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The Effects of Vagal Denervation on Cardiorespiratory and Behavioural Responses in the Newborn Lamb

by

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ABSTRACT

To date, few studies have investigated the role of vagal innervation on perinatal control of breathing. These studies had significant limitations including the compromise of upper airway function, anesthesia effects and/or tracheotomy. Furthermore, in previous studies the immediate newborn period was not monitored. Therefore, we performed bilateral intrathoracic vagal sympathetic denervation in newborn lambs to determine the role of the vagus nerve in the control of breathing during the immediate newborn period. We observed that vagal denervation led to respiratory failure as evidenced by hypoxemia and respiratory acidosis. Denervation also led to changes in breathing patterns as compared to sham operated lambs including increased expiratory time, and decreased respiratory rate, minute ventilation, and lung and respiratory system compliance. Our data showed no significant differences in sleep state intervals, or in surfactant aggregates between the sham operated and vagally denervated animals. Our results suggest that vagal input is critical for the maintenance of normal breathing patterns and gas exchange during the immediate newborn period.

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1. INTRODUCTION

1.1. Significance

Breathing becomes continuous at birth as opposed to episodic fetal breathing movements however, mechanisms of the establishment/maintenance of continuous breathing remain unknown. Disorders of breathing are a major cause of infant mortality and morbidity, thus the understanding of the mechanism responsible for the establishment and maintenance of breathing in the neonate is not only important from a physiological point of view, but also for improving pediatric health.

Vagal denervation studies have consistently shown that vagal innervation plays a more important role in the control of breathing in the newborn as compared to the adult (29,35,37,40,118). The vagus nerve (cranial nerve X) is a major nerve of the parasympathetic nervous system. Approximately 75% of all parasympathetic nerve fibers reside in vagus nerves passing to the thoracic and abdominal regions of the body (120). The vagus nerve supplies parasympathetic nerves to the heart, lungs, esophagus, stomach, and entire small intestine, proximal half of the colon, liver, gallbladder, pancreas, and upper portions of the ureters (50).

Parasympathetic vagal receptors are denser in central than peripheral airways and their activity is responsible for a number of responses. In the pharynx and larynx the vagus nerve serves the skeletal muscles where it is involved in modulating the length and width of glottic aperture. Cholinergic stimulation via branches of the

vagus nerve causes contraction of smooth muscle in the airway wall, increased secretions from bronchial mucus glands and goblet cells and rapid, shallow breathing (7,14,54,130). Afferent information arises from sensory receptors and not only communicates to the central nervous system but is also responsible for the activation of local reflexes causing the release of tachykinins from nerve endings in the airway wall. These tachykinins, substance P and neurokinin A, can cause bronchoconstriction, increased submucosal gland secretion and increased vascular permeability (130).

In the lungs, the sparse adrenergic (sympathetic) innervation of airways is denser in peripheral than in central airways. Bronchial adrenergic (β_2) receptors are stimulated by circulating catecholamines (epinepherine/norepinepherine) and cause relaxation of bronchial smooth muscle, whereas stimulation of less important α-adrenergic receptors results in bronchoconstriction (130). There is also a third type of neural airway control referred to as the non-adrenergic, non-cholinergic receptor system. These efferent fibers run in the vago-sympathetic trunk and when stimulated result in the release of inhibitory neurotransmitters vasoactive intestinal peptide (VIP) and nitric oxide (NO) causing relaxation of bronchial smooth muscle (130).

1.2. Pulmonary Vagal Afferent Fibers

The pulmonary vagal afferent receptors consist of both myelinated and unmyelinated fibers. The myelinated receptors are called the pulmonary stretch

receptors (PSR) and the rapidly adapting receptors (RAR). Unmyelinated fibers include the J (juxta-capillary) receptors and C-fibers which innervate bronchial receptors (96).

1.2.1. Pulmonary Stretch Receptors

PSR's, also known as the slowly adapting pulmonary receptors (SAR), primarily respond to mechanical stimulation of the lung as a result of lung inflation. They are located within smooth muscle and stimulation will result in a reflex termination of inspiration called the Hering-Breuer reflex. Thus, the activity of PSR's play a very important role in control of inspiratory time (T_I). It has also been shown that increased PSR activity significantly increases expiratory time (T_E) (9,132). Smooth muscle contraction will also stimulate PSR activity. This stimulation will reflexly decrease efferent outflow to smooth muscle, providing a powerful feedback loop to limit smooth muscle contraction (103). Thus, the Hering-Breuer reflex, which is more evident in early childhood, effects both the depth and duration of the inspiratory and expiratory phases in newborn mammals (126).

