Transvascular fluid dynamics in the pulmonary vasculature in horses at rest and during exercise

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2010

Acta Universitatis agriculturae Sueciae
2010:55

ISSN 1652-6880
ISBN 978-91-576-7468-5

Print: SLU Service/Repro, Uppsala 2010

Abstract

Vengust, M. 2010. *Transvascular fluid dynamics in the pulmonary vasculature in horses at rest and during exercise.* Doctoral dissertation. ISSN 1652-6880, ISBN 978-91-576-7468-5

Maximal exercise results in a marked increase in cardiac output (Q) with consequent adaptations in pulmonary macro- and microvasculature. These adaptations change pulmonary hemodynamics and increase fluid and solute movement between the pulmonary circulation and the pulmonary interstitium (across the lung). The purpose of this study was to determine pulmonary circulation transvascular fluid fluxes in a quantitative manner during exercise in horses. This was determined during exercise at 80% VO_{2max} on a high-speed treadmill until fatigue without any medication, with acetazolamide (Acz) treatment, and with furosemide (Fur) treatment.

Acetazolamide, a carbonic anhydrase (CA) inhibitor, has several effects on pulmonary vasculature and erythrocytes, which influence pulmonary circulation transvascular fluid fluxes and electrolyte changes across the lung. These mechanisms are expressed through its ability to reduce vascular smooth muscle tone and contractility, and to attenuate hydration/dehydration of CO_2 via the CA, Jacobs-Stewart cycle and chloride shift (Hamburger shift) inhibition.

Furosemide causes diuresis. The consequence of diuresis is a decrease in plasma volume, right ventricular preload, and Q, which results in reduction in transmural hydrostatic pressures in pulmonary vasculature. Reduction of transmural hydrostatic forces is the mechanism by which Fur is believed to attenuate exercise induced pulmonary hemorrhage. Furosemide has also a dilatory effect on the pulmonary vasculature, and it may affect the chloride shift across the erythrocyte membrane.

Resting, exercise, and recovery arterial and mixed venous blood were sampled from race fit standarbred horses. Blood (BV) and erythrocyte volume (EV) changes across the lung were calculated from changes in plasma protein, hemoglobin and hematocrit. Cardiac output was calculated using Fick equation. Fluid flux across the lung was quantified based on changes in BV and EV across the lung. Integrative physicochemical systems approach was used to describe acid base changes across the lung.

The overall findings of these studies showed that approximately 12 L/min or 4 % of Q moves from the pulmonary circulation into the pulmonary interstitium during exertion in horses. This volume, which left the pulmonary circulation, was derived primarily from the reduction of erythrocytes' volume across the lung. Acetazolamide attenuated transvascular fluid fluxes in the pulmonary circulation

through attenuation of the erythrocyte volume changes. It did not change Q. Furosemide did not affect erythrocyte volume changes and transvascular fluid fluxes in the pulmonary circulation, but reduced Q. Cardiac output during exercise is indicative of pulmonary capillary recruitment and/or dilatation coupled with the increase in the pulmonary surface area. From the results of our studies we conclude that pulmonary circulation transvascular fluid fluxes are regulated by erythrocyte volume regulation. Hydrostatic transmural gradients across the pulmonary vasculature have a minor effect on pulmonary circulation transvascular fluid fluxes during exercise in horses.

Keywords: pulmonary circulation, pulmonary hemodynamics, water transport, acetazolamide, furosemide, Chloride shift, Jacobs-Stewart cycle, erythrocyte volume regulation, exercise.

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To all that gave me a hand, a smile, a hope, advice…

Contents

List of Publications

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

I Vengust M, Staempfli H, Viel L, Heigenhauser G. (2006) Transvascular fluid flux from the pulmonary vasculature at rest and during exercise in horses. *J. Physiol.* 570, 397-405.

II Vengust M, Staempfli H, Viel L, Heigenhauser G. (2006) Effects of chronic acetazolamide administration on fluid flux from the pulmonary vasculature at rest and during exercise in horses. *Equine Vet J Suppl.* 36, 508-515.

III Vengust M, Staempfli H, Nunez de Moraes A, Teixeiro-Neto F, Viel L, Heigenhauser G. (2010) Effects of chronic acetazolamide administration on gas exchange and acid-base control in pulmonary circulation in exercising horses. Accepted for publication in *Equine. Vet. J. Suppl.*

IV Vengust M, Kerr C, Staempfli H, Pringle J, Heigenhauser G, Viel L. (2010) Effect of furosemide on transvascular fluid fluxes across the lung in exercising horses. Accepted for publication in *Equine. Vet. J.*

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

I I declare that most of work for this paper was performed by me.

II I declare that most of work for this paper was performed by me.

III I declare that most of work for this paper was performed by me.

IV I declare that most of work for this paper was performed by me.

Abbreviations

A_{tot}	weak electrolyte concentrations
BV	blood volume
CA	carbonic anhydrase
COPD	chronic obstructive pulmonary disease
EIPH	exercise induced pulmonary hemorrhage
EV	erythrocyte volume
FiO_2	fraction of inspired oxygen in a gas mixture
Fur	furosemide
HPV	hypoxic pulmonary vasoconstriction
$J_{V\text{-}A}$	pulmonary circulation transvascular fluid fluxes
PPA	pulmonary artery pressure
PVR	pulmonary vascular resistance
Q	cardiac output
SID	strong ion difference
VCO_2	rate of elimination of carbon dioxide
VO_{2max}	maximal oxygen uptake
VO_{2peak}	peak oxygen uptake

Introduction

Pulmonary circulation fluid dynamics adaptation to exercise

Maximal exercise results in marked increase in cardiac output (Q) (Bevegard et al. 1963). Depending on the rate of rise of Q, pulmonary macro- and microvascular pressures increase accordingly (Wagner et al. 1986; Groves et al. 1987, Schaffartzik et al. 1993, Newman et al. 1993). In concert with an increased macro- and microvascular pressures, blood flow redistribution occurs across the lung through the capillary recruitment and consequent increase in the pulmonary surface area (Bake et al. 1968, Hlastala et al. 1996). Changes in pulmonary hemodynamics during exercise increase fluid and solute movement across the alveolar-capillary barrier (Dexter et al. 1951, Johnson et al. 1960). Such adaptations can be associated with the development of clinically apparent pulmonary edema (McKechnie et al. 1979) or the subclinical perivascular edema and/or parenchymal interstitial edema that worsens the pulmonary gas exchange in dogs (Younes et al. 1987), small ruminants (Coates et al. 1984), pigs (Schaffartzik et al. 1993), humans (Schaffartzik et al. 1992, McKenzie et al. 2005) and horses (West et al. 1993).

Healthy animals and humans are capable of eliminating excessive lung fluid during strenuous exercise even when the pulmonary capillary hydrostatic pressure exceeds the edemagenic threshold (Wagner et al. 1986, Groves et al. 1987, Newman et al. 1988, Erickson et al. 1992, Newman et al. 1993, Manohar, 1993). Most of the excessive lung fluid is eliminated via the pulmonary lymph system (Staub et al. 1967, Mitzner and Sylvester 1986). During strenuous exercise the lung lymphatic flow rises several-fold above the baseline (Coates et al. 1984, Newman et al. 1988 and 1993).

During intense exercise horses develop substantial hypoxemia (Wagner et al. 1989; Dempsey and Wagner 1999), which is believed to be the major mechanism for altered pulmonary fluid fluxes. Hypoxemia results in pulmonary vasoconstriction (hypoxic pulmonary vasoconstriction, HPV) of small pulmonary arteries, which increases pulmonary microvascular pressure and can, in turn, affect pulmonary capillary water permeability (Mairbaurl et al. 2002). Similar adaptations to hypoxemia are observed in high-altitude intolerance in humans and animals (Hecht et al. 1962, Maggiorini et al. 2001, Swenson et al. 2002) and other chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and obstructive sleep apnoea (Weitzenblum et al. 1981, Weir and Olschewski 2006).

Hypoxemia stimulates a rise in the intracellular Ca_2^+ concentration in pulmonary vasculature smooth muscle cells, which results in their consequent vasoconstriction (Jabr et al. 1997). Hypoxic pulmonary vasoconstriction can be induced with (Furchgott and Zawadzki 1980, Stenmark and Mecham 1997) or without intact endothelium (Murray et al. 1990) via various mechanisms, such as changes in membrane potential, increase in free cytosolic Ca_2^+, increases in Ca_2^+ sensitivity of contractile apparatus, and myosin light chain phosphorylation (Harder et al. 1985, Madden et al. 1985, Mauban et al. 2005).

