\text{FiO}_2$  was 0.3 than when it was 0.85 (Cuvellez et al. 1990). However, decreasing the  $\text{FiO}_2$  to 0.5 in horses anesthetized with inhalant anesthesia did not change  $\text{Qs/Qt}$  (Hubbell et al. 2011), thus, merely decreasing the  $\text{FiO}_2$  may not eliminate the problem in all horses. Adding helium to the inspiratory mixture (added as a carrier gas in place of nitrogen) did decrease the  $\text{P(A-a)O}_2$  (Staffieri et al. 2009) but most anesthetic machines are not equipped to deliver helium. Moreover, in all of the studies listed here,  $\text{PaO}_2$  was lower in horses receiving  $\text{FiO}_2$  of anything  $<0.95$ . Decreased  $\text{PaO}_2$  is not likely to be concerning as long as arterial oxygenation stays in the normal range. However, low  $\text{FiO}_2$  may result in a  $\text{PaO}_2$  in the hypoxemic range, necessitating a therapeutic increase in  $\text{FiO}_2$  (Nyman et al. 1990). In some patients, low  $\text{PaO}_2$  can be predicted by low pulse pressure, emergency procedure, and dorsal positioning (Whitehair and Willits 1999) and this set of criteria describes a major portion of the equine anesthesia caseload, the horse with abdominal compromise or 'colic'. Colic patients are highly likely to be hypoxemic and may not tolerate lower  $\text{FiO}_2$ . Thus decreasing the  $\text{FiO}_2$  may not always be the answer for improving oxygenation in the anesthetized horse.

#### 1.4 Treating hypoxemia in anesthetized horses: Changing perfusion

With changes in ventilation often ineffective at improving oxygenation and often deleterious for the cardiovascular system, the question arises: can we alter *perfusion* instead of *ventilation* to achieve improvement in oxygenation in anesthetized horses?

##### 1.4.1 Systemically administered drugs

Administration of the positive inotrope dobutamine improved cardiac output and, thus, likely improved pulmonary perfusion but did not improve  $\text{PaO}_2$ . (Swanson and Muir 1986). Although disappointing, this outcome is not wholly surprising since improving blood flow to the entire lung would increase flow

not only to the ventilated lung regions, but to the non-ventilated lung regions as well, thereby maintaining, or perhaps even exacerbating, the degree of  $Q_s/Q_t$ . Clenbuterol administered intravenously (Gleed and Dobson 1990, Keegan et al. 1991) and albuterol administered as an inhalant (Robertson and Bailey 2002) have been used with some success to increase  $PaO_2$  in anesthetized horses. Although inhaled albuterol may relieve bronchoconstriction and improve ventilation, the most likely mechanism of action of both drugs is increased pulmonary blood flow as both heart rate and cardiac index are increased during administration of the drugs. As with dopamine, this can also worsen hypoxemia secondary to a more profound  $V_A/Q$  mismatch (Dodam et al. 1993), which would explain why this treatment commonly fails. Furthermore, both compounds can cause tachycardia and/or profound sweating in the horse.

Systemically administered vasodilating drugs, which are used some in human medicine to relieve pulmonary arterial hypertension (PAH) and improve oxygenation, may also cause hypotension and, as previously mentioned, hypotension is as deleterious as hypoxemia to the anesthetized horse (Grandy et al., 1987, Richey et al., 1990, Young and Taylor, 1993, Duke et al., 2006). Furthermore, systemic vasodilating drugs can worsen  $Q_s/Q_t$  as described above for dobutamine and the buterols. In human medicine, a selective pulmonary vasodilator, nitric oxide (NO) is often delivered as an inhaled gas (iNO) to selectively dilate the pulmonary vessels and improve oxygenation without affecting the systemic vasculature (Ichinose, Roberts and Zapol 2004).

#### 1.4.2 Nitric oxide (NO)

Nitric oxide (NO) is a small molecule of soluble gas that was identified in 1987 as the elusive 'endothelium-derived relaxing factor' (EDRF) (Palmer 1987). Intriguingly, before this discovery, NO was not viewed as a useful molecule but instead was known primarily as a toxic air pollutant. However, after 1987, the importance of NO as a biological molecule quickly became apparent and NO was named 'Molecule of the Year' by Science magazine in 1992 and in 1998, Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were jointly awarded the Nobel Prize in Physiology or Medicine for their discovery of 'nitric oxide as a signaling molecule in the cardiovascular system' (Zetterstrom 2009).

#### 1.4.3 NO production, mechanism of action and termination

NO and the amino acid citrulline are formed from L-arginine and oxygen by one of the nitric oxide synthase (NOS) enzymes (*Figure 4*). There are 3 major forms of NOS: 1) endothelial NOS (eNOS), which is a calcium-dependent

NOS that is constitutively produced in the endothelium; 2) neural NOS (nNOS), another calcium-dependent NOS that is constitutively produced in the brain and in a variety of peripheral nerves; and 3) inducible NOS (iNOS), a calcium-independent NOS that is not constitutive but that is induced by inflammatory and immunological processes (Ray, Chakraborti and Gulati 2007).

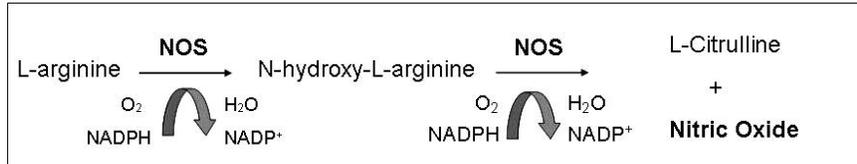


Figure 4. Formation of NO and citrulline from L-arginine. NOS can be eNOS, iNOS or nNOS.

Because NO is uncharged, the molecule is readily diffusible and, once formed, diffuses out of the cell where it was generated and into the target cell. The role of NO as EDRF is the best described, and perhaps the most important, function of the molecule. As EDRF, NO is continuously released into the vascular by the endothelium in response to various stimuli. NO causes an increase in cyclic guanosine monophosphate (cGMP) in the target cell and this leads to a relaxation of vascular smooth muscle and subsequent vasodilation (Stamler et al. 1994, Ichinose et al. 2004). Through these mechanisms, endogenous NO regulates both systemic and pulmonary vascular tone (Stamler et al. 1994, Blitzer et al. 1996). After inducing smooth muscle relaxation, NO rapidly diffuses into the bloodstream and immediately reacts with oxygenated hemoglobin to form methemoglobin and nitrate. The majority of the NO is excrete as nitrate in the urine (Ichinose et al. 2004).

#### 1.4.4 Endogenous NO in the equine pulmonary system

Endogenous NO is present in fairly high amounts in the upper airways of mammals and has been measured in the exhalate of humans (Hedenstierna and Hogman 1998, Cikach and Dweik 2012), primates (Schedin et al. 1997) and elephants (Schedin et al. 1997). Inhalation of endogenous NO from the nasopharyngeal region may play a role in homeostatic V<sub>A</sub>/Q matching in humans (Sanchez Crespo et al. 2010). Although NO was not measured in equine exhalate in one study (Schedin et al. 1997), results show that exhaled NO is released from the airways of the horse and may contribute to the regulation of pulmonary vascular tone during exercise (Mills et al. 1996). NO was also measured in the exhalate of anesthetized horses and the concentrations were different depending on whether the horse was

anaesthetized with halothane or a combination of ketamine, guaiphenesin and romifidine (Marlin et al. 2001). Tissue studies have also been used to identify endogenous NO in the equine pulmonary vasculature. Using isolated equine pulmonary arteries MacEachern et al. (MacEachern, Smith and Nolan 2004), determined that NO is involved in the hypoxic pulmonary vasoconstriction (HPV response) and Pelletier et al (Pelletier et al. 1998) determined that regional differences in endothelium-mediated relaxation are caused by regional differences in the magnitude of the endothelial release of nitric oxide. In the intact horse, administration of the NOS inhibitor N(omega)-nitro-L-arginine methyl ester (L-NAME) in standing horses caused a significant rise in pulmonary arterial pressures, indicating that endogenous NO plays a primary role in control of pulmonary vascular tone (Manohar and Goetz 1998).

Although manipulation of endogenous pulmonary NO may be therapeutically effective in some species (Brown et al. 1992, Rafiei, Aghadavoudi and Hojjat 2012), the ability to therapeutically manipulate endogenous NO in the pulmonary system of the horse is unclear. *Decreased* production of endogenous NO using the NOS inhibitor L-NAME had no effect on oxygenation in strenuously exercising horses (Manohar and Goetz 1998). Also, *increased* production of endogenous NO through administration of the NO precursor L-arginine failed to improve PaO<sub>2</sub> in anesthetized horses (Lerche 2006). If neither decreased or increased endogenous NO production had any effect on oxygenation, perhaps exogenously administered NO may be a better therapeutic choice in hypoxemic horses.

#### 1.4.5 Inhaled exogenous NO

As an exogenously administered inhaled gas, NO causes selective dilation of pulmonary blood vessels in ventilated areas, which causes reduction of pulmonary vascular resistance and improved blood flow to the lung regions exposed to the inhaled NO (Frostell et al. 1991, Frostell et al. 1993). This allows a redistribution of pulmonary blood flow to the better ventilated alveoli, thus decreasing venous admixture and improving PaO<sub>2</sub>. NO absorbed into the pulmonary capillaries is rapidly scavenged by hemoglobin and inactivated (Bloch et al. 2007), thus limiting the systemic impact. The first reports of the use of iNO in humans and research animals occurred in the late 1980's and early 1990's (Frostell et al., 1991, Frostell et al., 1993). Although primarily licensed for treating PAH in neonates, iNO is now widely used to treat a wide variety of pulmonary diseases that cause pulmonary hypertension and hypoxemia (Ichinose et al., 2004, Griffiths and Evans, 2005, Bloch et al., 2007, Creagh-Brown et al., 2009).

As with many exogenously administered compounds, iNO also has the potential to cause adverse effects. High concentrations of NO can react with oxygen to form the toxic gas NO<sub>2</sub>. This gas acts mainly as a respiratory mucosal irritant but high dosages may induce more toxic effects (Weinberger et al. 2001). In some species, abrupt cessation of inhaled NO (iNO), such as might occur at the end of an anesthetic period, can cause a 'rebound effect', which is a rapid and potentially drastic decrease of PaO<sub>2</sub> and increase of pulmonary arterial pressure (PAP) to values that are significantly worse than pre-iNO levels and the rebound effect may be related to a compensatory increase in the circulating concentration of the potent endogenous vasoconstrictor endothelin-1 (ET-1) (Chen et al. 2001, McMullan et al. 2001, Wedgwood et al. 2001, Pearl et al. 2002).

#### 1.4.6 Inhaled nitric oxide in horses

In spite of the fairly wide-spread use of iNO in humans and the potential utility of iNO in horses, very little of the gas has been used in equine research and none has been used in clinical patients. In fact, prior to the work presented here, a total of 6 papers regarding the use of iNO in horses had been published. In the first report of iNO in the horse, the gas caused bronchodilation in horses with experimentally-induced bronchoconstriction, but no markers of oxygenation were measured (Sweeney, Tomasic and Russell 1999). Inhaled NO was also used to reduce pulmonary hypertension in maximally exercising horses, but again no indices of oxygenation were measured (Kindig et al. 2001). In the first report of iNO used to improve oxygenation in horses, PaO<sub>2</sub> was not increased in horses maintained with inhalant anesthesia and continuous delivery of iNO (CiNO) (Young et al. 1999). However, CiNO did improve PaO<sub>2</sub> and SaO<sub>2</sub> in foals with experimentally-induced pulmonary hypertension (Lester, DeMarco and Norman 1999).

Then, in 2001 and 2002 Heinonen et al. (Heinonen et al. 2001, Heinonen et al. 2002), demonstrated that iNO delivered as a pulse (PiNO) to anesthetized horses significantly increased PaO<sub>2</sub> and decreased Qs/Qt without causing adverse effects. Timing of the pulse was critical for these changes and PaO<sub>2</sub> was increased and Qs/Qt decreased if the pulse was delivered during the early portion of the inspiration but was less effective, or not effective, if delivered throughout a greater percentage of inspiration or during the mid to latter part of the breath rather than at the beginning. The advantage of PiNO is that a lower dose of iNO is utilized, making the accumulation of exhaled NO and the production of NO<sub>2</sub> less likely. PiNO is easy to utilize since a tank of medical grade NO can be connected directly to the breathing system without the need

for adapting the anesthesia machine. Also, the amount of PiNO mixed with oxygen is very small and does not impact the  $FiO_2$ . Because of its efficacy, safety and ease of use, PiNO has the potential to revolutionize equine anesthesia. However, PiNO use must be moved from the anesthesia research laboratory to anesthesia in a clinical setting prior to its use in equine patients.

### 1.5 Preparing PiNO for clinical use in anesthetized horses

In the efficacy and safety studies by Heinonen et al. (Heinonen et al. 2001, Heinonen et al. 2002), PiNO was delivered to anesthetized horses for intervals of 5 minutes. Unfortunately, the duration of equine surgery is rarely less than one hour and more likely to be 2-3 hours. Although iNO is used for long duration therapy in humans (Bloch et al. 2007, Ichinose et al. 2004, Barst et al. 2012), there is some evidence that prolonged duration therapy does not always provide a sustained improvement in oxygenation (Day, Allen and Witte 1997). The sustainability of the response to long-term delivery of PiNO, the minimum effective pulse length for long-duration delivery, and the potential accumulation of excess NO and production of  $NO_2$  in the anesthesia circuit with prolonged administration all need to be determined. Also, surgery in the horse is done in both lateral and dorsal recumbency and the effects of PiNO in different recumbencies delivered for surgical durations are unknown. As stated in the introduction, horses positioned in lateral recumbency are less likely to experience hypoxemia than those positioned in dorsal recumbency (Nyman et al. 1988, Day et al. 1995) and it is possible that the degree of  $V_A/Q$  mismatch and, thus, the magnitude of the response to PiNO could be insignificant in lateral recumbency. Conversely, horses in lateral recumbency develop high  $P(A-a)DO_2$  (Nyman et al. 1988, Day et al. 1995) and  $Q_s/Q_t$  (Nyman and Hedenstierna 1989) and PiNO delivery may improve pulmonary function even in the presence of adequate  $PaO_2$ . Furthermore, in the previous studies utilizing PiNO in anaesthetized horses (Heinonen et al. 2001, Heinonen et al. 2002) there were no control groups and it is possible, albeit unlikely, that oxygenation improved with time rather than with treatment.

The effects of discontinuing PiNO in anesthetized horses has been briefly examined following the 5 minute delivery times but has not been examined following long-term delivery and has not been evaluated following discontinuation of PiNO, isoflurane and 100% oxygen. As stated, the anesthetic recovery period is the most dangerous phase of anesthesia for the horse (Johnston et al. 2002, Wagner 2008a) and any factors that further jeopardizes the recovery period would be unacceptable. If the previously described rebound effect occurs following PiNO use in horses, it could limit

the usefulness of this technique during anesthesia. Because the recovery phase is so important in the horse, assessment of the impact of PiNO on recovery is critical.

Finally, the mechanism of action of PiNO is generally, but not precisely, known and understanding the mechanism is an important aspect of safe and effective use of the therapy. The matching of the distribution of pulmonary blood flow and ventilation can be determined by the multiple inert gas elimination technique (MIGET) but MIGET does not provide regional anatomical data (Wagner 2008b). The actual spatial distribution (or redistribution) of blood flow in the lungs can be visualized in two dimensions by perfusion scintigraphy, which could be used to determine the spatial redistribution of blood flow in anesthetized horses.



## 2 Aims of the thesis

The hypothesis was that PiNO would improve oxygenation in anesthetized horses through spatial redistribution of pulmonary blood flow resulting in decreased shunt and better matching of perfusion to the ventilated regions of the lung without causing adverse effects either during anesthesia or in recovery.

With the goal of introducing PiNO for clinical use in isoflurane-anesthetized horses, the aims of our study were to determine the:

1. range of PiNO pulse durations that provide the maximal effect at lowest dose,
2. efficacy and safety of uninterrupted long-term administration of PiNO in horses positioned in dorsal and lateral recumbency,
3. safety and physiologic effects of PiNO discontinuation in anesthetized horses,
4. safety and physiologic effects of PiNO discontinuation on the anesthetic recovery period, and
5. ventilation/perfusion matching and spatial distribution of perfusion during PiNO.



## 3 Materials and Methods

### 3.1 Horses

Six healthy Standardbred horses (2 mares and 4 geldings) with a mean weight of 488 kg (range 450-510 kg) and a mean age of 5 years (range 4-6 years) were used in studies I and III. Ten healthy Standardbred horses (8 mares and 2 geldings) with mean weight of 513 kg (range 476-550 kg) and a mean age 9 years (range 5-13 years) were used for studies II and IV. Three additional healthy Standardbred horses (2 mares and 1 gelding) with a mean weight of 504 kg (range 458-581 kg) and a mean age of 6 years (range 3-8 years) were used in the MIGET portion of study 2. Three healthy Standardbred horses (1 mare and 2 geldings) with a mean weight of 513 kg (range 459-562 kg) and the mean age of 9 years (range 8-9 years) were used for Study V.

