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Scanning electron microscopy after corrosion casting of hypoxic pulmonary vasoconstriction in rats and subacute pulmonary edema after unilateral lung hypoxia in pigs

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SCANNING ELECTRON MICROSCOPY AFTER CORROSION CASTING OF HYPOXIC PULMONARY VASOCONSTRICTION IN RATS
and
SUBACUTE PULMONARY EDEMA AFTER UNILATERAL LUNG HYPOXIA IN PIGS

by

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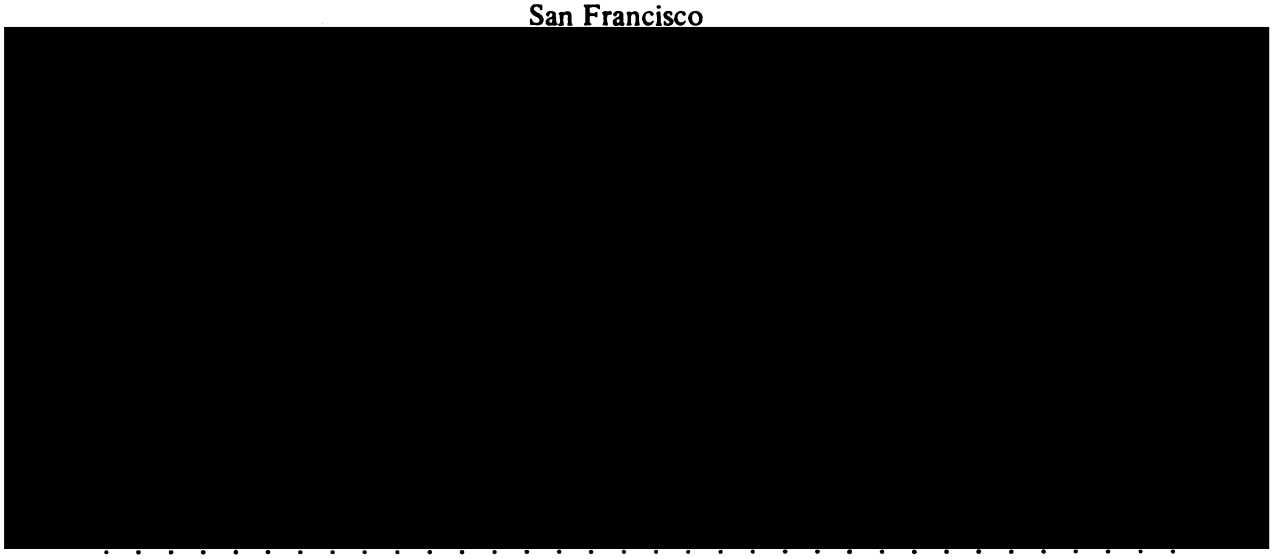

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SCANNING ELECTRON MICROSCOPY AFTER CORROSION CASTING OF HYPOXIC PULMONARY VASOCONSTRICTION IN RATS AND SUBACUTE PULMONARY EDEMA AFTER UNILATERAL LUNG HYPOXIA IN PIGS

by

Mary Stafford

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I. INTRODUCTION

A. Problem

High altitude pulmonary edema (HAPE), a sometimes fatal accumulation of water in the lungs, was first described in a clinical report in North America thirty years ago. Today, the incidence of HAPE continues to rise as the popularity of trekking and climbing to the highest peaks without acclimatization or supplemental oxygen continues to rise. Symptoms of headache, dizziness, shortness of breath, lethargy and nausea, sometimes felt upon rapid ascent to altitudes above 2,000 meters, describe an illness known as acute mountain sickness (AMS). AMS often precedes HAPE. This association has helped identify the circumstances and symptoms that lead to the occurrence of HAPE. Although this information is used to educate climbers about the risk of HAPE, it has not decreased the incidence of high altitude pulmonary edema. Despite the increased awareness, the pathophysiology of the disease remains unknown.

In the nineteenth century, death from high altitude pulmonary edema may have been mistaken for pneumonitis (Mosso, 1898). In 1913 Ravenhill described it as a "cardiac" type of "puna" or mountain sickness involving frothy sputum and lung rales. In North America the first case of HAPE was reported by Houston in 1960. Since then many more cases have been documented with detailed descriptions of the circumstances and symptoms accompanying them (Fred, et al.., 1962; Aria-Stella and Saldana, 1962; Aria-Stella and Kruger, 1963; Hultgren et al.., 1964; Singh et al.., 1965; Grover et al.., 1979; Hackett et al.., 1980; Dickenson et al.., 1983; see also review by Schoene, 1985).

Three types of pulmonary edema have been identified: (1) increased-permeability edema (noncardiogenic) which develops after acute lung injury and is characterized clinically by

elevated protein concentration in lymph derived from the lung relative to plasma (Staub, 1974); (2) increased-pressure edema (hydrostatic or cardiogenic) often caused by left ventricular failure or elevated pulmonary venous pressure; and (3) primary lymphatic obstruction (see review by Albertine, 1985). Although high altitude pulmonary edema is described as increased-permeability edema (Schoene, 1985), it may belong in a special category yet unidentified (Staub, 1984 and Ward, et al.., 1989).

At first the clinical picture of HAPE appeared similar to increased-pressure edema. However, while the pulmonary artery pressure is always greatly increased, the pulmonary artery "wedge" pressure which is elevated in left ventricular failure, remains normal (Hultgren et al.., 1968, 1970 and 1978). In chest radiographs a nonuniform distribution of edema seems to characterize HAPE (Grover et al.., 1979). In some X-rays white patches in the middle of the lung field suggested that edema might be forming around the larger vessels (Hultgren et al.., 1964) and some hemorrhagic cuffs in these areas were found at autopsy (Singh et al.., 1965). Hypotheses for possible pathophysiologic causes of HAPE include: (1) increased permeability edema caused by hypoxic constriction of some pulmonary vessels leading to overperfusion and leakage from nonconstricted vessels (Hultgren and coworkers, 1966) and (2) transarterial leakage of plasma and/or blood into the perivascular spaces caused by hypertensive overdistension and subsequent damage to pulmonary artery walls upstream to the constricted arterioles (Whayne and Severinghaus, 1968). More recently, elevated lung lymph to plasma protein concentrations have been measured in bronchalveolar lavage (BAL) fluid from climbers with HAPE suggesting some type of increased-permeability edema (Schoene, 1985).

