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INFLUENCE OF INHALED AMILORIDE ON LUNG FLUID CLEARANCE IN RESPONSE TO NORMOBARIC HYPOXIA IN HEALTHY INDIVIDUALS

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Running Title: The Necessity of ENaC in Lung Fluid Clearance

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Abstract (200 word limit)

Aim: To investigate the role of epithelial sodium channels (ENaC) on lung fluid clearance in response to normobaric hypoxia, twenty healthy subjects were exposed to 15 hours of hypoxia (FiO_2=12.5%) on two randomized occasions: 1) inhaled amiloride (A) (1.5mg/5ml saline); and 2) inhaled saline placebo (P). Changes in lung fluid were assessed via chest CT for lung tissue volume (TV), and the diffusion capacity of the lung for carbon monoxide (DLCO) and nitric oxide (DLNO) for pulmonary-capillary blood volume (V_C). Extravascular lung water (EVLW) was derived as TV-V_C and changes in the CT attenuation distribution histograms were reviewed.

Results: Normobaric hypoxia caused 1) a reduction in EVLW (change from baseline for A vs. P, -8.5±3.8 vs. -7.9±5.2%,$p<0.05$), 2) an increase in V_C (53.6±28.9 vs. 53.9±52.3%,$p<0.05$) 3) a small increase in DLCO (9.6±29.3 vs. 9.9±23.9%,$p>0.05$), and 4) CT attenuation distribution became more negative, leftward skewed, and kurtotic ($p<0.05$).

Conclusion: Acute normobaric hypoxia caused a reduction in lung fluid that was unaffected by ENaC inhibition via inhaled amiloride. Although possible amiloride-sensitive ENaC may not be necessary to maintain lung fluid balance in response to hypoxia, and it is more probable that normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of healthy individuals. The observed reduction in interstitial lung fluid means alveolar fluid clearance may not have been challenged.

Keywords: chest computed tomography (CT), diffusion capacity of the lungs for carbon monoxide and nitric oxide (DLCO/DLNO), epithelial sodium channels (ENaC)

Acronym List:

ASL: Airway surface liquid BSA: body surface area BMI: body mass index BP: blood pressure Cl^- : chloride
CRU: clinical research unit CT: computed tomography
DLCO: Diffusion capacity of the lungs for carbon monoxide
DLNO: Diffusion capacity of the lung for nitric oxide DM: alveolar-capillary membrane conductance EBC: exhaled breath condensate
EBCNa: exhaled breath condensate sodium concentration
EVLW: extravascular lung water
ENaC: epithelial sodium channels
EPI: epinephrine
FVC: forced vital capacity
FEV_1: forced expiratory volume in one second of the FVC
FEF_{25-75}: forced expiratory flow at 25-75% of the FVC
FiO_2: fraction of inspired oxygen
HAPE: high-altitude pulmonary edema
HR: heart rate
LLS: Lake Louise Score
Hb: Hemoglobin
Hct: hematocrit
MEFV: maximal expiratory flow volume
Na^+: sodium
NE: norepinephrine
PAP: Pulmonary arterial pressure
PV: plasma volume
Q: cardiac output
TR: tricuspid regurgitation
TV: tissue volume
VA: Alveolar volume
VC: Pulmonary-capillary blood volume
VO_{2MAX} : maximal exercise capacity

Introduction

Pulmonary edema results from an imbalance between forces driving fluid into the alveoli, namely Staring's Law of fluid filtration and the integrity of the alveolar-capillary barrier and the biological mechanisms for its removal, primarily active sodium (Na+) transport, which osmotically drives water reabsorption from the alveolar space, and lymphatic drainage. Hypoxic pulmonary vasoconstriction plays a role in the development of high-altitude pulmonary edema (HAPE) (Motley and others 1947; Sartori and others 2004; Swenson 2013). The increase in capillary hydrostatic pressure results in an increase in net filtration of fluid from the capillary to the interstitial space. Fluid that has been filtered, but not reabsorbed from the interstitial space, is then removed by the pulmonary lymphatics. Stimulation of increased lung lymph flow can occur in response to beta-2 adrenergic receptor stimulation mediated by increases in catecholamines, and increases in ventilation. These events will deform the tissue lympathic vessels attach to, and thereby facilitate pumping and production of lymph (Ikomi and others 1991; Mahe and others 1991; Pearse and others 2005; Zawieja 2009). Previous work by our group demonstrated that exposure to normobaric hypoxia increases ventilation and catecholamines and reduces lung fluid (Snyder and others 2006; Snyder and others 2008). The close proximity of the capillaries to the alveoli allows for optimal gas exchange, but this closeness also subjects the alveoli to potential fluid infiltration in conditions such as higher pulmonary artery pressure and increased pulmonary vascular resistance which can result in fluid accumulation in the interstitial space and if not cleared cause fluid to build up in the alveoli. Because of the inverse and exponential relationship between rate of diffusion and membrane thickness, increases in airway surface liquid (ASL) that are not quickly reabsorbed will increase the distance across the alveolar-capillary membrane and greatly impact rate of diffusion and alveolar-capillary membrane conductance (D_M). Lungs must

be kept moist, but not wet for effective and efficient gas diffusion. The principal determinant of ASL depth is the mass of salt on the airway surface (Boucher 1999).

Active transport of Na+ from the airspace through epithelial sodium channels (ENaC) and then across the basolateral membrane by Na+/K+ ATPase is believed to be the primary determinant of alveolar fluid clearance by creating an osmotic gradient, with ENaC-mediated Na+ absorption being the rate limiting step (Matthay and others 2002; Matthay and others 1996). The importance of ENaC in keeping the lungs moist, but not wet is supported by evidence which suggests: 1) mice with a non-functional ENaC will demonstrate a failure to thrive in neonates due to an inability to clear amniotic fluid from their lungs following birth (Hummler and others 1996); 2) individuals with pseudohypoaldosteronism, characterized by a loss of ENaC function, have been shown to have excess ASL (Kerem and others 1999); and 3) the pathologically dry lungs of patients with cystic fibrosis is partially due to hyperaborption of Na+ by ENaC (Mall and others 2004).

Evidence for ENaC's role in alveolar fluid clearance is abundant, but less clear is the evidence that it is necessary for maintaining lung fluid balance, and if impairment of channel function is sufficient to cause pulmonary edema. Further, there is evidence for impairment in Na+ transport in individuals susceptible to HAPE. For example, Sartori et al. demonstrated that baseline nasal potential difference was lower and the amiloride-sensitive Na^+ transport reduced in mountaineers susceptible to this condition (Sartori and others 2004). Sodium transport was shown to be further reduced with altitude exposure in HAPE susceptible individuals, although this reduction with altitude was not in amiloride-sensitive Na+ flux. In contrast, amiloride administration in rats caused a decrease in Na^+ transport with no additional decrease with

exposure to hypoxia. These data suggest that the reductions in ENaC activity were responsible for the reduction in Na+ transport with hypoxia (Tomlinson and others 1999a) and this hypoxia induced reduction in Na+ transport has been suggested to be mediated by downstream reduction in ENaC channel expression (Gille and others 2014). Additionally, prophylactically taken beta 2-agonist's can prevent HAPE in the susceptible subjects during altitude exposure (Sartori and others 2002) and stimulate amiloride-dependent lung fluid clearance in hypoxia exposed rats (Vivona and others 2001) by reversing the hypoxia-mediated reduction in Na+ transport. Therefore, the purpose of this study was to determine if ENaC is necessary for lung fluid clearance in response to normobaric hypoxia in healthy humans. We hypothesized that ENaC inhibition by amiloride would result in greater lung fluid accumulation evidenced by 1) an increase in lung tissue density (measured via computed tomography (CT)) and estimated extravascular lung water (EVLW), 2) an elevation in exhaled breath condensate Na+, where an increase in Na+ would suggest an increase in ASL depth as water as water follows salt, and 3) a reduction in diffusion capacity of the lungs due to alveolar fluid accumulation increasing the diffusion distance across the alveolar-capillary membrane.

Materials and Methods

Subjects

^Twenty-three healthy non-smoking adults of average fitness ($V0_{2PEAK}$ 106% predicted) agreed to participate in this study. The protocol was reviewed and approved by the Mayo Clinic Institutional Review Board, all participants provided written informed consent prior to study and all aspects of the study were performed according to the declaration of Helsinki. Exclusion criteria included 1) cardiovascular or pulmonary abnormalities; 2) history of renal disease; 3) obese (BMI >30); 4) pregnancy; 5) hospital contact restrictions or an inability to exercise. Two participants were 'screen failures', one due to illness and the other due to hospital contact restrictions. In addition, one subject was removed from the hypoxic tent after four hours due to nausea and general malaise. As such, the data reported reflects the results of the twenty subjects who completed the study.