### 3.2 Treatment Groups

In studies I, III and V, all horses received PiNO and all of the horses were in one group. In Study II there were two groups and the horses were divided so that 6 horses were in the control group (C group) that received inhalant anesthesia but no PiNO and 6 received inhalant anesthesia plus PiNO (PiNO group). Two horses were anesthetized in both groups and they were randomized in a cross-over design and anesthetized at intervals of no less than four weeks between studies. In study IV all horses were recovering from anesthesia (isoflurane, 100% oxygen discontinued) and were divided into two groups: 6 horses recovering from anesthesia + PiNO delivery and 6 horses recovering from anesthesia that did not include PiNO delivery. As with study II, 2 horses were used in both groups and they were randomized in a cross-over design and anesthetized at intervals of no less than four weeks between studies.

### 3.3 Anesthesia

A standard anesthetic protocol was used for Studies I, II, III and V; horses in study IV were anesthetized with same technique but data were not collected during anesthesia. Food but not water was withheld for 12 hours prior to anesthesia. On the day of the procedure, a complete physical exam was done to insure that all horses were healthy. After the exam,  $0.03 \text{ mg/kg}^{-1}$  acepromazine (Plegicil; Pharmacia & Upjohn Animal Health, Sweden) was administered intramuscularly to each horse. Approximately 30 minutes after the injection of acepromazine 7.5% guaifenesin (Myolaxin; Chassot & Cie AG; Switzerland) was infused IV until the horse developed ataxia (approximately  $100 \text{ mg/kg}^{-1}$ ). Anesthesia was then induced with the intravenous (IV) administration of a bolus of thiopental sodium (4 to  $5 \text{ mg/kg}^{-1}$ , Pentothal Natrium; Abbott, Sweden). The horse was intubated with a 24-mm cuffed endotracheal tube and placed in left lateral recumbency on a padded table. The endotracheal tube was connected to a semiclosed circle rebreathing system that was attached to a large-animal anesthesia machine. Anesthesia was maintained with spontaneous breathing of oxygen ( $\text{FiO}_2 > 0.95$ ) and isoflurane (Forene; Abbott, Sweden) delivered through an out-of-circle, agent-specific, precision vaporizer. Throughout the study, end-tidal isoflurane concentrations of 1.5% to 1.7% (approximately 1.2-1.25 times the minimum alveolar concentration of isoflurane in horses) were maintained. The gas monitor was calibrated before each research period by use of a commercially prepared calibration gas. All horses were allowed to equilibrate under anesthesia for 45 minutes (Studies I, II and III) or 60 minutes (Study V), during which time they were instrumented for data collection.

### 3.4 Instrumentation (Studies I, II, III, V)

All horses were instrumented with ECG electrodes placed for lead II analysis and measurement of heart rate. The skin over the facial artery was clipped free of hair and aseptically prepared and a 20-gauge, 5-cm catheter was introduced percutaneously for measurement of arterial blood pressure and for collection of arterial blood samples for blood gas analysis. An area over the right jugular vein was clipped free of hair and aseptically prepared; an introducer kit was used to place a 7-F thermodilution catheter through the jugular vein and into the pulmonary artery for measurement of pulmonary arterial blood pressure and for collection of mixed venous blood samples for blood gas analysis. A pig-tail, multiport catheter was introduced by the same technique into the same jugular vein, advanced to the right ventricle, and retracted into the right atrium for injection of saline (0.9% NaCl) solution for cardiac output (Qt)

determination. Catheters were positioned by use of pressure-tracing guidance and simultaneous ECG monitoring and were locked in position via a Luer-lock adapter. Systolic, diastolic, and mean arterial blood pressure (ABP) values (SAP, DAP, MAP, respectively) and systolic, diastolic and mean arterial pulmonary pressure (SPAP, DPAP, MPAP, respectively) were measured by use of pressure transducers positioned at the level of the sternal manubrium, which (Qt) was considered to correspond to the level of the right atrium. Cardiac output was determined by use of a thermodilution technique in which a bolus of 20 mL of cold (0°C) saline solution was injected rapidly by hand through the pig-tailed catheter. A minimum of 3 injections was performed, and the data were averaged at each time period. Cardiac output, systemic arterial blood pressures, heart rate (HR), FiO<sub>2</sub>, respiratory rate (RR), tidal volume (TV), partial pressure of end-tidal carbon dioxide (PETCO<sub>2</sub>), and end-tidal isoflurane concentration were recorded from a standard anesthesia monitor (AS/3- AM™ anesthesia monitor; Datex-Ohmeda, Finland).

### 3.5 Instrumentation (Study IV)

The area over the artery was clipped free of hair and aseptically prepared and a 20-gauge, 5-cm catheter was introduced percutaneously into the facial artery for collection of arterial blood samples for blood gas analysis. An area over the right jugular vein was clipped free of hair and aseptically prepared and an introducer kit was used to place a 7-F thermodilution catheter through the jugular vein and into the pulmonary artery for collection of mixed venous blood samples for blood gas analysis. Heart rate was counted by manual palpation of the facial artery and respiratory rate was counted by watching thoracic excursions. Other commonly used anesthetic monitors (eg, ECG, pulse oximeter, cardiac output computer) were not utilized in the recovery portion of the study as the presence of an abundance of monitoring equipment in the recovery stall was deemed dangerous.

### 3.6 PiNO Delivery

A standard delivery technique, described below, was used for studies I, II and V. In Studies III and IV PiNO was delivered in the method described but data were collected following PiNO cessation. Once completely instrumented, the horses were allowed to equilibrate under anesthesia for 45 minutes (Studies I and II) or 60 minutes (Study V), data were then collected and used as anesthesia baseline data (T45). After baseline data collection, PiNO delivery commenced. The PiNO was delivered during the initial portion of inhalation

during each breath by use of a proprietary device that was designed at the Datex-Ohmeda Research Unit, Helsinki, Finland specifically for pulsed delivery of iNO during spontaneous breathing. The device was activated by negative pressure and delivered a volumetric dose into the endotracheal tube at the onset of inspiration in the same manner as previously described (Heinonen et al. 2001, Heinonen et al. 2002). The delivery device was connected to a port located at the proximal end of the endotracheal tube. The NO was supplied in a cylinder of 2,000 ppm medical grade NO in N<sub>2</sub> (AGA AB, Sweden). In Study I and the MIGET portion of Study II, the dose of PiNO was measured using an AD-lite™ (Datex-Ohmeda, Finland) and expired NO was monitored with a chemiluminescent analyser prototype (Datex-Ohmeda, Finland) connected between the Y-piece and the point of NO administration. The analyzer was calibrated with the mixture 100 µL/L NO in N<sub>2</sub> (Aga AB, Lidingö, Sweden) and with room air depleted of NO with a charcoal absorber. The monitor was used to determine the end-tidal and peak expired NO fractions.

### 3.7 Methods for MIGET (Studies II (MIGET) and V (MIGET))

The distribution of ventilation and perfusion was estimated by a multiple inert gas elimination technique (MIGET; Wagner et al. 1974; Hedenstierna et al. 1987) in Study II (this portion of the study designated Study II (MIGET)) and Study V (designated Study V (MIGET)). After anesthesia as described above, an infusion of six inert gases (sulphur hexafluoride, ethane, cyclopropane, enflurane, ether and acetone; combined and dissolved in isotonic sodium chloride solution) was begun into the jugular vein at 30 ml\*min<sup>-1</sup>, during which time the horses were instrumented as described above. After 60 minutes of infusion and instrumentation, arterial and mixed venous blood samples were drawn, and mixed expired gas was collected from a heated mixing box connected to the expiratory limb of the large animal circle (*Figure 5 a & b*). Gas concentrations in the blood samples and exhalate were measured by a gas chromatograph (Hewlett Packard 5890 series II, GA, USA). From the blood- and mixed expired gas the V<sub>A</sub>/Q was calculated according to the original technique (Hedenstierna et al. 1974). Blood flow (Q<sub>t</sub>) and standard deviation of its logarithmic distribution (log SDQ), tidal volume (V<sub>T</sub>) and standard deviation of its logarithmic distribution (log SDV), right to left vascular shunt % of the Q<sub>t</sub> (Q<sub>S</sub>; perfusion of lung regions with V<sub>A</sub>/Q<0.005), and dead space % of the V<sub>T</sub> (V<sub>D</sub>; ventilation of lung regions including apparatus dead space with V<sub>A</sub>/Q>100) were measured.



Figure 5. a (on left) view of heated mixing box (at left of photo); b (on right) view of anesthesia machine modifications to an open breathing system for MIGET exhalant gas collection. Note that the exhalation limb of the breathing circuit is not connected to the anesthesia machine. It connects to the heated mixing box.

### 3.8 Methods for scintigraphy (Study V)

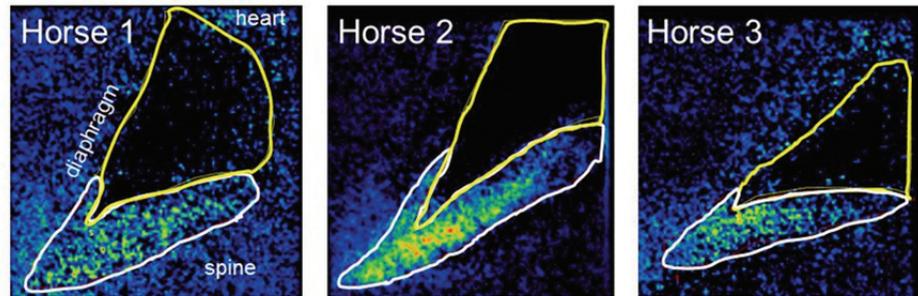
Perfusion scintigrams of the caudal lung fields were made using a large field of view gamma camera fitted with a general purpose low energy collimator (Picker SX 300, Picker International Inc., Cleveland, OH, USA) at the same times that the MIGET was determined. An aliquot of 500 Mbq of  $^{99m}\text{Tc}$ -macroaggregated human serum albumin (MAA: Macroaggregated albumin, TechneScan LyoMAA, Mallinckrodt AB, Sweden) was prepared using standard methods (Votion and LeKeux 2003) and injected slowly over a period of 1 minute, which included 3 breaths in Horses 1 and 2 and 8-10 breaths in horse 3, to ensure even distribution throughout the lung (Votion and LeKeux 2003). Images were acquired and processed using a nuclear medicine program controlling the gamma camera (Hermes Medical Systems<sup>®</sup>, Hägersten, Sweden). Each study was made as a dynamic acquisition at a rate of 2 seconds per frame (picture) for one minute, with a resolution of 256x256 pixels. Pulmonary perfusion was studied in each horse at prePiNO, PiNO and postPiNO. Neither the camera head nor the horses were moved between studies.

As each 2-second frame had a poor signal-noise ratio due to relatively few scintillation points, the edges of the lungs were poorly defined. All frames were filtered with a medium resolution Metz (fast fourier transform) filter to remove the high frequency components (noise) and produce definite margins of the lungs. In order to eliminate motion unsharpness due to breathing, frames acquired during the expiratory pause were identified as the smallest-sized lung field and frames larger than these (caused by breathing) were removed from

the data set. Twenty of the expiratory images from each study were randomly chosen so that each study then contained 20 frames, which were summed as a single static 10 second image. This process was repeated for each horse at PrePiNO, PiNO and PostPiNO.

To compensate for the decay of the tracer  $^{99m}\text{Tc}$ , the static expiratory images collected at pre-PiNO were corrected for the loss of radioactivity during the time from their acquisition to the time of the acquisition of images collected at PiNO. Also, the images acquired at PostPiNO included background radioactivity from the injections at PrePiNO and PiNO. This background activity was subtracted from the images acquired at PiNO and PostPiNO.

To produce images that showed the redistribution of blood flow, the activity in each pixel in the PiNO image was subtracted from the pixel activity in the pre-PiNO image (*Figure 6*). The resulting functional image identified the areas that increased and decreased in activity due to NO inhalation. The border between the areas of increase and decrease was a well-defined narrow zone where the perfusion did not change. Regions of interest (ROIs) were drawn by hand around the two areas of change. The ROIs were copied and pasted onto the images just described and the total activity in each ROI for the 9 images was recorded. All ROIs were drawn and measured 4 times. Averaged counts for each ROI in the images of the PiNO and PostPiNO studies were plotted as percentage change from the baseline study.



*Figure 6.* Functional images of the caudal lung of the three horses. The left border is defined by the diaphragm, the lower border by the spine, and the right border by the edge of the field of view of the gamma camera. The yellow line defines the ventral region of interest (ROI), in which counts increased after inhalation of nitric oxide (NO), indicating increased blood flow. The white line defines the dorsal ROI, in which counts, and flow decreased. The images were made by subtracting the NO treatment image from the baseline image. This created pixels with negative numbers (black) where flow increased, and colored pixels where flow decreased, scaled according to the relative color scale for each image. To facilitate drawing the ROIs, especially at the isocount line between them, the color scales were adjusted to enhance the contrast between the two ROIs.

Each filtered study for each horse was again examined frame-by-frame and the frames at maximal inspiration were extracted and saved as a summed static image. This data group was not limited to 20 frames because the data was used only to calculate the area of a ROI, not radioactivity within a ROI. ROIs around the entire lung field of each of these static images were drawn automatically as an isocount line at a threshold of 15% of the maximum counts/pixel. The areas as a number of pixels were recorded for peak inspiration and expiration and the difference between them was averaged for each horse. This number gave an indication of the relative tidal volumes.

### 3.9 Blood gas analysis (Studies I-V)

Arterial and mixed venous blood samples were obtained for assessment of arterial pH (pHa), mixed venous pH (pH $\bar{v}$ ), partial pressure of oxygen in arterial blood (PaO<sub>2</sub>), saturation of hemoglobin with oxygen in arterial blood (SaO<sub>2</sub>), partial pressure of oxygen in mixed venous blood (P $\bar{v}$ O<sub>2</sub>), saturation of hemoglobin with oxygen in mixed-venous blood (S $\bar{v}$ O<sub>2</sub>), partial pressure of carbon dioxide in arterial blood (PaCO<sub>2</sub>), and partial pressure of carbon dioxide in mixed venous blood (P $\bar{v}$ CO<sub>2</sub>). Arterial and mixed venous oxygen saturation were measured with a standard electrode technique (ABL 500; Radiometer, Denmark) and blood haemoglobin (Hb) concentrations were measured spectrophotometrically (OSM 3; Radiometer, Denmark). Samples for blood gases were stored on ice until analysis. Blood gas values were corrected for atmospheric pressure but not body temperature and P50 in the horse.

### 3.10 Endothelin-1 analysis (Studies II-IV)

Mixed venous blood was collected in chilled tubes containing EDTA (final concentration, 10mM) and centrifuged at 4°C for 10 minutes to separate the plasma which was subsequently analyzed for ET-1 concentration. Acid ethanol was added to precipitate the protein. The precipitate was analyzed for ET-1-like immune reactivity by use of a radioimmunoassay involving antiserum raised against ET-1 in rabbits, which are immunized for antibody production at the laboratory performing the assays. The detection limit of the assay was 1.91 picomol, the intraassay variation was 6% and the inter-assay variation was 14%. The cross-reactivity of the E1 antiserum was as follows: ET-1, 100%; ET-2, 27%; ET-3, 8%; and big ET-1, 0.14%. The plasma concentration of ET-1 was expressed as picomol per mL of plasma.

### 3.11 Calculated data (Various studies)

Shunt fraction ( $Q_s/Q_t$ ) was calculated using the following formula:

$$Q_s/Q_t = (C_c'O_2 - C_aO_2)/(C_c'O_2 - C\bar{v}O_2)$$

where  $C_c'O_2$ ,  $C_aO_2$ , and  $C\bar{v}O_2$  are oxygen content of capillary, arterial, and mixed venous blood, respectively. These values were calculated as:  $C_xO_2 = (1.36 \times \text{haemoglobin concentration} \times SxO_2) + (0.003 \times PxO_2)$

Alveolar-arterial oxygen difference  $P(A-a)O_2$  as calculated using:

$$PAO_2 - PaO_2$$

where  $PAO_2$  is the partial pressure of oxygen in the alveoli.

Partial pressure of alveolar oxygen ( $PAO_2$ ) was calculated using:

$$FiO_2 - (PACO_2/R)$$

where  $PaCO_2$  was used as  $PACO_2$  (the partial pressure of carbon dioxide in the alveoli) and  $R$  is the respiratory quotient (0.8).  $FiO_2$  is the fraction of oxygen in the inspired air.

### 3.12 Determination of the PiNO Pulse Length

In order to determine the most effective duration of pulse delivery for each horse (Studies I and II), a flow sensor detecting gas flow was fitted onto the endotracheal tube to trigger the delivery of iNO during the first portion of inspiration in three separate predetermined steps of increased pulse duration. The durations were designed to correspond to approximately 20%, 30%, 45% and 60% of the total inspiratory time in Study I. Because the 20% inspiratory time was fairly ineffective, it was not used in Study II so only 30%, 45% and 60% were used in Study II. The horses were allowed to equilibrate at each pulse length for 15 minutes and then data were collected before the subsequent pulse length was initiated. Pulse duration was not randomized. The pulse length that corresponded to the highest (peak)  $PaO_2$  in the individual horses was delivered for the remainder of each study. In Study II (MIGET), PiNO was pulsed at 30% and 60% of the inspiratory time for 15 minutes each. Based on the data collected in Studies I and II PiNO45% was chosen for Study V.