B. Background

Pathophysiologic studies and clinical evidence have not been definitive in establishing the

cause of high altitude pulmonary edema. In chronological order the following experiments were performed to test the various hypotheses which Milledge (1983) and others (Ward et al.., 1989) believe are not mutually exclusive. The clinical evidence is also reported.

Hultgren and coworkers (1966 and 1978) produced experimentally overperfusion pulmonary edema in dog lungs by surgical resection of the lung and transfusion of blood to increase flow through the lung. Overperfusion of the pulmonary vascular bed occurred when alveolar hypoxia was induced to cause vasoconstriction in some areas, diverting blood flow such that the remaining vessels were overperfused. They showed that non-uniform vasoconstriction in the lungs resulted in high flow in less constricted areas, leading to edema in these areas.

Whayne and Severinghaus (1968) observed edema and hemorrhagic cuffs after exposing rats to 8% O_2 and 10 minutes of swimming exercise. They postulated that hypertensive over-distension and damage to the pulmonary arterial wall upstream to constricted arterioles might promote transarterial leakage of plasma or blood into the perivascular spaces.

Iliff (1971), using excised dog lungs, demonstrated that arterial walls less than 200 micrometers in diameter slowly leaked dye and formed perivascular cuffs at normal pulmonary artery pressures when airway pressure was elevated above pulmonary artery pressure. Thus, compression of venous circulation caused arterioles to leak. This supported Severinghaus' hypothesis that transarterial leakage caused HAPE.

Bland et al.. (1977) were unable to produce increased fluid filtration with hypoxia and elevated pulmonary artery pressures in adult sheep. However, they found increased fluid filtration under the same circumstances in newborn lambs. The type of edema found in the lambs did not fit the category of increased-permeability edema because lymph to plasma

protein ratios decreased (Bressack and Bland, 1980). In 1978, Ohkuda and coworkers found increased leakage and protein permeability after perfusion of microemboli to produce uneven pulmonary artery obstruction in sheep. In this way he supported Hultgren's original hypothesis and proposed that high vascular pressure and high blood flow through a restricted microvascular bed caused the leakage.

In 1980 Hackett et al.. reported four clinical cases of high altitude pulmonary edema in patients with congenital absence of the right pulmonary artery. They maintained that high pressure and high flow caused the edema. In 1981, Mitzner and Sylvester studying pressure-flow relationships and fluid filtration rates in isolated perfused pig lungs showed that hypoxic pulmonary vasoconstriction caused increased fluid filtration because of increased critical closing pressures.

In 1983, Landolt and coworkers were unable to reproduce Hultgren's results. Using anesthetized sheep, they found no change in lymph flow or the lymph:plasma protein concentration ratio after 65% lung resection and blood transfusion to maintain cardiac output with or without alveolar hypoxia. They questioned whether HAPE was in fact a type of increased permeability edema. On the other hand, samples of BAL fluid from high altitude pulmonary edema victims on Mt. McKinley contained elevated concentrations of high molecular weight proteins compared to controls (Schoene, 1984 and 1985). This suggests that HAPE is a type of increased permeability edema, but the mechanism is not revealed in these studies. Ultrastructural studies have not shown explicit evidence for microvascular damage (see review by Albertine, 1985).

C. Objectives

To further investigate what may cause high altitude pulmonary edema, I performed two

separate studies. I tested the hypothesis that high altitude pulmonary edema may be the result of leakage from pulmonary arteries caused by overdistension of the vessels proximal to or upstream from the site of terminal arterial constriction, as proposed by Severinghaus (1971). I used the same rat experimental model as Whayne and Severinghaus (1968). However, I used 6% O_2 instead of 8% O_2 during 10 minutes of swimming exercise. Then I injected methacrylate into the rat lungs immediately following the hypoxic exercise. In this way I could observe sites of possible leakage in simulated high altitude pulmonary edema and observe acute hypoxic vasoconstriction in three dimensions using scanning electron microscopy (SEM). I did not identify the leakage sites in this hypoxic pulmonary edema model but I did observe non-uniform vasoconstriction of the smaller sized pulmonary arteries.

The objective of the second study was to determine whether high flow and/or high pressure underlies hypoxic pulmonary edema in pigs. This study was based on preliminary results of another study done in this lab (Milledge et al.., 1968) using unilateral hypoxic ventilation and microembolization of lung capillaries in dogs. If pulmonary edema is caused by high capillary flow, it should be greater in the less anoxic left lung. If increased pulmonary arterial pressure causes edema, edema should be greater in the hypoxic lung or the same in both lungs. Briefly, in this experiment I used a tracheal divider to induce asymmetric hypoxia in anesthetized pigs. In a closed chest preparation, the right lung was ventilated with nitrogen and carbon dioxide (more hypoxic, low flow, high pressure) and the left lung was ventilated with air and carbon dioxide (less hypoxic, overperfused) in a closed chest preparation. After three hours the pigs were killed with an overdose of pentobarbital as the lung lobes were being perfused with 3% buffered glutaraldehyde for histologic examination. The lung lobes were removed and prepared for extravascular wet/dry weight determinations (Pearce et al.., 1965). My results indicate that subacute edema forms in both the right and left lung and therefore leakage occurs on both the high pressure side and the high flow side. Also, the site

of leakage appears to be greater in the smaller sized vessels.

II. METHODS

A. Rat Experiments

Two groups of male Long Evans rats weighing approximately 150 grams were used in the experiment. The control group (n=3) was given room air to breathe during rest before pulmonary artery perfusion of substances for clearing and preservation of vessels. In the experimental group (n=2) hypoxia and pulmonary hypertension were induced by supplying a gas mixture of 6% O_2, 5% CO_2 in N_2 to breathe during and after swimming in 37°C H_2O for 10 minutes before pulmonary artery perfusion with normal saline to wash the blood out followed by buffered glutaraldehyde to fix the internal vessel wall (Whayne and Severinghaus, 1968).