Protocol

At an initial screening visit, 1) height and weight were measured, 2) a blood draw was taken to rule out anemia and 3) a pregnancy test was completed in female subjects. Next, baseline pulmonary function was measured before each subject performed a maximal exercise capacity test on a cycle ergometer. Those without any of the exclusion criteria were then exposed to ~15hours of normobaric hypoxia in a double-blind, crossover, and randomized fashion of two experimental conditions: 1) nebulized amiloride (A) (1.5mg in 5ml normal saline), and 2) saline placebo (P). The experimental conditions were performed on different occasions separated by > 3 days. Changes in lung fluid from before to after hypoxic exposure were assessed via chest CT

for lung tissue volume (TV), exhaled breath condensate sodium concentration (EBCNa) for ASL sodium flux, and the diffusion capacity of the lungs for carbon monoxide (DLCO) and nitric oxide (DLNO) for the determination of pulmonary-capillary blood volume (V_C). Extravascular lung water (EVLW) and changes in the CT attenuation distribution histograms were reviewed. A summary of the hypoxic exposure visit is provided in Figure 1.

Normobaric Hypoxia Exposure

For both treatment visits, subjects arrived at the Mayo Clinic Clinical Research Unit (CRU) at 13:00 and a venous catheter was placed in a brachial or antecubital vein. Following 30 minutes of quiet rest, a baseline blood draw was taken for measurement of complete blood count, serum catecholamines and plasma sodium and chloride. Baseline measurement of lung fluid and pulmonary arterial pressure were taken. Subjects were then transferred to the hypoxic tent (FiO_2 12.5%, P_AO_2 91.5mmHg) (Colorado Altitude Training, Boulder, CO) at approximately 16:00. Vital signs, including heart rate (HR), blood pressure (BP), respiratory rate, respiratory sounds, acute mountain sickness symptoms (modified Lake Louise Score (Savourey and others 1995); see supplemental material), as well as tent temperature, barometric pressure and CO_2 level were assessed and recorded every two hours by a CRU nurse assigned to the patient. Although we did not measure urine output as part of the initial protocol, we observed and received feedback from the first six subjects that within a few hours of the initial nebulization the participants seemed to urinating more frequently on one visit compared to the other visit. Due to the known diuretic effects of oral amiloride in the kidney, and the known short surface half-life and subsequent absorption of nebulized amiloride across the lung epithelium (Knowles and others 1990b; Mentz and others 1986; Noone and others 1997), we thought this may have been a sign that the local

nebulization amiloride was having quite rapid systemic effects. As such, we modified the protocol to record fluid input and output in all remaining subjects, so net fluid output could be quantified. Subjects wore a wrist pulse oximeter (Nonin WristOx 3100, Nonin Medical, Inc., Plymouth, MN) to allow for continuous HR and peripheral oxygen saturation (SpO_2) monitoring during their time in the tent. The subjects remained in the tent overnight for a total of 15.3 ± 0.9 hours. If subjects needed to use the rest room, they were fitted with a portable mask connected to a gas reservoir attached to a cylinder of the hypoxic gas (12.5% O_2) until returning to the hypoxic tent. At around 6:00 the following morning (14.6 ± 0.9 hours post entry to the tent), the blood draw was repeated and followed by EBC collection inside the tent. A mask and gas reservoir were used to keep the subjects hypoxic during the CT and DLCO and DLNO measurements. Hemoglobin (Hb) and hematocrit (Hct) measured from the complete blood count completed by the Mayo Clinic Clinical Core Laboratory (Sysmex XE5000) were used to estimate the change in plasma volume (PV) using the following equation (Dill and Costill 1974):

$$\Delta\%PV = \left(\left(\frac{Hb_{t1}}{Hb_{t2}}\ x\ \frac{100 - Hct_{t2}}{100 - Hct_{t1}}\right) - 1\right) x\ 100$$

Epinephrine (EPI) and norepinephrine (NE) were measured by the Mayo Clinic Clinical Research Unit immunochemical core laboratory using High Performance Liquid Chromatography.

Drug Administration

The randomization and preparation for the administration of nebulized amiloride (1.5mg in 5mL saline) and nebulized saline placebo (5mL saline) was performed by the Mayo Clinic CRU pharmacy ensuring that the study investigators, technicians, nursing staff and subjects were blinded. Amiloride and saline were nebulized using standard apparatus (ReliaMed) connected to

a room air supply flowing at 8L/min. Each treatment was administered at three time points during exposure to hypoxia: 1) upon entering the tent at 16:00; 2) 21:00; and 3) 4:00 the following morning. The investigators were unblinded after all subjects had completed the study.

Chest CT Assessment of Lung Fluid

Chest CT measurements were performed before and 15.5 ± 0.9 hours following hypoxic exposure for both treatment visits. The CT protocol followed what has been previously used in our laboratory (Johnson and others 2012; Snyder and others 2006). Briefly, the same scanner (GE LiteSpeed spiral CT scanner, GE Healthcare) was used for all CT scans. A scout scan was performed on the baseline visit of each overnight stay to determine the location and size of the lungs. The non-contrast chest CT scan was obtained with 2.5 mm thick slices with a 1.2mm overlap initially and then reconstructed to 1.25mm with a 0.6mm overlap. Before the subject was removed from the scanner a mark was placed on the subject's skin to designate the anatomical location of the start of the scan and the table height and number of slices were recorded. The baseline scout scan was repeated for each hypoxic exposure visit (placebo and amiloride), but the table height and number of slices was kept consistent with what was done the first hypoxic exposure visit. A member of the study team was with the subject in the scanning room, and instructed the subject to take a maximal inhalation and hold their breath at the total lung capacity. At this time the study team signaled the radiology technician to complete the scan, and once through the scanner the subject was told they could relax and return to normal breathing. Although a gated spirometer was not used to control lung volumes, the difference between baseline and post exposure to hypoxia CT derived air volumes was on average less than 5%.

The CT images were then analyzed using custom image analysis software (Apollo, VIDA Diagnostics). The analyses were completed by a lab member blinded to the condition of the

subject's CT scan. The software segments the image to separate lung tissue from surrounding structures. In each picture element, the lung density was assumed to be a linear combination of air which has a Hounsfield units = -1000, and lung tissue which has the density of water, HU= 0. As such, an element at -600 HU represents 40% tissue, and -300 HU would represent 70% tissue. A histogram analysis of the picture elements within the lung tissue area was performed to obtain a mean lung density in HU and a tissue volume by summation of all the elements in the lung fields. The density and tissue volumes (TV) can also be determined for individual lobes of the lung. Two different methods were used to assess lung water from the CT. First, an estimation of extravascular lung water (EVLW). Since the tissue volume measured from the CT scan consists of lung tissue, blood and water, we subtracted the pulmonary capillary blood volume obtained from the DLCO and DLNO measures to remove the blood component ($EVLW = TV - V_C$). If we then assume tissue volume remains relatively constant between the pre and post scans, any change in the EVLW describes changes in lung fluid. Second, differences in EVLW between study conditions were estimated using a histogram analysis approach. Lung interstitial tissue was segmented from surrounding tissue, large airways, and blood vessels using segmentation algorithms built in MATLAB (Mathworks, Inc., Natick, MA). CT attenuation distributions were generated from the segmented images. Mean, skew, kurtosis, and full-width half-max (FWHM) were calculated from these distributions (Chase and others 2016). When the attenuation becomes less attenuated or more negative, more skewed to the left and/or more kurtotic this collectively suggest less fluid as the attenuation distribution is becoming less dense, and shifting away from water's attenuation of 0 HU. Previous work has demonstrated a strong positive correlation between attenuation and extravascular lung water (Scillia and others 1999; Shaker and others 2004).

Measurement of Diffusion Capacity of the Lungs for Carbon Monoxide, Nitric Oxide and Assessment of Cardiac Output

^Before and 16.0+0.9 hours following hypoxic exposure for both treatment visits, DLCO and DLNO and cardiac output (Q) were measured simultaneously with the subjects in an upright seated position using the rebreathing technique with a 5-liter anesthesia bag containing 0.7% acetylene, 9% helium, 0.3% carbon monoxide ($C_{18}O$), 40 PPM NO (diluted immediately before the test in the bag from an 800 PPM gas mixture) and 35% O_2, at a respiratory rate of 32 breaths/minute as described previously (Hsia and others 1995; Snyder and others 2006; Snyder and others 2005; Wheatley and others 2015; Wheatley and others 2011a; Wheatley and others 2011b; Wheatley and others 2013). The volume of gas placed in the bag was a standardized volume of 1575mL for all resting measures to ensure the bag did not collapse during inhalation, but also did not cause an unnecessary excess of gas in the bag during the maneuver. Bag volume was reduce to 1050mL in one subject. At the end of a normal expiration (functional residual capacity), the subjects were switched into the rebreathe bag and instructed to nearly empty the bag with each breath for 8-10 consecutive breaths. The maneuver was performed in triplicate before and after hypoxic exposure (performed immediately following completion of the CT scan).