### 3.13 Data Collection (see time lines at the bottom of this section)

#### 3.13.1 Studies I and II

All data including HR; SAP, MAP and DAP; SPAP, MPAP and DPAP;  $FiO_2$ ; RR; TV; Hb; ETiso; pH<sub>a</sub> and pH<sub>v</sub>;  $PaO_2$ ;  $P\bar{v}O_2$ ;  $SaO_2$ ;  $S\bar{v}O_2$ ;  $P(A-a)DO_2$ ; and

Qs/Qt were measured or calculated following 45 minutes of equilibration after commencement of isoflurane anesthesia prior to NO administration (T45) and after each 15 minute increase in PiNO (T60, T75, T90 and T105 for Study I; T60, T75 and T90 for Study II ) and then every 15 minutes for the duration of the study (T105, T120, T135 and T150). Cardiac output was determined at T45, T105 and T135. Mixed venous blood samples were collected for ET-1 analysis at T45 and T150.

### 3.13.2 Study II (MIGET)

Data were collected after 60 minutes of anesthesia equilibration without PiNO (baseline). Following baseline data collection, NO delivery commenced as described above. NO was pulsed during the first 30% and 60% of inspiration, respectively and data were collected 15 minutes after equilibration at each percentage of PiNO (PiNO30% and PiNO60%). The order of delivery was not randomized.

### 3.13.3 Study III

Data including HR; SAP, MAP and DAP; SPAP, MPAP and DPAP; FiO<sub>2</sub>; RR; TV; Hb; ETiso; pHa and pH $\bar{v}$ ; PaO<sub>2</sub>; P $\bar{v}$ O<sub>2</sub>; SaO<sub>2</sub>; S $\bar{v}$ O<sub>2</sub>;[A-a]DO<sub>2</sub>; and Qs/Qt were measured or calculated at the time that the PiNO was discontinued (T0) and every minute for 10 minutes (T1 through T10), followed by every 5 minutes for 20 minutes (T15 through T30) for a total of 30 minutes of data collection. All data were collected or calculated at PT0, PT1, PT5, PT10, PT25, and PT30 minutes. At all other times, all data were measured or calculated except for pH $\bar{v}$ , P $\bar{v}$ O<sub>2</sub>, S $\bar{v}$ O<sub>2</sub>, Qt, P(A-a)O<sub>2</sub>, and Qs/Qt. Venous blood samples for ET-1 analysis were collected at PT0, PT10 and PT15 minutes.

### 3.13.4 Study IV

All data including HR; RR; pHa and pH $\bar{v}$ ; PaO<sub>2</sub>; P $\bar{v}$ O<sub>2</sub>; SaO<sub>2</sub>; S $\bar{v}$ O<sub>2</sub>; P(A-a)O<sub>2</sub>; and Qs/Qt were measured or calculated at the time that the isoflurane, oxygen and PiNO were discontinued (T0) and every two minutes for 10 minutes (T2-T10), followed by every 5 minutes for 20 minutes (T15-T30) for a total of 30 minutes of data collection.

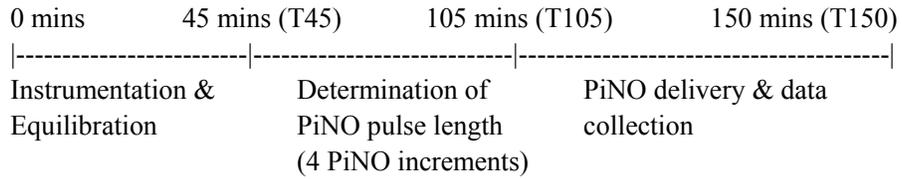
### 3.13.5 Study V

All data including HR; SAP, MAP and DAP; SPAP, MPAP and DPAP; RR; TV; pHa, pH $\bar{v}$ ; PaO<sub>2</sub>; P $\bar{v}$ O<sub>2</sub>; SaO<sub>2</sub>; S $\bar{v}$ O<sub>2</sub>; Qt; and ET-1 concentration were measured or calculated following 60 minutes of anesthesia equilibration (PrePiNO), following 30 minutes of PiNO delivery (PiNO) and 30 minutes

after PiNO cessation (PostPiNO). At the same times, MIGET and scintigraphy examinations were performed.

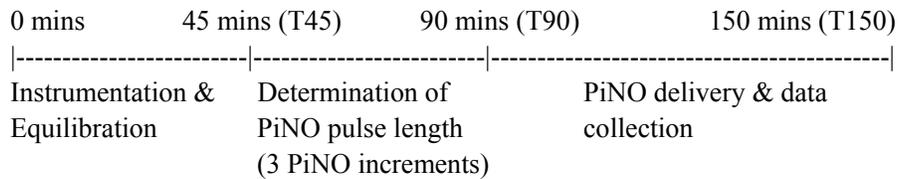
### 3.14 Time lines for data collection

#### 3.14.1 Study 1: Long term delivery of PiNO in *dorsally* recumbent horses



Data collected at times (T): T45 (anesthesia baseline), T60, T75, T90, T105, T120, T135, T150

#### 3.14.2 Study II: Long term delivery of PiNO to *laterally* recumbent horses



Data collected at times (T): T45 (anesthesia baseline), T60, T75, T90, T105, T120, T135, T150



### 3.15 Data Analysis

Studies I-IV: Data were assessed for normal distribution using the Shapiro-Wilk test. Repeated measures ANOVA was used to compare data within and between groups. The Bonferroni significant difference test was used for post hoc comparisons.

Study V: Data were assessed for normal distribution using the Shapiro-Wilk test. Repeated measures ANOVA was used to compare physiologic data within and between groups. For each horse, measurement and event (PrePiNO [baseline], PiNO [treatment] and PostPiNO [recovery]), the ratio Dorsal/(Dorsal+Ventral) was calculated for the counts in each ROI. This ratio was compared between time points using a mixed-effects ANOVA with horse as a random factor and event as a fixed factor. The Bonferroni significant difference test was used for post hoc comparisons..

Data from II (MIGET) and V (MIGET) were presented as descriptive data only.

For all statistical calculations, a software package (GraphPad Prism, GraphPad Software, CA, USA) was used and a value of  $p < 0.05$  was considered significant.

## 4 Results and Discussion

### 4.1 Preparing for Clinical Use: Efficacy of PiNO

PiNO pulsed in the first 30-45% of the inhalation phase of the breath caused an increase in arterial ( $\text{PaO}_2$  and  $\text{SaO}_2$ ) and mixed venous ( $\text{P}\bar{\text{v}}\text{O}_2$  and  $\text{S}\bar{\text{v}}\text{O}_2$ ) oxygenation and a decrease in  $\text{Qs}/\text{Qt}$  in horses anesthetized long term in dorsal (Study I) or lateral (Study II) recumbency, anesthetized following discontinuation of PiNO (Study III), recovering from anesthesia following the discontinuation of isoflurane, 100% oxygen and PiNO (Study IV) and during short-term delivery in dorsal recumbency for the scintigraphy study (Study V). PiNO also improved  $\text{V}_\text{A}/\text{Q}$  matching as determined by MIGET (Studies II and V) and caused a redistribution of blood from dependent to non-dependent lung regions as determined by scintigraphy (Study V). Thus, PiNO is efficacious and provides beneficial changes in gas exchange and pulmonary function during and after isoflurane anesthesia in healthy horses.

#### 4.1.1 Determination of effective pulse duration

In humans, iNO has traditionally been delivered continuously so that it is inhaled throughout the entire inspiratory period (Barst et al. 2012). However, more recently, PiNO has been utilized as pulse of iNO delivered at the beginning of inspiration (Channick et al. 1996, Ivy et al. 1998, Kitamukai et al. 2002, Ivy et al. 2003, Pepke-Zaba and Morrell 2003, Vonbank et al. 2003, Barst et al. 2012) and this technique was used in our Studies I, II and V and prior to data collection in Studies III and IV. To determine the shortest duration of PiNO delivery that would effectively increase  $\text{PaO}_2$  and decrease  $\text{Qs}/\text{Qt}$ , the pulses were delivered at different percentages of the inspiratory time in Studies

I and II (*Table 1*). Based on the efficacy of pulses in these studies, PiNO45% was chosen for Study V.

Study Number	Pulse length as a percentage of the duration of inhalation
I	20%, 30%, 43% and 73%
II	30%, 45% and 60%
II (MIGET)	30% and 60%
V	45%

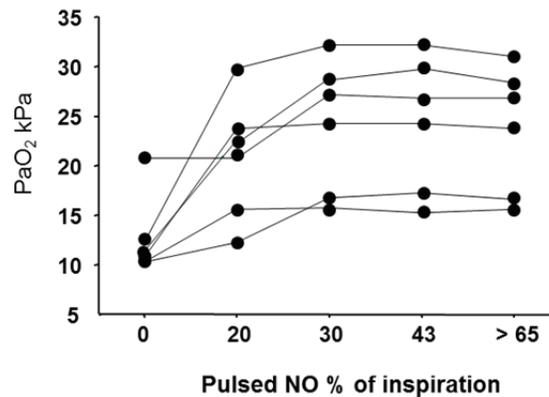
*Table 1.* Studies in which iNO was delivered beginning with the onset of inhalation. Pulse length is described as percentage of the duration of the inhalation phase of the breath.

Administration of PiNO at all pulse lengths improved PaO<sub>2</sub> and Qs/Qt compared to baseline (*Table 2; Figure 7*) in all horses but one (Study II (MIGET) Horse 3).

Study Number		PaO <sub>2</sub> in kPa and [mmHg]	Qs/Qt (%)	# of horses with maximum Improvement (total # of horses in the study)
I	Baseline	12.6±4.1 [95±31]	32 ± 2	
I	20%	20.8±6.2 [156±47]	29 ± 3	0 (6)
I	30%	24.2±6.6 [182±59]	23 ± 4	4 (6)
I	43%	24.3±6.7 [182±50]	22 ± 4	2 (6)
I	73%	23.9±6.6 [179±50]	24 ± 2	0 (6)
II	Baseline	26.9±10.8 [202±81]	28 ± 7	
II	30%	38.5±7.3 [289±55]	22 ± 2	4 (6)
II	45%	37.2±8.1 [279±61]	22 ± 3	2 (6)
II	60%	36.9±8.8 [277±66]	23 ± 7	0 (6)
II (MIGET)	Baseline	18.7±5.2 [140±39]	39 ± 5	
II (MIGET)	30%	30.4±4.6 [228±35]	30 ± 3	3 (3)
II (MIGET)	60%	27.7±0.2 [194±2]	33 ± 3	0 (3)

*Table 2.* Effects of differing pulse lengths on PaO<sub>2</sub> and Qs/Qt. Baseline = no PiNO

However, the pulse length producing the peak effect varied among the individual horses (*Figure 7*). In Study I, the delivery of PiNO20% improved PaO<sub>2</sub> but the improvement was not significant in all horses. The maximum increase in PaO<sub>2</sub> and decrease in Qs/Qt occurred at PiNO30% in 4 horses and at PiNO45% in 2 horses. In Study II, the maximum increase of PaO<sub>2</sub> occurred at PiNO30% in 4 horses and PiNO45% in 2 horses. Qs/Qt was not significantly different among those pulse lengths. Both PiNO73% (Study I) and PiNO 60% (Studies II and II (MIGET)) not only failed to provide a further increase in PaO<sub>2</sub> in most horses but actually caused a slight decrease in PaO<sub>2</sub> and increase in Qs/Qt in 2 horses in Study I (*Figure 7*) when compared to the PiNO45%. In Horse 3 Study II (MIGET), PiNO60% caused a deterioration in PaO<sub>2</sub>, Qs/Qt and log SDQ to values below (PaO<sub>2</sub>) or above (Qs/Qt, log SDQ) baseline (prePiNO) values. However, that horse had a fairly high PaO<sub>2</sub> at baseline and the PaO<sub>2</sub> values were never in the hypoxemic range nor were the changes significant.



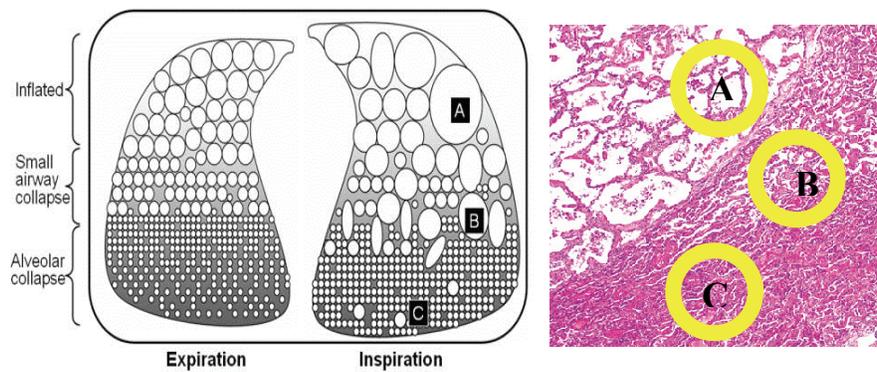
*Figure 7.* Individual horse (n=6) response to PiNO administered in four different pulse lengths (average 20%, 30%, 43% and >65% of the inspiratory time; Study I) on arterial oxygen tension (PaO<sub>2</sub>).

In addition to the pulse lengths listed in the table, data that was not published in Study II (MIGET) was collected when PiNO was delivered during the middle 50-85% of the breath. With this delivery, there was a decrease in PaO<sub>2</sub> and an increase in Qs/Qt compared to PiNO30% and PiNO60% in two horses and no change in the third horse. Thus, we have shown that a short pulse of PiNO is optimal for improved pulmonary function and that the short pulse needs to be synchronized with the beginning of inhalation. These results are similar to those from previous studies in which iNO pulsed into the first phase

of inhalation was more effective than iNO pulsed during the late phase of inhalation (Heinonen et al. 2001, Heinonen et al. 2002). Based on this body of work, we believe that it is the pulsed-delivery – and the duration and timing of that pulse – that is crucial for maximum effects from PiNO.

CiNO does improve PaO<sub>2</sub> in some human patients but fails to provide improvement in others (Ichinose et al. 2004, Griffiths and Evans 2005, Bloch et al. 2007, Creagh-Brown, Griffiths and Evans 2009). Just as CiNO did increase PaO<sub>2</sub> in conscious foals with experimentally induced pulmonary hypertension (Lester et al. 1999) but failed to increase PaO<sub>2</sub> in adult halothane-anesthetized horses (Young et al. 1999). Based on the success of the pulse delivery technique in our studies, we propose that PiNO might provide a more consistent response than CiNO. PiNO has recently been introduced in human medicine and appears to be as equally efficacious and safe as CiNO in a review of initial trials (Barst et al. 2012). The rationale for the success of PiNO is based on the probable distribution of iNO within the lungs and the fact that both timing and duration of the pulse should result in iNO distribution to aerated lung regions (Heinonen, Hogman and Merilainen 2000, Heinonen, Merilainen and Hogman 2003).

PiNO delivered in the early phase of inhalation will selectively reach the lung regions that are ventilated but poorly perfused. Because alveoli that are distended are easier than collapsed alveoli to fill with air, gases entering the airways during the initial phase of inhalation move easily into the well-ventilated alveoli. Once these alveoli are filled, inhaled gases begin to move into alveoli that are in the so-called transitional or border zone (the zone between ventilated and atelectatic alveoli; *Figure 8*).



*Figure 8.* From a sketch on the left and a histological slice of tissue on the right we see alveoli that are A=inflated; B=border zone; C=atelectatic (Lapinsky and Mehta 2005).

Transitional or border zone alveoli are not fully distended and have a fairly high closing volume, which means that they do not open with every breath (low  $V_A/Q$ ). Thereby, these alveoli do not consistently participate in gas exchange. CiNO allows distribution to all alveoli that can be opened, including those that are intermittently open and poorly ventilated, whereas delivery of PiNO during the early phase of inhalation allows selective NO distribution only to the consistently open, well-ventilated alveoli. The iNO-mediated vasodilation and increased blood flow to well-ventilated alveoli improves PaO<sub>2</sub> because blood flowing to these alveoli becomes fully saturated with oxygen with every breath. Conversely, the iNO-mediated vasodilation and increased blood flow to intermittently ventilated alveoli could fail to increase, and could even decrease, the systemic PaO<sub>2</sub> since blood perfusing those alveoli could return to circulation without becoming fully saturated with oxygen. Furthermore, these areas of poor ventilation would normally not receive a high percentage of blood flow because of hypoxic pulmonary vasoconstriction (HPV). Even a small uptake of NO with subsequent vasodilation could potentially counteract the local HPV, thereby increasing perfusion in the low  $V_A/Q$  and shunt regions of the lung with a subsequent decrease in PaO<sub>2</sub>. These effects could potentially contribute to the failure of NO to improve oxygenation during CiNO. The PaO<sub>2</sub> impact of perfusion of low  $V_A/Q$  regions of the lung is demonstrated by the alveoli on the left in *Figure 1* in the introduction.

However, the shortest pulse of 20% of the inhalation time used in Study I was only moderately effective and this could be due to the fact that the pulse is actually too short. The earliest percentage of delivered PiNO fills the deadspace (ventilated portions of the airway that do not participate in gas exchange) and reaches only a small number of ventilated alveoli, thus making a minimal impact on PaO<sub>2</sub>. Conversely, with the longest pulse lengths, (73% in Study I and 65% in Study II), the iNO, in addition to being distributed to ventilated lung regions, could reach into the boundary zone between ventilated and atelectatic lung regions and this could explain why these longer pulse lengths failed to further improve pulmonary function when compared to the intermediate duration iNO pulses. In Studies I and II the initial improvement of PaO<sub>2</sub> and Qs/Qt with pulse lengths of 30%-45% followed by the deterioration in the same parameters with the longer pulse lengths supports the theory that iNO delivered as a pulse in the first part of inhalation reaches the alveoli that are actively participating in gas exchange, while continuing the delivery reaches transitional or border zone alveoli.