Starling's hypothesis/principle

Study of water movement across the capillary wall is based on Starling's hypothesis (Starling, 1896a), which states that a balance between the hydrostatic and oncotic pressures across capillary walls holds the blood within a systemic circulating system of water-permeable vessels. In subsequent work by Landis (1927) Starling's hypothesis was summarized with the following equation:

$$J_v/A = L_p((P_c - P_i) - (\pi_c - \pi_i)), \quad (1)$$

where J_v/A is relationship between the filtration or reabsorption rate of fluid per unit area of capillary wall, L_p is the hydraulic permeability of the capillary wall, P_c is capillary pressure, P_i is hydrostatic pressure of the interstitial fluid, π_c is oncotic pressure of plasma and π_i is oncotic pressure of the interstitial fluid.

Later, the work of Pappenheimer and Soto-Rivera (1948) transformed Starling's hypothesis into Starling's principle. They estimated the quantitative relations between the arterial, venous and capillary pressures and demonstrated how these pressures could be estimated in isolated

perfused hindlimbs of dogs and cats. Starling's principle states that fluid movements across microvascular walls are determined by differences in hydrostatic and oncotic pressure.

Starling's principle is used to explain fluid movement across the systemic circulation. However, it is less successfully utilized to understand fluid dynamics across the pulmonary circulation (Starling, 1896b; Michel, 1997; Effros and Parker, 2009).

A quantitative approach to acid-base chemistry

The application of a physicochemical approach to the regulation of acid-base status in intra- and extracellular space clarifies the link between fluid and electrolytes in physiological aqueous solutions (Stewart 1978, Stewart 1981, Stewart, 1983). It quantifies the relative contributions of three independent variables: strong ion difference (SID), weak electrolyte concentrations (A_{tot}), and PCO_2 to changes in dependent variables ($[H^+]$, $[HCO_3^-]$) in aqueous solutions. Changes in $[H^+]$ can be achieved only by changing one or more of these three independent variables. The system is constrained by three fundamental physical laws: conservation of mass, electro-neutrality and the equilibrium constraints on dissociation reactions.

Strong ions are by definition electrolytes that, based on their K_A, completely dissociate in physiological aqueous solutions. The net effect of the presence of strong ions can be expressed in terms of the difference between the total concentration of strong base cations and strong acid anions. This is termed strong ion difference (SID):

$$[SID] = \Sigma[\text{strong cations}] - \Sigma[\text{strong anions}] \quad (2)$$

Weak electrolytes are only partially dissociated in H_2O. A_{tot} is used to express the total available anionic charge of the weak electrolytes, which consist of associated (HA) and dissociated (A^-) forms:

$$[A_{tot}] = [A^-] + [HA] \quad (3)$$

Carbon dioxide, a major end product of cell metabolism is moderately soluble in H_2O. The amount of dissolved CO_2 (dCO_2) is directly proportional to its partial pressure (PCO_2) in the gas phase and its solubility coefficient (SCO_2):

$$dCO_2 = SCO_2 (PCO_2) \quad (4)$$

Dissolved CO_2 reacts with H_2O to form carbonic acid (H_2CO_3), which further dissociates into H^+ and HCO_3^- (hydration of CO_2); HCO_3^- then further dissociates to form H^+ and CO_3^{2-}:

$$H_2O + CO_2 \leftarrow^{CA}\rightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow H^+ + CO_3^{2-}. \quad (5)$$

During exercise CO_2 moves down its partial pressure gradient from a working muscle into the circulation and is then removed via the respiratory system.

Pharmacologic modulation of pulmonary transvascular fluid fluxes

Pulmonary fluid dynamics can be selectively altered to determine in detail the events specific for physiology of pulmonary transvascular fluid fluxes or to determine the events associated with the pathophysiology of lung diseases. For the purpose of this thesis pulmonary transvascular fluid fluxes were studied after acetazolamide and furosemide administration. Both drugs have very specific pharmacological activities and are used extensively clinically and experimentally to cause changes in pulmonary circulation that can alter transvascular fluid fluxes.

Acetazolamide

Acetazolamide (N-(5Sulfamoyl-1,3,4-thiadiazol-2yl) acetamide), a carbonic anhydrase inhibitor, has several clinical and investigational applications (Pocker and Watamori 1973). It has been previously used in horses experimentally to evaluate the effect of acidosis on exercise responses (Rose et al. 1990, Hodgson et al. 1991). Acetazolamide also reduces HPV and effects pulmonary vascular resistance (PVR) (Lloyd 1966; Rudolph and Yuan 1966, Morray et al. 1988).

Carbonic anhydrase catalyzes the reversible reaction involving the hydration/dehydration of CO_2 (Maren 1967) as shown by:

$$CO_2 + H_2O \leftarrow^{CA}\rightarrow H_2CO_3 \leftrightarrow H^+ + HCO^-_3 \quad (6)$$

Carbonic anhydrase (CA) catalyzes the hydration/dehydration of CO_2 and enhances the Jacobs Stewart cycle and chloride exchange several folds (Maren 1967, Kifor et al. 1993). Jacobs-Stewart cycle (Jacobs and Stewart 1942) enables optimal CO_2 elimination across the lung by transformation of intravascular HCO_3^- to molecular CO_2. Bicarbonate is transported into erythrocytes in exchange for Cl^- by band 3-mediated anion exchange

(Bretcher 1971). Hydrogen ion combines with intraerythrocytic HCO_3^- to generate molecular CO_2, which diffuses across the erythrocyte membrane and capillary endothelium into the alveolar space (Jacobs and Stewart 1942; Maren 1967).

Carbonic anhydrase is metabolically closely interlinked with the activity of anion exchanger (AE1) forming a capnometabolon (Kifor et al. 1993). Therefore, inhibition of CA reduces the hydration/dehydration reaction of CO_2 (Swenson and Maren, 1978) and decreases AE1 transport activity (Sterling et al. 2001). Impaired CO_2 dehydration reaction and attenuation of Cl^- movement across the erythrocyte membrane may reduce transvascular fluid fluxes across the lung.

In horses (Rose et al. 1990, Hodgson et al. 1991) and humans (Kowalchuk et al. 1992, Kowalchuk et al. 1994), CA inhibition impairs CO_2 transport and its elimination in lungs. Moreover, chronic CA inhibitors cause a state of systemic acidosis by blocking renal reabsorption of bicarbonate (metabolic acidosis) and tissue CO_2 retention (respiratory acidosis) (Swenson 1998, Swenson 2000). An inadequate or absent hydration/dehydration reaction increases the rate of rise of P_aCO_2 and reduces rate of rise of VCO_2 during exercise (Swenson and Maren, 1978; Rose *et al.* 1990, Hodgson *et al.* 1991, Kowalchuk *et al*. 1992, Kowalchuk *et al*. 1994).

Independent of CA inhibition (Shimoda et al. 2007), Acz also reduces HPV (Deem et al. 2000, Hohne et al. 2004). Deem et al. (2000) observed that Acz reduces HPV by 50% and reduced the rate of rise by 40% in isolated blood perfused rabbit lung. More recently Hohne et al. (2004) reported a complete inhibition of HPV in dogs exposed to FiO_2 of 0.10 after treatment with Acz. Acetazolamide prevents a rise in $[Ca_2^+]$ in response to hypoxia in pulmonary artery smooth muscle cells (Hohne et al. 2007).

Acetazolamide, through CA inhibition, results in venous and arterial hypercapnia. Reports on the effects of hypercapnia and acidosis on pulmonary vasoconstriction are contradictive. Gordon et al. (1999) reported that hypercapnic acidosis in intact newborn piglets has no effect on hypoxic pulmonary artery pressure (PPA) under acute conditions, whereas 60-80 min of sustained acidosis resulted in a marked increase in both baseline and hypoxic pulmonary vascular resistance (PVR). Extracellular acidosis has been shown to increase PVR in isolated dogs' pulmonary lobes (Lloyd 1966), and in both, calves (Rudolph and Yuan 1966) and children (Morray et al. 1988), with congenital heart disease and associated pulmonary hypertension.

The slowing of the rate of CO_2 hydration/dehydration by Acz (Swenson and Maren, 1978) also results in increase in intracellular CO_2 and intracellular $[H^+]$. Raffestin and McMurtry (1987) reported that increase in pulmonary smooth muscle intracellular $[H^+]$ also decreases PVR in isolated rats' lungs. However, Shimoda et al. (2007) reported that the effect of increased intracellular $[H^+]$ on PVR is independent of mechanisms that involve pulmonary smooth muscle cell intracellular acidification or a change in its membrane potential.