The rate of disappearance of acetylene from the exhaled gas mixture during rebreathing is used to assess pulmonary blood flow. Since acetylene does not bind to hemoglobin, the rate of disappearance of acetylene is limited primarily by the rate at which a new volume of blood is transported through the lungs. Because all the blood in the pulmonary circulation per minute is equal to the volume of blood in the systemic circulation per minute, the measure of the

disappearance of acetylene provides a reliable measure of cardiac output and has previously been validated in our laboratory using direct Fick during exercise (Johnson and others 2000; Liu and others 1997).

The diffusing capacity of the lungs for carbon monoxide is based on the contribution of both the membrane conductance and the hemoglobin binding and described by the equation developed by Roughton & Forester (Tamhane and others 2001).

$$\frac{1}{DLCO} = \frac{1}{DM_{CO}} + \frac{1}{\theta_{CO} \cdot V_C}$$

The rate of disappearance of the gases with each breath is calculated from the slope of the exponential disappearance for each gas with respect to helium using custom software(Snyder and others 2005). Unlike DLCO, DLNO is theoretically based solely on membrane conductance as nitric oxide is scavenged 8000 times faster by hemoglobin than O_2 so its uptake into the blood is nearly instantaneous. Although currently being debated, DLNO has been considered a relatively direct measure of membrane conductance (D_{MNO}) as the diffusion resistance of the blood is trivial (Hsia 2002; Hsia and Raskin 2005; Roughton and Forster 1957b; Tamhane and others 2001), but not infinite, and for our purposes of comparing change in response to a stimulus gives reliable results (Coffman and others 2016). Using this assumption, the D_{MNO} value is used to calculate the D_M for carbon monoxide (D_{MCO}) by adjusting for differences in diffusion constants based on molecular weight and solubility between the two gases as described previously using an alpha ratio of 2.2 (Tamhane and others 2001; Wheatley and others 2010b). Pulmonary-capillary blood volume (V_C) is then calculated from the DL_{CO} measured by subtracting the resistance to diffusion associated with alveolar-capillary barrier (D_{MCO}) and correcting for differences in the rate of uptake and binding to hemoglobin ($1/\theta$) due to differences in Hb concentrations and the alveolar pressure of oxygen as described previously using the Roughton and Forester 2.5 Θ_{CO}

equation (Roughton and Forster 1957a; Tamhane and others 2001; Wheatley and others 2010b). This technique has been validated in our laboratory and used extensively for studies in other clinical populations (Olson and others 2006; Snyder and others 2006; Snyder and others 2008; Wheatley and others 2011a; Wheatley and others 2011b).

Pulmonary Function Testing

Baseline spirometry was assessed on the screening visit according to American Thoracic Society guidelines (Medical Graphics CPXD, Minneapolis, MN) to determine forced vital capacity (FVC), forced expiratory volume in one second of the FVC (FEV_1) and forced expiratory flow at 25-75% of the FVC (FEF_{25-75}). Before and after hypoxic exposure on visits 2 and 3 subjects repeated FVC maneuvers following each of the diffusion capacity measurements (Miller and others 2005). Predicted values for all pulmonary function measures were based on predicted equations from NHANES III (Hankinson and others 1999).

Exhaled Breath Condensate (EBC)

Exhaled breath condensate samples were collected using a Jaeger EcoScreen cooling unit (Cardinal Health, Yorba Linda, CA) as we have previously described (Wheatley and others 2010a). During the 20 minute collections, subjects sat wearing a nose clip and breathed through a mouthpiece so all their exhaled breath could be directed to the Teflon condenser inside the EcoScreen cooling unit. Collections were made at baseline and the next morning following hypoxic exposure before subjects were removed from the tent. Samples were frozen at -80°C and then batch analyzed with quantification of chloride completed using ion chromatography and sodium measured with inductively-coupled plasma mass spectrometry.

Pulmonary Arterial Pressure

Pulmonary arterial pressure was calculated from the tricuspid regurgitation (TR) velocity as described previously (Yock and Popp 1984) using the equation AP=A4V , where P is the pressure and V (m/s) is the tricuspid regurgitant velocity. The same sonographer performed the echocardiographic measures at baseline and after the fifteen hours of hypoxic exposure being performed before the subject was removed from the tent. There were three sonographers who performed these measurements on the subjects, all of them using the following methods for their assessment. Color Doppler was used to locate the tricuspid regurgitation jet. Data reported are from sixteen out of twenty subjects for whom a jet could be visualized and successfully measured. The maximal velocity was determined by careful application of the continuous wave sampler within and parallel to the regurgitation jet.

Statistical Analysis

The SPSS statistical software package (v.22; SPSS, Inc., Chicago, IL) was used for all statistical analyses. Two-factor repeated measure ANOVA was used to evaluate the main effects of normobaric hypoxia, drug (amiloride vs. placebo) and their interaction on the measures of lung fluid and systemic response to the conditions. Paired samples t-tests were performed between percent change from baseline to post exposure to hypoxia metrics (LLS, urine input/output) for the two treatments, with an alpha level of 0.05 used to determine statistical significance. All values presented are mean ±SD unless otherwise stated.

Results

Subject characteristics for the twenty subjects who participated in this study are provided in Table 1.

Changes in Lung Fluid in Response to Normobaric Hypoxia and Amiloride

Normobaric hypoxia did not change DLCO, DLNO, or alveolar-capillary membrane conductance (D_M) for both amiloride and placebo conditions (Figure 2). By contrast, hypoxic exposure caused an increase in pulmonary capillary blood volume (V_C) (hypoxia effect $p<0.01$); the magnitude of increase in Vc was not different in amilioride vs. placebo (54±29% vs. 54±52%, p= 0.52) (Figure 2). There was a reduction in CT derived tissue volume in response to hypoxic exposure (hypoxia effect p=<0.01) that was similar between amiloride and placebo conditions (-49.3±25.7 vs. -46.1±31.2 mL, p=0.69) (Figure 3). This decrease in tissue volume was not uniform across the lungs, with a minimal reduction (~2 ml) in the mid-right lobe, a 10 to 13 ml reduction in the left lobes and upper right lobe, and a trend for a larger decrease, especially with amiloride, in the lower right lobe (~16ml) (Figure 4). There was a similar and significant decrease in EVLW from before to after hypoxic exposure (hypoxia effect $p<0.01$) with amiloride and placebo (-8.5±3.8% vs. -7.9±5.2%, p=0.53) (Figure 3). CT attenuation distributions showed the same trend for EVLW. Distribution average was shifted more negative, more leftward skewed, and more kurtotic after hypoxic exposure in both groups suggesting clearance of fluid from the lungs due to the shift towards less attenuation (hypoxia effect $p<0.05$, Table 3). There was no difference in these changes between amiloride and placebo conditions ($p>0.05$). Additionally, there was a decrease in plasma volume with hypoxic exposure (hypoxia effect

p<0.01) for both conditions amiloride vs. placebo (-9.2±9.7 vs. -11.0+11.0 p= 0.52) suggesting that the decrease in EVLW was not just a shift of fluid from the interstitial to vascular space.

Although there was a decrease in interstitial lung fluid with hypoxia and no effect of amiloride in the gross measures of changes in EVLW or on diffusion capacity or alveolar-capillary membrane conductance, a measure of alveolar fluid, there were still signs of ENaC inhibition. First, utilizing EBC Na+ to assess changes in alveolar lung fluid suggested a trend for a decrease with placebo, but an increase with amiloride as was expected with amiloride inhibiting ENaC mediated-sodium absorption at the level of the alveolar epithelium. Second, in the fourteen subjects fluid input and output was recorded and although the pairwise comparison was not significant (p =0.44, Table 4), review of the individual responses under each condition shows the variability, and demonstrates that in eight subjects fluid loss was greater with amiloride compared to only four participants where fluid loss that was greater with the placebo than with amiloride, and two participants who showed no real difference between conditions (Figure 5).

Systemic Responses to Normobaric Hypoxia Exposure

The systemic responses to the normobaric hypoxia exposure are presented in Table 4. There was no change in cardiac output with hypoxic exposure (hypoxia effect p>0.05) and no difference between conditions amiloride vs. placebo (p=0.99), and the increase in systolic pulmonary arterial pressure was small (hypoxia effect p=0.02) and not different between amiloride vs. placebo visits (p=0.41). Hypoxic exposure caused a significant increase in HR with normobaric hypoxia (hypoxia effect p<0.01) that was not different between conditions (p=0.23). There was a trend for a reduction in norepinephrine concentration from pre- to post-hypoxia in

the amiloride condition (p=0.46), with no other change in catecholamine concentration was observed. Under both experimental conditions, there was no change in respiratory rate, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{75}, suggesting hypoxic exposure had minimal to no effect on lung and airway function (hypoxia and condition effect $p>0.05$). Hypoxic exposure caused a significant and sustained reduction in SpO_2 (hypoxia effect $p<0.01$) that was similar between amiloride and placebo conditions (86±3 vs. 85±3%, p=0.29). Over the course of the hypoxic exposure (~15 h), SpO_2 decreased below 80% for only 18.3±16.1 min and 15.8±15.3 min in amiloride and placebo, respectively. No individual presented with signs of HAPE, subjects demonstrated mild altitude sickness with low modified Lake Louise Scores.