#### 4.1.2 Indices of efficacy and physiologic data

PiNO administered to hypoxemic horses improved arterial ( $\text{PaO}_2$  and  $\text{SaO}_2$ ) and venous ( $\text{P}\bar{\text{v}}\text{O}_2$  and  $\text{S}\bar{\text{v}}\text{O}_2$ ) oxygenation and decreased  $\text{Qs}/\text{Qt}$ . Since inhaled NO is rapidly inactivated by hemoglobin in the pulmonary circulation, systemic effects of the gas should be minimal to absent and this is evidenced by our data in which no changes in  $\text{Qt}$  or ABP occurred. Minor respiratory depression (as evidenced by increased  $\text{PaCO}_2$  and decreased  $\text{pHa}$ ) did occur in all horses and was exacerbated in the PiNO horses. All other physiologic variables had minor or insignificant changes.

##### *PaO<sub>2</sub> and SaO<sub>2</sub>*

Partial pressure  $\text{O}_2$  ( $\text{PaO}_2$ ) dissolved in blood and the percentage of hemoglobin saturated with  $\text{O}_2$  ( $\text{SaO}_2$ ) are commonly used to evaluate pulmonary function since the primary role of the lung is to bring  $\text{O}_2$  into the body while eliminating  $\text{CO}_2$  from the body.

##### Effects during anesthesia

PiNO increased both  $\text{PaO}_2$  and  $\text{SaO}_2$  in anesthetized horses in Studies I, II and V. The results of Study II are particularly interesting because the inclusion of the control group allowed an assessment of the magnitude of the improvement in oxygenation with PiNO. In this study, both the  $\text{PaO}_2$  and the  $\text{SaO}_2$  were significantly higher in the PiNO horses than in the C horses throughout the entire anesthetic period (*Figure 9*). In these laterally-recumbent horses, both  $\text{PaO}_2$  and  $\text{SaO}_2$  were fairly high at baseline so the values for these parameters were not significantly greater than baseline at any point in the PiNO horses. However, both  $\text{PaO}_2$  and  $\text{SaO}_2$  were significantly lower than baseline in the C horses at the last 3 data collection times. Our horses were fairly deeply anesthetized and anesthetic depth affects ventilation so the difference between the groups could be due to impaired ventilation. However the C horses were actually ventilating more effectively (as determined by  $\text{PaCO}_2$ , see **section** on ‘other physiologic parameters’) than the PiNO horses at these time points. The continued decline in  $\text{PaO}_2$  could also be due to the slow onset of absorption atelectasis. Although the high  $\text{FIO}_2$  could cause absorption atelectasis in both groups, the PiNO-mediated redistribution of blood away from the atelectatic regions would decrease the impact of those regions on the  $\text{PaO}_2$ .

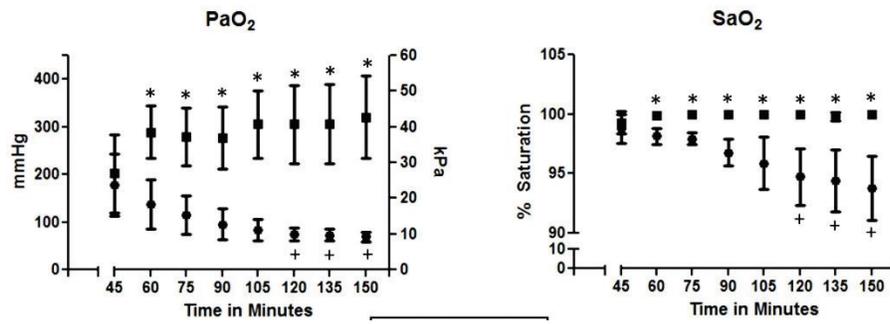


Figure 9. PaO<sub>2</sub> and SaO<sub>2</sub> from laterally recumbent isoflurane anesthetized horses (Study II). Data collection began 45 minutes after anesthesia was induced and continued for 150 minutes. Horses indicated with a square received PiNO beginning immediately after data collection at time 45. Horses indicated with a circle received the same anesthetic protocol but did not receive PiNO. Asterisks indicate significant differences between groups. Crosses indicate significant difference from baseline (Time 45) measurements. Significance = p<0.05.

As expected, PaO<sub>2</sub> was very low at baseline in the dorsally recumbent horses (Figure 10) and was increased by PiNO administration. The increase in PaO<sub>2</sub> was sustained over a long duration in both Study I and Study II. The efficacy of PiNO over a prolonged duration (150 minutes) is important because the duration of equine surgical procedures can be quite long.

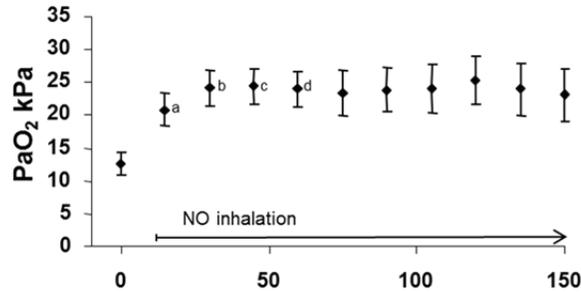
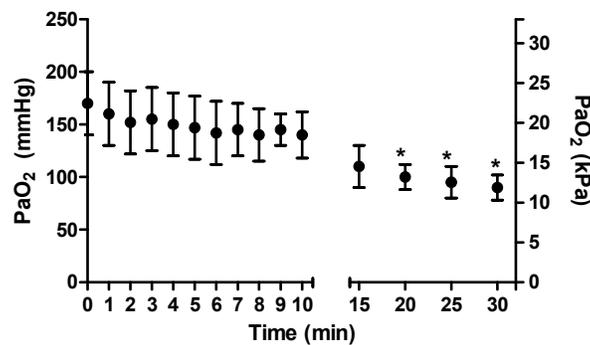


Figure 10. PaO<sub>2</sub> from dorsally recumbent isoflurane anesthetized horses (Study I). Data collection began 45 minutes after anesthetic stabilization and continued for a total of 150 minutes. a=PiNO20%, b=PiNO30%, c=PiNO43%, d=PiNO60%. PaO<sub>2</sub> was significantly higher than baseline at all times. Significance = p<0.05.

### Effects during recovery

The positive impact of PiNO on oxygenation continued even after the cessation of PiNO in both Studies III and IV. Following discontinuation of PiNO while the horses were still anesthetized (Study III; *Figure 11*), the PaO<sub>2</sub> and SaO<sub>2</sub> dropped slowly but steadily. The PaO<sub>2</sub> did decrease below baseline but not to the hypoxemic range. The decrease was probably due to increased perfusion to the atelectatic area with a worsening of Qs/Qt following withdrawal of the PiNO. These horses, in comparison to the Study IV horses were still receiving isoflurane and FiO<sub>2</sub> >0.95 so the decline of PaO<sub>2</sub> in this group could also be due to anesthesia-related hypoventilation, anesthesia-related impairment of HPV or a slow development of absorption atelectasis. This study was done as a precursor to Study IV to first test the safety of discontinuing PiNO in anesthetized horses before moving to a true recovery study where all anesthetic support would be discontinued.



*Figure 11.* Gradual decrease in PaO<sub>2</sub> following discontinuation of PiNO during isoflurane and FiO<sub>2</sub> >0.95 oxygen administration (Study III). PiNO was discontinued at Time 0 and data was collected for 30 minutes. \*=significant difference (p<0.05) from Time 0.

The results in Study IV were surprising and exciting. In Study IV, both the PaO<sub>2</sub> and the SaO<sub>2</sub> were significantly higher in the NO horses than in the C horses throughout the entire recovery period (*Figure 12*) with no decrease over time in either group. This prolongation in oxygenation could have a major positive effect on recovery and was unexpected as PaO<sub>2</sub> returned to pre-iNO baseline within 5 minutes after cessation of PiNO in a previous study (Heinonen et al. 2001). The difference could be that the long-duration delivery results in a more prolonged effect as both the arterial and venous blood become optimally saturated with oxygen. Neither P $\bar{v}$ O<sub>2</sub> nor S $\bar{v}$ O<sub>2</sub> were reported in those studies.

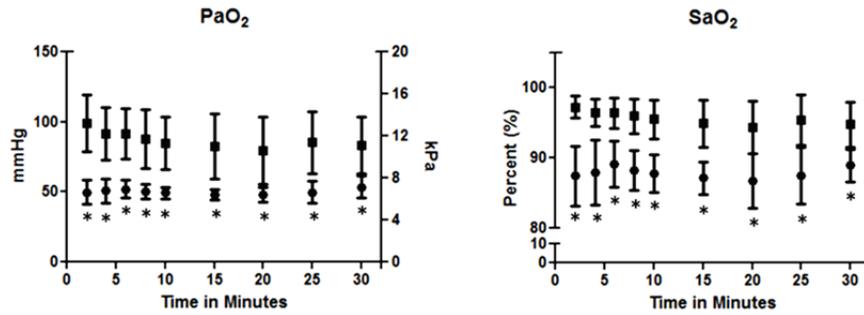


Figure 12. PaO<sub>2</sub> and SaO<sub>2</sub> from horses recovering from isoflurane anesthesia (Study IV). Horses indicated with a square received PiNO during anesthesia and horses indicated with a circle received the same anesthetic protocol but did not receive PiNO. Asterisks indicate significant differences between groups. Significance = p<0.05.

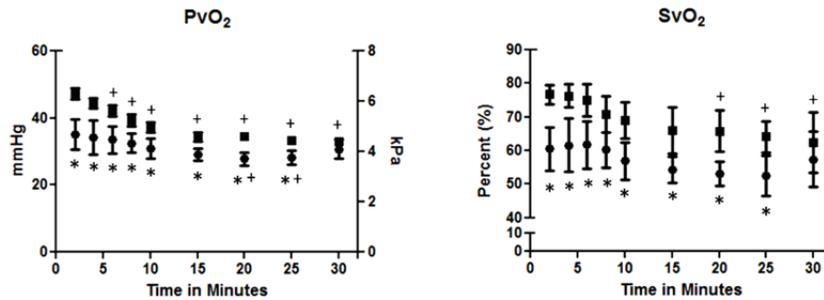
### *P $\bar{v}$ O<sub>2</sub> and S $\bar{v}$ O<sub>2</sub>*

Venous oxygen parameters may not appear as useful as arterial oxygen parameters but, in fact, venous oxygen parameters describe oxygenation at the tissue level, where oxygen is required as a metabolic substrate for all homeostatic processes. The balance of oxygen delivery (DO<sub>2</sub>) and oxygen uptake (VO<sub>2</sub>) at the tissue level can be calculated if the Qt is measured, but can also be estimated by the S $\bar{v}$ O<sub>2</sub>. This is an extremely important parameter as adequate oxygen delivery to the tissues is the goal of resuscitative therapy yet the end-point can be difficult to determine since Qt and ABP do not indicate actual DO<sub>2</sub>. In fact, even with normalization of standard goal directed therapy (eg, MAP>70mmHg), DO<sub>2</sub> may not be adequate (Scalea et al. 1990, Bannon et al. 1995, Pope et al. 2010, Hosking et al. 2011). With the calculation of CaO<sub>2</sub>, DO<sub>2</sub> can be determined but this does not determine the utilization of oxygen (VO<sub>2</sub>) at the cellular level and an imbalance of delivery and consumption, leading to cellular hypoxia, can exist without being identified. Fortunately, P $\bar{v}$ O<sub>2</sub> and S $\bar{v}$ O<sub>2</sub> can be used to estimate the balance between DO<sub>2</sub> and VO<sub>2</sub> (Reinhart and Bloos 2005). S $\bar{v}$ O<sub>2</sub> is commonly utilized in human medicine as a prognostic index in critical care patients (Pope et al. 2010). Normal S $\bar{v}$ O<sub>2</sub> values are roughly 70-75% (Hosking et al. 2011) and values below 60-65% have been correlated with increased morbidity and mortality in patients with certain diseases (Hosking et al. 2011). Hyperoxia (S $\bar{v}$ O<sub>2</sub> >90%) is also associated with increased mortality, presumably due to decreased tissue utilization of oxygen (Pope et al. 2010).

In our studies, both  $P\bar{v}O_2$  and  $S\bar{v}O_2$  were increased during PiNO delivery (Studies I and II) and decreased as PiNO effects waned (Studies III and IV). The magnitude of the impact of PiNO on venous parameters is most obvious in Studies II and IV because the PiNO horses can be compared to the C horses.

#### Effects during Anesthesia

In Study II (*Figure 13*), both  $P\bar{v}O_2$  and  $S\bar{v}O_2$  were greater in the PiNO horses than in the C horses at the last 3 ( $S\bar{v}O_2$ ) or 4 ( $P\bar{v}O_2$ ) time points and the  $P\bar{v}O_2$  was greater than baseline at the last two time points. One horse in the C group had a  $S\bar{v}O_2$  in the upper 60% range at the last 3 time points.



*Figure 13.*  $P\bar{v}O_2$  and  $S\bar{v}O_2$  from laterally recumbent isoflurane anesthetized horses (Study II). Horses indicated with a square received PiNO during anesthesia and horses indicated with a circle received the same anesthetic protocol but did not receive PiNO (C group). Asterisks indicate significant differences between groups. Crosses indicate significant difference from baseline measurements. Significance =  $p < 0.05$ .

#### Effects during Recovery

In the recovery study (Study IV; *Figure 14*) both  $P\bar{v}O_2$  and  $S\bar{v}O_2$  decreased within the first 10 minutes after cessation of anesthesia and PiNO and then stabilized. The decrease was significant from baseline only in the PiNO group. However, in spite of significant decrease from baseline, both  $P\bar{v}O_2$  and  $S\bar{v}O_2$  were higher in the PiNO horses than in the C horses until the very end of the recovery period.  $S\bar{v}O_2$  average values in the PiNO horses were in the 60% range at T10-30. In the C horses  $S\bar{v}O_2$  average was in the 60% range from T2-8 and in the 50% range from T10-30 (lowest value average 7 kPa [53 mmHg] at T25), meaning that the  $S\bar{v}O_2$  was in the possible critical range in the C horses from the time that 100% oxygen was discontinued. The highest  $S\bar{v}O_2$  in the C horses (average 8.3 kPa [62 mmHg]) occurred immediately after

cessation of FiO<sub>2</sub> 0.95 oxygen and anesthesia, whereas the lowest S $\bar{v}$ O<sub>2</sub> in the PiNO horses (average 8.4 kPa [63 mmHg]) occurred at T30 when the horses were struggling to stand.

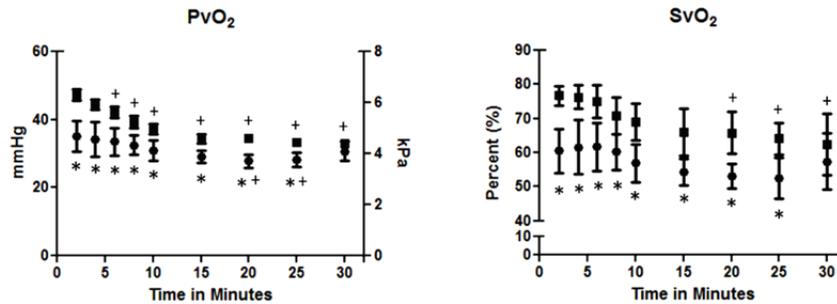


Figure 14. P $\bar{v}$ O<sub>2</sub> and S $\bar{v}$ O<sub>2</sub> from horses recovering from isoflurane anesthesia (Study IV). Horses indicated with a square received PiNO during anesthesia and horses indicated with a circle did not receive PiNO (C group). Asterisks indicate significant differences between groups. Crosses indicate significant difference from baseline measurements. Significance = p<0.05.

#### General comments

Decreased P $\bar{v}$ O<sub>2</sub> and S $\bar{v}$ O<sub>2</sub> is caused by: 1) decreased DO<sub>2</sub> due to ventilatory (ie, hypoxemia leading to decreased PaO<sub>2</sub> and SaO<sub>2</sub>) or cardiovascular (eg, decreased Qt, hemoglobinemia, anemia, hypovolemia, hemorrhage or shock) factors, and/or 2) increased VO<sub>2</sub> due to factors that increase oxygen consumption (eg, hypermetabolism, hyperthermia, shivering, pain, movement or seizures). By analyzing each of these components, the cause of the differences between the PiNO and C horses can be determined. First, the Qt in horses in Study II did not change during the study. In Study IV, due to the waning effects of inhalant gases and the beginning of spontaneous movement, we would expect the cardiac output to increase, not decrease, during recovery from anesthesia. Second, none of the horses in either group were hypermetabolic, hemoglobinemic, anemic, hypovolemic, hemorrhaging, in shock, hyperthermic, in pain or seizing. Thus, the significant differences in the S $\bar{v}$ O<sub>2</sub> between the two groups of horses were due to the fact that the C horses were more hypoxemic than the PiNO horses throughout the entire study period in both Studies II and IV. The decline of S $\bar{v}$ O<sub>2</sub> that occurred in Study IV regardless of the initial oxygenation status, was most likely due to increased VO<sub>2</sub> due to the initial shivering that the horses were experiencing in early recovery followed by movement and standing in mid to late recovery. All

horses had minor movements (lifting the head or moving a limb) by T10-15 and all had attempted to attain sternal recumbency or to stand by T 20.