1.2.2. Rapidly Adapting Receptors (RAR's)

The second receptors in the myelinated group are the rapidly adapting receptors (RAR's) or irritant receptors; first discovered by Knowlton and Larabee (1). RAR's are stimulated by large inflations or deflations as well as inhaled irritants such as noxious gases, cigarette smoke, dust and cold air stimulate these receptors. In extrapulmonary airways, they are found throughout the airway in between epithelial

cells (27,113). Whereas in the bronchial tree, they are mainly found in the major airways with 55-70% of them located within 1 cm of the hilum (5,84). Distribution of the RAR's in the airway and lungs does not parallel the distribution of PSR's (96).

Although RAR's are mainly mechanoreceptors, their response can also be altered by chemical stimulation. Histamine, serotonin and prostaglandin F2 have all been found to stimulate RAR responses (36). The major effect of RAR stimulation is to induce bronchoconstriction and rapid, shallow breathing (27). As a result of their responses, RAR's are believed to provide the major afferent input for the airway defense reflexes (27).

1.2.3. Juxta-Capillary Receptors and C-Fibers

Compared to myelinated fibers, the unmyelinated fibers have been shown to outnumber the myelinated fibers 4:1 in pulmonary vagal branches in cats (27). The first type of unmyelinated vagus fiber is the J or Juxta-capillary receptor. This afferent receptor system was first described by Paintal in 1973 (97). Together with the other type of unmyelinated fibers, the C-fibers, these two receptors comprise the unmyelinated C-fiber afferent system (27). The difference between the two receptors is that J-receptors are found in the parenchyma whereas C-receptors are found in the bronchi (96). J and C receptors also differ in their blood supply. J receptors, also called pulmonary C-fibers, are perfused by the pulmonary circulation whereas bronchial C-fibers are perfused by the bronchial circulation

(27).

It has been shown that C-fibers are not primarily mechanoreceptors since lung hyperinflation only minimally stimulates a minority of receptors, and lung deflation has no effect on C-fiber activity (26). During normal breathing, the C-fibers have been shown to play a major role in the initiation of inspiration through the shortening of expiratory time (103). Stimulation of receptors by various chemicals such as histamine, prostaglandins, phenyl diguanide and capsaicin, is highly species specific (13,14,18,26,27). Stimulation of the unmyelinated fibers results in rapid and shallow breathing, bronchoconstriction, and increased airway secretion often accompanied by marked cardiovascular depressor effects (27). Thus, along with RAR's, these fibers also play a role in airway defense reflexes (27).

1.3. Vagus and the Perinatal Control of Breathing

To date, there have been very few studies that have investigated the role of vagal innervation on perinatal control of breathing. In one of the earliest studies in this area, Coombs and Pike examined the effects of cervical vagotomy in anaesthetized, tracheotomized kittens. In their study unilateral vagotomy resulted in no change in breathing patterns, whereas bilateral vagal denervation resulted in death within a few hours preceded by gasping and dyspnea, even in kittens 40-50 days old. In contrast, bilateral cervical vagotomy of adult cats resulted in a reduction in respiratory rate however the animals could live indefinitely (29).

In 1968, Schwieler revisited the effects of the vagal denervation on respiration during postnatal development. In particular, he investigated the differences between newborns and adults with respect to changes in the respiratory pattern and levels of P_aCO_2 and pH post vagotomy at different postnatal ages (118). In animals which were vagally denervated, anaesthetized and had tracheal cannulas inserted, they found that T_I increased in newborn and adult cats and rabbits. However, post vagotomy, animals less than three weeks of age exhibited greater respiratory pattern changes than animals more than four weeks old (118). In fact, the respiratory pattern changes in the young animals always resulted in respiratory failure. These findings agree with the results by Coombs and Pike indicating that the effects of vagotomy on respiration are much more evident in newborns than in mature animals.

Evidence also suggests that in animals less than two weeks of age, ventilation decreased causing increases in P_aCO_2 and decreases in pH which did not result in any effective ventilatory stimulation. In adult animals, however, these effects were not seen. Rather, adult animals show a constant arterial PCO_2 and pH (118).

In 1980, Duron and Marlot examined cervically vagally denervated, tracheotomized kittens between 1 day and 5 weeks of age which were either anaesthetized or decerebrated. Animals were decerebrated to avoid the effects of anaesthesia on breathing patterns (37). They observed that, in both anaesthetized and decerebrate animals, inspiratory and expiratory duration increased while

respiratory frequency decreased compared to controls. Compared to decerebrate animals, anaesthetized animals had a prolonged T_E which often led to death due to apnea. Postnatal development was found to be associated with increased expiratory activity, and a decrease in the effects of bilateral vagotomy on breathing patterns (37).

Fedorko et al. investigated the respiratory effects of cervical vagotomy in anaesthetized and non-anaesthetized rats from 1 day old to adults. In the unanaesthetized 1-6 day old pups, vagotomy resulted in decreased minute ventilation and breathing frequency while tidal volume (V_T), T_E and T_I all increased. When these animals breathed 100% oxygen similar effects of vagotomy on breathing patterns were seen, namely a decreased minute ventilation in the denervated group (40). When anaesthetized animals were studied, similar changes were seen in breathing variables as in the unanaesthetized animals. When the pup's breathing patterns were then compared to adult breathing patterns similar increases in T_I and V_T/kg were observed, however, a significant difference was seen with respect to T_E and ventilation. The change in T_E of pups post vagotomy was much greater than in adults and the changes in minute ventilation in adults were small to non-existent compared to the pups (40).