After swimming and before PA perfusion both groups of rats were anesthetized with pentobarbital (60 $mg \cdot kg^{-1}$), tracheostomized, attached to a modified ventilator (Bird, Mark2) and ventilated at 50-60 breaths per minute. The hypoxic rats were kept hypoxic during the time it took the pentobarbital to anesthetize them and to tie a cannula into the pulmonary artery via the right ventricle. These procedures took 20-25 minutes. O_2, CO_2 and N_2 were delivered via flow meters to the swimming and recovery chambers. The gases were monitored continuously with a mass spectrometer (Perkin Elmer 1100).

During perfusion (Figure 1), normal saline (10-20 ml) was used to wash the blood out of the lung first. Next, 3% buffered glutaraldehyde (10 ml) was perfused to fix the lung tissue. These perfusates were maintained at 37°C by a circulating water bath (Heto, Denmark Type 01T623). During normoxia the saline was bubbled with room air. During hypoxia the saline

was equilibrated with 6% O_2, 5% CO_2 and N_2. The objective of the technique was to keep the tissues as physiologic as possible until the moment of fixation.

Finally, methacrylate (Mercox CL-2R) was perfused. We held the perfusion pressure constant at 25 cmH_2O and the airway pressure constant at 10-15 cmH_2O throughout the procedure. Catalyst was added to the methacrylate prior to its perfusion. The methacrylate to catalyst ratio was approximately 20:1. This mixture allowed ample time for perfusion before the methacrylate became solid.

Once the methacrylate hardened (10-15 minutes), we excised the lungs and soaked them in 30% NaOH overnight at 100°C. They were rinsed in tap water for at least 6 hours to remove any remaining tissue (Figure 2). Under a dissecting microscope the pulmonary arterial tree could be followed from the cannula which remained tied to the cast with suture. The veins were carefully cut away.

Initially, the methacrylate casts were frozen and then fractured as recommended by Murakami (personal communication) to obtain pieces for mounting on aluminum SEM specimen stubs. Later, most casts were cut while submerged in water to obtain reproducible planes of section for mounting them on specimen stubs. The specimens could not be cut in room air even after freezing because the Mercox was hard and difficult to cut. To prevent scattering the specimens were placed under water while being cut.

Once mounted, each of the lung casts was sputter coated (Hummer Sputter Coater V, Technics Instruments Co.) with 200 Angstroms of gold and observed under a scanning electron microscope (Cambridge Stereoscan 150) at 10 or 20 kilovolts. For control and hypoxic rats one lobe of the lung was put together from these SEM photomicrographs. From these pictures the vessels were grouped according to size and counted. The number of vessels that

were constricted was compared to the total number of vessels in that group. The results were plotted graphically showing percent vessels constricted versus vessel size.

B. Pig Experiments

We studied two groups of pigs to determine whether high flow or high pressure causes hypoxic pulmonary edema. The control group (n=3) breathed room air after a tracheal divider was inserted. Approximately 30 minutes after intubation the lungs were perfused and removed. In the experimental group (n=4), the right lung was ventilated with N_2 and CO_2 to produce unilateral hypoxic pulmonary hypertension while the left lung was ventilated with air and CO_2 for 3 hours in a closed chest preparation (Figure 9).

The pigs (approximately 20 kg) were given atropine (0.44 $mg \cdot kg^{-1}$ I.M.) 15 minutes prior to administration of xylazine (Rompum, 2 $mg \cdot kg^{-1}$ I.M.) and ketamine (Vetalar, 20 $mg \cdot kg^{-1}$, I.M.). Then ketamine (same dose as above) was administered as needed via an ear vein until the femoral vein was exposed and a catheter was inserted for intravenous drug infusions throughout the remainder of the experiment. Anesthesia was maintained with sodium pentobarbital (35 $mg \cdot kg^{-1}$, I.V.) and titrated according to the blood pressure throughout the experiment. With the pig in the supine position a Swan-Ganz catheter was placed in the pulmonary artery via the jugular vein. Pulmonary artery pressure was continuously monitored from the catheter. A tracheostomy was performed and a tracheal divider inserted (Leymed Robert Shaw, double lumen). Separation of the tracheal divider was verified by ventilating one lung with 100% oxygen and the other lung with room air. Using a mass spectrometer (Perkin Elmer 1100), inspired and expired gases were sampled for O_2, CO_2, and N_2. The sampled gas values were collected and stored in the computer (Digital, PDP 11/44) and displayed on a CRT screen at the experiment site. When no mixing of the gases occurred at expiration

from each tracheal tube, separation of the tracheal divider was considered complete. The lungs were ventilated separately with a dual piston pump (Harvard) delivering constant volume to each lung with 5 cm H_2O positive end expiratory pressure to prevent atelectasis. Flaxedil (1 $mg \cdot kg^{-1}$ I.V.) was given to the animal after it was put on the ventilator to prevent respiratory movement. The ventilator rate and volume were adjusted to achieve normal blood gases (approximately 20 $ml \cdot kg^{-1}$ tidal volume). Airway pressure was monitored continuously from each endotracheal tube. In the hypoxic group baseline systemic arterial and pulmonary arterial pressures and continuous inspired and expired gases were established. Indomethacin (5 $mg \cdot kg^{-1}$, I.V.), a cyclooxygenase inhibitor, was given to block endogenous prostacyclin synthesis in the lung (Kadowitz, et al..,1975; Meyrick, et.al., 1985).

Thirty minutes after the administration of Indomethacin, the right lung was made hypoxic by adding N_2 to the inspired gas. Inspired gases were adjusted to maintain the hypoxic level. Pco_2 was increased as needed to keep the P_aco_2 approximately normal (38-40 mmHg). The left lung continued to receive room air. The hemodynamic data included assessment of baseline levels of systemic blood gases, systemic and pulmonary artery pressures for 30 minutes. Blood gases were drawn every 10-15 minutes during the first hour of hypoxia and every 30 minutes thereafter for three hours. Blood for analysis was drawn into heparinized plastic syringes, placed on ice or immediately analyzed for gas tensions and pH using Radiometer type electrodes calibrated with known gas mixtures.

At the end of the experiment the pigs were killed by administering sodium pentobarbital at five times the anesthetic dose. The chest was opened and the lung lobes were clamped and removed for extravascular wet/dry lung weight determinations (Pearce, et al..; 1965). The lobes were fixed by perfusion for light microscopy.