Acetazolamide activity through CA inhibition, HPV reduction and influence on PVR can influence pulmonary circulation macro- and microvascular pressures. The purpose of this study was to ascertain whether indeed a reduction in pulmonary vascular pressures could influence pulmonary circulation transvascular fluid fluxes.

Furosemide

Furosemide (Fur) (4-chloro-N-[2-furylmethyl]-5-sulfamoylanthranilic acid) is a rapidly acting loop diuretic, which is used extensively to modulate fluid balance throughout the body (Kirkendall and Stein 1968, Kim et al. 1971). Loop diuretics are actively secreted into the proximal renal tubules and inhibit the active re-absorption of electrolytes in the thick ascending limb of the loop of Henle (Odlind 1979). These drugs act on the luminal surface of the epithelial cells to inhibit Na^+, K^+, and Cl^- transport, which leads to a reduced renal interstitial hypertonicity, the reduction of water absorption, and diuresis (Greger and Wangemann 1987).

Furosemide is widely prescribed for management of racehorses experiencing exercise induced pulmonary hemorrhage (EIPH) (Arthur 1991). It is usually administered at least 4 h before race-time, and the dose, depending on the administrative racing jurisdiction, is limited to 250 or 500 mg without adjustment for body weight (Arthur 1991). Hinchcliff et al. (2009) reported that Fur at a dose of 500 mg decreased the incidence and the severity of EIPH in Thoroughbreds racers. The effect of Fur on EIPH is dependent on the dose and the time of administration before the exertion (Lester et al. 1999). Premedication with furosemide may help decrease or prevent from occurrence of EIPH through 1) the effect of diuresis on blood and plasma volume (Hinchcliff et al. 1991, Hopper et al. 1991, Hinchcliff and McKeever 1998), 2) Fur direct effect on the pulmonary vasculature (Lundergan et al. 1988, Greenberg et al. 1994, Hinchcliff and McKeever 1998), and 3) furosemide-induced bronchodilatation (Olsen et al. 1992a, Rubie et al. 1993, Almirall et al. 1997). Additionally, Fur can also influence erythrocytes' fluid release across the lung. Thus the various

beneficial effects of Fur on EIPH, as discussed further in detail, may influence pulmonary circulation transvascular fluid fluxes.

The diuretic effect of Fur causes a reduction in blood and plasma volume in horses (Hopper et al. 1991, Hinchcliff et al. 1991, 1996), which decreases the right atrial pressure and pulmonary arterial pressure during exertion (Manohar et al. 1994, Parker et al. 1995). These physical alterations translate into reduction in pulmonary artery pressure and diminished transmural hydrostatic pressures in pulmonary capillaries (Manohar 1993, 1994, Manohar et al. 1994, Hinchcliff et al. 1996). Thus, the reduction in transmural pulmonary capillary pressure (intra-capillary pressure minus the perivascular (alveolar) pressure) is believed to attenuate stress failure of pulmonary capillaries and thereby reduces the incidence and severity of EIPH (Manohar 1993, 1994, Manohar et al. 1994, West et al. 1993, Manohar and Goetz 1996). Reduction in transmural (hydrostatic) pulmonary capillary pressure can also affect pulmonary circulation transvascular fluid fluxes based on the Starling's hypothesis.

Direct pulmonary vasodilatation and improved pulmonary compliance effects of Fur (and other diuretic drugs) is probably related to 1) prostaglandin release and the initial pressor actions to activation of the renin-angiotensin system (Silke 1993, Lundergan et al. 1988) and 2) to an effect on $Na^+/K^+/Cl^-$ co-transport or chloride-mediated refilling of intracellular calcium stores (Greenberg et al. 1994). It should be emphasized that hemodynamic properties of Fur are beneficial at rest and in patients with mild physical impairments due to ventricular dysfunction. The actions during exercise, however, are more varied and less beneficial (Silke 1993). Nevertheless, it is prudent to evaluate the option that pulmonary vasodilatation and improved pulmonary compliance caused by treatment with Fur may cause changes in pulmonary circulation transvascular fluid fluxes in horses during submaximal exercise.

Furosemide has a weak bronchodilator effect when inhaled in asthmatic humans (Bianco et al. 1988) or given intravenously to horses with (Rubie et al. 1993) or without the pulmonary obstructive disease (Olsen et al. 1992a). The bronchodilatory effect of furosemide is mediated through prostanoid production (Olsen et al. 1992b, Rubie et al. 1993, Almirall et al. 1997). Bronchodilation reduces the effect of exercise induced alveolar hypoxia and consequent pulmonary vasoconstriction of small pulmonary arteries, which increase pulmonary microvascular pressure (Mairbaurl et al. 2002). Increased pulmonary microvascular pressure reduces transmural hydrostatic forces, which are traditionally believed to be the regulator of pulmonary fluid dynamics.

The nonselective inhibition of $Na^+/K^+/2Cl^-$ by Fur was also observed in erythrocytes; however, inhibition of $Na^+/K^+/2Cl^-$ cotransport does not

influence erythrocyte volume regulation (Kracke and Dunham 1987). Furosemide can, however, affect the erythrocyte fluid volume by attenuating the chloride shift (Hamburger shift) across the erythrocyte membrane (Bretcher 1971, Lambert and Lowe 1980, Guizouarn et al. 2001). Erythrocyte volume regulation greatly contributes to pulmonary circulation transvascular fluid dynamics; therefore, Fur attenuation of the chloride shift may have significant effect in vascular fluid fluxes across the lung.

Problem identification

Paper I

At the outset of this thesis work fluid movement across the pulmonary circulation at rest and during exercise had not been quantified. Lymph flow studies had provided some evidence on pulmonary circulation transvascular fluid fluxes. Even though the pulmonary lymph flow is mixed with lymph flow from nonpulmonary tissues (Demling and Gunter 1982; Drake *et al*. 1986) and the conducting airways (Wagner et al. 1998), increased exchange of fluid across pulmonary capillaries correlates with increased lymph flow from lungs (Coates et al. 1984)

Fluid movement across the pulmonary circulation cannot be quantified using the Starling's equation. This is because of several factors, including inability to define forces governing these events in lung compartments, lung compartments are not fully recognized/determined and water transport across the membrane is also regulated independently of solute transport (aquaporins) (King et al. 2004). In addition, pulmonary circulation is protected from fluid leakage/ edema formation by several mechanisms, which are properties solely found in the pulmonary vasculature and lacking in systemic vessels (Effros and Parker 2009). Based on that, a simple Starling model cannot be readily applied to study pulmonary circulation transvascular fluid fluxes (King et al. 2004, Effros and Parker 2009). Therefore, we conducted a study where fluid movement across the across the lung was quantitatively assessed by comparison of blood volume differences between mixed venous blood and arterial blood (across the lung) in horses at rest and during exercise.

Paper II

Hypoxemia indirectly contributes to fluid accumulation in the pulmonary interstitium (Mairbaurl et al. 2002), which is most often seen in HPV of the acute high-altitude intolerance in humans (Maggiorini et al. 2001, Swenson

et al. 2002). Hypoxic pulmonary vasoconstriction can be managed with carbonic anhydrase inhibitors, such as Acz. Acetazolamide exerts its therapeutic effects through reduction or prevention of vasoconstrictory effects of hypoxemia on pulmonary microvasculature (Schoene et al. 2001). It will also cause CO_2 retention and acidosis. It has been documented that hypercapnia can *per se* impair gas exchange with its influence on pulmonary arterial pressure (Brimioulle et al. 1991). Together with increased $[H^+]$ it has a prominent role in generalized edema formation in human COPD patients (Karadag et al. 2004); however, it should not result in accumulation of fluid in lungs (Haberkern and Bland 1981).

The ability of Acz to reduce or prevent vasoconstrictory effects of hypoxemia on pulmonary microvasculature is already established (Schoene et al. 2001). However, CO_2 retention and acidosis influence on lung fluid balance in horses (and other mammals) at rest and during exertion (under hypoxemic conditions) is not known. Thus, the research plan to study this was undertaken in paper II.

Paper III

Effects of CO_2 retention and acidosis on acid base and electrolyte balance across the lung in pulmonary circulation in horses or other animals or a human at rest and during exertion have also not yet been described. A quantitative approach to acid-base chemistry was used to determine the electrolyte changes across the lung. Another objective was to link pulmonary gas exchange and electrolyte changes to pulmonary circulation transvascular fluid fluxes.