In a more recent study, Delacourt et al. examined the effects of vagal afferents on the diphasic ventilatory response to hypoxia in newborn lambs approximately 15 days old (35). The main difference between this and previous work is that the right

vagal trunk was sectioned below the emergence of the recurrent laryngeal nerve sparing disturbance to upper airway function. The left vagus was sectioned in the neck causing left vocal cord paralysis. Previcus studies had all performed vagotomies in the cervical region resulting in total vocal cord paralysis and possible abolishment of upper airway function. Another difference between this study and previous ones is that previous studies were performed on intubated, anaesthetized newborns, whereas Delacourt et al. performed studies on awake newborn lambs with either an intact (used mask), or bypassed (used endotracheal tube) upper airway.

Delacourt et al. observed that "vagally denervated newborn lambs with intact upper airways had a weaker response to hypoxia compared to unvagotomized lambs, whereas the mode of response to hypoxia was diphasic (initial increase in minute ventilation followed by a reduction in minute ventilation) in both groups. Blunting of response to hypoxia disappeared in the intubated lambs, which shows the important role played by vagal afferents in enhancing the ventilatory response to hypoxia through the control of laryngeal dynamics" (35).

Previous studies on the effects of vagal denervation in newborns on breathing had one or more of the following limitations. Firstly, vagotomy was usually performed in the cervical region resulting in compromise of upper airway function and vocal chord paralysis. Secondly, in many studies, animals were examined while under effects of anaesthesia and tracheotomized. It is known that anaesthesia results in

respiratory depression and tracheotomy bypasses the upper airway which plays an important role in respiration. The final limitation of previous studies is that the immediate newborn period was not monitored. All previous studies investigated the effects of vagotomy at least one day after birth. Since the role of the vagus changes with development, to examine its effects at birth we must look at its role as close to birth as possible. Thus the role of the vagi in establishing and maintaining breathing and pulmonary gas exchange has not yet been elucidated.

A number of these limitations were circumvented in a recent study by Wong et al. In this study, vagal denervation or sham surgery was performed prenatally at 130-132 days of gestation and animals were studied after spontaneous vaginal delivery at 140-142 days of gestation (term=147+/- 2 days). Vagal denervation was performed intrathoracically, below the origin of the recurrent laryngeal nerves thereby avoiding the paralysis of upper airway muscles (133). In this study, vagotomized lambs could not establish effective gas exchange and developed respiratory failure exhibited by severe hypoxemia and respiratory acidosis. In contrast, sham operated animals established effective alveolar ventilation (133).

Breathing patterns also differed between sham and vagally denervated animals. In sham operated lambs breathing frequency decreased approximately 20% over the first 30 minutes of life. Vagotomized lambs, on the other hand, decreased breathing frequency by approximately 50% within the first 30 minutes of life. Additionally, lung lavage showed poor surface activity (a measure of surfactant

function) in the vagotomized animals and very good surface activity in the sham operated lambs.

In a similar study by Hasan et al. (unpublished observations), analysis of broncho-alveolar lavage samples showed the vagally denervated animals to have significantly lower amounts of large aggregates (active surfactant) and higher amounts of small aggregates (inactive surfactant) compared to sham operated newborns. Vagally denervated animals also had significantly lower mRNA expression of surfactant proteins SP-A, and SP-B. This study thus shows that vagal afferents are vital for establishment of continuous and adequate gas exchange at birth. Although studies by Wong et al, and Hasan et al. overcame previously mentioned limitations vagotomy was performed antenatally, thus the effects seen may be influenced by variables present antenatally or at birth such as lung liquid absorption, surfactant secretion, changes in pulmonary blood flow, and hormonal surges.

At birth, the fetal circulation switches from a serial type to a parallel type. The major cause of this shift is the great rise that occurs in systemic vascular resistance in the absence of the placenta and a decrease in pulmonary vascular resistance leading to closure of the ductus arteriosus, ductus venosus, and foramen ovale (23,61,112). Pulmonary vascular resistance is also lowered by lung expansion in the immediate newborn period (12,34,65-67,123).

In the studies by Wong et al., autopsy showed the lungs to be atelectic. However, neither pulmonary blood flow or lung volume values were recorded. Thus it is possible that absence of lung expansion as evidenced by the atelectasis resulted in reduced pulmonary blood flow and eventual respiratory failure in these lambs. Recently, this question was investigated by Hasan et al., and it was shown that pulmonary blood flow does not decrease. Rather, the blood flow was higher in the vagotomized lambs. Respiratory failure then, is not due to the lack of increase in pulmonary blood flow in the immediate newborn period.