For perfusion fixation of the lobes the main pulmonary artery was ligated. Plastic tubing (3/4" i.d., 1/8 " o.d.) was inserted and tied in place in the distal portion. The base of the heart was excised to allow flow of the perfusates through the lung and out through the left ventricle. The lung was perfused with isotonic saline (37°C) to wash the blood out and then with 3% buffered glutaraldehyde by gravity flow (50 cm H_2O) while airway pressure was set at 15 cm H_2O after the lobes had been clamped off. When perfusion was complete the lobes were removed and submerged in beakers of the same fixative for 1-3 weeks before sectioning. Inflation pressure was adjusted to 15 cm H_2O and maintained during the first 12 hours of fixation.

Pulmonary edema was quantified three ways: the edema ratio, histologic evidence of cuffing and the Pearce technique. First, to measure the vessels for calculation of the edema ratio, the lobes were cut into blocks 1 cm x 1 cm x 1 mm. The sections were embedded in methacrylate (JB4 System) and stained with hematoxylin and eosin. The finished slide held a section 5 micrometers thick. The tissues were examined and photographs taken with the aid of an American Optical microscope. A photo montage was made by photographing several parts of each slide and putting the pictures together. For each montage every artery was counted. The vessel was considered an artery only if it was located next to an airway.

The diameter of the pulmonary artery was measured and expressed as the mean ± s.d. of five diameters taken from the center of the vessel. The vessel was excluded from the study if the length of the diameter perpendicular to the maximum measured diameter was less than half the maximum measured length. Interstitial thickness was expressed as a mean ± s.d. of five distances around the vessel from the arterial endothelium to the outermost interstitial area appearing to have fluid accumulation.

The edema ratio was calculated for each vessel as ER = (cuff area - artery area)/artery area (Michel, 1982). The vessels were grouped according to their diameters: less than 50 micrometers, 50-100 micrometers, 100-150 micrometers, 150-200 micrometers, 200-250 micrometers and greater. For each category the interstitial thickness was measured separately for the left and right lungs in the hypoxic pigs. For controls, left and right lung arterial diameters and interstitial thickness were grouped together. The values for the three groups, the left and right lung experimental groups and the control group, are reported as mean ± s.d.. Analysis of variance was used to determine statistical significance ($p<0.05$) between the experimental lungs (high pressure, right lung and high flow, left lung) and the control lungs (Zar,J. H., 1974).

Second, histological evidence for cuffing was determined as the number of arteries that had edema cuffs surrounding them compared to the total number of arteries counted in one montage. This cuff thickness determination was justified as similar measurements were made in the control group. In this way, "normal" cuff thickness was compared to those with "edema" cuffs.

Finally, the Pearce technique was used to determine the extravascular wet/dry weight ratios. Statistical analyses were carried out using the Student's Paired-t test ($p<0.05$ was regarded as significant) for the extravascular wet/dry weight ratios and the histologic results of cuffing.

III. RESULTS

A. Rat Experiments

SEM micrographs of a normoxic lung (Figures 3 and 4) were different from the SEM

micrographs of hypoxic lungs (Figures 5 and 6). In normoxic lungs smooth vessel walls were observed. In the hypoxic lungs two types of apparent constriction are seen. In the larger vessels a corrugated type constriction was seen and in the smaller vessels a sausage type constriction was seen (Figure 7).

In areas of apparent constriction the ratio of diameters of adjacent maximum to minimum was 1.12 ± 0.05 S.D. in all vessels less than 150 micrometers in diameter. Constriction appeared in approximately 10% of the vessels 150- 300 micrometers in diameter, 30% of the vessels 100-150 micrometers in diameter, 50% of the vessels 50-100 micrometers in diameter and in 80% of the vessels 30-50 micrometers in diameter (Figure 8).

B. Pig Experiments

Edema cuffs were expressed as edema ratios (ER= (cuff area - artery area)/ (artery area)). The edema ratio was larger, but not significantly, for the hypertensive, hypoxic right lung (3.08 ± 1.4, n=75 arteries) versus the lower pressure, hypoxic left lungs (2.77 ± 1.2, n=89 arteries). These edema ratios were significantly larger ($p<0.01$, ANOV) than for the normoxic, control lungs (1.8 ± 0.4, n=88 arteries) (see Figure 10).

Histologic results from the right lungs (high pressure, n=4) are compared to those of the left lung (overperfused, n=4) and expressed as number of edema cuffs present divided by the total number of arteries counted in that pig lung section (Figure 11). The proportion of cuffed pulmonary arteries was always greater in the high pressure right lung compared to the high flow left lung in pigs ($p<0.05$, Paired-t Test).

The results of the extravascular wet/dry weight measurements using the Pearce technique are shown in Figure 12. For the control group (n=3) the left lungs were had a ratio of 3.4 ±

0.26 and for the right lungs the ratio was 3.24 ± 0.15. After asymmetric hypoxia (n=4) the overperfused left lungs had a wet/dry weight ratio of 3.8 ± 0.37 compared to 3.8 ± 0.26 in the hypoxic right lung (n.s.).

The hemodynamic data before and after hypoxia are summarized in Table 1. In this table the pulmonary artery and systemic arterial pressures are shown with the arterial blood gas measurements before and after hypoxia. The mean systemic arterial pressure was calculated as diastolic pressure plus one third times pulse pressure (Pmean = diastolic + 1/3 (systolic - diastolic). In all animals hypoxia caused a decrease in the arterial oxygen tension. There was no significant change in carbon dioxide tension although pH became more acidic. The mean systemic arterial pressure was elevated during hypoxia.

IV. DISCUSSION

A. Rat Experiments

In the first study on rats we observed uneven hypoxic pulmonary vasoconstriction. We observed two types of constriction and documented the size of vessels most affected. The types of constriction I saw with SEM were consistent with light microscopic and electron microscopic studies of muscle and elastic lamina presence in normal rat lungs (Meyrick and Reid, 1979; Cook et al.., 1975; Hislop and Reid; 1978). The type of constriction I saw in the larger sized vessels (greater than 150 micrometers) is compatible with the presence of elastic lamina surrounding these vessels. The type of constriction I saw in the smaller sized vessels (less than 150 micrometers) may represent constriction of the partially muscular arteries described by Meyrick, Hislop and Reid (1978).