Discussion

In this study we demonstrated that 1) there was a reduction in lung fluid, specifically interstitial lung fluid, with exposure to normobaric hypoxia and 2) the use of nebulized amiloride to inhibit ENaC did not affect lung fluid regulation. The results of this study replicate our laboratory's prior findings that exposure to normobaric hypoxia as well as hypobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals (Snyder and others 2006; Snyder and others 2008; Taylor 2013), but did not follow our original hypothesis that ENaC inhibition by amiloride would result in greater lung fluid accumulation. The novel findings in this study was the observation that lung fluid regulation was unaffected by ENaC inhibition via inhaled amiloride. The following discussion will highlight the potential mechanisms of lung fluid removal, the importance of ENaC and the ability of hypoxia to challenge alveolar fluid clearance.

First, what is mediating removal of fluid with exposure to hypoxia?

Consistent with our laboratory's previous findings, we demonstrated a reduction in interstitial lung fluid through CT derived measures of EVLW with exposure to hypoxia. However, in this current study alveolar fluid clearance rate appeared to be unchanged as there was no change in DLCO or DM with exposure to hypoxia. One possible explanation for this is that although in both studies subjects were kept in hypoxia until and between all measurements, in the current study the DLCO gas mixture used for post hypoxia measurements was the same as baseline where the oxygen concentration was 35%, where as in the previous study a special hypoxic DLCO gas mixture was used where the oxygen concentration was 18%. Since the change previously observed was not drastic (+10%), the potential of reoxygenation over the 10 breaths of the non-hypoxic DLCO gas may have limited our ability to measure a change with hypoxia in the current study. The results of these studies seem to highlight that the observed fluid reduction is predominantly interstitial fluid removal. As such, lymphatic drainage is potentially of greater importance and the primary mediator of the observed reduction in lung fluid. Previous work in sheep and dogs has shown that lymph flow increases 10-40% with hypoxia (Levine and others 1988; Martin and others 1986) and the increases in ventilation experienced with hypoxia also facilitate pumping and production of lymph (Ikomi and others 1991; Mahe and others 1991; Pearse and others 2005; Zawieja 2009). Additionally, in our previous study we observed an increase in exhaled nitric oxide with normobaric hypoxia exposure (Snyder and others 2006; Van Iterson and others 2017). In the thoracic lymphatic duct of rat, initiation of spontaneous contraction of the phasically non-active segments results in nitric oxide mediated relaxation of these segments. This reduction in lymphatic vessel tone improves diastolic filling of the vessels and although contraction rate is reduced, lymphatic contractions are stronger making overall lymphatic pumping more efficient (Gashev 2008; Gasheva and others 2006). As such, we

hypothesize that the reduction in interstitial lung fluid observed in this study and in previous work in response to normobaric hypoxia is primarily driven by increases in lymphatic fluid clearance mediated by 1) increases in minute ventilation likely elevated due to increases in tidal volume, since we did not observe an increase in respiratory rate and 2) increases in NO mediating relaxation of the lymphatics such that they can more efficiently clear any excess interstitial fluid that is not reabsorbed.

Is impairment of ENaC function really insufficient to cause pulmonary edema in response to hypoxic exposure?

First, at least two types of Na^+ channels have been identified to exist in the alveolar epithelium each with very different regulation, and quite often opposite response to the same stimuli (Eaton and others 2004; Trac and others 2017). ENaC is composed of three homologous subunits: a-ENaC, P-ENaC and y-ENaC. It is the ratio and combination of these subunits that can produce channels with varying conductances and regulatory properties. When a channel is composed of all three subunits then the channel has high Na^+ selectivity and falls into the highly selective channel (HSC) type. In contrast, nonselective cation channels (NSC), or amiloride insensitive channels, are composed of at least one a-ENaC subunit and at least one acid-sensing ion channel 1(ASIC1a) and the channel has low Na+ selectivity or no selectivity, making it likely to secrete K+ rather than absorb Na+(Trac and others 2017). Hypoxia can cause a shift from HSC to NSC as

hypoxia reduce HSC or ENaC channelsm, but increase NSC expression (Jain and others 2001; Trac and others 2017), and reduces sodium transport across the airway epithelium (Tomlinson and others 1999b). In rats it was demonstrated that amiloride caused a greater drop in transepithelial Na+ flux, measured by nasal potential difference (NPD), than hypoxia alone. With hypoxia and amiloride there was no additional reduction in Na^+ current, suggesting that the

reduction in Na+ with hypoxia was amiloride-sensitive ENaC mediated (Tomlinson and others 1999a). Further, prior work has demonstrated that total or mean NPD is reduced in HAPE-prone subjects prior to altitude exposure, suggesting reduced resorption, with only Sartori et al showing a significant reduction in the amiloride-dependent Na+ transport (Mairbaurl and others 2003; Sartori and others 2004). Upon ascent to altitude results continued to conflicted at times, as Sartori et al observed a further decreased in total NPD, specifically only the amiloride-insensitive Na+ current by ~30%, with no change in the amiloride sensitive Na+ current, and this was only HAPE-prone subjects (Sartori and others 2004). In contrast, Mairbaurl et al found that total NPD became more positive due to increased chloride secretion, occurring in response to nasal dryness, and an observed increase in the amiloride insensitive Na+ current in both control. The amiloride-dependent Na+ reabsorption decreased in control subjects, while remained unchanged in HAPE-prone individuals (Mairbaurl and others 2003). Additionally, previous cell and tissue work has found that the 40-50% of the Na+ and airway fluid clearance occurs through amiloride-insensitive channels (O'Brodovich and others 2008; Sakuma and others 2006), with one study in human ATII cells demonstrating the amiloride-insensitive made up 70% of the fluid transport (Fang and others 2006). Data is conflicting as to which channel Na+ is moving through to mediate fluid clearance, but recent work by Trac et al. demonstrated that NSC reduction through knocking down either a-ENaC or ASIC1a reduces alveolar fluid clearance and causes wetter lungs. Further, unlike with ENaC (HSC) where its expression and numbers decrease with hypoxia, NSC increase expression in response to hypoxia and albeit likely not as effectively they are able to assist in preventing alveolar edema (Trac and others 2017). Focusing on the human in vivo and in vitro work as well as the results of this study suggest that although present, amiloride sensitive Na+ transport is not the sole means of alveolar fluid clearance, especially in response to

426 normobaric or hypobaric hypoxia. Measurement of nasal potential difference was not performed
427 in this study limiting our ability to directly assess respiratory transepithelial baseline Na+
428 transport and the effects amiloride administration had on this in response to the normobaric
429 hypoxia exposure.
430 Second, one has to also question whether the amiloride dose sufficient for inhibition -
431 how much reached the alveoli and how long was it acting locally on the airway epithelia before
432 being absorbed and circulated systemically. Nebulized amiloride was originally developed for
433 potential use in individuals with cystic fibrosis, where it was hoped it could inhibit the
434 pathological hyperabsorption of Na+ that occurs through ENaC in these individuals.
However,
435 nebulized amiloride showed very poor efficacy in clinical trials and this was attributed to its
low
436 potency and short half-life duration on the airway epithelia (Graham and others 1993; Hirsh
437 2002; Knowles and others 1990a; Kohler and others 1986; Pons and others 2000). Understanding
438 these limitations of amiloride, but wanting to inhibit ENaC locally with a nebulized amiloride
439 dose FDA approved, amiloride was administered three times (five and seven hours apart) during
440 the subject's hypoxia exposure. Even with this repeat dosing, drug delivery may have been
441 limited by the aerosol droplet size (larger portion of droplets being outside the respirable
range
442 of 1-5pm), and the lack of a standardized pattern of breathing, which could have reduced
443 alveolar deposition such that the required concentration for effective blockade of 10pmol/L in
444 the alveoli may not have been reached, and ENaC blockade was then only partial (Noone and
445 others 1997; Schulz 1998). The timeline line of SpO_2 and change from baseline (A SpO_2) every
446 two hours during the hypoxia exposure shows a trend from a drop in SpO_2 following the

amiloride administrations, but this occurs with both placebo and amiloride (Figure 6). Although complete ENaC blockade was unlikely, the results suggest amiloride was having an inhibitory

effect locally as there was a trend for higher EBC Na+, a non-invasive assessment of airway surface liquid composition, with amiloride compared to placebo. This measurement has its limitations as although the composition of EBC is considered to be a dilute surrogate marker of ASL composition, one cannot be certain what region(s) of the lung the droplets are being formed. Future work should follow up with nasal potential difference measurements to provide an additional measure of changes in ion flux in the airway epithelium in response to hypoxia with and without amiloride. We also have signs that the nebulized amiloride was being absorbed across the epithelia and acting on the kidneys to cause diuresis as there is a trend for a higher net urine output with amiloride. This observed diuretic effect aligns with earlier pharmacokinetic work showing that after aerosol delivery, amiloride plasma concentration peaks by 30 minutes and 50% of amiloride has been excreted by four to six hours post administration (Noone and others 1997).