Using the integrated physicochemical systems approach, it is possible within each fluid compartment to describe the influence of three independent variables, strong ion difference (SID), PCO_2 and total concentrations of weak acids and bases (A_{tot}) on $[H^+]$ and $[HCO_3^-]$, which are considered dependent variables (Stewart 1983). In paper III we hypothesized that Hamburger (chloride) shift and the Jacobs-Stewart cycle play a critical role in acid base homeostasis across the lung.

Paper IV

Furosemide may attenuate EIPH through a reduction in transmural hydrostatic pressures in pulmonary capillaries, which is attributed to decrease in plasma volume and right ventricular preload as a result of diuresis (Hinchcliff et al. 1991; Hinchcliff and McKeever 1998), dilatory effect of Fur on the pulmonary vasculature (Lundergan et al. 1988, Olsen et al. 1992a, Greenberg et al. 1994, Hinchcliff and McKeever, 1998) and

furosemide-induced bronchodilatation (Rubie et al. 1993, Almirall et al. 1997).

In addition, Fur may affect the chloride shift (Hamburger shift) across the erythrocyte membrane via the Cl^-/HCO_3^- exchanger (AE1, Band 3) (Bretcher 1971, Lambert and Lowe 1980), which is a part of the erythrocyte volume regulation mechanisms (Guizouarn et al. 2001). Thus, the action of Fur to decrease transmural hydrostatic pressures in pulmonary capillaries and attenuation of the chloride shift are both potential mechanisms by which Fur may reduce or attenuate fluid fluxes across the lung. Therefore, in conducting the experiments for paper IV, we hypothesized that pre-exercise treatment with Fur will attenuate transpulmonary fluid fluxes in horses during intense exercise.

Aims of the study

- To quantify transvascular fluid fluxes in the pulmonary circulation at rest and during exercise in horses,
- To quantifiably determine adaptation of transvascular fluid fluxes in the pulmonary circulation at rest and during exercise in horses after treatment with the carbonic anhydrase inhibitor acetazolamide,
- To determine blood gas and electrolyte adaptation across the lung during exercise in horses after treatment with acetazolamide,
- To determine the interrelation between acid base and electrolyte balance and pulmonary circulation transvascular fluid fluxes,
- To quantifiably determine adaptation of transvascular fluid fluxes in the pulmonary circulation at rest and during exercise in horses after treatment with furosemide.

Materials and methods

Six race fit Standardbred horses were used for each study. Different horses were recruited for each experiment with owner/trainer consent. The study protocols were approved by the Animal Care committee according to the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, Ontario). All horses were returned to owners healthy and resumed their normal training and racing activities.

All studies (paper I to IV) had similar experimental protocols for exercise regimens and sampling, apart from administration of Acz (Paper II, III) or furosemide (Paper IV).

Pre-Experimental Protocol

Horses were familiarized to the treadmill over a period of one week. During the first three days horses were given repeated walking exercise on the treadmill for 20 min. daily (15 min. walk, 5 min. slow pace) at 10% treadmill inclination, followed by two days of light exercise with the respiratory mask. Before every treadmill exercise horses were weighed, fitted with a safety harness, hobbles, and heart rate meter (Equistat Model HR-8 A, EQB Inc., Unionville, PA, USA).

On day six peak O_2 uptake (VO_{2peak}) was determined for each horse, which comprised three treadmill exercise periods: warm-up, incremental exercise, and recovery. During the warm-up period the horses walked on horizontal treadmill with no incline for 5 min at 2-3 m/s and then trotted for 5 min at 4-5 m/s. At the end of this 10 min warming-up period the treadmill was inclined to 10% and the speed increased to 8 m/s. The incremental exercise then consisted of a stepwise increase of velocity of 1 m/s every 60 s. An open flow through system was used for collection of pulmonary gases throughout the entire exercise protocol. Peak O_2 uptake was determined as the point at which no further increase in VO_2 occurred,

despite an increase in speed, or a level of exercise where the horse could no longer maintain pace with the treadmill speed. Three days following the determination of VO_{2peak}, the horses were hand walked for 15 min daily. The experimental protocol was then conducted on the fourth day.

Experimental protocol

Prior to experiment, the of the mid cervical region over both jugular veins was clipped, desensitized with lidocaine 2.5% and prilocaine 2.5% cream (lidocaine 2.5% and prilocaine 2.5%; AstraZeneca Pharmaceuticals LP, Wilmington, DE, USA) and aseptically prepared. Pulmonary Swan-Ganz catheter (Baxter Healthcare Corp., Irvine, CA, USA) and a 150 cm long central venous polyethylene blood catheter (#240, Becton Dickinson, Sparks, MD, USA) were placed aseptically via the left and right jugular vein into the pulmonary artery for mixed central venous blood sampling and core body temperature measurements. Correct catheter placement was ascertained by observing characteristic pressure waveforms on an oscilloscope (Criticare 1100, Criticare Systems Inc., Waukesha, WI, USA). A 20 Gauge catheter (Insyte-W, Infusion Therapy Systems Inc., Sandy, UT, USA) was inserted into the facial or transverse facial artery. A 30-cm long extension tubing with a three way stopcock was connected to intravenous and arterial catheters. Catheters and extension tubing were sutured to the skin.

Horses stood still on the treadmill until their heart rate reached their resting value and then were warmed-up as described in the pre-experimental protocol of the VO_{2peak} determination. Following the warm-up treadmill speed was set to the velocity and slope inducing 80% of the VO_{2peak}. Horses were then exercised until fatigued. During recovery horses were walked on the treadmill at 1.7 m/sec for 15 min.

Acetazolamide treatment experiment (paper II and III)

Horses were given Acz (Apo-acetazolamide, Apotex Inc., Ontario, Canada) orally at a dose of 10 mg/kg of BW TID for three days or were not treated (control). Acetozolamide tablets were crushed and mixed with a mixture of molasses and corn oil, and administered orally with a Toomey syringe (C.R. Bard Inc., Covington, GA, USA). Thirty min. before the treadmill protocol horses were given 30 mg/kg of BW of Acz mixed in 8 ml of tap water/kg of BW via a nasogastric tube or were administered tap water only (control).

Furosemide treatment experiment (paper IV)

Horses were given 250 mg of furosemide i.v. or placebo intravenously four hours prior to the treadmill protocol.

Pulmonary gas collection

Pulmonary gas exchange was measured at rest, during exercise, and during the recovery period. Before exercise the respiratory mask was fitted on the horse's nose. An open flow through system was used for collection of expired gases throughout the entire exercise protocol (Wagner *et al*. 1989). The expired gas was drawn into the O_2 analyzer (Ametek, Model S-3A/1, Pittsburgh, PA, USA), which measured the concentration of inspired O_2. Inspired O_2 was recorded in 10-second periods throughout the experiment. For analysis the average of three measurements in a 30 second period was used, which coincided with blood sampling intervals.

Blood sampling and blood analysis

Resting arterial and mixed venous blood samples were collected simultaneously under anaerobic conditions twice in a five-minute interval. Further sampling was performed in 60 s intervals during the exercise period until fatigue. During the recovery period sampling was performed immediately after the treadmill was stopped (0 min) and then at one, two, three, five, 10, and 15 minutes into the recovery period. Prior to each sampling 10 mL of blood was withdrawn from catheters and discarded. Blood samples were collected into lithium-heparinized syringes (S-Monovette, Sarstedt AG and Co, Nümbrecht, Germany), stored on ice, and analyzed in duplicate with the Stat Profile M Analyzer (Nova Biomedical Corporation, Waltham, MA, USA) immediately after the treadmill protocol ended. Stat Profile M Analyzer uses conductivity for hematocrit, conductivity/reflectance for haemoglobin and O_2 saturation analysis, ion selective electrodes for analysis of Na^+, K^+, Cl^-, as well as for pH and PCO_2; amperometry for PO_2 and La^-. Blood O_2 content was calculated from the O_2 saturation and the Hb concentration using standard equations. Total plasma protein was measured using a clinical refractometer (Attago 331, Attago, Tokyo, Japan). The pH, PCO_2, HCO_3^- and PO_2 sample values were corrected for the horses' core body temperature (Model COM-2, Baxter Healthcare Corp., Irvine, CA, USA).

For erythrocyte and whole blood electrolyte analyses, blood samples were repeatedly frozen (–80^{o}C) and thawed (room temperature) to induce erythrocyte lyses.