Figures 5 and 6 confirm physiologic evidence for vasoconstriction in the arteries (see

review by Dawson, 1984) even though the site of hypoxic constriction has been long debated (Kapanci, et al.., 1974; Fishman, 1976; see Staub 1963, 1974 and 1984 for reviews). We did not look systematically at the veins. Also, the quantitative results we obtained from these micrographs (Figure 8) confirm results obtained by Nagasaka, et al..(1984) in his micropuncture measurements of the lung indicating vasoconstriction of the pulmonary arteries during hypoxia.

Murakami (1971) first used the low-viscosity polyester resin known as methacrylate injection medium (Mercox (R)) in a SEM study. Since then, it has been used extensively to obtain injection replicas of various microvasculature (Hodde et al.., 1977; Hijiya and Okada, 1978; Ohtani, 1980). Methacrylate corrosion casts are reported to be less fragile than those vascular casts injected with latex (Sobin, 1965; Nowell, 1980) although the latex method has since been improved (Frasca,1978). In fact, Kendall and Eissman (1980) used latex to look at normal human pulmonary capillaries with excellent results.

I found the methacrylate casts were also fragile. Although Mercox is a hard medium it was difficult to fracture into an appropriate size for mounting on the specimen stubs. The small arteries seemed particularly fragile in this preparation so I submerged the specimens in a beaker of water while cutting them into smaller pieces. The Mercox was not an ideal medium in this respect but it was easy to coat with gold for SEM inspection. In a recent review of microvascular corrosion casting of the lung, Schraufnagel (1987) suggests presoaking the casts in alcohol for 15 minutes prior to cutting. This softens the medium to allow for even cutting.

The viscosity of the methacrylate was not adjusted to that of blood in my studies. Thus, it may have been too viscous to reveal leakage sites between endothelial cells. Mercox (R) has

a viscosity of 27 centipoise, approximately 10 times that of blood. However, a greater degree of shrinkage of the specimen occurs when it is diluted to the viscosity of blood and that is a major criticism of the dilution technique (Weiger et. al.,1986). Another casting material that has a viscosity similar to blood is Araldite and it has been used successfully in liver casts (Hanstede and Gerrits, 1982). Araldite may be advantageous to try in hypoxic lung corrosion casts since its viscosity is that of blood. However, Mercox has less shrinkage, hardens quickly, adheres well to endothelial cell walls and produces the most accurate replicas (Schraufnagel, 1987).

B. Pig Experiments

There was slightly more edema on the high pressure side (right lung) than on the side that received greater flow (left lung); however the difference was not significant. There was a significant difference between both hypoxic groups and the control group after calculating the edema ratio for all vessels included in the study. This calculated ratio normalizes the apparent edema cuffing according to the vessel size. Thus, the problem that larger cuffs appear around larger vessels because of the greater distensibility of the interstitial space there is taken into account (Michel RP, 1982).

We found a greater proportion of cuffed arteries in the right lung (low flow, high pressure) compared to the left lung (high flow) ($p<0.05$, paired-t test) when counting perivascular cuffing of arteries in each montage prepared from photographs taken from the slides prepared for histology. The cuffing around these vessels was measured and counted by an unbiased observer.

There was no difference between extravascular wet/dry lung weights using the technique developed by Pearce and coworkers (1965) because we did not induce severe edema

characterized by alveolar flooding. This technique has been used to determine increases in lung weight for other types of edema formation so it is useful in identifying fluid increases. Perhaps, three hours of hypoxia was not enough time to cause alveolar flooding in these experiments because there was evidence of subacute interstitial edema. Histologic observations and edema ratio measurements gave us quantitative differences between the two groups even though we measured no differences determining the extravascular wet/dry lung weights for each lung.

The pig was used as the experimental animal for two reasons. First, it has been shown to have one of the stronger vasoconstrictor response to hypoxia compared to other species, e.g. dogs, rabbits, cats and ferrets, especially in isolated lung preparations (Peake et al.., 1981). Second, the lung of the 12 week old pig is structurally similar to the human adult lung (Rendas et al.., 1978) although remodeling of the peripheral pulmonary vasculature occurs sooner in pig than in human (Reid, 1979). I used pigs weighing approximately 20 kilograms between 9 and 12 weeks old.

The systemic arterial pressure was elevated after hypoxia in my study. I administered Indomethacin ($5mg \cdot kg^{-1}$, IV), the cyclooxygenase inhibitor, 30 minutes prior to hypoxia to block prostacyclin synthesis in the lung in an effort to sustain the elevated pulmonary arterial pressure that occurs with hypoxia but eventually fades. Meyrick and coworkers (1985) used repeated injections of Indomethacin in sheep and found hypertension after 2 weeks. There is evidence that it works over a shorter time course (Kadowitz et al.., 1975; Rubin and Lazar, 1981; and Brigham and Ogletree, 1981). Also, Gordon et al..(1986) using an arterial-venous occlusion technique in isolated lungs of two week old lambs found that Indomethacin increased the vascular resistance of the small muscular pulmonary arteries more than upstream or downstream lung segments during hypoxic pulmonary vasoconstriction.

Several studies in the 1970's indicate methodologic problems with my results. Benumof and Wahrenbrock (1975) found less hypoxic pulmonary vasoconstriction with elevated lung vascular pressures in isolated dog lungs. Tucker et al.. (1975) found decreased hypoxic pulmonary vasoconstriction with increases in pulmonary blood flow in dogs as well. Yet, these earlier studies in dogs are at variance with more recent work in isolated pig lungs (Sylvester et al..,1983) and in an *in situ* hanging lung model (Cheney, 1987) which support my results.

C. Future Research

Since the results of these studies are inconclusive I would like to propose other studies that may help determine the site of leakage in high altitude pulmonary edema. For example, more corrosion casting using Mercox adjusted to blood viscosity and SEM viewing of corrosion casts comparing hypoxic and normoxic rat lungs in a blinded study. Vascular labeling with Monastral blue is another technique that could be utilized to study vascular leakage during simulated high altitude exposure in animals.