The second possible cause of respiratory failure in vagally denervated lambs is surfactant deficiency. Pulmonary surfactant is responsible for lowering surface tension which helps stabilize the lung. It is composed of approximately 80% glycerolphospholipids, 10% cholesterol and 10% proteins as well as surfactant proteins SP-A, SP-B, SP-C and SP-D (20,24,82,104,117). Phospholipids and proteins are secreted from type II cells in the lungs and result in formation of tubular myelin, a substance that eventually migrates to the surface of lung epithelium to form surfactant. Surfactant can be divided into two populations; large aggregates, which are made up of surface active proteins and phospholipids of surfactant, and small aggregates which are comprised of degraded and inactive surfactant proteins and phospholipids.

A number of studies have shown that vagotomy can interfere with surfactant synthesis or release and that this is largely due to changes in breathing patterns

associated with vagotomy (2,30,31,44,62,93-95). Studies have also shown that cholinergic stimulation may be involved in surfactant secretion, and that large inflations of the lungs are a potent stimuli for surfactant secretion (80).

In previous studies by Wong et al. (133), and Hasan et al. (unpublished observations), vagally denervated animals had decreased levels of large aggregates and increased levels of small aggregates compared to sham operated lambs as well as lower levels of SP-A, and SP-B mRNA in vagally denervated compared to sham operated animals. These changes in the surfactant system are likely to contribute to the respiratory failure in vagally denervated newborn lambs. Although this evidence suggests that surfactant deficiency may, in part, be responsible for respiratory failure, it is not known if this is a primary event due to synthesis and secretion of surfactant, or a secondary event due to inhibition from plasma proteins and lipid membranes of red cells.

Another explanation for respiratory failure in the vagally denervated lambs may be the lack of vagal feedback either from myelinated or unmyelinated fiber afferent systems thus effecting lung volume and respiratory patterns leading to respiratory failure.

1.4. Vagus and the Sleep States

Previous studies have shown a relationship between vagal feedback, respiration and the three sleep states, non-REM, REM, and arousal (114).

1.4.1. NREM Sleep (Quiet Sleep)

NREM sleep is characterized by reduced overall and cerebral metabolism, reduced cerebral blood flow, reduced activity in a majority of brain neuronal sites, reduced heart rate and arterial blood pressure, reduced brain and body temperature. During much of the sleep period, there is also reduced release of adrenal cortical hormones. Thus NREM sleep is a state of lowered metabolism and rest (114).

1.4.2. REM Sleep (Active Sleep)

During REM sleep, cerebral blood flow and metabolism are increased, the majority of neuronal sites in the brain show increased discharge, brain temperature rises, and skeletal muscles exhibit hypotonia. Overall systemic metabolism of this state as determined via oxygen consumption is low. Autonomic patterns of this state are variable; the heart rate and blood pressure rise slightly relative to NREM levels and also show more variability. There are also intermittent twitches in fine distal muscles and in the brain. These twitches give rise to the extraocular eye muscle twitches that embody REM sleep. Overall, the REM sleep state can be described as a sleeping body with a highly activated brain (114).

1.4.3. Sleep State and Respiration

Several studies have addressed the relationship between sleep state and respiration. In 1953, Aserinky et al. showed that children have a higher respiratory rate during REM sleep than during NREM sleep. These results were also seen in

newborn infants (6,106). Three subsequent studies showed that minute ventilation and variability of ventilation also increased during REM compared to NREM sleep (17,57,58).

Previous studies have also shown a relationship between vagal feedback, respiration and sleep states (39,41,45,73,99,100,110). A study by Farber et al. exhibited that although pulmonary inflation and deflation reflexes were equally active in REM and NREM sleep they were more variable in REM sleep (39). In contrast, Phillipson has shown that in dogs the Hering-Breuer reflex, as assessed by duration of apnea following lung inflation, is weak in REM sleep, but strong in NREM sleep and arousal (100). Studies by Finer et al., which also involved examination of the relationship between the vagus and sleep state showed that respiratory control mechanisms and sleep state are interdependent in full term infants (41). Thus, previous work suggests that the lung inflation reflex falls into a class of reflexes, like auditory reflexes, such that volume related vagal afferent inputs are not centrally decoded or acted upon during REM sleep (41).

Vagal denervation studies (both cervical and intrathoracic) by a number of investigators have confirmed that the vagal reflexes have a greater influence during the NREM state as compared to the arousal and REM sleep states. Studies by Jouvet, Phillipson et al. and Remmers et al. have shown that after vagotomy, breathing patterns and variability during REM sleep remained intact (71,100,110). On the other hand, bilateral cervical vagotomy in adult dogs

resulted in a significantly lower respiratory frequency during NREM sleep compared to animals with intact vagi or during the awake state with blocked vagi (99,100). A study by Foutz et al. in adult cats showed that vagal afferents function during each sleep state to terminate inspiration, however, during the awake and REM states separate but functionally equivalent mechanisms of central origin supplement the vagus in facilitating the termination of inspiration (45). They conclude that the absence of these mechanisms during NREM sleep accounts for the increased vagal influence during this state (45).