Although this study did not show that ENaC was necessary for preventing lung fluid accumulation, it does not discount the role of ENaC in regulating alveolar lung fluid clearance. ENaC has been demonstrated to be necessary for fetal alveolar lung fluid clearance, where knock out of alpha ENaC caused a failure to thrive in mice (Mall and others 2004), but this study and the work of others suggests that the role of amiloride-sensitive ENaC is not primary or solely responsible for maintaining lung fluid homeostasis in response to normobaric or hypobaric hypoxia. ENaC's role in lung fluid balance is alveolar fluid clearance and in response to normobaric hypoxia we do not observe that this role is challenged, such that it is not needed or necessary to maintain lung fluid balance. The current study and previous work have demonstrated that exposure to normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals (Snyder and others 2006; Snyder and others 2008)

meaning that even with amiloride inhibition of ENaC, complete or partial, alveolar fluid clearance is not really challenged as there is not a buildup of interstitial fluid that can potentially move into the alveoli. As such, we conclude that ENaC may not be necessary to maintain gross lung fluid homeostasis in response to normobaric hypoxia in healthy, non-HAPE susceptible individuals, but instead its role in more fine tuning and alveolar fluid balance and in this exposure there was no alveolar edema to prevent.

Third, was the hypoxic stimulus sufficient to challenge alveolar fluid clearance?

Exaggerated pulmonary hypertension plays an important role in the development of high-altitude pulmonary edema (HAPE) (Sartori and others 2000; Sartori and others 2004; Scherrer and others 1999). If the hypoxia stimulus is not sufficient, pulmonary arterial pressure would not be increased due to hypoxic pulmonary vasoconstriction and there would not be a large shift of fluid into the interstitial space (Maggiorini and others 2001). The estimated capacity of the lymphatics to absorb fluid is between 0.20-0.40mL/kg per hour for each pleural space (Shields 2009). Although the conditions (hypoxic tent, CRU environment, level of hypoxia) were the same between this study and our previous study, the degree of hypoxemia experienced by the subjects in the current study was slightly less, with an average SpO_2 around 85% overnight and less than 20 minutes at a SpO_2 less than 80% in the current study compared to an average of 82% overnight in the previous study. In the current study, participants demonstrated an increase in HR of less than 15 bpm, a small increase in PAP, no change in respiratory rate and no rise in catecholamines whereas in our previous work we saw an average 14 bpm increase in HR, a doubling of PAP, and an increase in both EPI and NE with 17 hours of normobaric hypoxia exposure (Snyder and others 2006). Mazzeo et al. demonstrated that in response to an acute high altitude exposure, there is a rapid (within 4 hr) and significant increase in arterial EPI

concentrations (Mazzeo and others 1994). Hypoxia directly stimulates the adrenal medulla to release EPI into the circulation, with the increase in EPI concentration directly related to the severity of hypoxia exposure (i.e. the decline in arterial O_2). In calves, Bloom et al. demonstrated that only in response to intense hypoxia (arterial PO_2 17.1±2.8mmHg) did the adrenal medulla secrete physiologically effective amounts of catecholamines (Bloom and others 1977). With an average peripheral desaturation greater than 80%, it is unlikely that there was a severe decline in arterial O_2 (80% SpO_2 = PaO_2 ~50mmHg), and as such not a strong enough stimulus for EPI release from the medulla. Alveolar fluid clearance, where ENaCs plays a role, would only be challenged when net fluid balance is disrupted such that there is more fluid moving from the pulmonary vessels to the interstitial space than can be removed by the lymphatic vessels; as then this excess fluid has the potential to shift into the alveolar space. With no change in catecholamines and no increase in pulmonary arterial pressure and a reduction in lung fluid in the current study, the hypoxia exposure likely did not challenge alveolar fluid clearance such that amiloride mediated impairment in alveolar transepithelial Na^+ transport would compromise lung fluid clearance. Additionally, further work is needed to evaluate the role of ENaC in lung fluid, both alveolar and interstitial, to determine if these observations also hold true in HAPE- susceptible individuals.

Conclusion

Acute normobaric hypoxia caused a reduction in lung fluid volume that was unaffected by ENaC inhibition via inhaled amiloride, suggesting amiloride-sensitive ENaC were not necessary to maintain a balance between lung fluid accumulation and lung fluid clearance. We demonstrate a reduction in lung fluid, and as such it is likely that alveolar fluid clearance, where ENaC would be involved, was not significantly challenged. It is possible amiloride-sensitive

ENaC may not be necessary to maintain lung fluid balance in response to hypoxia, but it is more probable that normobaric hypoxia promotes lung fluid clearance rather than accumulation for the majority of individuals.

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Tables

Table 1

Population Demographics

	All	Male	Female
n	20	11	9
Age (years)	27±5	29+6	25+3
Height (cm)	172±8	176+7	166+4*
Weight (kg)	71+13	80+8	58+6*
BMI (kg/m^2)	24±3	26+3	21+3*
BSA (m^2)	1.8+0.2	2.0+0.1	1.6+0.1
VO_{2PEAK} (% predicted)	106+19	102+20	111+17
FVC (% predicted)	104+15	106+15	102+16
FEV_1 (% predicted)	102+15	104+17	99+14
FEF_{27-75} (% predicted)	97+22	101+25	93+18
Hemoglobin (g/dl)	14.0+1.2	15.0+0.8	13.1+0.8*

FVC=forced vital capacity; FEVi=forced expiratory volume after one second of FVC; FEF_{25-75}= forced expiratory flow at 25-75% of FVC. Data are presented as mean±SD. * $p<0.05$ vs. Males

Embedded table for figure 3

Table 2: Absolute Changes	Placebo		Amiloride	
	Baseline	Post	Baseline	Post
Tissue Volume (mL) *	834+137	788+145*	837+137	788+144
Extravascular lung water (mL)	797+137	736+143*	802+137	736+142*

*$p<0.05$ hypoxia effect

Table 3 Differences in EVLW using a histogram analysis of CT attenuation distributions

	Placebo		Amiloride	
	Baseline	**Post**	**Baseline**	**Post**
Average (HU)	**-889.0+23.4**	**-894.2+21.1***	**-886.3+26.5**	**-898.3+21.3***
Skew	**4.3+0.6**	**4.6+0.79***	**4.3+0.60**	**4.6+0.69***
Kurtosis	**30.9+8.0**	**35.3+10.1***	**31.2+8.9**	**35.0+10.0***
FWHM (HU)	**58.9+12.8**	**57.8+14.9**	**61.0+12.1**	**57.2+13.4**

FWHM = full width half-max. Data are presented as mean+SD.* $p<0.05$ vs. baseline

Table 4 Systemic Responses to Normobaric Hypoxia

Systemic Response to Normobaric Hypoxia						
	Placebo			Amiloride		
	Baseline	Post	Difference	Baseline	Post	Difference
Cardiac Output (L/min)	4.6+1.4	4.2+1.1	-0.3+1.0	4.2+0.9	3.8+1.3	-0.3+1.1
Systolic Pulmonary Artery Pressure (mmHg)	15.9+9.9	17.6+11.5	3.6+5.3	10.6+7.2	16.3+11.5*	6.0+11.0
HR (bpm)	67±9	78+14*	10+9	65+12	79+15*	14+12
SpO2 (%)	98+1	92+4*	-11+5	99+1	92+3*	-14+4
EPI (pg/mL)	32.5+25.7	32.7+24.1	0.20+33.0	36.4+50.0	31.0+24.4	-5.4+47.5
NE (pg/mL)	242.2+82.5	207.0+101.4	-35.2+92.6	246.1+133.1	184.9+63.1*	-61.3+114.5
Respiratory Rate (breath/min)	15.9+1.6	16.7+1.8	0.8+1.8	15.7+1.8	16.8+1.6	1.1+2.5
FVC (L)	4.9+0.3	4.7+0.3	-0.11+0.05	4.8+0.3	4.8+0.3	-0.08+0.05
FEV_1 (L)	3.9+0.2	3.9+0.2	-0.02+0.04	3.9+0.2	3.9+0.2	-0.03+0.05
FEV_1/FVC (%)	81.7+1.4	83.3+1.4	0.6+0.8	82.1+1.4	82.7+1.4	1.6+0.4
FEF_{25-75} (L/sec)	3.9+0.2	4.1+0.2	0.2+0.1	3.9+0.2	4.0+0.2	0.02+0.10
FEF_{75} (L/sec)	1.9+0.1	2.1+0.1	0.1+0.1	2.0+0.1	2.0+0.1	0.1+0.1
PEF (L/sec)	9.0+0.6	8.7+0.6	-0.2+0.2	8.6+0.5	8.6+0.6	0.03+0.2
EBC Na^+ (mmol/L)	0.71+0.29	0.58+0.25	-9.7+43.2%	0.61+0.21	0.72+0.51	20.1+92.5%
Fluid Input-Output (mL)	-136.3+732.4			-272.8+319.6		
Modified Lake Louise Score	1.0+1.2			1.1+1.2		
Percent of Time SpO2<80%	15.8+15.3			18.3+16.1		