Recently, Townsley et al.. (1989) has been using microvascular casting of the lung to observe microvascular injury induced by high pressure and oleic acid. In these studies the viscosity of the methylmethacrylate was adjusted to blood viscosity (approximately 1.8 centipoise (cPs)). The results suggest that the two different types of lung perturbations produce different sites of microvascular injury. For example, using three different experimental groups Townsley found leak sites in the larger extraalveolar vessels only after high venous pressure (85-88 cm H20) and after oleic acid treatment at low venous pressure (15-20 cm H_2O). The extraalveolar vessels did not leak during normal pressures while the alveolar capillaries leaked at all pressures. However, the methacrylate was diluted to a viscosity less than blood and shrinkage of specimens may be a problem in these studies. Perhaps utilizing

Townsley's corrosion casts method in my experiments of hypoxia with exercise in rats, the viscosity of the methacrylate would not be problematic.

Another study to determine the site of leakage in HAPE would be to use a vascular label. When colloidal suspensions, carbon black and mercuric sulfide, became unavailable for use as vascular labels, Joris and coworkers (1982) demonstrated the use of Monastral blue as an acceptable substitute for labeling permeable vessels. They found that the pigment adheres to the vessel walls at sites of vascular leakage. This would be advantageous in my studies as it would not diffuse away from the leakage site as occurs with Evans blue dye. Iliff (1971) had demonstrated that small pulmonary arteries as well as veins stained using Evans blue. It is not known whether the dye diffused into the arterial perivascular space from the veins (although the veins were collapsed by the inflation pressure) or whether the arteries themselves leaked. Now Monastral blue may be a better label to test the site of leakage.

Simulating neurogenic inflammation by vagal nerve stimulation, McDonald (1988) successfully identified specific sites of increased tracheal vascular permeability in detailed light and electron microscope studies using Monastral blue labeling. He found that most of the Monastral blue was trapped in the basement membrane of venules 7-80 micrometers in diameter. These vessels are known to have gaps between adjacent endothelial cells. These venular changes occurred with degranulation of epithelial secretory cells suggesting mediation by sensory axons ending nearby. However, McDonald and coworkers (1988) subsequently found that the vascular permeability they observed was caused not by direct sensory stimulation but by mediators released by epithelial cells in response to substance P or other tachykinins. Although only veins leaked in McDonald's studies, it is possible that arteries smaller than 80 microns could leak between gaps of endothelial cells during HAPE. A pilot study using Iliff's (1968) lung model with different arterial and airway pressures (i.e. Zone I,

Zone II and Zone III lung conditions) during normoxia should be performed. The results of the Evans Blue dye should be compared with Monastral blue as a label for vascular leakage. This study would determine if Monastral blue would be useful to try in another study using hypoxia with exercise to simulate HAPE in rats and to identify the site of vascular leakage.

Horseradish peroxidase (HRP) is another vascular label. Using perfusion fixation and HRP application as a blood tracer, Hucker and coworkers in 1976 demonstrated the time sequence and site of fluid leakage in the pulmonary vessels following intracranial pressure elevation to simulate neurogenic pulmonary edema.

V. CONCLUSION

Using SEM and corrosion casting of the lung after hypoxia, we observed uneven hypoxic pulmonary vasoconstriction in rats. We observed two types of constriction and documented the size of vessels where it appears most frequently. In the pig experiments, we determined that high pressure and high flow in the hypoxic pig lung contribute significantly to subacute edema cuff formation compared to the normoxic control group.

There has been renewed interest in identifying the site of hypoxic pulmonary vasoconstriction (Hakim et al.., 1983; Marshall and Marshall 1983a; and Marshall 1983b), its role in fluid balance (Sylvester et al.., 1983; Dawson et al., 1983) and its role in the development of chronic hypertension (see Meyrick and Reid, 1983; Meyrick et al.., 1985 for reviews). Perhaps new developments in these areas will increase our understanding of the etiology of high altitude pulmonary edema.

The results of this microvascular corrosion casting of the pulmonary vasculature during hypoxia is unique (Stafford et al.., 1983). The other corrosion casting studies cited in the discussion observed normal vasculature except that by Townsley and coworkers (1988, 1989). It

is my belief that further development of corrosion casting, viewing of replicas with SEM as well as more controlled studies as suggested by Schrafnagel (1987) comparing the normal lung with those made hypoxic will provide a technique to identify the leakage site in high altitude pulmonary edema.

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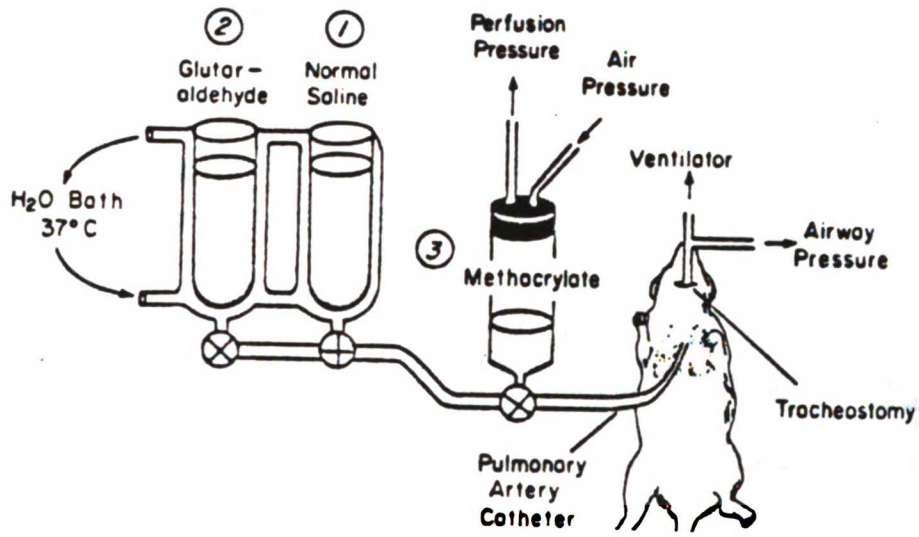
FIGURES

RAT EXPERIMENT



Legend Figure 1:

Set-up for experimental perfusion of rat lungs.

Legend Figure 2:

A methacrylate cast of the pulmonary arteries of a rat before the specimens were cut and mounted for gold coating.