Although Foutz performed his studies in adult cats, Praud et al. also observed that NREM sleep is characterized by increased vagal influence in neonates (unpublished observations). Thus although previous studies on the role of vagal afferents and sleep state were mainly performed in adult animals, the role of the vagus on respiration appears similar in neonates during the different sleep states.

It is clear that, in the past, much attention has been paid to the role of vagal afferents and sleep state. However, most of these studies concentrated on adult animals. Thus the role of the vagus on respiration during the different sleep states in the immediate newborn period has been of limited extent.

1.5. Rationale

To determine the role of the vagus nerve in control of breathing during the immediate newborn period, we performed bilateral intrathoracic vagal denervation in newborn lambs to exclude the variables present antenatally or at birth.

We examined the effects of bilateral intrathoracic vagotomy on pulmonary mechanics and function tests, surfactant secretion, sleep stages and blood gas tensions in newborn lambs. To ensure that the period observed is the immediate newborn period, lambs were studied within 24 hours of birth. Based on previous studies we hypothesize that:

1. Vagal denervation will result in inadequate gas exchange.
2. Vagal denervation of newborn lambs will result in detrimental effects on breathing patterns.
3. Intrathoracic vagal denervation of newborn lambs will result in an increased amount of small aggregates and a decreased amount of large aggregates suggesting a decreased surface tension reducing ability of pulmonary surfactant.

1.5.1. Specific Aims

Specific Aim 1:

To investigate the role that vagal afferents play in gas exchange of newborn lambs to test the hypothesis that vagal denervation will result in inadequate gas exchange.

Specific Aim 2:

To investigate the effect of intrathoracic vagal denervation of newborn lambs on breathing patterns to test the hypothesis that vagal denervation of newborn lambs will result in detrimental effects on breathing patterns.

Specific Aim 3:

To investigate the role that vagal afferents play in surfactant secretion from type II cells to test the hypothesis that intrathoracic vagotomy of newborn lambs will result in increased amount of small aggregates and a decreased amount of large aggregates suggesting decreased surface tension reducing ability.

CHAPTER 2: METHODS

2.1. Implantation of Arterial and Venous Catheters

Lambs were anaesthetized for surgery using 4% halothane in oxygen for induction and 1.5% halothane for maintenance. Observing sterile techniques, a 2 cm incision lateral to the trachea and immediately below the thyroid cartilage was made in the neck to gain access to the jugular vein and carotid artery. A 60 cm polyvinyl catheter (1mm ID, 2mm OD, Portex, Hythe, Kent, UK) was inserted into the jugular vein by making a small incision in the vessel and advancing the catheter 7 cm. Thereafter, the carotid artery was isolated and a similar catheter was implanted. Both catheters were secured in place using size 0 silk, and the incision sewn closed using size 0 silk. The arterial catheter was used to draw blood samples for arterial pH and blood gas tension analysis to investigate pulmonary gas exchange. This catheter was also used to record blood pressure and heart rate, while the venous catheter was used to administer antibiotics and fluids post-operatively.

2.2. Intrathoracic Sectioning of the Vagi and Implantation of Diaphragmatic Electrodes

The lamb was placed on its left side to gain access to the incision point which is located at the fourth intercostal space and a 2 cm lateral incision was made. The vagus runs alongside the phrenic nerve and is located along the left atrium caudal to the pulmonary artery. After isolating the vagus from the phrenic nerve, a 1 cm

portion of the vagus was sectioned.

To minimize occurrence of intrapleural air, a size 8 Fr. chest tube was inserted through a small incision made at the sixth intercostal space, and a purse string suture was placed around the incision site. Next, the ribs adjacent to the incision site were sewn together using size 2 silk and the incision site was closed in layers using size 0 silk. Finally, suction was attached to the chest tube (20 cmH_2O).

The procedure was repeated on the right side of the lamb. The right vagus nerve is located caudal to the azygous vein. The nerve was isolated and a 1 cm portion was sectioned. A chest tube was inserted as aforementioned, ribs adjacent to the incision site were sewn together and the incision site closed in layers using size 0 silk. The two chest tubes were attached via a "Y" connector and suction was applied under water seal.

Three diaphragmatic electrodes were implanted through a 2 cm lateral incision made parallel to the ribs at the level of the tenth intercostal space into the costal diaphragm. Surgical techniques for the implantation of electrodes have been given in detail previously (56). All electrode wires (AS 633 Cooner, Chatsworth, CA) for diaphragm and sleep staging were soldered to a connector (Lemo S.A., Switzerland). The ribs adjacent to the incision site were sewn together using size 2 silk and the incision site sutured in layers using size 0 silk.