SpO_2= peripheral oxygen saturation; HR= heart rate; EPI= epinephrine; NE= norepinephrine; FVC=forced vital capacity; FEV_1=forced expiratory volume after one second of FVC; FEF_{25-75}= forced expiratory flow at 25-75% of FVC; FEF_{75}= forced expiratory flow at 75 % of FVC; PEF= peak expiratory flow; EBC Na^+= exhaled breathe condensate Na^+; Modified Lake Louise Score is out of 30 and averaged over their tent exposure; Percent of time SpO_2<80%= percent of time in tent that nonin wrist stats dropped below 80%. Data are presented as mean±SD.* $p<0.05$ vs. baseline

Figure Legends

Figure 1 Hypoxia Visit Schematic

Figure 2 Diffusion Capacity of the Lungs for Carbon Monoxide (DLCO) and Nitric Oxide (DLNO) in Response to Normobaric Hypoxia

Pre-hypoxia (white bars) to Post-hypoxia (black bars) for placebo and amiloride. Panel A: Diffusion capacity of the lungs for carbon monoxide (DLCO); Panel B: Diffusion capacity of the lungs for nitric oxide (DLNO); Panel C: Alveolar-capillary membrane conductance (DM); Panel D: Pulmonary-capillary blood volume (V_C). Percent change listed for each above, placebo vs. amiloride respectively; * $p<0.05$ vs. baseline

Figure 3 Changes in CT assessed Lung Tissue Volume (TV) and Calculated Extravascular Lung Water (EVLW) After Normobaric Hypoxia

Difference from post hypoxia to pre hypoxia for placebo (white bars) and amiloride (black bars) for tissue volume (ATV) and extravascular lung water (EVLW = TV - V_C). Percent change from baseline is listed for EVLW. And absolute change is found in table 2.

Figure 4 CT Tissue Volume Changes Stratified by Lung Lobe after Normobaric Hypoxia

Difference from post hypoxia to pre hypoxia for placebo (white bars) and amiloride (black bars) for tissue volume for lung lobes: LL= lower left; UL= upper left; UR= upper right; MR= middle right; LR= lower right.

Figure 5 Individual Fluid Input-Output in Response to Normobaric Hypoxia

Each line represents a subject, with the fluid input-output plotted for the amiloride visit and the Placebo visit and line connecting the two to show how the responses differed between conditions. Negative fluid loss greater in placebo vs. amiloride condition (dashed lines); negative fluid loss with amiloride and a positive with placebo or less fluid gain with amiloride (black lines). No difference in I/O between conditions (grey lines).

Figure 6 SpO_2 and change from Baseline ($ASpO_2$) During the Normobaric Hypoxia Exposure

The average SpO_2 noted by the CRU nurse every two hours in subjects during their amiloride visit (black squares) and placebo visit (open black circles). Black arrows represent the time when amiloride/placebo was nebulized. The change in SpO_2 from baseline every two hours is presented for amiloride (grey squares) and placebo (open grey circles). Standard deviation is not presented on figure to keep figure clear. Amiloride SD: mean ±4.4; range (1.2-5.6). Placebo SD: mean ±4.4; range (3.2-5.9). Amiloride delta SD: mean ±5.1; range (3.9-6.0). Placebo delta SD: mean ±6.3; range (4.9-8.1).

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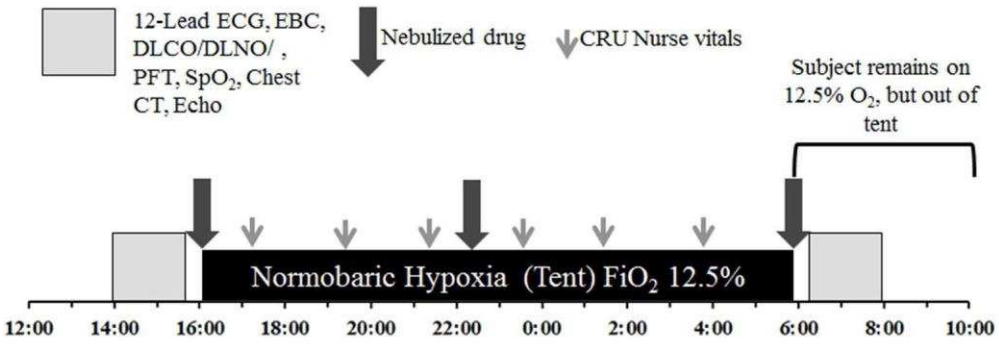

Figure 1 Hypoxia Visit Schematic 211x74mm (96 x 96 DPI)

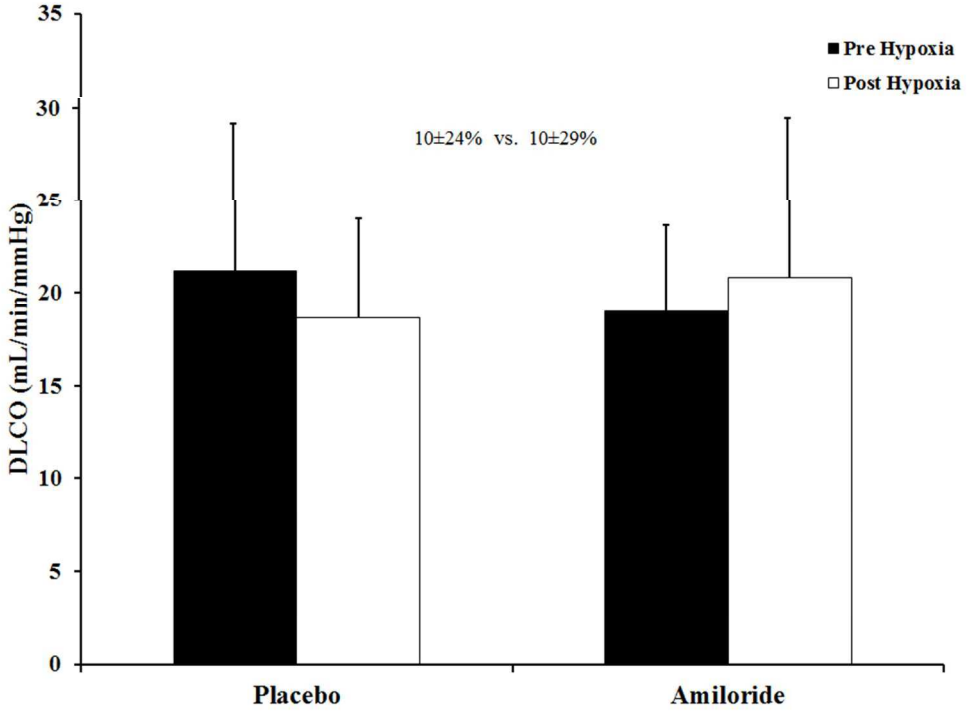

Figure 2A Diffusion Capacity of the Lungs for Carbon Monoxide (DLCO) in Response to Normobaric Hypoxia

240x174mm (96 x 96 DPI)