Legend Figure 3:

Representative picture of a normoxic pulmonary artery from a rat between 150 and 300 micrometers in diameter. The scale bar is 100 micrometers. Note the smooth appearance of the vessel casts. Small indentations represent individual endothelial cell nuclei.

Legend Figure 4:

A 30-60 micron diameter normoxic vessel from a rat lung. Despite some tissue debris, the vessels are smooth and exhibit nuclear indentations.

Legend Figure 5:

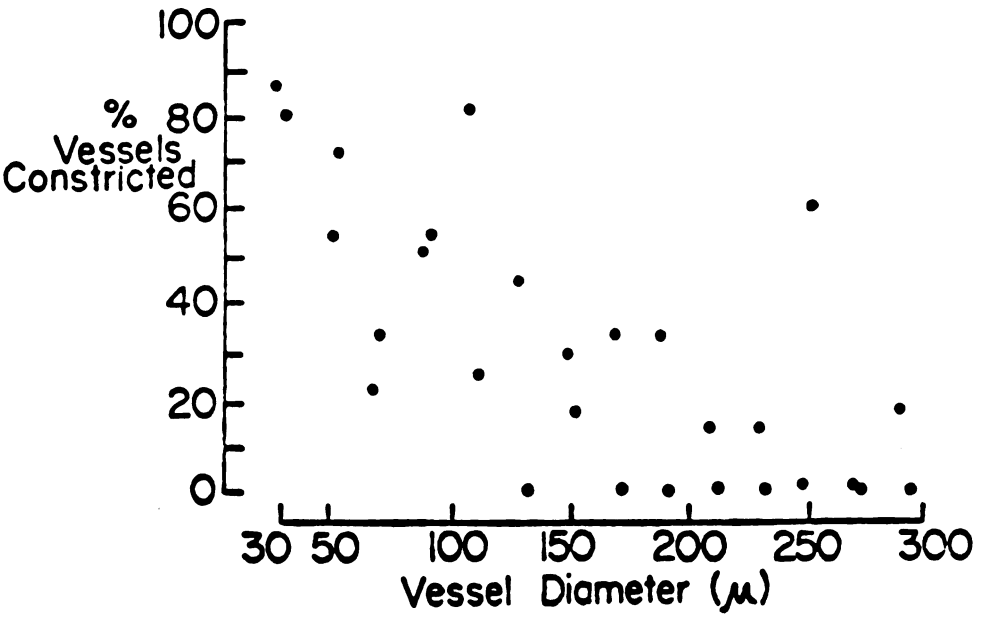
Hypoxic pulmonary arterial vasoconstriction in rat vessel casts larger than 100 microns was evident as corrugations along the casts.

Legend Figure 6:

Hypoxic pulmonary vasoconstriction in rat vessel casts less than 100 micrometers in diameter.

Legend Figure 7:

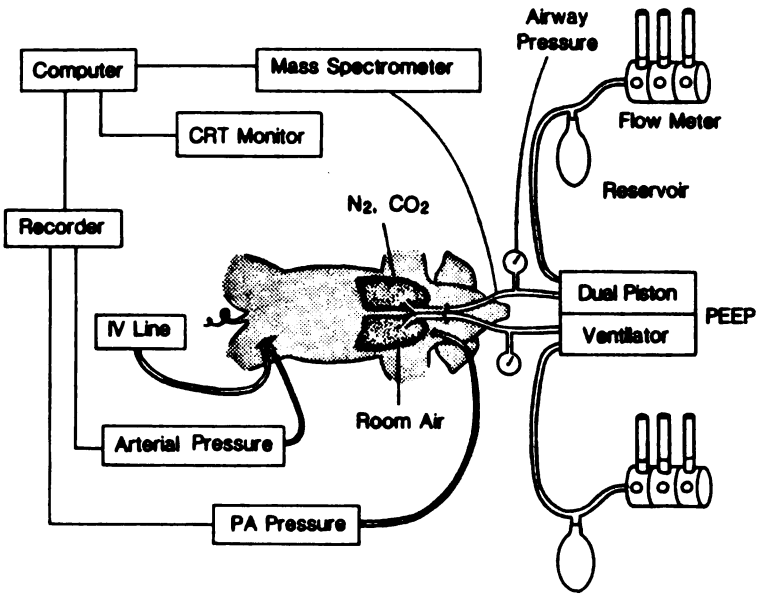
Two types of apparent constriction seen in rat vessel casts of hypoxic pulmonary arteries. Corrugations present in vessel casts greater than 100 micrometers and sausage shaped vessels in casts less than 100 micrometers.



Legend Figure 8:

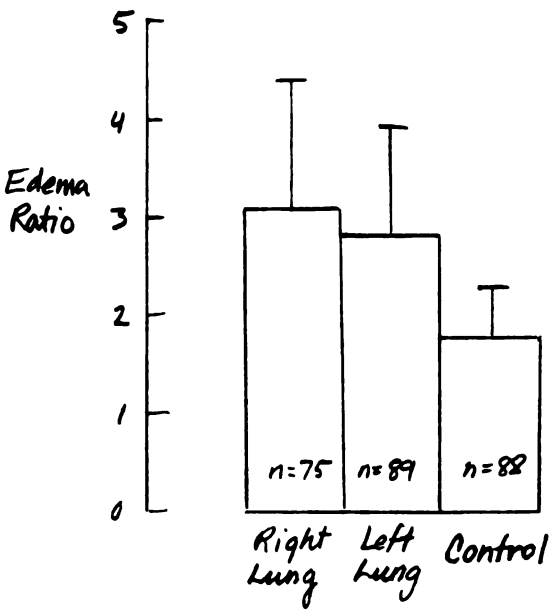
Percent pulmonary arterial vessel casts constricted versus vessel diameter (micrometers) in rats.

PIG EXPERIMENT



Legend Figure 9:

Set-up for pig experiments.



Legend Figure 10:

Results of the edema ratio for right and left lung pulmonary arteries in the control and experimental groups in pigs.