Although the prognostic value of  $S\bar{v}O_2$  for morbidity and mortality has not been established in the horse and all of these horses recovered without incident, these were healthy horses and the outcome could potentially be different in unhealthy horses. Horses anesthetized for surgical correction of colic had a greater negative oxygen balance and decreased muscle oxygenation than healthy horses (Edner et al. 2007). Since decreased tissue oxygen, or ischemia, is implicated in post-anesthetic myopathy and neuropathy (Wagner 2008a), improved tissue oxygenation during the recovery period could potentially decrease the incidence of these morbidities and perhaps improve the safety of equine anesthesia.

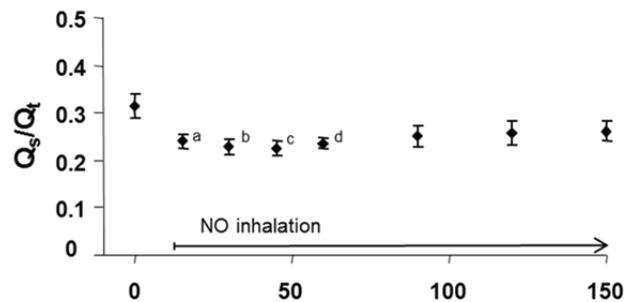
Because lactate is produced during anaerobic metabolism, plasma lactate concentrations can also be used to evaluate tissue oxygen delivery. Increased lactate is a predictor of morbidity and mortality in both humans (Bakker and de Lima 2004, Mikkelsen et al. 2009) and horses (Tennent-Brown et al. 2010, McCoy et al. 2011). Measurement of lactate in our studies would have allowed an interesting comparison to  $S\bar{v}O_2$ .

#### *Qs/Qt*

The PiNO-induced decrease in  $Qs/Qt$  was used as a major determinant of improved pulmonary function in our studies because  $Qs/Qt$ , as calculated by the classic Berggren equation (Berggren 1942), is widely considered to be the 'gold standard' of pulmonary function calculations (Nelson 1993). As stated in the introduction,  $Qs/Qt$  is the ratio of the portion of blood shunted from the venous circulation to the arterial circulation without being oxygenated ( $Qs$ ) to the total blood flow ( $Qt$ ). In addition to the atelectasis that causes intrapulmonary shunt in horses, intrapulmonary shunt can also be caused by diffusion impairment and by blood flow to areas of consolidation (eg, tumors or abscesses). A normal intrapulmonary shunt in humans is <5% (Johnson 2004) and this fits with the shunt identified in standing horses (1%) (Hedenstierna et al. 1987). A shunt of 5-15% would be considered mild, 15-30% would be 'major' and >30% would be 'severe' in humans (Johnson 2004). If  $Qs/Qt$  is >50%, the  $PaO_2$  is not likely to improve with oxygen therapy. As can be seen by our data, anesthetized horses commonly develop  $Qs/Qt$  that would be considered 'severe' for humans, further emphasizing the dramatic adverse effects of anesthesia on equine respiratory physiology.

### Effects during anesthesia

PiNO delivery decreased Qs/Qt in all studies. In dorsally recumbent horses (Study I; *Figure 15*), the Qs/Qt decreased with the first dose of PiNO (PiNO20%) from 32% at baseline and remained at an average of 25% throughout the entire duration of the 2.5 hour study. This is lower than the Qs/Qt that often occurs in dorsally recumbent horses and the horses in dorsal recumbency in V (MIGET) were more representative with a Qs/Qt of just over 50% at baseline. In spite of the magnitude of the Qs/Qt, the horses did respond to treatment and Qs/Qt decreased to just over 30% during PiNO administration. The Qs/Qt increased back to baseline 30 minutes after PiNO cessation.



*Figure 15.* Qs/Qt from dorsally recumbent isoflurane anesthetized horses (Study I). a=PiNO20%, b=PiNO30%, c=PiNO45%, d=PiNO60%. PaO<sub>2</sub> was significantly higher than baseline at all times. Significance = p<0.05.

In laterally recumbent horses (Study II; *Figure 16*), Qs/Qt was again decreased with the first dose of PiNO (PiNO30%) and was significantly lower in the PiNO than the C horses at all times throughout the duration of the study except at baseline. However, the Qs/Qt data were more variable in this study than in Study I, which kept the values from reaching a statistical difference from baseline values. The Qs/Qt was in the 30% range from T45-T75 and 40% range from T90-T150 in the C horses and in the 20% range in the PiNO horses at all times.

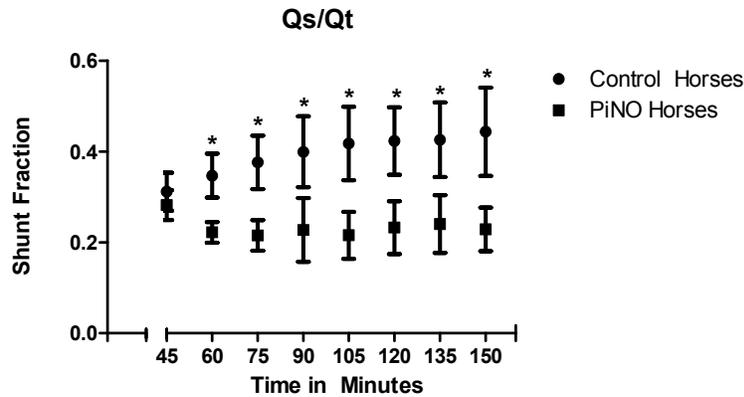


Figure 16. Qs/Qt from laterally recumbent isoflurane anesthetized horses (Study II). Horses indicated with a square received PiNO beginning immediately after data collection at time 45. Horses indicated with a circle received the same anesthetic protocol but did not receive PiNO. Asterisks indicate significant differences between groups. Significance =  $p < 0.05$ .

#### Recovery effects

The improvement in pulmonary function was sustained in recovery (Study IV; **Figure 17**) and the Qs/Qt in the PiNO horses (range 13-17% at various time points) was lower than the Qs/Qt in the C horses (range 30-48% at various time points) throughout the entire recovery period. The Qs/Qt of the control group slowly decreased but did remain significantly greater than the PiNO group. The decrease was potentially due to improved ventilation and re-expansion of alveoli with low  $V_A/Q$  or to the waning effects of the high intra-operative  $FiO_2$  with a subsequent decrease of the degree of absorption atelectasis. The prolonged effect on Qs/Qt is a major finding in our work since Qs/Qt is a marker of pulmonary function. If pulmonary function is improved throughout recovery, horses would be less likely to become hypoxemic, even if recovery was prolonged. The improvement in pulmonary function could be an indication to administer PiNO even in horses that are only marginally hypoxemic.

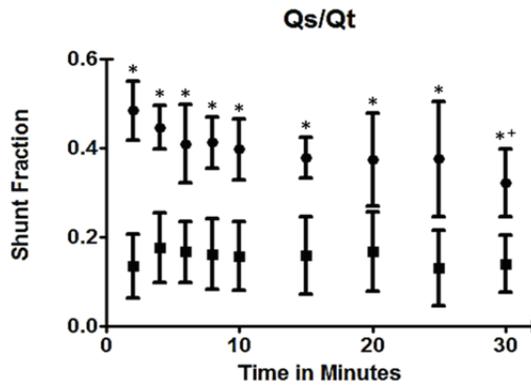


Figure 17. Qs/Qt (shunt fraction) from horses recovering from isoflurane anesthesia (Study IV). Horses indicated with a square received PiNO during anesthesia and horses indicated with a circle received the same anesthetic protocol but did not receive PiNO. Asterisks indicate significant differences between groups. Crosses indicate significant difference from baseline measurements. Significance =  $p < 0.05$ .

However, in Study III (Figure 18), the Qs/Qt gradually increased to a point that was significantly higher than baseline (mean 40%) but not abnormally high for dorsally recumbent horses. These horses were still anesthetized with isoflurane and  $FiO_2 > 0.95$  oxygen so the increase maybe due to a gradual return to a high percentage of  $V_A/Q$  mismatch or to the continued effect of a high  $FiO_2$  on absorption atelectasis, or both.

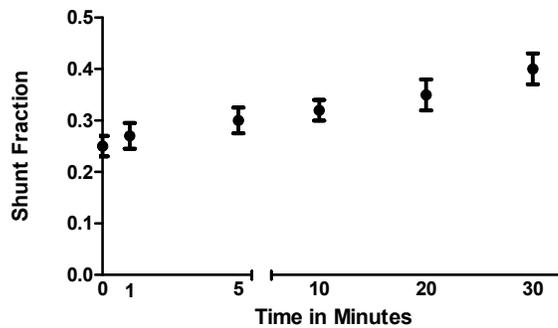


Figure 18. Gradual increase in Qs/Qt (shunt fraction) in horses anesthetized with isoflurane and oxygen following discontinuation of PiNO during anesthesia (Study III). PiNO was discontinued at Time 0 and data was collected for 30 minutes. \*=significant difference ( $p < 0.05$ ) from Time 0.

### General comments

Although it is considered the gold standard for Qs/Qt calculations, there are some limitations to the Berggren shunt equation. Primarily, the formula does not allow a differentiation between intrapulmonary and extrapulmonary shunting since venous admixture from the Thebesian and pulmonary veins is included in the Qs/Qt value. Also, the Berggren equation does not allow differentiation of Qs/Qt from very low  $V_A/Q$  regions nor does it allow the identification of diffusion impairment. Differentiation of various causes of Qs/Qt can be important in determining the mechanism of action of drugs or techniques designed to improve pulmonary function, such as the delivery of PiNO. Finally, the equation is based on some measurements (arterial and mixed venous blood gas values) but also on some calculations (pulmonary capillary oxygen content) and errors in calculation or errors in assumptions (like  $PaCO_2 = PACO_2$ ) can lead to errors in Qs/Qt values. The most precise way to determine true intrapulmonary Qs/Qt is with the MIGET (Wagner 2008b).

### *P(A-a)O<sub>2</sub>*

Shunt fraction can also be estimated in clinical practice by a variety of oxygen tension indices that use a comparison of the driving pressure ( $FiO_2$  or  $PAO_2$ ) for diffusion of oxygen into the pulmonary capillary blood to the actual  $PaO_2$  (Johnson 2004). Indices include  $P(A-a)O_2$ ,  $PaO_2/FiO_2$ ,  $PaO_2/PAO_2$  and others. Oxygen tension indices are often used to assess pulmonary function in clinical patients since the indices can be calculated with data obtained from an arterial blood gas ( $PaO_2$  and  $PaCO_2$ ) with no need to collect mixed venous blood as is required to calculate Qs/Qt. In Studies II, III and IV we calculated  $P(A-a)O_2$  as a marker of pulmonary function. PiNO delivery decreased  $P(A-a)O_2$  in all studies and cessation of PiNO resulted in a slow increase in the  $P(A-a)O_2$  values. In studies II and IV the  $P(A-a)O_2$  was larger in the C horses than in the PiNO horses. Thus, the  $P(A-a)O_2$  values in our study paralleled the Qs/Qt values, as found in other equine studies (Marntell et al. 2005b). However, values for  $P(A-a)O_2$  can be skewed by factors other than pulmonary function, including the  $FiO_2$  and the  $PaCO_2$ . In our studies, the horses were on various  $FIO_2$  (1 in Studies II and III; roughly 0.3 in Study IV) and increased  $FiO_2$  causes an increased  $P(A-a)O_2$  that seems to exceed the actual increase in Qs/Qt (Staffieri et al. 2009, Johnson 2004). Also, the horses were hypercarbic in many of the studies and hypercarbia will falsely decrease the value for  $P(A-a)O_2$  due to a lower calculated  $PAO_2$ . Finally, the true Qs/Qt value can be skewed because  $PaCO_2$  is used as equivalent to  $PACO_2$ , and this assumption is not always true (Robertson and Hlastala 1977).  $P(A-a)O_2$  has failed to correlate to Qs/Qt in equine studies. For instance,  $P(A-a)O_2$  significantly increased

following detomidine sedation in standing horses ( $0.5 \pm 0.4$  pre-sedation,  $2.2 \pm 0.7$  post-sedation) but the change in  $Q_s/Q_t$  was insignificant ( $1.1 \pm 0.3$  pre-sedation,  $1.3 \pm 0.4$  post-sedation) (Nyman et al. 2009). Therefore, we based the conclusions regarding the magnitude of shunt in our studies on  $Q_s/Q_t$ .

*Ventilation-perfusion distribution measured by MIGET*

The gold-standard for measuring intrapulmonary  $Q_s/Q_t$  and  $V_A/Q$  matching is the MIGET, which uses the solubility of six inert gases to determine the  $V_A/Q$  relationships from  $Q_s/Q_t$  ( $V_A/Q=0$ ) to  $V_D/V_T$  ( $V_A/Q=\infty$ ). A major advantage of  $Q_s/Q_t$  determined with MIGET is that the extrapulmonary venous admixture is not included in the  $Q_s/Q_t$  values and the percentage of areas of low  $V_A/Q$  can be differentiated from  $Q_s/Q_t$  (Wagner 2008b). MIGET was first described in the 1970s by Wagner (Wagner 2008b) and has since been used in a variety of species (Frans et al. 1993, Schmekel et al. 1994, Batchinsky et al. 2006, Strang et al. 2010) to determine  $V_A/Q$  matching. MIGET was adapted for the horse in 1987 by Hedenstierna & Nyman (Hedenstierna et al. 1987) and was used to evaluate pulmonary function in conscious standing horses. In that study,  $Q_s/Q_t$  was  $1.2 \pm 0.4\%$  and  $\log SDQ$  was  $0.41 \pm 0.05$ . The RSS was an average of 4.9. MIGET has also been used to determine the  $V_A/Q$  relationships in horses: anesthetized with halothane (Nyman and Hedenstierna 1989); sedated with detomidine and butorphanol (Nyman et al. 2009); sedated with romifidine and butorphanol with or without acepromazine followed by tiletamine-zolazepam anaesthesia (Marntell et al. 2005a); conscious, sedated and anesthetized breathing either  $FiO_2$  of 0.21 or  $>0.95$  (Marntell et al. 2005b); anesthetized receiving clenbuterol (Dodam et al. 1993); during heavy exercise (Wagner et al. 1989, Seaman et al. 1995) and prolonged exercise (Hopkins et al. 1998); breathing helium during exercise (Erickson et al. 1994); with red cell hypervolemia (Funkquist et al. 1999); with chronic (Nyman et al. 1991) or mild (Nyman et al. 1999) bronchiolitis; and with a history of *Rhodococcus equi* pneumonia as foals during high intensity exercise as adults (Funkquist et al. 2002).

Other values of particular interest obtained from the MIGET include the standard deviation of the logarithmic distribution of perfusion ( $\log SDQ$ ) and the standard deviation of the logarithmic distribution of ventilation ( $\log SDV$ ), which are dimensionless numbers that describes the degree of dispersion or  $V_A/Q$  match/mismatch. In humans, normal  $\log SD$  values range 0.4–0.6, moderate disease may cause an increase 1.0 while severe disease would result in  $\log SD$  of 1.5–2.5 (Wagner 2008b). The residual square of sums (RSS) which describes the ‘goodness of fit’ or precision of the model and is a quality control

assessment. Acceptable RSS values are <6. MIGET was used in dorsally recumbent horses receiving PiNO for 5 minute intervals (Heinonen et al. 2001). In that study Qs/Qt was 32±2, 22±2, and 30±3 in spontaneously breathing horses before, during and 15 minutes after PiNO administration and 26±4, 19±4 and 25±4 in horses with IPPV at the same time periods. LogSDQ prePiNO was 0.46-1.15 in individual horses and decreased to 0.37-0.79 during PiNO (indicating more efficient matching of V<sub>A</sub>/Q) with a return to baseline postPiNO. RSS for this study was 0.3-5.6. These data are comparable to the data obtained in our studies. MIGET was used in Studies II (lateral recumbency) and V (dorsal recumbency and scintigraphy). Results from Study V (MIGET) will be discussed in the ‘mechanism of action’ section. In Study II (MIGET) (Table 3; Figure 19), PaO<sub>2</sub> increased while Qs/Qt and logSDQ decreased in all horses with PiNO30%. In horses 1 and 2, PiNO60% caused minimal changes from PiNO30%. As previously mentioned, in horse 3, PiNO60% caused deterioration in PaO<sub>2</sub> compared to PiNO30% and values returned towards baseline values. The marked improvement in arterial oxygenation during PiNO in horses 1 and 2 was due to an evident reduction of Qs/Qt with minimal impact on low V<sub>A</sub>/Q areas. Compared to baseline, Qs/Qt decreased in all three horses during PiNO30% and was lower in two horses and higher in one horse during PiNO60%. Log SDQ decreased during PiNO and the RSS of all studies indicated a good fit of the data to the predicted data.