Calculations

Plasma volume changes across the lung ($\Delta PV_{v\text{-}a}$) were calculated from changes in plasma protein [PP] at the same time point from central venous to arterial blood (across the lung) (Dill and Costill 1974):

$$\%\Delta PV_{v\text{-}a} = (([PP_v] - [PP_a]) / [PP_v]) \times 100, \quad (7)$$

where $[PP_v]$ is the plasma protein concentration in venous and $[PP_a]$ the plasma protein concentration in arterial blood. To account for changes in plasma volume relative to hematocrit (Hct), the equation 1 was adjusted for changes in the Hct across the lungs.

$$\%\Delta_{Hct}PV_{v\text{-}a} = (([PP_v] \times (1\text{-}Hct_v) - [PP_a] \times (1\text{-}Hct_a)) / [PP_v]) \times 100 \quad (8)$$

where $\%\Delta_{Hct}PV_{v\text{-}a}$ is the plasma volume change relative to Hct changes from central venous (Hct_v) to arterial (Hct_a) blood.

Changes in erythrocyte volume ($\Delta EV_{v\text{-}a}$) across the lungs were calculated from changes in hemoglobin [Hb] and hematocrit [Hct] in venous ($[Hb_v]$, $[Hct_v]$) and arterial blood ($[Hb_a]$, $[Hct_a]$) (Costill *et al*. 1974):

$$\%\Delta EV_{v\text{-}a} = ((([Hb_v] / [Hb_a]) \times (Hct_a / Hct_v)) - 1) \times 100 \quad (9)$$

For calculation of the blood volume (BV) the $\%\Delta EV_{v\text{-}a}$ was adjusted for Hct changes across the lung:

$$\%\Delta_{Hct}EV_{v\text{-}a} = ((([Hb_v] / [Hb_a]) \times (Hct_a / Hct_v) \times Hct_a) - Hct_v) \times 100 \quad (10)$$

Blood volume changes across the lung were then measured from $\Delta_{Hct}PV_{v\text{-}a}$ and $\Delta_{Hct}EV_{v\text{-}a}$:

$$\%\Delta BV_{v\text{-}a} = ((([PP_v] \times (1\text{-}Hct_v) - [PP_a] \times (1\text{-}Hct_a)) / [PP_v]) \times 100) + ((([Hb_v] / [Hb_a]) \times (Hct_a / Hct_v) \times Hct_a) - Hct_v) \times 100 \quad (11)$$

Cardiac output (L/min) was calculated based on the Fick principle using VO_2 and blood O_2 content from central venous and arterial blood. Fluid flux ($J_{V\text{-}A}$ L/min) across the lung was then quantified based on Q and $\%\Delta BV$:

$$J_{V\text{-}A} = (Q \times \%\Delta BV). \quad (12)$$

Plasma $[H^+]$ was calculated from the measured pH as the antilog.

Strong ion difference was calculated as the sum of strong cations minus the sum of the strong anions:

$$[SID] = ([Na^+] + [K^+]) - ([Cl^-] + [La^-]) \quad (13)$$

Plasma [A_{tot}] was calculated using a conversion factor of 0.21 mmol/L of plasma protein (Staempfli *et al.* 1999).

Erythrocyte ion concentrations were calculated from whole blood ($_{wb}$) and plasma ($_{p}$) ion concentration according to Buono and Yeager (1986) and McKelvie *et al.* (1991).

$$_{e}[ion] = (_{wb}[ion] - (_{pl}[ion] \times (1 - Hct))) \times Hct^{-1} \quad (14)$$

All venoarterial differences for plasma ions and proteins were corrected for $\Delta PV_{v\text{-}a}$ using the equation (McKenna *et al*. 1997):

$$[Ion]_{V\text{-}A} = ([Ion]_{v} / (1 + (\Delta PV_{V\text{-}A})) - [Ion]_{a} \quad (15)$$

A similar correction was made for erythrocyte ion concentration using $\Delta EV_{V\text{-}A}$ (McKenna *et al*. 1997).

Study design and statistical analysis

A cross over design was used when comparisons were made between control and treatment, with horses serving as their own control. The furosemide study was done in a double-blinded fashion

All data were normally distributed as verified with Shapiro-Wilks test.

Effects of treatment and exercise (time) as well as treatment and exercise (time) interaction on variables were analyzed using a two-way repeated-measures ANOVA. Treatment and exercise (time) were considered as repeated factors.

Pair-wise comparison with Bonferroni adjustment was used only to assess statistical significance of differences between rest and exercise times and between parameters of different treatments at the same specific time point.

Overall effect of treatment with Acz or Fur was defined at rest, during exercise (from first minute to fatigue) and recovery (from first minute of recovery till the end of recovery). Repeated-measures two-way ANOVA was used to compare overall effects between treatments.

A statistical significance level of $P < 0.05$ was used and data are expressed as means ±SE.

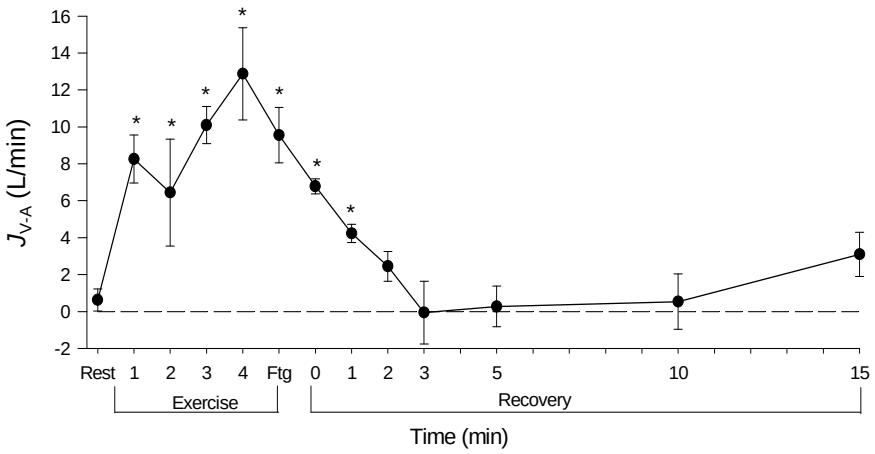
Statistical analysis was performed with SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Transvascular fluid flux from the pulmonary vasculature at rest and during exercise in horses (paper I)

Transvascular fluxes in lungs of horses (hereafter identified as $J_{V\text{-}A}$) during exercise reached approximately 4% of the Q or 12 L/min (figure 1).

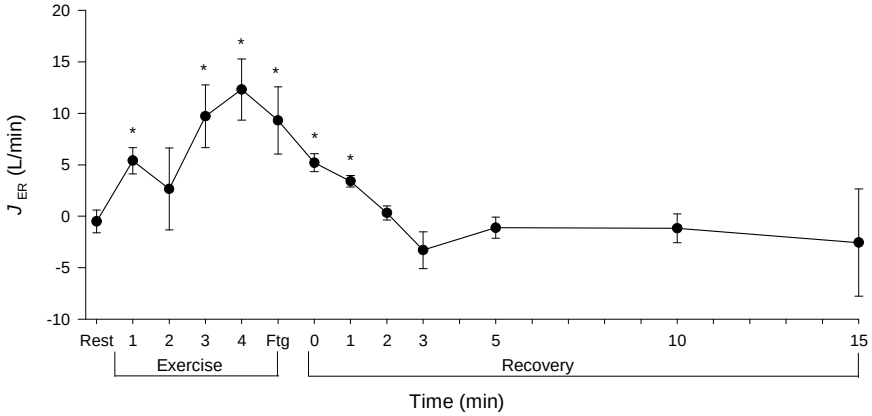
*Figure 1. Fluid flux from the pulmonary vasculature ($J_{V\text{-}A}$) at rest, during exercise and recovery. Values are means ± SE (n=6). *, different from rest (P < 0.05). Ftg, fatigue.*



In the present study it was established that erythrocyte volume decrease (erythrocyte volume release) across the lung was an important contributor to whole fluid flux from the pulmonary vasculature (figure 2). This was surprising, because this study's hypothesis was derived from the ability of

horses to develop and sustain high pulmonary macro- and microvascular pressures during exercise (Slonim et al. 1954; Elkins and Milnor, 1971; Wagner et al. 1986; Younes et al. 1987; Newman et al. 1993). Those are derived from increases in PPA during exertion (Erickson et al. 1992; Manohar, 1993; Wagner et al. 1989, Wilkins et al. 2001), which translates into a relatively high pulmonary capillary pressure (Sinha et al. 1996) and consequent increase in the transvascular fluid filtration across the lung (Sinha et al. 1996).