PROPORTION OF CUFFED/TOTAL PULMONARY ARTERIES IN PIGS

	RIGHT LUNG vs	LEFT LUNG
CONTROL	4/41=0.10	5/34=0.15
HYPOXIC	25/40=0.63	15/48=0.31
	15/26=0.58	18/40=0.45
	26/35=0.74	28/70=0.40
	36/56=0.64	23/57=0.40

Legend Figure 11:

Histologic results of edema cuffing around pulmonary arteries in the right and left lungs for control and experimental groups in pigs.

RESULTS EVLW/EVLD IN PIGS

	RIGHT LUNG	LEFT LUNG
CONTROL		
1	3.32	3.64
2	3.06	3.12
3	3.33	3.43
mean±sd	3.24±0.15	3.40±0.26
HYPOXIC		
1	3.92	3.97
2	3.94	3.86
3	3.97	4.06
4	3.42	3.24
mean±sd	3.81±0.26	3.8±0.37

Legend Figure 12:

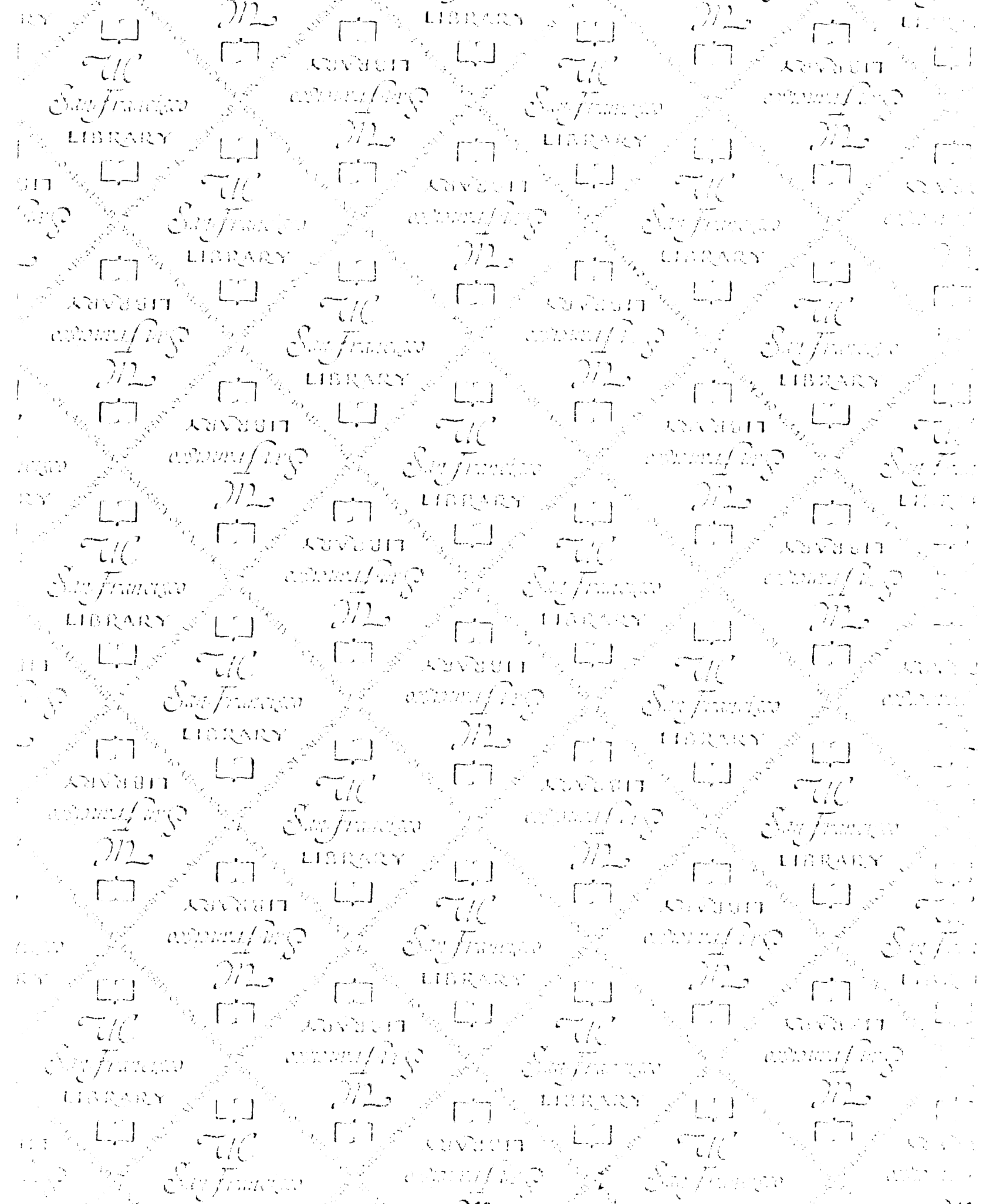
Results of the extravascular wet/dry lung weight measurements in pigs using the Pearce technique.

Table 1: Hemodynamic Data Pigs

Pig		Ppa (mmHg)	Psa (mmHg)	P_aO_2 (mmHg)	P_aCO_2 (mmHg)	pH
1	control	13 (n=2)	95 (n=2)	95.3	42.9	7.456
	hypoxic	23±3 (n=6)	103±11 (n=6)	30.5±4.1 (n=8)	40.5±1.6 (n=8)	7.348±0.05 (n=8)
2	control	13.7 (n=2)	99±13 (n=2)	160.5	37.5	7.558
	hypoxic	20±3 (n=8)	101±16 (n=5)	34.3±8.9 (n=7)	33.7±3.0 (n=8)	7.404±0.090 (n=7)
3	control	18±2 (n=2)	95±3 (n=3)	80.2	42.3	7.428
	hypoxic	32±6 (n=13)	113±6 (n=12)	34.4±2.2 (n=8)	46.5±2.0 (n=8)	7.307±0.020 (n=8)
4	control	9±1 (n=2)	95±14 (n=2)	81	31.3	7.478
	hypoxic	27±6 (n=10)	126±23 (n=9)	30.4 (n=8)	44.5±11.2 (n=9)	7.287±0.14 (n=8)
mean	control	13±4	96±2	104.3±38.1	38.5±5.4	7.480±0.05
mean	hypoxic	26±5	111±12	32.4±2.3	41.3±5.6	7.337±0.05

Legend Table 1:

Hemodynamic data in pigs before and after hypoxia.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