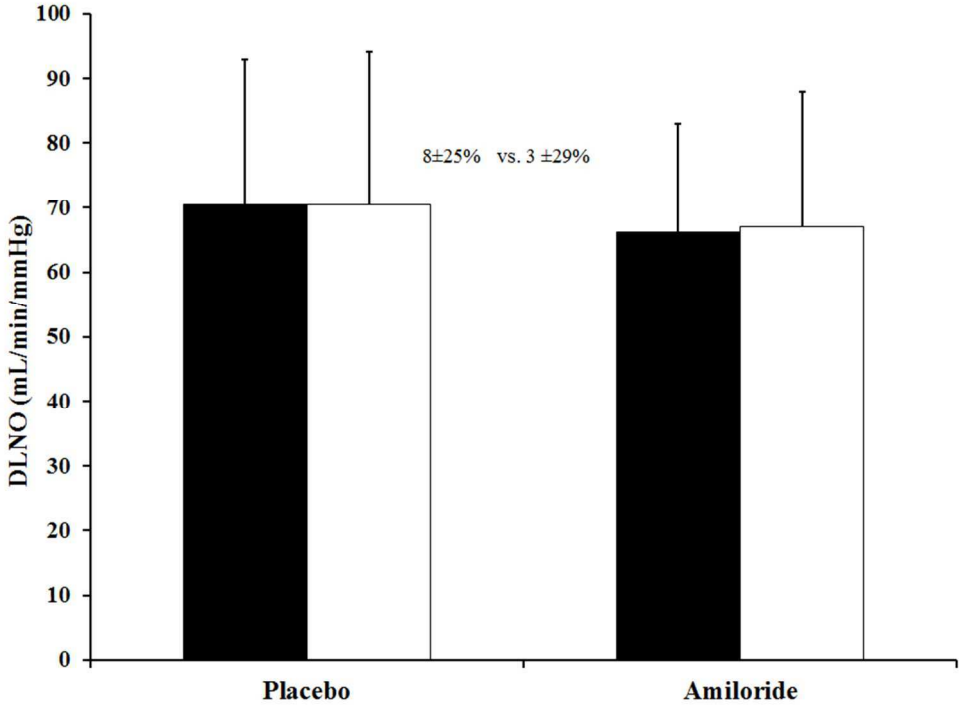

Figure 2B Diffusion Capacity of the Lungs for Nitric Oxide (DLNO) in Response to Normobaric Hypoxia

240x174mm (96 x 96 DPI)

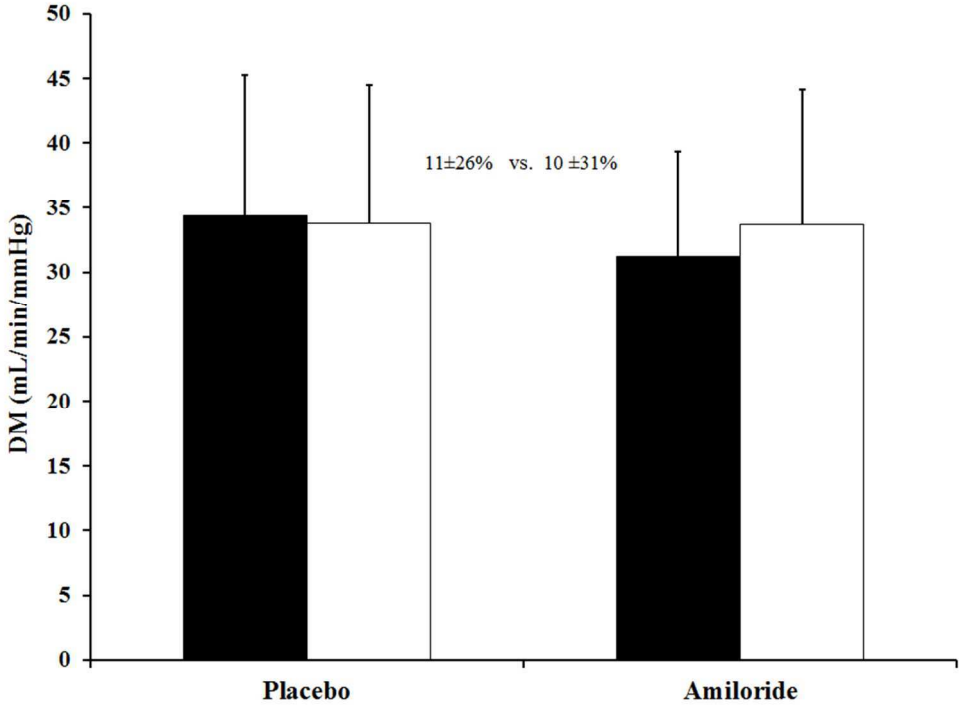

Figure 2C Alveolar-capillary membrane conductance (DM) in Response to Normobaric Hypoxia

240x174mm (96 x 96 DPI)

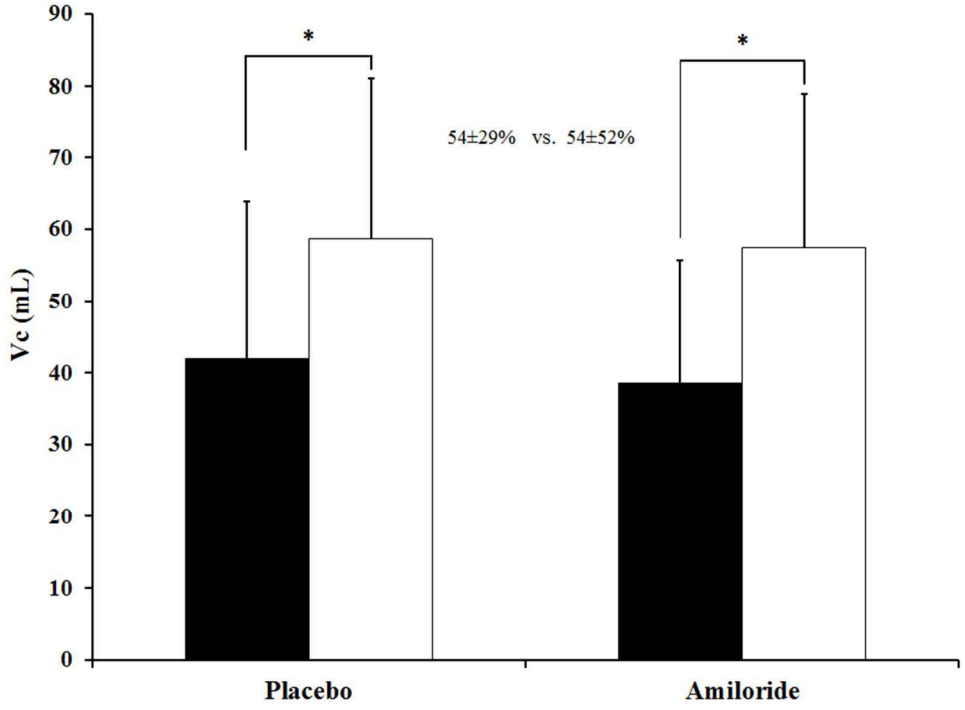

Figure 2D Pulmonary-capillary blood volume (VC) in Response to Normobaric Hypoxia

240x174mm (96 x 96 DPI)

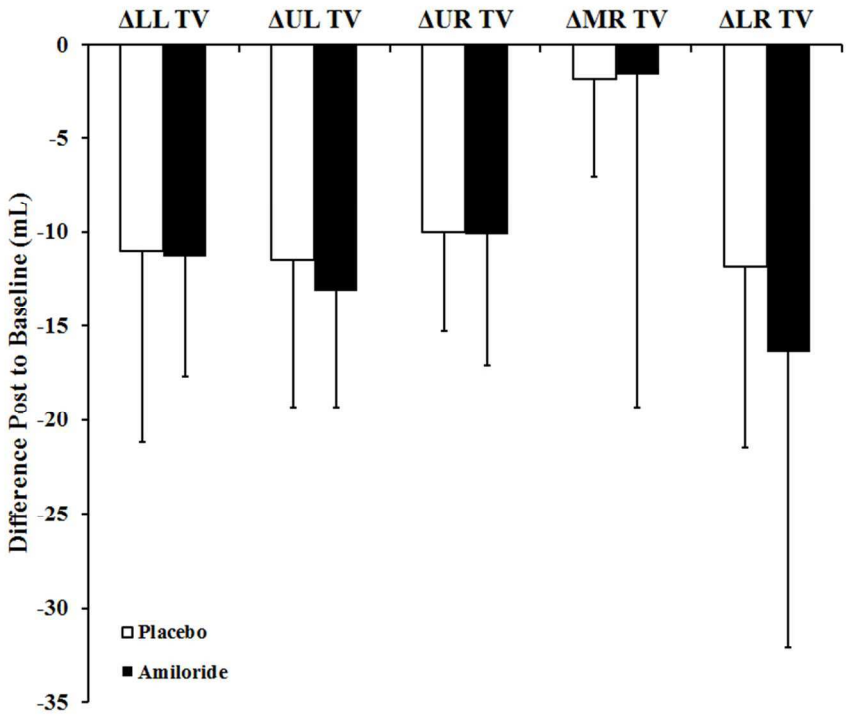

Figure 3 Changes in CT assessed Lung Tissue Volume (TV) and Calculated Extravascular Lung Water (EVLW) After Normobaric Hypoxia

240x174mm (96 x 96 DPI)