*Figure 2. Fluid flux across the lung from erythrocytes (J_{EV}) at rest, during exercise and recovery. Values are means ± SE (n=6). *, different from rest (P < 0.05). Ftg, fatigue.*



In paper I our results were interpreted classically, through changes in pulmonary circulation transmural hydrostatic forces. We concluded that the pulmonary circulation transvascular fluid fluxes are caused by 1) pulmonary capillary recruitment and/or dilatation coupled with the increase in the pulmonary surface area (Bake et al. 1968, Hlastala et al. 1996), 2) increased pulmonary microvascular pressures (Sinha et al. 1996) and 3) changes in the pulmonary transcapillary gradients (Bland and McMillan 1977), and, as determined in this study, erythrocyte volume decrease/erythrocyte fluid release. Pulmonary transvascular fluxes increase as a function of the net transvascular driving pressure: hydrostatic pressure gradient minus protein osmotic pressure gradient (Starling 1896a,b, Bland and McMillan 1977). Hydrostatic pressures are traditionally accepted to be the important determinant of pressure and fluid dynamics in the pulmonary circulation. However, the equilibrium between fluid and solutes in pulmonary vasculature during exercise became altered with active fluid

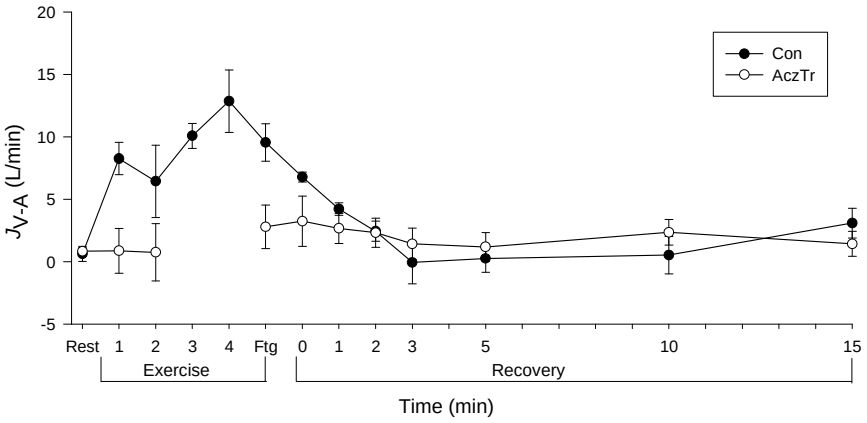
release from erythrocytes. The dilution of plasma protein reduced plasma colloid osmotic pressure gradient, which, assisted with the increased hydrostatic pressure, favored the fluid out of the pulmonary vasculature restoring the transvascular gradient to its equilibrate state. Therefore, oncotic forces have an important function along with hydrostatic forces in the extent of fluid movement from the pulmonary vasculature during exercise.

Effects of chronic acetazolamide administration on fluid flux from the pulmonary vasculature at rest and during exercise in horses (paper II)

After quantification of pulmonary transvascular fluid fluxes in paper I the objective was put forward to better define the physiologic mechanisms related to $J_{V\text{-}A}$ through evaluating its response to treatment with Acz (AczTr). Acetazolamide reduces or prevents vasoconstrictory effects of hypoxemia on pulmonary microvasculature (Schoene et al. 2001). Hypoxemia is common in horses during sub- and maximal exercise. In addition, chronic Acz administration causes CA inhibition, which in turn induces hypercapnia. Hypercapnia *per se* and in combination with metabolic acidosis can express vasoconstrictory or vasodilatory effects on pulmonary vasculature (Brimioulle et al. 1991), and thereby influence pulmonary transvascular fluid fluxes. Disorders that may cause hypercapnia are mostly associated with diseases of lungs, the airways, or both. However, hypercapnia not only arises from the respiratory system but also from diseases affecting the neural, muscular, chest-wall, and circulatory components of the respiratory system. Regardless of the etiology, animals and humans still exercise, at intensities that cause a variable degree of hypoxemia, under pathologic conditions that can enhance or cause severe hypercapnia. Understanding of the effect of hypoxemia, hypercapnia and metabolic acidosis on pulmonary circulation transvascular fluid fluxes is also important for more severely ill patients where lung water dynamics can be compromised even in the absence of exercise.

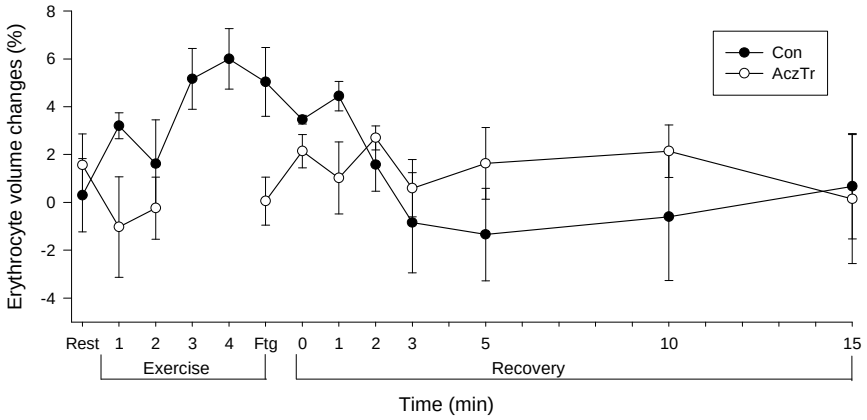
At rest $J_{V\text{-}A}$ was not different between Con and AczTr. However, during exercise, $J_{V\text{-}A}$ increased to a similar level that was reported in paper I. Transvascular fluid fluxes were attenuated in AczTr. Cardiac output was not different between control and AczTr (figure 3).

Figure 3. Fluid flux from the pulmonary vasculature (J_{V-A}) at rest, during exercise and recovery in control (Con) and chronic acetazolamide treatment (AczTr). Values are means ± SE (n=6). Ftg, fatigue.



In our first publication we determined that erythrocytes, through their volume regulation, contribute significantly to pulmonary circulation transvascular fluid fluxes. In this study the erythrocyte fluid release across the lung was diminished by chronic treatment with Acz (figure 4).

Figure 4. Erythrocyte volume changes across the lung at rest, during exercise and recovery in control (Con) and chronic acetazolamide treatment (AczTr). All values are means ± SE (n=6). Ftg, fatigue.



Because Q was not affected by Acz it is possible to conclude that the effects of hypoxemia, hypercapnia and acidosis together on pulmonary vasoconstriction during exercise did not have a significant effect on pulmonary circulation pulmonary macro- and microvascular pressures, which would consequently diminish pulmonary circulation transvascular fluid fluxes. However, chronic treatment with Acz affected erythrocyte volume regulation/erythrocyte fluid release across the lung. The mechanism affecting the fluid release from erythrocytes during exercise after AczTr cannot be determined from data gathered in this study. However, there are some mechanisms that, when challenged with hypoxemia, hypercapnia and acidosis, could potentially disable erythrocytes from reducing their volume across the lung, including:

1. Increased intracellular [H^+] in the erythrocytes would stimulated Na^+/H^+ pump on the erythrocyte plasma membrane, which contributes to fluid accumulation in erythrocytes and the erythrocyte volume increase to the extent that K^+/Cl^- co-transport, a part of the system that contributes to erythrocyte volume decrease (Honess et al. 1996, Speake et al. 1997, Gibson et al. 2000), could not compensate for on the passage through the lung capillary bed. K^+/Cl^- co-transport should be activated/stimulated and the Na^+/H^+ pump disabled/depressed in blood passing pulmonary capillaries (Honess et al. 1996, Speake et al. 1997, Gibson et al. 2000),
2. Erythrocyte volume regulation could have been affected by prevention of fluid release from the pulmonary circulation into pulmonary interstitial (due to possible direct effect of Acz on pulmonary vascular wall permeability) causing osmolality forces in plasma unfavourable for erythrocytes to complete their volume decrease/fluid release, and
3. Slowing the rate of $CO_2/HCO_3^-/H^+$ interconversion increased the fraction of total CO_2 in the erythrocyte preventing erythrocyte fluid release, because of increased intracellular osmolality.

Regardless of the cause or combination of causes, pulmonary circulation transvascular fluid fluxes commence simultaneously with respiratory gas exchange. They can be affected by factors that influence or interfere with respiratory gas exchange mechanisms. However, pulmonary circulation transvascular fluid fluxes appear to be quite refractory to changes in pulmonary circulation pressures when fluid dynamics across the lung are subject to an acute event such as exercise.