2.3. Implantation of Sleep State Electrodes

2.3.1. Nuchal EMG (EMG_{NK})

A 3 cm incision was made in the dorsal neck region. The neck was then dissected until the nuchal muscles were located. At this point, a pair of electrodes were sewn into the right nuchal muscle. The electrodes were secured in place by anchoring them to the nuchal skin.

2.3.2. Electrocorticogram (ECoG)

A 6 cm "U shaped" incision was made 0.5 cm above the coronal sutures. An 18 gauge needle was used to drill 2 holes 0.5 cm above the lamboidal suture line and 3 cm apart through the skull to the level of the dura. The ocular and cortical electrodes were tunneled simultaneously through the nuchal incision and exteriorized at the cranial incision. The ECoG electrode contained 3 wires; two for measurement of electrocortical activity, and one for grounding purposes. Electrodes were advanced into these holes and secured into place using tissue glue and a rubber stopper. Finally, the ground wire was sewn into the cranial skin.

2.3.3. Electro-oculogram (EOG)

Two 0.5 cm incisions were made along the superior and inferior orbital ridges. Electrodes were tunneled subcutaneously from the cranial incision site and the EOG electrodes were sewn into the orbicularis oculi muscles above and below the eye. After insertion of sleep state electrodes, incisions in the neck, head and

above and below the eye were sutured using size O silk.

2.4. EXPERIMENTAL DESIGN (Figure 1)

2.4.1. Pulmonary Function Testing (PFT)

Pulmonary function testing was performed using a 4.5 or 5 Fr. endotracheal tube (ETT) one hour prior to surgery, and during recovery, 6, and 24 hours post-operatively, where recovery refers to the time when the animal was spontaneously breathing. Prior to intubation, each lamb was administered 25 mg/kg of chloral hydrate for sedation.

To perform the pulmonary function tests, we used a Fleisch pneumotachograph (size 00), a Hans-Rudolph flow occluder (Hans Rudolph, Kansas City, MO), and Validyne pressure transducers (DP45-32-A-3-5-S-4-D, DP45-14-A-3-5-S-4-D Validyne Engineering, Northridge, CA). The data was recorded on a PC (Dell 233 MHz) and also displayed on a chart recorder (Gould Brush 2800s). To analyze the data, we utilized the Anadat, Labdat and Auto programs (Version 5.2, RHT-Info Dat Inc., Montreal, QU). Variables analyzed include tidal volume, respiratory rate, minute ventilation, inspiratory and expiratory times and static and dynamic compliance and resistance.

Tidal volume, and respiratory rate were calculated directly from breathing through the ETT. Dynamic compliance and resistance, which is lung compliance and pulmonary resistance values calculated over a series of breaths through the ETT differs from static compliance and resistance. Static compliance and resistance of

the respiratory system reflects respiratory system properties, and is measured by occluding airflow at end inspiration in lambs breathing through an endotracheal tube.

2.4.1.1. Static Respiratory System Compliance and Respiratory System Resistance:

Static respiratory system compliance (C_{RS}) and resistance (R_{RS}) were calculated by the method of Lesouef et al (76). In lambs, compliance and resistance were obtained after occluding a single breath at end inspiration for 300 msec and then obtaining the plateau airway pressure ($P_{plateau}$) and flow (F_{max}). $P_{plateau}$ is established by the occlusion and is the driving pressure in the mouth which is responsible for the measured expiratory airflow. F_{max} is the maximum airflow immediately after the occlusion.

10 passive expirations were analyzed in each subject for each period of pulmonary function testing. The flow during expiration after the occlusion was measured and integrated to obtain volume. From this, a flow-volume curve was constructed, and compliance and resistance were calculated over the linear portion of the flow-volume curve. The linear portion of the flow-volume curve is represented by the formula,

$$\text{Flow} = (1/\text{Tau})(\text{Volume}) + V_{extr}$$

Where V_{extr} represents the total passive expiratory volume which is the volume at the zero flow intercept, and 1/Tau represents the slope of the straight line where Tau = R_{RS} * C_{RS}. Finally then,

$R_{RS} = P_{plateau}/F_{max}$

$C_{RS} = V_{extr}/P_{plateau}$

2.4.1.2. Dynamic Lung Compliance and Pulmonary Resistance

In contrast to static respiratory system compliance and resistance, dynamic compliance measures lung compliance and pulmonary resistance since the driving pressure is P_{TP} ($P_{AIRWAY\ OPENING} - P_{PLEURAL}$). It was calculated over a series of 10 breaths based on the equation:

$$P_{TP} = (1/C_{dyn} * V_T) + (R_{dyn} * Flow) + Pc$$

Where C_{dyn} is dynamic lung compliance, and R_{dyn} is dynamic pulmonary resistance. Pressure and airflow was recorded, V_T was calculated from flow by integration over time and P_c is the positional constant pressure in the esophagus, an arbitrary number that depends on the pressure transducer position and the amplifier baseline.