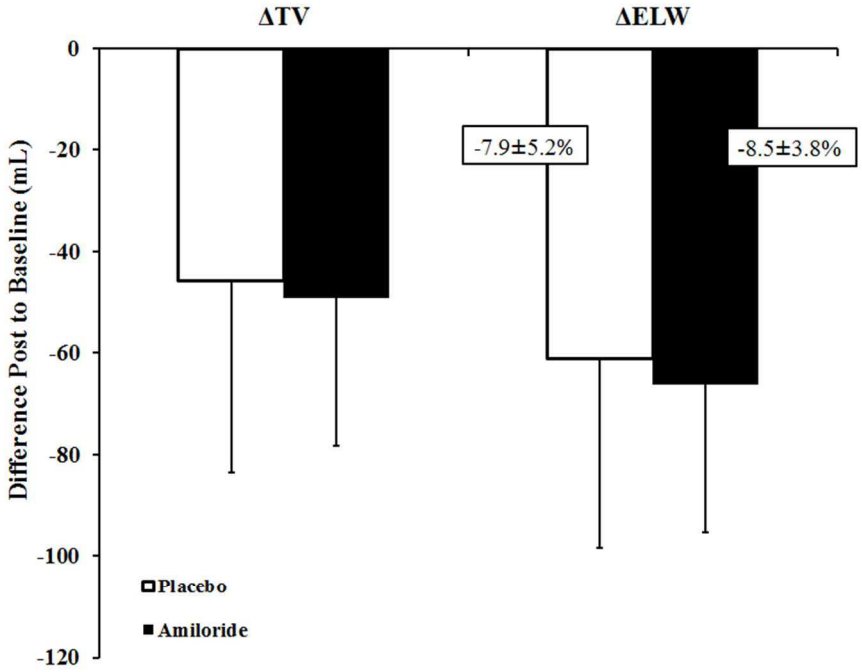

Figure 4 CT Tissue Volume Changes Stratified by Lung Lobe after Normobaric Hypoxia

240x174mm (96 x 96 DPI)

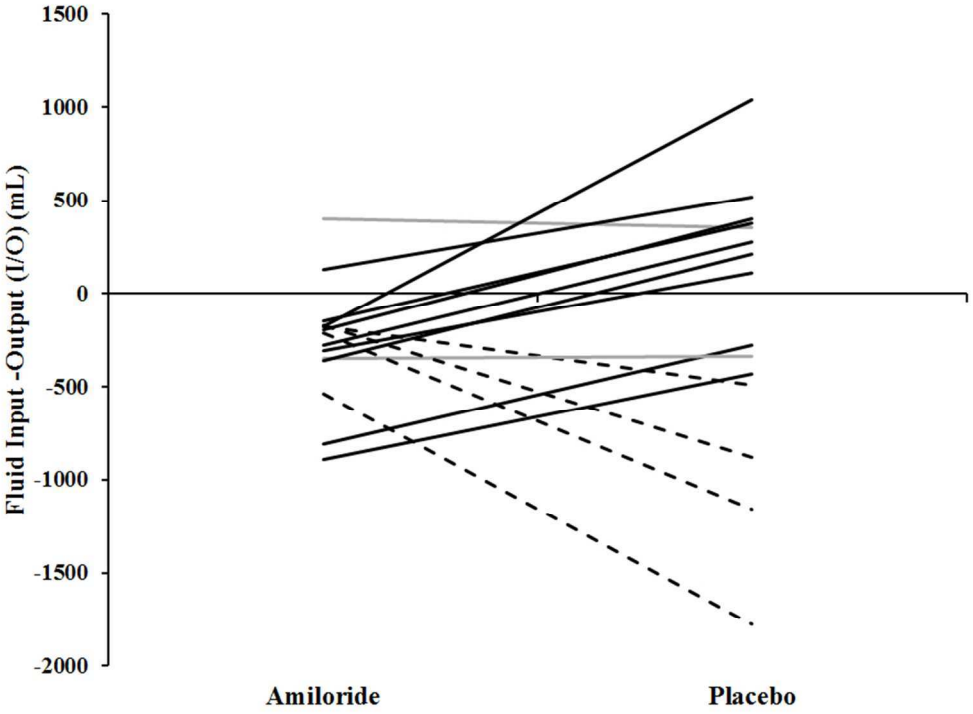

Figure 5 Individual Fluid Input-Output in Response to Normobaric Hypoxia

240x174mm (96 x 96 DPI)

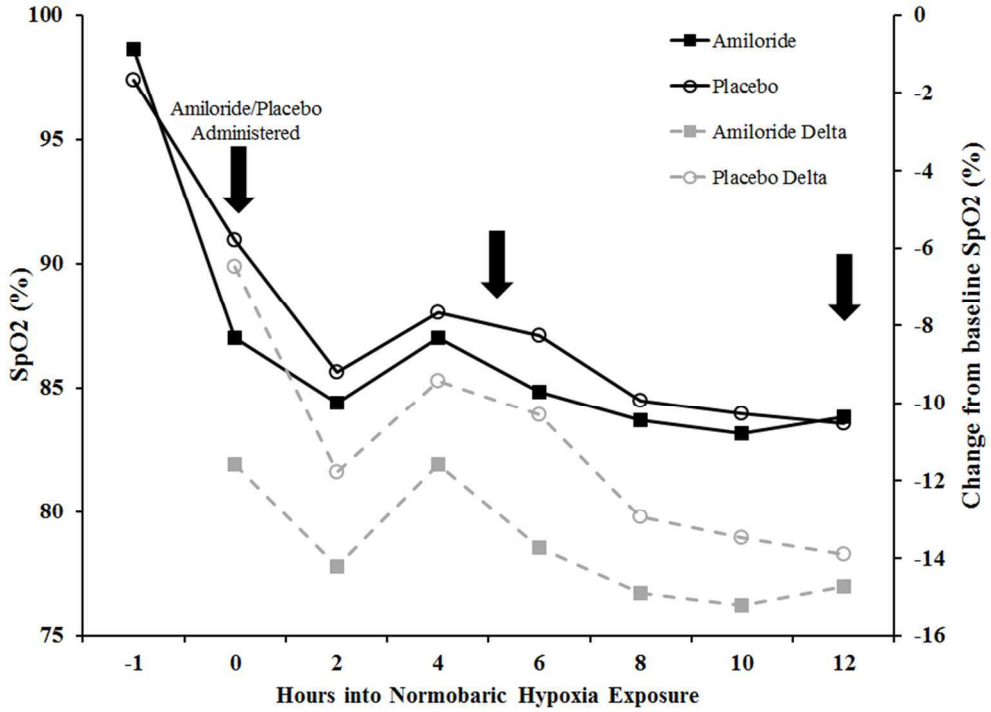

Figure 6 SpO2 and change from Baseline (ΔSpO2) During the Normobaric Hypoxia Exposure

241x174mm (96 x 96 DPI)

Supplement:

High Altitude Symptoms Worksheet: Lake Louise **A**cute **M**ountain **S**ickness Questionnaire

COLUMN 1		COLUMN 2		COLUMN 3	
1. Headache		**5. Difficulty sleeping**		**9.Cough**	
No headache	0	*Slept well as usual*	0	*No change from usual*	0
Mild headache	1	*Did not sleep as well as usual*	1	*More than usual*	1
Moderate headache	2	*Woke many times, poor night's sleep*	2	*Significantly more than usual*	2
Severe, incapacitating	3	*Could not sleep at all*	3	*Unable to stop coughing*	
2. GI (stomach)		**6. Short of breath at rest**		**10. General health**	
No problems	0	*Breathing as usual*	0	*I feel OK*	0
Poor appetite, nausea	1	*Mildly short of breath*	1	*A little ill but can do everything*	1
Moderate nausea, vomiting	2	*Moderately short of breath*	2	*Somewhat ill, limited*	2
Severe N & V, incapacitating	3	*Severely short of breath*	1 3	*Feel bad, can't function normally*	3
3. Fatigue/weak		**7.Edema/swelling (hands, arms, face, feet)**			
Not tired or weak	0	*No swelling*	0		
Mild fatigue/weakness	1	*Swelling in 1 spot*	1		
Moderate fatigue/ weakness	2	*Swelling in 2 spots*	2		
Severe F/W, incapacitating	3	*Swelling in multiple spots*	3		
4. Dizzy/lightheaded		**8. Change in mental status**			
Not dizzy	0	*No problems*	0		
Mild dizziness	1	*A little slow of thinking*	1		
Moderate dizziness	2	*Definitely confused at times*	2		
Severe, incapacitating	3	*Very confused and lethargic*	3		
Column 1 Total		**Column 2 Total**		**Column 3 Total**	

Column 1	
Column 2	
Column 3	
Overall total score	

Study Stopping Criteria

If number in any grey box is circled, STOP STUDY

If total score is >25, STOP STUDY