Effects of chronic acetazolamide administration on gas exchange and acid-base control in pulmonary circulation in exercising horses (paper III)

In this study we evaluated 1) effects of CO_2 retention and acidosis on acid base and electrolyte balance across the lung and 2) the interrelation between acid base and electrolyte balance and pulmonary circulation transvascular fluid fluxes. We hypothesized that Hamburger shift and the Jacobs-Stewart cycle play a critical role in acid base homeostasis and volume changes across the lung.

In this study we used the integrated physicochemical systems approach to describe acid base changes across the lung in pulmonary circulation in exercising horses without (control) and with CA inhibition (with related metabolic and respiratory acidosis). The physicochemical systems approach was used to describe the influence of three independent variables, strong ion difference (SID), PCO_2 and total concentrations of weak acids and bases (A_{tot}) on $[H^+]$ and $[HCO_3^-]$, within each fluid compartment (Stewart 1983).

The full data arrangement in tables in this study was very extensive. For a comprehensive review of results please refer to paper III. In summary however, the following most significant conclusions can be made:

1. Chronic CA inhibition affected exercise time to fatigue: exercise duration at 80% VO_{2peak} was significantly shorter in AczTr (2.6 ±0.2 min) compared to Con (4.7 ±0.2 min, $P < 0.0001$),
2. Chronic CA inhibition greatly influenced the acid base homeostasis across the lung,
3. Acid base disturbance across the lung was related to retention of CO_2 and inhibition of Cl^- flux across the erythrocyte membrane,
4. Volume regulation of erythrocytes and acid base changes across the lung are linked to each other, and
5. A_{tot} and La^- have no influence on acid base status across the lung.

During exercise AczTr attenuated the hydration/dehydration reaction and slowed the equilibration between CO_2 species in pulmonary capillaries, which further on increased P_aCO_2 (Swenson 1998, Swenson 2000). The slowing of the CA reaction means that equilibration between CO_2 species is not complete during the transit through the pulmonary capillary. The reflection of these activities is also inhibition of HCO_3^- transport. The decrease in the rate of HCO_3^- dehydration after treatment with Acz increased the erythrocyte $[HCO_3^-]$ and consequently reduced the plasma $[HCO_3^-]$ (Swenson 1998, Swenson 2000).

Erythrocyte volume decreased across the lung during exercise was attenuated by AczTr. There are several active and passive mechanism that regulate erythrocyte volume, which are manifest as the erythrocyte regulatory volume increase in peripheral tissues and by the erythrocyte regulatory volume decrease across the lung (Fievet et al. 1990, Gibson et al. 1993, Gibson et al. 1995, Honess et al. 1996, Speake et al. 1997; Juel et al. 1999, Gibson et al. 2000). Based on our results it appears that the major reason for incomplete or absent volume decrease across the lung in AczTr is due to depressed erythrocyte [Cl^-] efflux and slowed CO_2 dehydration/hydration reaction or Jacobs-Stewart cycle.

Plasma SID_{V-A} (plasma strong ion difference across the lung) had a positive value in Con and AczTr indicating a reduction in SID across the lung. This was in greater part driven by an influx of Cl^- from erythrocytes to initiate the Jacobs-Stewart cycle (Jacobs and Stewart 1942, Jennings 1989), which substantially decreases erythrocyte $[SID]_{V-A}$. Carbonic anhydrase inhibition prevented efflux of Cl^- from erythrocytes, hence the substantially decreased erythrocyte $[SID]_{V-A}$ in AczTr. With exception of La^-, other strong ions showed the tendency to counteract changes caused by CO_2 retention and Cl^- efflux attenuation.

At rest plasma $[H^+]_{V-A}$ was similar in Con and AczTr, indicating that elimination of CO_2 was increased due to stimulated ventilation kinetics (Swenson and Maren 1978, Ward et al. 1983, Kowalchuk et al. 1994). During exercise in Con, despite a concomitant plasma SID decrease across the lung, plasma $[H^+]_{V-A}$ remained strongly positive (indicating a $[H^+]$ reduction in plasma across the lung). This was due to a substantial PCO_2 reduction and Cl^- efflux (and SID increase) across the lung. Conversely, in AczTr plasma $[H^+]_{V-A}$ was affected by CO_2 species retention and erythrocyte Cl^- shift impairment and was lower than in Con.

CO_2 and Cl^- changes in erythrocytes across the lung appear to be the major contributors to acid-base and ions balance, as well as volume changes across the lung, in exercising horses, and most likely also in other mammalian species. Therefore, the hypothesis that chloride shift and the $CO_2/HCO^{3-}/H^+$ interconversion are important for pulmonary circulation transvascular fluid fluxes was confirmed.

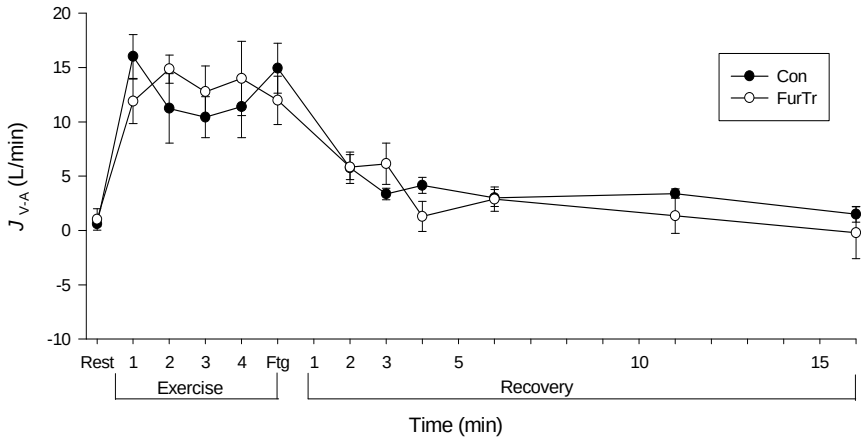
Effect of furosemide on transvascular fluid fluxes across the lung in exercising horses (paper IV)

Furosemide is prescribed for management of racehorses experiencing EIPH (Arthur 1991). The dose used in this study was 250 mg administered

4 hours before exercise, according to limits imposed in Canada and some US horse racing jurisdictions. Treatment with Fur attenuates the exercise-induced rise in pulmonary capillary blood pressure, which is believed to decrease the occurrence of EIPH via the reduction of the transmural hydrostatic pressures in pulmonary capillaries (Manohar 1993, 1994, Manohar et al. 1994, Kindig et al. 2001).

In this study treatment with 250 mg of Fur (FurTr) four hours before the onset of exercise had no effect on $J_{V\text{-}A}$ and did not affect the duration of exercise at 80% VO_{2max} to fatigue (figure 5).

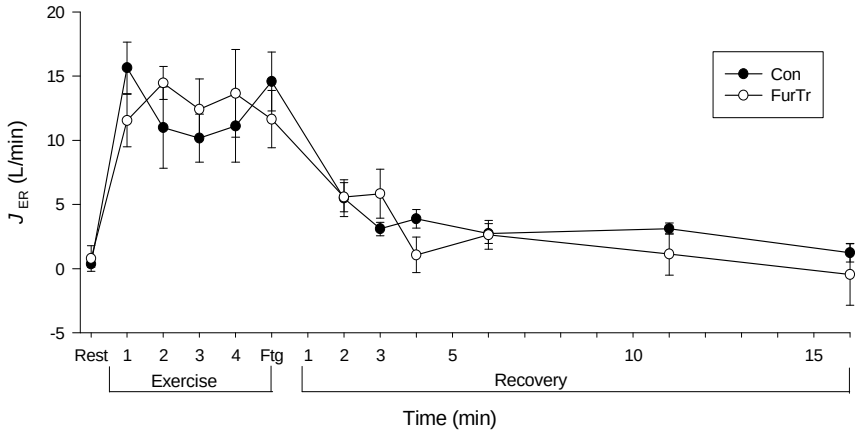
Figure 5. Fluid flux from/into the pulmonary vasculature ($J_{V\text{-}A}$) at rest, during exercise and recovery. Values are means ± SE (n=6). Ftg: fatigue. Positive values indicate decrease of $J_{V\text{-}A}$ across the lung. Negative value indicate increase of $J_{V\text{-}A}$ across the lung.



As well, treatment with Fur did not affect erythrocyte fluid release (figure 6).