To calculate C_{dyn} and R_{dyn}, we used the computerized method of multiple linear regression which has been proven effective for clinical use and trend monitoring (3). Linear regression estimates the coefficients of the linear equation, involving

one or more independent variables that best predict the value of the dependent variable. Thus the outcome of P_{TP} (the dependent variable) can be predicted from coefficients of $1/C_{dyn}$ and R_{dyn} and independent variables such as volume and flow, as described in the equation $P_{TP} = (1/C_{dyn} * V_T) + (R_{dyn} * Flow) + Pc$. Here the calculated P_{TP} is compared with the real measured P_{TP} and computer calculates the correlation as follows. An initial value for C_{dyn} and R_{dyn} is assumed. By stepwise adjustment of $1/C_{dyn}$ and R_{dyn}, a new P_{TP} and new correlation is calculated and compared with the previous. If the change made an improvement in the correlation, another change in the same direction is performed, until the optimum correlation is achieved. Otherwise a change in opposite direction is made and the correlation evaluated. If there is no improvement in correlation either way, then the last values were taken as the true coefficients. The regression coefficient must be greater than 0.99.

2.4.2. Medications

Sedation: Chloral hydrate (25 mg/kg) was used to sedate the lambs for pulmonary function testing.

Antibiotics: Two doses of 25 mg/kg of cefazolin sodium in saline (Ancef, Smith Kline Beecham Pharma Inc., Oakville, ON) and 2.5 mg/kg gentamicin sulfate (Garamycin injectable, Schering Canada Inc., Pointe-Claire, QC) was administered. One dose was administered immediately after surgery, and another dose was given 8 hours post–operatively.

Morphine: 0.15 mg/kg was administered 6 hours post-operatively after pulmonary

function testing.

Euthanol: 5 ml of euthanyl (MTC Pharmaceuticals, Cambridge, ON) was used to sacrifice the lamb.

Fluids: 10% Dextrose was continuously infused intravenously at 90 ml/kg/day to prevent hypoglycemia and dehydration.

2.4.3. Arterial Blood Gas Tensions, pH, and Body Temperature

Arterial blood was drawn every 60 minutes or more frequently if clinically indicated (ex. every 20 minutes during apneic episodes) for measurement of arterial pH, and blood gas tensions. Rectal temperature was continuously recorded (Physitemp Instruments Inc., Clifton, New Jersey) (Table 1).

2.4.4. Sleep States, EMG_{DI}, Blood Pressure, Heart Rate, Esophageal Pressure

Post-operatively, the lamb was connected to a recording apparatus (Neurolog System; Medical Systems, Greenvale, NY). The ECoG, EOG, EMG_{NK} and EMG_{di} signals were amplified and filtered appropriately with frequency ranges: 0.5-40 Hz, 5-40 Hz, 50 Hz - 1 KHz, 50Hz – 1 KHz respectively. Blood pressure readings were obtained via the arterial catheter and recorded using a pressure transducer (Statham P23 ID; Gould Inc., Instrument Division, Cleveland, OH). Esophageal pressure was obtained by placing a 8 Fr. feeding tube in the mid-esophagus. Esophageal pressure data was recorded using a pressure transducer (Statham P23 ID; Gould Inc., Instrument Division, Cleveland, OH). All electrophysiologic signals, blood pressure and esophageal pressures were recorded on an eight-

channel recorder (Gould Brush 2800s) and on a video cassette recorded using an eight-channel Neurocorder (DR-886; Neurodata Instruments Corporation, New York, NY). The data was then analyzed on a PC computer.

2.4.5. Post-Mortum Analysis

After completing the studies, animals were sacrificed using Euthanyl. The lamb's chest was opened on the right side at the level of the fifth intercostal space. The right and left vagus nerves were examined to assure complete denervation and the phrenic nerves were assessed to ensure that they were left intact. Next, the right middle lobe was sectioned and one piece, approximately 1 cm^3, was used for scanning electron microscopic examination of alveolar type II cells and lamellar bodies (see 2.4.5.c.).

2.4.5.a. Broncho-Alveolar Lavage (BAL)

To perform the broncho-alveolar lavage, the trachea was exposed and a 4 Fr. ETT connected to a bag of chilled saline was advanced through a small tracheal incision. Approximately 2.5 cm of the tube was advanced and secured into place above the carina. 25 ml/kg of saline was drained into the trachea and lungs, and then drawn back using a 60 cc syringe until no more liquid lavage could be obtained. This procedure was repeated until five lavages were performed. The lavage was centrifuged for 8 minutes at 150 x g to isolate cells and debris which were discarded. The supernatant was then centrifuged for 20 minutes at 40,000 x g (Ti 60 rotor, Beckman centrifuge) to separate the lavage into large and small

surfactant aggregates. Large aggregates were resuspended in 15 ml of saline and analyzed for total phospholipids using Bartlett's method (8). Surface tension lowering properties were assessed using the Captive Bubble technique developed by Schürch et al. (115,116).