Time Variable	Study II (MIGET)			Study V (MIGET)		
	PrePiNO	PiNO30%	PiNO60%	PrePiNO	PiNO	PostPiNO
Log SDQ	0.79±0.10	0.70±0.07	0.66±0.02	1.00±0.14	0.83±0.13	0.98±0.09
Qs/Qt%	39±8	30±5	33±6	51±5	32±6	51±8
PaO <sub>2</sub> kPa	18.7±9.0	30.4±4.6	27.7±0.4	6.7±0.8	16.8±6.3	8.9±1.9
PaO <sub>2</sub> mmHg	140±67	228±35	208±3	50±6	126±47	67±15

Table 3. Physiologic data excerpted from Study II (MIGET) and Study V (MIGET).

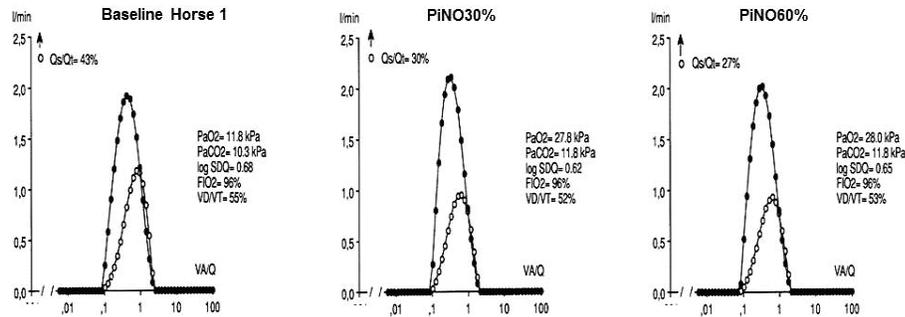


Figure 19. Ventilation-perfusion distribution in one horse (Study II (MIGET)). Data from one horse is presented as an example of the changes induced by PiNO. The ventilation-perfusion distribution ( $V_A/Q$ ) presented as a log scale and the ventilation and perfusion in  $L \text{ min}^{-1}$  as open and closed circles, respectively.

One limitation of the MIGET is that it can be used to determine the degree of matching of ventilation with perfusion but it cannot provide information on the actual localization of the changes in perfusion (Wagner 2008b), which will be discussed in a later section.

#### *Pulmonary arterial pressure (PAP)*

A primary goal of iNO therapy in humans is to improve oxygenation by decreasing the PAP in patients with pulmonary arterial hypertension (PAH) (Ichinose et al., 2004, Griffiths and Evans, 2005, Bloch et al., 2007, Creagh-Brown et al., 2009). Inhaled NO can relieve PAH in horses, as evidenced by the fact that iNO decreased PAP in exercising horses with pulmonary hypertension (Mills et al. 1996, Kindig et al. 2001). However, PAP was measured in Studies II and horses in those studies did not have lower PAP during PiNO administration as would have been expected when considering the mechanism of action of iNO (Table 3). PAP did slowly increase in Study III and this will be further discussed in the safety section. Failure of PiNO to decrease PAP in horses also occurred in earlier studies from our laboratory (Heinonen et al. 2001, Heinonen et al. 2002).

Although no significant changes in overall PAP were detected, the lung is a very heterogeneous organ and  $V_A/Q$  matching can vary in different regions of the lung. The inability to detect a global change does not exclude the fact that regional differences might occur. Also, the well-ventilated areas of the lung were poorly perfused but not necessarily hypertensive in our studies so a reduction of PAP was not necessary for improved gas exchange. In healthy human volunteers, breathing endogenous (nasal) or exogenous iNO, the iNO

caused a redistribution of pulmonary perfusion (Sanchez Crespo et al. 2010). Because these patients were probably not afflicted with pulmonary hypertension (although PAP was not measured), the redistribution of the blood flow represents a movement of blood in response to vasodilation in well ventilated areas with a subsequent improvement in  $V_A/Q$  matching rather than a decrease in PAP.

Redistribution of flow is an important concept for the use of PiNO in anesthetized horses. Rather than being used to improve oxygenation by reduction of PAP, in anesthetized horses PiNO would be used to improve oxygenation through redistribution of blood flow from low to high  $V_A/Q$  regions in patients that are likely to have normal pulmonary blood pressures. This may further add to the safety of PiNO in horses because it is possible that a lower dose of iNO is required to improve oxygenation than is required to relieve PAH (Griffiths and Evans 2005). Another potential reason for the lack of decreased PAP during PiNO in our horses is that anesthesia may have caused a decrease PAP, thereby masking any increase that might have been detectable in the absence of anesthesia. Finally, there is a potential that we are evaluating the wrong parameter. In healthy conscious human volunteers, blockade of endogenous NO by L-NG-monomethyl Arginine citrate (L-NMMA) caused an increase in pulmonary vascular resistance by almost 40% but no change in PAP because of a concurrent decrease in Qt (Stamler et al. 1994). Although Qt remained steady in our horses, the Qt in anesthetized horses is lower than the Qt in conscious horses and this could mask or blunt any changes in pulmonary vascular resistance (PVR). A final possibility for the lack of change in PAP is that pulmonary hypertension is mediated by HPV and there are reasons that alterations in HPV might have affected our results. HPV is discussed in detail in a subsequent section.

Study II	PAP PrePiNO (T45)	PAP range (lowest to highest) during PiNO
PiNO horses	26.8± 8.8	23.7± 7.6 (T105) to 25.7± 7.1 (T60)
Control horses	26.7± 4.4	23.3± 7.5 (T60) to 28.0± 6.5 (T120)

*Table 4.* Pulmonary artery pressure in Study II (horses anesthetized in lateral recumbency with isoflurane and oxygen without (C) or with PiNO).

## *Other physiologic variables*

### Cardiovascular data

HR was measured in all studies and ABP and Qt were measured in all studies except Study IV. HR was slightly but significantly lower during the iNO treatment in Study I, slightly higher PostPiNO than PrePiNO in Study V, but was not different between groups or over time in Study II. ABP increased in the first 30 minutes of data collection in Studies I and II in all groups. Qt also increased slightly over time in Study I but did not change and was not different between groups in Study II. Because the ABP increased in the C group as well as the PiNO groups, we do not attribute the increase to PiNO-mediated systemic changes but rather to normal adaptation to inhalant anesthesia as has been previously described (Steffey et al. 1987) or to hypercarbia-mediated cardiovascular stimulation (see respiratory data below). In Study III, the HR increased slowly over time but the ABP did not change so presumably the SV decreased. The increase in the HR may have been in response to the declining PaO<sub>2</sub>. In Study IV, there was no difference in HR between groups. ABP, MPAP and Qt were not measured during recovery due to the danger of having the equipment in the recovery stall as the horse regained consciousness.

A limitation that could affect clinical use is that, at various times, horses in all studies were hypotensive and the hypotension was not treated. Hypotension, which can alter PaO<sub>2</sub> by decreasing pulmonary blood flow, is generally treated in anesthetized horses by the administration of positive inotropic drugs, like dobutamine. However, the administration of dobutamine also increases Qs/Qt, possibly due to increased blood flow in dependent atelectatic lung areas (Swanson and Muir 1986). Hypotension would, by necessity, be treated in clinical patients and treatment could potentially decrease the effectiveness of PiNO.

### Respiratory data

During anesthesia (Studies I, II and V), all horses had varying degrees of respiratory depression as indicated by increased PaCO<sub>2</sub> and respiratory acidosis. There were no statistically significant differences in RR or TV in either Study I or II but the minor differences that did occur contributed to the hypercarbia and respiratory acidosis. Both PaCO<sub>2</sub> and pH<sub>a</sub> were significantly different in the PiNO group than the C group at time points towards the end of anaesthesia in Study II. Horses in both groups experienced anesthesia-induced respiratory depression and suppression of the hypercarbic respiratory drive. However, we are unclear as to the mechanism of the difference between the PiNO and C groups. The most likely cause is increased ventilation due to

hypoxemic respiratory drive in the C horses, however, hypoxemic respiratory drive reportedly does not occur until the PaO<sub>2</sub> is <8 kPa (60 mmHg) and the lowest PaO<sub>2</sub> in the C group was approximately 9.2 kPa (69 mmHg) at T150. However, this value for initiation of hypoxemic drive is for humans and the value for horses is unknown, so perhaps hypoxemic respiratory drive occurs at a higher PaO<sub>2</sub> in horses. In Study V, the RR was rapid in one horse and this is discussed in the ‘mechanism of action’ section. In study III, the RR rate did not change but the TV increased at the final time points, causing a decrease in the PaCO<sub>2</sub>. The increase in the TV may have been in response to the declining PaO<sub>2</sub>.

In Study IV, no differences in RR occurred over time or between groups. The RR increased quickly after cessation of anesthesia (from 3-5 breaths/min at the end of anesthesia to 11-13 breaths/min in recovery) and remained stable throughout recovery in both groups. PaCO<sub>2</sub> was higher at Time 2 than at any other time point in both groups of horses but this difference was only significant in the PiNO horses and there were no other differences within or between groups over time. The horses were mildly hypercarbic and acidemic throughout recovery but the PaCO<sub>2</sub> was lower and the pH<sub>a</sub> was higher when compared to values measured during anesthesia in studies I and II. These results are due to the increased RR, which is an expected response in horses recovering from the respiratory-depressant effects of anesthesia.

The fact that hypercarbia was fairly common but was not treated could be a limitation that might affect clinical use of PiNO. Hypercarbia, which can alter gas exchange as described by the alveolar gas equation, is generally treated using mechanical ventilation. However, as stated in the introduction, positive pressure ventilation has been shown to decrease total blood flow and to alter V<sub>A</sub>/Q and Q<sub>s</sub>/Q<sub>t</sub>, thus hypercarbia was not treated in our study. Hypercarbia would, by necessity, be treated in clinical patients and treatment could potentially decrease the effectiveness of PiNO.

## 4.2 Preparing for Clinical Use: Safety of PiNO

Discontinuation of PiNO pulsed in the first 30-45% of the inhalation phase of the breath did not cause a rapid and/or dangerous ‘rebound’ increase of PAP or Q<sub>s</sub>/Q<sub>t</sub> nor a decrease of PaO<sub>2</sub> in anesthetized horses (Study III) or horses recovering from anesthesia (Study IV). Plasma concentrations of ET-1 did not increase during (Studies II, III and V) or after (Study IV) anesthesia. NO did not accumulate in the anesthesia breathing circuit (Studies I, II (MIGET), III) and N<sub>2</sub>O was not detected (Study III). Thus, PiNO should be considered safe to deliver during isoflurane anesthesia in healthy horses.

#### 4.2.1 'Rebound Effect'

As stated in the introduction, withdrawal of iNO can produce a precipitous decrease in PaO<sub>2</sub> with a concurrent increase in Qs/Qt and PAP that is potentially mediated by an increase of circulating ET-1 concentrations -1) (Chen et al. 2001, McMullan et al. 2001, Wedgwood et al. 2001, Pearl et al. 2002). Our results indicate that, following the withdrawal of PiNO, an ET-induced rebound effect does not develop in healthy isoflurane-anesthetized horses.

##### *Indices of rebound*

We used potential changes in oxygenation, Qs/Qt, PAP and ET-1 concentrations to monitor for a rebound effect.

##### Oxygenation, Qs/Qt and PAP

The lack of a rapid decrease in PaO<sub>2</sub> or SaO<sub>2</sub> (*Figure 6*) or a rapid increase in Qs/Qt (*Figures 12 and 13, above*) after the discontinuation of PiNO in horses still receiving isoflurane and FiO<sub>2</sub> > 0.95 (Study III) suggested that a rebound effect would not occur following PiNO discontinuation in a true anesthesia recovery situation. Study III was completed before attempting a true recovery situation because horses recovering from anesthesia can be quite dangerous and, if a rebound effect following PiNO discontinuation occurred, the horse could be hard to treat. In fact, not only did a rebound effect fail to occur following discontinuation of PiNO, isoflurane and FiO<sub>2</sub> > 0.95 in Study IV, PiNO delivered during anesthesia caused a prolonged beneficial effect that allowed both arterial and venous oxygenation (*Figures 5 and 8*) to remain higher and Qs/Qt (*Figure 11*) to remain lower in the PiNO group than the C group throughout most of recovery. The reason for the prolonged effect on oxygenation is unclear but could potentially be related to distant effects of PiNO (Hambraeus-Jonzon et al. 2001) or to the fact that the baseline oxygenation was very high, prolonging time to desaturation (Edmark et al. 2003).

One limitation is that PAP was not measured in Study IV because it was dangerous to have the monitoring equipment in the recovery stall as the horses were regaining consciousness. However, PAP measured in Study III slowly increased (baseline 14±3; range following discontinuation of PiNO 14±1 to 17±1) but was never significantly different from baseline nor higher than expected for horses under anesthesia and a slow, minor change does not fit the definition of rebound. Furthermore, decreasing oxygenation and increasing shunt are also hallmarks of a rebound effect and neither of these phenomena occurred in the horses in Study IV.

### ET-1

ET-1, an endogenous endothelium-derived peptide is an extremely potent vasoconstrictor. Like NO, ET-1 acts in close proximity to its site of synthesis. Also like NO, ET-1 may contribute to basal pulmonary vascular tone (Johnson et al. 2002). Increased ET-1 concentrations cause pulmonary vasoconstriction with subsequent PAH, and increased ET-1 is a component of the pulmonary vasoconstrictive response to hypoxia (ie, HPV), although whether it is a primary component or a supporting component is unknown (Moudgil, Michelakis and Archer 2005, Sylvester et al. 2012)(see more on HPV in the next section). Increased ET-1 may also be a component of the rebound phenomena and ET-1 was chosen as a marker for rebound because concentrations of the peptide increase during iNO administration (Chen et al., 2001, McMullan et al., 2001, Wedgwood et al., 2001, Pearl et al., 2002). Although the exact mechanism of the rebound effect is unknown, the increase in ET-1 and the concomitant decrease in NOS activity are likely contributors. As in other species, ET-1 is a potent vasoconstrictor in the pulmonary vessels in horses (Benamou et al. 2003, Benamou, Marlin and Lekeux 2001) and ET-1 concentrations increase in horses known to have diseases that cause pulmonary vasoconstriction, like recurrent airway obstruction (Benamou et al. 1998, Costa et al. 2009).

In our studies, plasma ET-1 concentrations did not increase either during (Studies II and V, *Table 5*) or after (Studies III and V, *Table 5* and Study IV *Figure 14*) PiNO administration. The ET-1 data from Study IV are quite interesting because the C group of horses, which were more hypoxemic than the PiNO group at all times, also had higher ET-1 concentrations at all times, although there was considerable variability in the data and significance was only reached at T2, T20 and T25.

<b>Study II</b>	<b>T45</b>		<b>T150</b>
ET-1 PiNO picomol/mL	6.62± 1.89		7.82± 1.16
ET-1 C picomol/mL	6.71± 2.04		7.38± 2.92
<b>Study III</b>	<b>0 mins (T0)</b>	<b>10 mins</b>	<b>30 mins</b>
ET-1 picomol/mL	4.40 ± 0.46	4.62 ±0.42	5.04 ±0.50
<b>Study V</b>	<b>PrePiNO</b>	<b>PiNO</b>	<b>PostPiNO</b>
ET-1 picomol/mL	5.59±0.51	7.65±1.91	5.49±2.43

*Table 5.* ET-1 concentrations during isoflurane anesthesia with or without PiNO (Study II), following cessation of PiNO in anesthetized horses (Study III) and before, during and after PiNO administration for 15 minutes (Study V). Data are presented as mean±SEM.

Although, ET-1 increases during PiNO, ET-1 also increases in response to both acute and chronic hypoxia (Ferri et al. 1995, Trakada and Spiropoulos 2001, Talbot et al. 2008). It is possible that the low doses of PiNO produced by pulse-delivery are too low to increase ET-1 concentrations, while the hypoxemia experienced by the C horses did increase ET-1 concentrations. A reduction of ET-1 concentration also occurs during acute lung injury, potentially because of anti-inflammatory properties of iNO (Busch et al. 2006). However, our horses were healthy and this is unlikely to be the case in our studies. Because PiNO has not previously been studied in-depth in anesthetized horses, we cannot rule out the fact that ET-1 may not increase in response to iNO in this species.

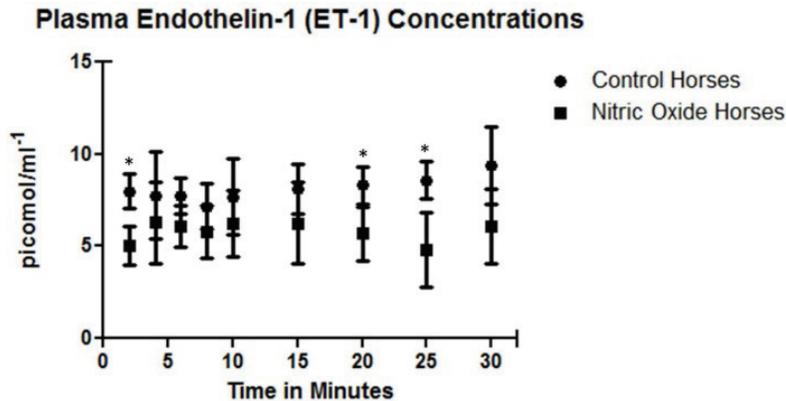


Figure 20. Plasma ET-1 concentrations from horses recovering from isoflurane anesthesia (Study IV). Horses indicated with a square received PiNO during anesthesia and horses indicated with a circle received the same anesthetic protocol but did not receive PiNO. Asterisks indicate significant differences between groups. Significance =  $p < 0.05$ .