However, treatment with Fur reduced Q during exercise: cardiac output increased from 20.9 ±6 L/min and 19.4 ±9 L/min at rest in control and FurTr, respectively, to 299.9 ±16.6 L/min and 247.9 ±13.9 L/min at the first min of exercise in control and FurTr, respectively ($P < 0.0001$). It returned to resting values during recovery. During exercise Q was lower in FurTr compare to Con ($P = 0.01$).

Figure 6. Erythrocyte fluid fluxes (J_{ER}) at rest, during exercise and recovery. Values are means ± SE (n=6). Ftg: fatigue. Positive values indicate decrease of J_{ER} across the lung. Negative values indicate increase of J_{ER} across the lung



This study suggests that the focus with regards to investigation into the initiation and maintenance of pulmonary circulation transvascular fluxes should in greater part shift from pulmonary circulation pressures to erythrocyte volume regulation. Most ion exchange mechanisms in erythrocytes are part of the erythrocyte volume regulation mechanisms (Guizouarn et al 2001). $Na^+/K^+/Cl^-$ co-transport, which carries the major diuretic effect of Fur, does not poses significant volume regulation properties, whereas chloride shift (Hamburger shift) across the erythrocyte membrane (Bretcher 1971, Lambert and Lowe 1980, Kracke and Dunham 1987) can contribute substantially to pulmonary transvascular fluid fluxes. Furosemide blocks anion exchange mechanisms (Hamburger shift) in erythrocytes (Lambert and Lowe 1980); however, water movement across the erythrocyte membrane in this study was similar in Con and FurTr. Therefore, at a dose of 250mg Fur activity on the erythrocyte anion exchange mechanisms also seems unlikely to be of significance in exercising horses four hours after the intravenous administration.

Cardiac output indicates the changes in pulmonary capillary recruitment and/or dilatation and the increase in the pulmonary surface area during exercise (Bake et al. 1968, Hlastala et al. 1996). Furosemide caused decrease in Q, which indicates a decrease in pulmonary capillary recruitment, less explicit capillary recruitment and consequent smaller transmural hydrostatic pressures compare to Con. However, this did not

affect $J_{V\text{-}A}$. It is then unlikely that horses affected by EIPH would benefit from treatment with 250mg of furosemide four hours before exercise if current etiology theory of the disease is relevant, because Starling forces most probably retain their effect on pre- and post pulmonary capillary vessels. Pulmonary capillaries are very resilient to leakage and changes in transmural hydrostatic forces (Effros and Parker, 2009). The filtration coefficient of the pulmonary capillaries is smaller than that of the extra-alveolar vessels (Schneeberger and Karnovsky 1976, Bhattacharya 1988, Maggiorini et al. 2001). Therefore, the dynamics of water movement in the pulmonary circulation are complex events encompassing gas exchange mechanisms, erythrocyte volume regulation and only then Starling's forces.

Methodological considerations (all papers)

In our studies we have measured total transvascular fluxes from the pulmonary circulation, which include fluid that contributes to increases in lung lymph flow and total lung water. Lung lymph flow studies implementing different experimental conditions such as exercise (Coates et al. 1984, Newman et al. 1988), left atrial balloon obstruction (Parker et al. 1981), hypoxia (Dauber and Weil 1983), or 100% O_2 inhalation (Newman et al. 1983) to induce pulmonary vascular pressure changes or increase lung vascular permeability, would require better defined attention to the uncertainty concerning the tissues drained by the lymphatics and the effect of the lymph nodes themselves on lymph constituents. On the other hand, gravimetric lung fluid dynamic studies only detect variations in the presence of lung water and are unable to account for alterations when changes are to be contributed to the vascular, interstitial, and/or cellular compartments in lungs (Lin et al. 1998). They also require static experimental conditions (Hanel et al. 2003). In contrast to detecting changes in lung fluid dynamics by lung lymph flow or pulmonary gravimetric studies we were able to measure the total fluid flux from pulmonary circulation from changes in BV in vivo and in dynamic experimental settings.

Plasma volume changes associated with exercise were calculated using plasma protein concentration as described by Dill and Costill (1974) (equation 7). Plasma volume changes were then calculated relative to Hct changes in venous and the arterial blood (equation 8) to account for the very unlikely event of: 1) relative plasma volume changes (Hct

decrease/erythrocyte removal) or 2) absolute plasma volume changes (plasma leakage from the pulmonary circulation).

Hemoglobin is confined strictly to erythrocytes and should not be affected by physiologically derived stress or by most pathologically derived alterations. Therefore, erythrocyte volume across the lung was calculated based on changes in Hb (equation 9) (Costill et al. 1974). Equation 9 was also adjusted for possible Hct changes across the lung when used for blood volume calculations.

Methods for calculation of blood volume changes used in this experiment are precise (coefficient of variance from 0.7 to 2.4%) and are able to measure small changes (Dill and Costill 1974, Costill et al. 1974, Harrison 1985). They provide accurate results acutely in exercising subject, which would perhaps include other animals and humans, without need for chronic instrumentation, or, potentially, at patient's bedside, with chronic instrumentation for continuous monitoring of volume changes across the lung.

In conclusion, methods for calculation of blood volume changes used in these experiments are simple and have been validated and published previously (Dill and Costill 1974, Costill et al. 1974, Harrison 1985).

Summary and conclusions

Regarding the pulmonary circulation transvascular fluid fluxes ($J_{V\text{-}A}$):

1. During exertion in horses approximately 4% of Q moves from pulmonary circulation into the pulmonary interstitium,
2. Erythrocyte volume regulation/fluid release across the lung is the most important contributor to $J_{V\text{-}A}$,
3. $J_{V\text{-}A}$ commence with respiratory gas exchange,
4. $J_{V\text{-}A}$ appear to be largely insensible to changes in pulmonary circulation transmural hydrostatic pressures during exercise,
5. CO_2 and Cl^- changes in erythrocytes across the lung appear to be the major contributors to acid-base and ions balance, as well as pulmonary circulation transvascular fluid fluxes,
6. Furosemide at the dose of 250 mg given four hours before exercise does not change erythrocyte volume regulation/fluid release across the lung,
7. Furosemide at the dose of 250 mg given four hours before exercise does not affect $J_{V\text{-}A}$ in pulmonary circulation despite the decreased Q,
8. Erythrocyte volume regulation/fluid release across the lung is more relevant to $J_{V\text{-}A}$ than pulmonary circulation transmural hydrostatic forces.
9. The data in this thesis is a paradigm change in the interpretation of pulmonary circulation transvascular fluid fluxes away from the traditional interpretation, which is based on Starling's principles, to major influences of the erythrocyte volume decrease across the lung via the Jacobs-Stewart cycle and chloride (anion) exchanger (Band 3 or AE1).

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Acknowledgments

I wish to acknowledge the support and guidance given by my supervisors, Dr. John Pringle, Dr. Laurent Viel and Dr. George Heigenhauser. I am lucky and greatly honored to be guided through this PhD program by these eminent individuals. They all left a permanent impression on my professional and personal life.

I would like to thank Dr. Henry Staempfli for his great help in my research and clinical progress. His famous saying “Medicine keeps us humble” will forever echo in my ears.

I am grateful to all that gave me their indispensible assistance and advice during the exercise studies (Dr. Wayne McDonell, Dr. Francisco Teixeira, Dr. Aury Moraes, Dr. Luis Arroyo, Dr. Heather Chalmers, Dr. Carolyn Kerr, Dr. Dorothee Bienzle, Gabrielle Monteith, Dan Schnurr).

I would like to thank Dr. Vojteh Cestnik for his support in my research and professional work at the Veterinary faculty Ljubljana.

Finally, but most importantly, I thank my beautiful Katarina, Eva and Petra for their support and inspiration through my PhD work. I am deeply indebted to Maria and Edgar who gave me loving attention, great encouragement, and a warm and cozy escape at all times. I am grateful to Tone, Gorazd, and Katjusa for their love, support and advice. I thank Barbara, Milena and Stane for their support.

Although I cannot share the happiness of achieving the PhD degree with my parents, Andrea and Mihael, they raised me to be the person I am, and to them I owe thanks for my success and happiness. **I dedicate this work to both of them**.

This study was supported by the:

- Ontario Ministry of Agriculture and Food– Equine Program,
- Department of Clinical Studies at the University of Guelph,
- Slovenian Research Agency grant P4-0053,

- Canadian institutes of health research,
- Department of Medicine at the McMaster University Medical Centre.

The overall PhD program of training was conducted within the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences. Most of the experimental work was conducted at the Ontario Veterinary College, University of Guelph. Some experimental procedures were performed at Department of Medicine, McMaster University Medical Centre.