The use of ET-1 concentrations as a marker for the rebound effect could also be a limitation of our study. Although the fact that the lack of adverse changes in arterial and venous oxygenation and Qs/Qt indicate that a rebound did not occur, we may be looking for the wrong marker as ET-1 may not be responsible for producing hypoxic pulmonary hypertension (Johnson et al. 2002) or a rebound effect in all species. Also, ET-1 can be difficult to consistently detect, even after stimuli that should cause an increase in circulating ET-1 (Morganti et al. 2000). Although hypoxia does seem to consistently produce an increase in ET-1 (Morganti et al. 2000), the difficulty in detection could increase the variability in results and could explain some of the inconsistent values that occurred in our study. Also, the assay that we used

has not been validated for the horse, thus our data can be compared within our study and but may be invalid when compared to other studies.

*Why no rebound?*

When compared to other studies, there are several components of our studies that might explain the lack of a rebound effect. The impact of ET-1 and endogenous NO have already been discussed. The other most likely components are the delivery method of the iNO, species studied, anesthetic drugs used, and health of study animals

PiNO delivery method

As stated above, there is the potential that the low dose of iNO delivered by pulse does not induce an increase in ET-1 concentrations and thus might not induce a rebound effect.

Horses and hypoxic pulmonary vasoconstriction (HPV)

In response to hypoxemia, systemic arteries dilate but pulmonary arteries constrict in a phenomena termed ‘hypoxic pulmonary vasoconstriction’ (HPV). The mechanism of HPV is not completely elucidated but it begins with low alveolar oxygen tension (low PAO<sub>2</sub>) and ends with contraction of the pulmonary arterial smooth muscle cells located primarily in resistance arteries of <200 mm (Moudgil et al. 2005). HPV is a locally-produced, endothelium-dependent protective mechanism designed to limit blood flow to hypoxemic areas of the lung in order to optimize V<sub>A</sub>/Q matching and decrease the impact of hypoxemic lung regions on PaO<sub>2</sub> (Nagendran et al. 2006).

The constrictive response of the pulmonary vasculature to hypoxia is highly variable among species and marked differences occur in both the magnitude of the HPV response and the oxygen level required to provoke the response (Peake et al. 1981). The horse appears to elicit a fairly weak HPV response, although not as weak as that of the dog (Elliott et al. 1991). HPV occurs in equine and bovine isolated pulmonary arterial rings but the HPV response in equine pulmonary vessels is approximately 33% less than the response in bovine vessels (MacEachern et al. 2004). In the intact animal, ponies develop only moderate HPV yet pigs develop profound HPV (Elliott et al. 1991). In one-lung ventilation models of hypoxemia, the pig develops profound HPV (Hambraeus-Jonzon et al. 2001) while the horse fails to develop any HPV (Lerche 2006) response. The cause(s) of the species differences are unknown and, in a 154 page review of HPV, the author offered only this theory, ‘The mechanisms underlying species variability in HPV are unknown; however, the ubiquity of the response and observations that differences are quantitative rather than qualitative argue against variability in the fundamental mechanisms

of HPV. More likely, species differences result from variability in mechanisms that modulate HPV (Sylvester et al. 2012).

Among other species, the rebound phenomena has been reported in humans (Ichinose et al. 2004), pigs (Chen et al. 2001) and sheep (Black et al. 1999, Ross et al. 2005). Pigs produce a profound HPV response and can experience a profound rebound effect. The HPV response in horses is less than that of the pig, and horses in our study did not have a rebound effect. To our knowledge, the HPV response in humans has not been directly compared with that of horses or pigs, but results of research in altitude-induced HPV suggest that there is great variability among individuals (Naeije 1997), which could further contribute to the fact that some humans respond to NO inhalation and some do not, and some develop rebound and some do not. So perhaps there is a species-specific intrinsic degree of vasoreactivity that predisposes each species to an inherent HPV response that is mirrored by an inherent rebound effect. However, sheep do not fit this theory as sheep have a minimal HPV response (Sylvester et al. 2012) and yet can have a profound rebound response (Black et al. 1999, Ross et al. 2005). Because the HPV response is very species-specific and because the species specificity cannot be explained, any direct species-species comparisons regarding HPV and pulmonary function in response to hypoxia is difficult.

#### Drugs used for Anesthesia

The sedatives and general anesthetic drugs used in our studies could also have affected our results. Horses were anesthetized with isoflurane and inhalant anesthetic drugs are known to affect HPV. Inhalant anesthetic drugs generally inhibit the HPV response whereas injectable anesthetic drugs may inhibit, maintain, or even potentiate the HPV response (Nagendran et al. 2006). However, a rebound response has been detected in conscious and anesthetized humans, conscious and anesthetized sheep and anesthetized pigs. Thus, because of the wide variety of anesthetics used and the inclusion of conscious subjects in clinical treatment and research trials, it is unlikely that the rebound effect or lack of a rebound effect could be totally attributed to the anesthetic protocol. Nevertheless, it could be argued that the inhalant anesthetic drug used in the present study blunted the HPV response, thereby decreasing the degree of pulmonary vasoconstriction and minimizing the effect of NO inhalation among the study horses. This would indeed decrease the likelihood of a rapid rebound response following cessation of NO inhalation but would also have limited the initial response to NO. Although the horses in our study responded to inhalation of NO, whether the response would have been greater if isoflurane had not been used cannot be determined.

Finally, the variety of effects caused by different anesthetic drugs could explain the fact that halothane-anesthetized horses failed to respond to NO inhalation (Young et al. 1999) and yet isoflurane anesthetized horses in our study did respond to NO inhalation. Whether or not the anesthetic drugs blunted the response to PiNO and the lack of a rebound effect is actually only important as a point of discussion and not as a limitation since the goal of our studies is to find a means to relieve hypoxemia in anesthetized horses. The anesthetic protocol that we used is a standard protocol used in clinical cases. Interestingly, different anesthetic protocols may affect endogenous NO differently, as inhalant anesthesia with halothane caused a significantly lower production of exhaled nitric oxide than romifidine-guaifenesin-ketamine caused (Marlin et al. 2001). This could contribute to the greater impairment of pulmonary function by inhalant anesthesia when compared to injectable anesthesia (Marntell et al. 2005b).

#### Rebound in health and disease

Another important difference (and potential limitation) of our study, compared with other investigations, is the fact that we included only healthy horses, whereas the most of the clinical patients and many of the research animals used in other studies were unhealthy. A rebound effect appears to be more likely in humans with pulmonary hypertension, acute respiratory distress syndrome, hypoxemic respiratory failure, pulmonary hypertension or during or following cardiothoracic surgery or lung transplantation. Among other animals with disease, pigs with endotoxemia (Chen et al. 2001) and neonatal sheep with pulmonary hypertension (Ross et al. 2005) can also develop the rebound effect. Because ET synthesis is increased in disease states (Sanai et al. 1996), (48) an ET-induced rebound effect may be more likely to develop in diseased patients than in healthy patients. Also, the etiology of the rebound effect was different following iNO withdrawal in lambs with normal pulmonary circulation and those with PAH (Ross et al. 2005). The type of disease may also affect the rebound response as rebound has not been reported in patients with chronic PAH or COPD (Vonbank et al. 2003, Barst et al. 2012). iNO does not cause a rebound effect in healthy humans breathing iNO for a short duration (Sanchez Crespo et al. 2010) or our healthy horses. Thus, PiNO delivery to horses with disease that might cause the rebound effect needs to be studied.

#### 4.2.2 Accumulation of NO in the circuit

With the delivery of PiNO rather than CiNO, the total dose and dose length of iNO is decreased and delivered during the first part of the breath, which decreases the chance of NO accumulation in the rebreathing system. The

delivery unit was placed at the close to the patient, at the junction of the endotracheal tube and the circle breathing system, thus minimizing the chance that inhaled NO will react with oxygen to form NO<sub>2</sub> since the likelihood of the reaction increases as the time NO and oxygen are in contact increase. In Studies I and II (MIGET), there were no detectable levels of NO in the circuit within 5 minutes after discontinuing administration, which virtually eliminates the chance that exhaled NO will be available to react with oxygen to create NO<sub>2</sub>. NO<sub>2</sub> was not detected in the circuit at any time in Study III. Gradual weaning from iNO alleviates the chance of a rebound effect (Bloch et al. 2007) and residual NO in the circuit could have produced an inadvertent 'weaning.' However, since no NO was detected in the circuit, the lack of a rebound effect was not the result of a weaning effect from retained NO.

In humans, dosages of 5-80 ppm of continuously delivered iNO are most commonly reported (Ichinose et al., 2004, Griffiths and Evans, 2005, Bloch et al., 2007, Creagh-Brown et al., 2009). With PiNO in humans, dosages of are reported as mls/breath (Barst et al. 2012). In our horses, the dose was based on delivery of PiNO for a certain percentage of the time of inhalation and this is the most practical way to deliver PiNO in the clinical setting with the current delivery method. In reality, the dose was administered 'to effect' since we based our therapeutic pulse length on the lowest duration pulse that would provide the greatest change in PaO<sub>2</sub> and Qs/Qt. The dose delivered to effect as a pulse rather than continuously results in a decreased amount of NO in the system and, thus, less accumulation.

Inhaled NO can cause adverse effects other than just the rebound effect and production of nitrous dioxide. Methemoglobinemia, pulmonary cellular proliferation (due to increased cGMP), DNA strand breaks, production of a cytotoxic oxidant (peroxynitrite), and blockade of platelet aggregation are all related to iNO (Weinberger et al. 2001). However, these adverse effects are uncommon and primarily caused by excessively high dosages. Even at the high end of clinically used dosages, side effects are uncommon. For instance, iNO of anything less than 100 ppm iNO is unlikely to cause clinically significant methemoglobin in children or adults with normal hemoglobin function (Barst et al. 2012).

### 4.3 Mechanism of action of PiNO

Because the classic mechanism for PiNO-induced improvement in PaO<sub>2</sub> and Qs/Qt is relief of pulmonary hypertension and our horses were not hypertensive, the question of mechanism of action remains unanswered.

#### 4.3.1 Contribution from areas of low V<sub>A</sub>/Q and Qs/Qt

Using MIGET in Study II, we suggested that improvement of pulmonary function is due to redistribution of the blood flow with subsequent improvement in V<sub>A</sub>/Q matching and decreased Qs/Qt. However, the question of whether redistribution occurred at individual alveoli scattered throughout the entire lung or by mass movement of blood flow away from the dependent atelectatic Qs/Qt area was not answered. For redistribution to occur in individual alveoli throughout the entire lung, alveoli with low V<sub>A</sub>/Q (low ventilation) and intermittent opening would have to be recruited to consistently participate in gas exchange. However, based on the fact that alveoli with low V<sub>A</sub>/Q are not likely the major cause of hypoxemia (Data from Study IIMIGET and (Nyman et al. 1990), it is unlikely that recruitment of these alveoli will make a major impact. Because the Qs/Qt was identified as a major contributor to hypoxemia in our studies (using Berggren shunt equation in Studies I-V and MIGET in Study II) and was defined as the primary cause of hypoxemia in a former study (Nyman et al. 1990), it may be more likely that blood perfusing the Qs/Qt is actually redistributed by a mass movement of blood from the atelectatic region to regions with ventilation. However, this would require blood to move against gravitational pull because the shunting occurs in the dependent lung regions (Nyman et al. 1990).

#### 4.3.2 Pulmonary blood flow distribution in the horse

To redistribute away from the dependent lung regions in the dorsally recumbent horse blood would also have to move against its normal perfusion pattern. As with many other mammalian species (Mure et al. 2000) (Nyren et al. 1999, Walther, Domino and Hlastala 1998) (Walther et al. 1997), pulmonary blood flow distribution in the horse may be somewhat controlled by gravity but gravity is not the strongest factor. In fact, although markedly heterogenous, distribution appears to be ‘opposite to gravity’ in the standing conscious horse lung, with flow increasing as the vertical distance up the lung increases (Hlastala et al. 1996). Blood flow appears to be preferentially directed to the caudo-dorsal regions of the lung as determined in anesthetized ponies maintained in the prone position (Jarvis et al. 1992), standing and exercising horses (Bernard et al. 1996, Erickson et al. 1999), and standing and exercising horses with or without respiratory disease (Harmegnies et al. 2002).

Distribution that is not solely controlled by gravity is also present in the anesthetized recumbent horse (Staddon and Weaver 1981, Dobson et al. 1985), prompting Dobson & Gleed to state, 'an unidentified factor overrides gravitational effects under our conditions of anaesthetization' (1996). The lack of gravitational control over perfusion distribution is also evident in other species. In the prone position, perfusion is distributed selectively to the dorsal diaphragmatic region but perfusion loses its selectivity and becomes more uniform when the person (Mure et al. 2000) or pig (Nyren et al. 1999) or dog (Walther et al. 1998) or sheep (Walther et al. 1997) is in the supine position. The uniformity of perfusion decreases  $V_A/Q$  matching and oxygenation in the supine position.

This preferential distribution 'against gravity' is likely due to the vasodilatory effects of endogenous NO. In humans, nitric oxide synthase expression and nitric oxide production are significantly higher in dorsal compared to ventral lung regions (Rimeika et al. 2004). In isolated swine arterial rings from various lung regions, NOS activity and vasorelaxation in response to endothelium-dependent induction of endogenous NO production was greater in dorsal than in ventral pulmonary arterial rings (Rimeika et al. 2006). Pelletier et al. (Pelletier et al. 1998) compared vasorelaxation of equine pulmonary arteries from the caudo-dorsal (non-dependent) and cranio-ventral (dependent) lung in response to endothelium-dependent induction of NO production. Profound relaxation occurred in caudo-dorsal vessels but only a slight relaxation, followed by a profound contraction, occurred in the cranio-ventral vessels. The spatial location of the responses likely explains the spatial distribution of pulmonary blood flow. However, the reason for the differing spatial responses is unknown. The following explanations have been offered: difference in responsiveness of vascular smooth muscle to NO; differences in the magnitude of the endothelial production or release of NO; differences in inactivation of NO by oxygen radicals or by NO diffusion to the vascular smooth muscle (Pelletier et al. 1998).

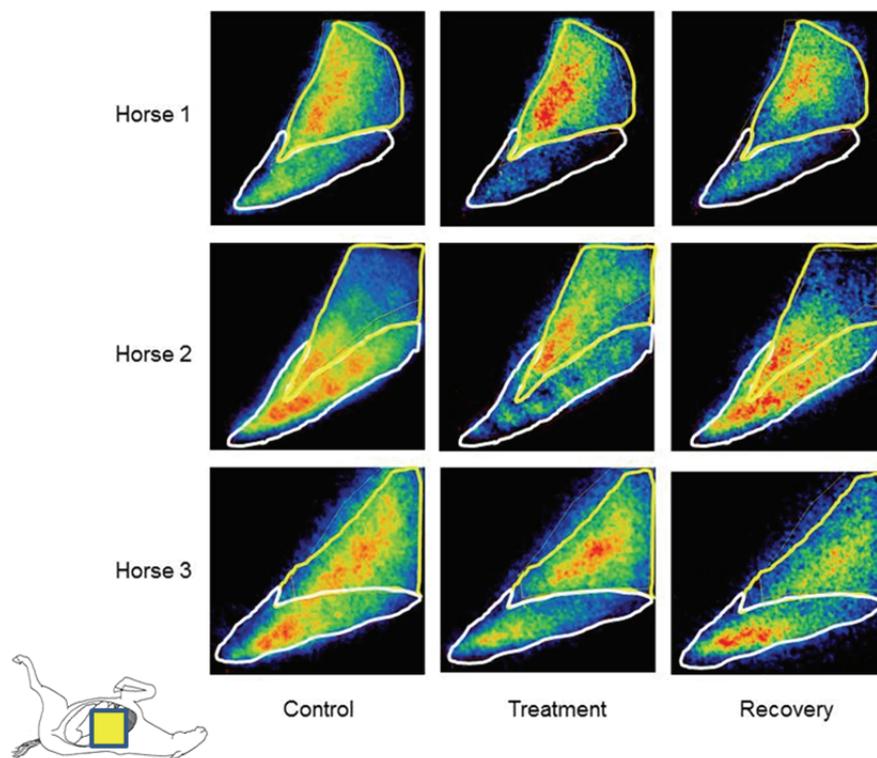
Regardless of the cause of the spatiality of the perfusion distribution, the preferential caudodorsal perfusion in the horse means that pulmonary perfusion in a horse in the dorsal or supine position will preferentially be distributed to the same area that has been made atelectatic by compression from the diaphragm and viscera. This again would necessitate a movement of blood up the vertical plane of the lung if our theory of mass movement is correct.

#### 4.3.3 Pulmonary blood flow redistribution: scintigraphy

The actual spatial distribution (or redistribution) of blood flow in the lungs can be visualized in two dimensions by perfusion scintigraphy. The small particles

of radioactive albumin injected during each examination are completely lodged in the pulmonary vascular bed in the first pass of the injected volume and the distribution and intensity of the radioactivity in the lung is proportional to the distribution and rate of the pulmonary blood flow. Scintigraphy has been used to study distribution or redistribution of blood flow in standing horses, exercising horses and horses with pulmonary disease (Harmegnies et al. 2002, Votion et al. 1999a, Votion et al. 1997, Votion et al. 1999b, Votion and LeKeux 2003, O'Callaghan et al. 1987, Bernard et al. 1996, Erickson et al. 1999, Hlastala et al. 1996).

In Study V, scintigraphic images indicated that, during PiNO delivery, the pulmonary blood flow was redistributed *en masse* from dependent, presumably non-ventilated, regions of the lung to non-dependent, presumably better ventilated, regions of the lung (Figure 21, 22, 23). The blood flow returned to the baseline regions 30 minutes following cessation of PiNO. This is a major finding in our study since a mass movement of blood flow away from gravity, although hypothesized to be the mechanism of action, is still somewhat surprising because of the large vertical gradient that the blood must move in the horse thorax and because of the fact that blood must move from the preferential area of distribution. We did not pursue the mechanism of the mass redistribution but it is likely two-fold, with PiNO-mediated vasodilatory effects in the non-dependent lung and PiNO-mediated vasoconstriction in the dependent lung. The latter mechanism is postulated on the fact that, in healthy pig lungs, iNO downregulates endogenous NO production and causes vasoconstriction in hypoxic lung regions distal to the lung regions receiving iNO (Hambraeus-Jonzon et al. 1998, Hambraeus-Jonzon et al. 2001). Conversely, in endotoxemic pigs, iNO causes an increase in endogenous NO production in lung regions distal to the lung regions receiving iNO. This may cause vasodilation in the distal segments, counteracting the iNO-induced increase in blood flow to the ventilated lung regions and potentially contributing to hypoxemia (Nilsson et al. 2010). This underscores the need to investigate PiNO in compromised horses.



*Figure 21.* Images of relative blood flow before (control), at end of treatment with PiNO delivered for 30 minutes (treatment) and 30 minutes after cessation of inhalation of PiNO (recovery). The left upper border is defined by the diaphragm, the lower border by the spine, and the right border by the edge of the field of view of the gamma camera. The yellow lines define the non-dependent ventral (with increased flow) and the white lines the dependent (dorsal) (with decreased flow) ROIs. They were copied from the functional images in *Figure 6*. The images were made by subtracting the NO treatment image from the baseline image. After inhalation of PiNO, the flow in the non-dependent ventral regions increases (colors more towards the red) and in the dependent dorsal regions decreases (colors more towards the blue). At 30 minutes after cessation of NO inhalation, flows have reverted to approximately the baseline. The field of view is indicated in yellow in the horse sketch to the right of the images. To facilitate drawing the ROIs, especially the isocount line between them, the color scales were adjusted to enhance the contrast between the two ROIs. The color scales have been adjusted in each image to maximize contrast between ROIs so the images are not directly comparable.

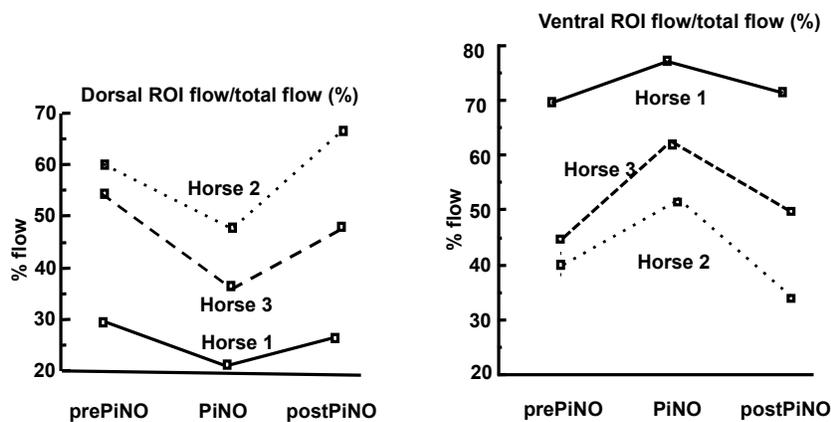


Figure 22. Relative flow in the dependent (dorsal) and nondependent (ventral) regions of interest (ROIs) as percentage of total flow in both ROIs. The percentage of blood flow to the nondependent lung regions increased while the percentage of blood flow to the dependent lung regions proportionately decreased during PiNO, and returned to baseline levels 30 minutes after cessation of PiNO.

A limitation of scintigraphy is that it is a two-dimensional process and the lung is a three-dimensional organ. Thus, we could determine the vertical movement of the blood but could not discern the magnitude of the redistribution horizontally across the lung. However, using CT and necropsy results, the horizontal distribution (not redistribution) was shown to be equally distributed across both lung fields in anesthetized horses (Nyman et al. 1990). For future studies it might be possible to use single photon emission computed tomography (SPECT), which provides a more complete 3-D picture of the redistribution of the blood flow in the lungs. Using SPECT in anesthetized pigs, the atelectatic dependent zone was shown to be uniformly distributed across the lung fields (Strang et al. 2010). The injection of fluorescent microspheres into the circulation can also be used to determine 3-D blood flow and this has been extensively used in horses (Dobson et al. 1985, Sinclair et al. 2000, Lerche 2006). However, limitations of this technique include the fact that final data collection requires sacrifice of the horse, microsphere size can skew data, tissue sampling size and location can skew data and drying of the lung can change the size and configuration, which can also skew the data.

#### 4.3.4 Pulmonary ventilation and blood flow redistribution: MIGET

Data were collected using MIGET at the same time as the scintigraphic scans and RSS was low in all studies, indicating a good fit of our model to the data. With MIGET we demonstrated that  $Q_s/Q_t$  decreased significantly during PiNO compared to PrePiNO which resulted in a prompt increase in  $PaO_2$  and  $SaO_2$  (Figure 23 and Table 2).  $V_A/Q$  matching (as determined by logSDQ) improved in all horses at PiNO but the improvement was not significant (Figure 24). Indices trended toward PrePiNO baseline at PostPiNO and were not different from PrePiNO.

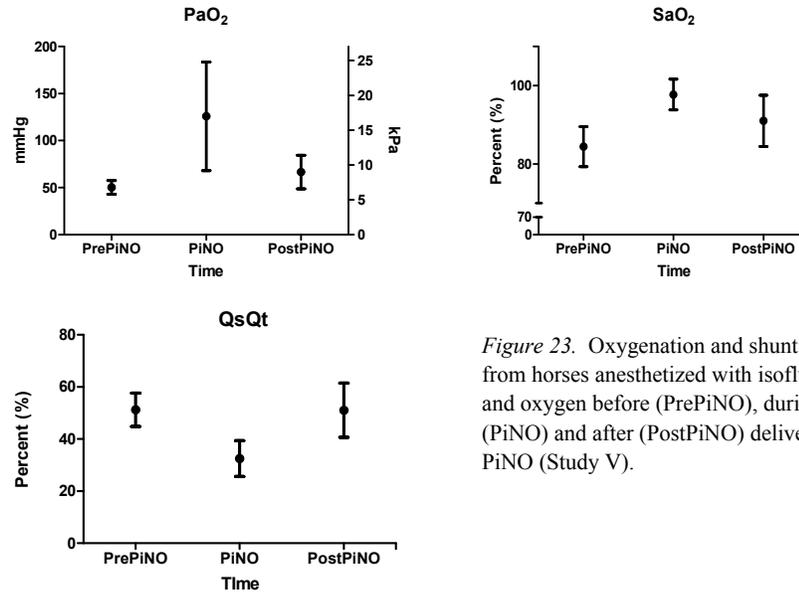


Figure 23. Oxygenation and shunt data from horses anesthetized with isoflurane and oxygen before (PrePiNO), during (PiNO) and after (PostPiNO) delivery of PiNO (Study V).

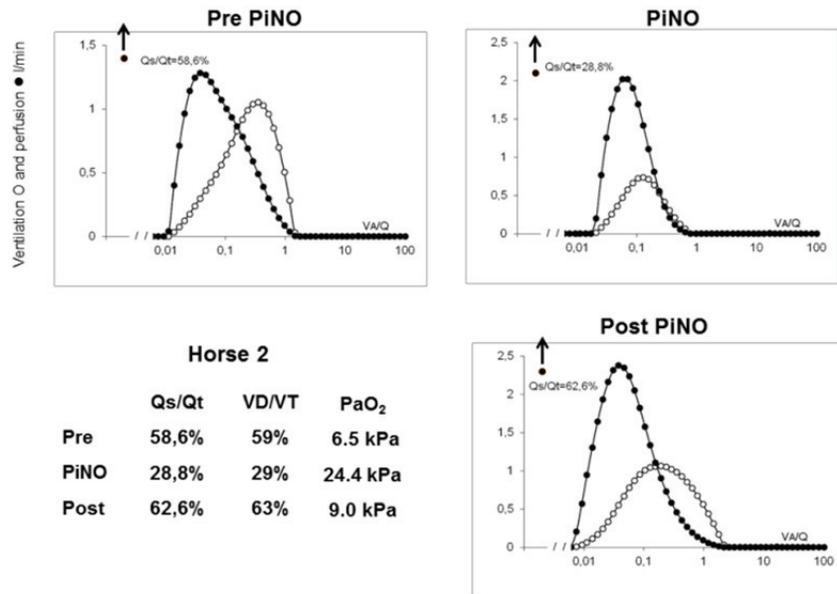


Figure 24. MIGET data from one horse anesthetized with isoflurane and oxygen before (PrePiNO; upper left graph), during (PiNO; upper right graph) and after (PostPiNO; lower graph) delivery of PiNO (Study V).

Changes in oxygenation,  $Q_s/Q_t$  and  $V_A/Q$  were minimal in horse 3 compared to horses 1 and 2, as can be seen by the individual data (Figure 25). This was due to the respiratory pattern of Horse 3, which unveiled a limitation of the PiNO delivery unit. The unit can deliver a pulse in a maximum of 8 breaths/min and Horse 3 was breathing 12 breaths/min, resulting in a delivery of PiNO in every other breath rather than every breath. The limitation is described in the operation manual of the unit: ‘The breath cycle shall be at least 7 seconds long and RR less than or equal to 8. With shorter breath time, false triggering may occur’. False triggering is activation of the unit without delivery of PiNO. This horse also had a very low tidal volume (as can be seen by scintigraphy in Figure 26) so the low tidal volume combined with the rapid respiratory rate meant that Horse 3 received a very low dose of PiNO and had a low, yet still measurable, response. The RR and TV were different from the other horses but were not outside the normal limits of anesthetized horses. However, because the RR and TV were outside the limits of the delivery unit, the pulsed dose was too small to achieve maximum effect. As with the delivery of PiNO20% (Study I), the small volume of PiNO probably reached the conducting airways but the delivery to the gas exchange regions of the lung was minimal.

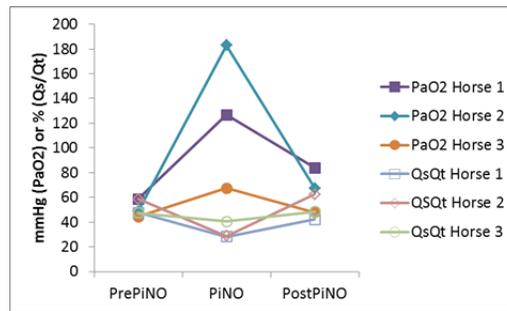


Figure 25 Response of individual isoflurane anesthetized horses to PiNO (Study V). The scale on the left is mmHg (for the PaO<sub>2</sub>) and percent (for the Qs/Qt). Note that PaO<sub>2</sub> goes up and Qs/Qt goes down during PiNO administration and then values return to or trend to baseline (PrePiNO) 30 minutes after PiNO cessation (PostPiNO). Horse 2, which had the largest Qs/Qt and was one of the most hypoxemic, had the most significant response to PiNO.

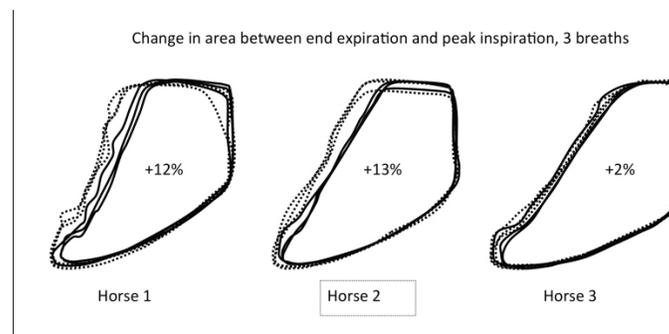


Figure 26. Change in area of the lungs between end expiration (solid lines) and peak inspiration (dotted lines). Horse 3 has minimal change in area, indicating a much lower tidal volume than the other two horses.

The major limitation of this portion of the project is that only 3 horses were studied. However, MIGET and scintigraphy were measured simultaneously, allowing us to determine that the distribution of the blood flow visualized with scintigraphy changed at the precise time that the Qs/Qt was decreased and the V<sub>A</sub>/Q more evenly matched during PiNO. The use of the MIGET to determine V<sub>A</sub>/Q matching has been validated as the gold standard technique in conscious (Hedenstierna et al. 1987) and anesthetized (Nyman and Hedenstierna 1989) horses and has been used to demonstrate changes in matching during PiNO (Heinonen et al. 2001). Scintigraphy has previously been shown to detect

changes in regions of blood flow in exercising horses and horses with lung pathology (Hlastala et al. 1996, Bernard et al. 1996, Erickson et al. 1999). Furthermore, all major parameters of interest ( $\text{PaO}_2$ ,  $\text{SaO}_2$ ,  $Q_s/Q_t$ , and pulmonary blood flow) trended significantly in the same direction in all three horses, even in the horse receiving a very low dose of PiNO. Because of the consistent changes in all 3 horses, the simultaneous measurement of MIGET and scintigraphy and the fact that both techniques are sensitive and specific for the indices we were measuring, we contend that our results are robust in spite of the low number of animals used.

## Conclusions

With our data, a major question – ‘can perfusion, rather than ventilation, be changed to improve oxygenation in the anesthetized horse?’ – was answered in the affirmative. Blood flow, as evidenced by scintigraphy, was redistributed to the ventilated regions against gravity and preferential perfusion distribution in response to PiNO. This redistribution decreased Qs/Qt and improved both  $V_A/Q$  matching and oxygenation in the anesthetized horse. This redistribution is an important concept for use of PiNO in anesthetized horses since it is this mechanism that provides the improvement in oxygenation.

With the goal of introducing PiNO for clinical use in anesthetized horses, we have extended previous work which indicated that PiNO should be delivered in the early portion of inhalation. The most effective pulse-length of PiNO was 30-45% beginning with inspiration and we have proven that the response to uninterrupted delivery of the pulse is sustained over at least 2.5 hours in both the dorsally and laterally recumbent horse. This duration is an important difference from previous studies where PiNO was delivered for only 5 minutes. In our studies, the PiNO-mediated increase in oxygenation ( $PaO_2$ ,  $SaO_2$ ,  $P\bar{v}O_2$ ,  $S\bar{v}O_2$ ) and decrease in Qs/Qt was sustained and stable throughout the entire anesthetic period. Perhaps most importantly, we have shown that there is no deleterious or dangerous rebound response in healthy horses following withdrawal of PiNO and, in fact, PiNO has a prolonged effect that lasts throughout the 30 minute recovery time. The improved oxygenation and pulmonary function (as evidenced by decreased Qs/Qt) during recovery are major reasons to use PiNO in equine anesthesia since improved oxygenation during the recovery period could potentially improve recovery from anesthesia and decrease the incidence of ischemia-related conditions which are so prevalent in the horse.

We are confident that PiNO is ready for clinical use.



## 5 Future Research and Limitations

We have shown that redistribution of perfusion, rather than ventilation is the answer to improving oxygenation in the anesthetized horse and that redistribution of perfusion can best be produced by utilizing PiNO. However, the mechanism that initiates the redistribution is unknown and perhaps could be elucidated in future studies.

The major limitation of our study ‘preparing PiNO for clinical use’ is that we have used PiNO only in healthy horses. Thus, exploring the effects of PiNO in patients with abdominal compromise and endotoxemia is critical. Currently, PiNO is being used in compromised horses in the equine clinic at SLU (Nyman, personal communication, October 2012) and the results are positive.

Perhaps the most interesting, and beneficial, research yet to be done would answer the question, ‘what is the real impact of anesthesia-induced hypoxemia in the horse? There is an argument that horses recover from anesthesia even if they are hypoxemic so why do we need to improve oxygenation? The argument continues that horses are incredible athletes and at maximum exercise are profoundly hypoxemic yet they recover rapidly and without adverse impact. But does the brief exercise-induced hypoxemia of the conscious exercising horse relate to the prolonged drug-induced hypoxemia of the anesthetized horse? Do horses really recover from hypoxemia during anesthesia ‘without incident’ or do we not know what ‘incidents’ to look for? Are we too focused on the early recovery in the recovery room without measuring the long term outcome? We have the power to decrease morbidity during and after anesthesia, especially in those horses that are compromised when they arrive in the induction stall. To improve the outcome of equine anesthesia, we need:

- a validated scoring system to assess the duration and quality of recovery and we need to use it in the clinics;

- outcomes measurements that assess long term effect of anesthesia and surgery, i.e. postoperative wound infections, myopathies, neuropathies, inflammation, etc...
- pre-, intra- and postoperative prognostic markers for tissue hypoxemia are (eg lactate and venous oxygen saturation) that are correlated to outcome.

Finally, we need to refine the equipment for clinical use (ie, mechanical ventilation, different breathing rates, etc...).

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