2.4.5.b. Lung Isolation and Light Microscopy

Following the lavage, the lungs were removed from the chest wall and were preserved by inflating them with formalin at a pressure of 25 cmH_2O. Once preserved, the lungs were stored at room temperature until they were examined by light microscopy for perivascular and alveolar edema. For light microscopy, tissues were processed through paraffin and standard 5 μm sections were stained with hematoxylin and eosin.

2.4.5.c. Right Middle Lobe Preparation – Scanning Electron Microscopy

The unfrozen section of the right middle lobe was cut into two pieces and fixed for scanning electron microscopy. One piece of right middle lobe was placed in a small dish. A 1 cc syringe attached to a 25 gauge needle was filled with 1% osmium tetroxide in fluorocarbon. The osmium was then randomly injected into the lobe and left for an hour (until the entire lobe appeared black). The lobe was postfixed in 2.5% Gluteraldehyde, and refrigerated until was analyzed by scanning electron microscopy for presence or absence of type II cells and lamellar bodies. The lung tissue was then embedded in epon, sectioned and stained with uranyl acetate/lead citrate and examined using a Hitachi 450 SEM.

2.5. Statistical Analysis

The effects of intrathoracic vagal denervation and sham surgery on pulmonary gas exchange, breathing patterns, heart rate, blood pressure, and of surface tension of the broncho-alveolar lavage was analyzed using repeated measures of the analysis of variance across time. If a significant effect of time was observed, Tukey's test was performed to determine where the differences were across time but within a given group. A Chi-square test was performed to compare the numbers of animals in each group. Arterial blood gas data (P_aO_2, P_aCO_2) and pH between the two groups was compared using the Wilcoxin Rank-Sum statistic. Differences in sleep state, post-operative course and surfactant aggregates were analyzed using an independent student's T-test. All values are given as Mean $\pm$ SD and statistical significance was considered as $P<0.05$. The study protocol was approved by the animal care committee of the University of Calgary.

CHAPTER 3: RESULTS

3.1. Post-Operative Course

Post-operatively, vagally denervated animals were unable to establish pulmonary gas exchange and spontaneous breathing and had to be manually ventilated and placed on supplemental oxygen while sham-operated lambs were quickly able to establish regular breathing patterns. The onset of spontaneous breathing and the time until animals were awake and alert (determined by the time when animals opened their eyes, and lifted their head) post-operatively was significantly higher in vagally denervated lambs as compared to sham operated animals (Table 1).

Additionally, over the 24 hour course of the study, denervated animals required oxygen (7/8) and manual ventilation (5/8) while sham operated animals did not require these interventions. Because of the increased requirements for oxygen and manual ventilation vagally denervated animals could not be extubated until approximately 165 minutes post-operatively whereas sham operated animals were able to be extubated around 97 minutes post-operatively (Table 1).

All denervated animals required additional heating via a heat lamp as body temperatures 30 minutes post-operatively averaged 36.6 °C (average body temperature is 39.0°C). Mean duration of supplemental heating was 482±420 minutes in vagally denervated animals. Low body temperatures were maintained

over the study course as we observed that removal of the supplemental heat source resulted in a diminishing of body temperature in vagally denervated animals. In contrast only 2 sham operated animals required supplemental heating for an average duration of 93±67 minutes and mean body temperature 30 minutes post-operatively was 38.2 °C (Table 1).

Denervated animals developed respiratory failure approximately 20 hours post-operatively as evidenced by prolonged apneic periods and gasping. In contrast, sham operated lambs maintained normal breathing patterns throughout the study course. Due to the differences in breathing patterns, the mean duration of the study was less than 18 hours in denervated compared to 24 hours in sham operated animals as 6 of the denervated animals succumbed to respiratory failure prior to 24 hours post-operatively.

3.2. Arterial pH and Blood Gas Tensions

Arterial gas tensions and pH exhibited marked differences between sham operated and vagally denervated animals (Figure 2). Arterial pH values exhibited no significant differences between sham and denervated lambs until 20 hours post-operatively where pH dropped significantly in the denervated lambs to 7.2±0.2 vs. 7.3±0.1 in sham operated lambs (Figure 2A).

Differences in arterial carbon dioxide tensions between the two groups appeared late in the study (Figure 2B). By 16 hours post-operatively, CO_2 levels of the

denervated animals versus the sham operated animals was, respectively, 49.3±13.9 mmHg versus 39.4±4.8 mmHg. It was also around this time period that the denervated lambs began to show prolonged apneic periods and respiratory distress.

Arterial oxygen tensions remained significantly lower in denervated lambs immediately after surgery (27.3±2.2 vs. 50.5±18.4 mmHg in denervated versus sham operated lambs respectively) and from two hours post-operatively until the end of the study as compared to the sham operated lambs whose oxygen tensions remained higher over the study period (Figure 2C).

3.3. Respiratory Pattern and Pulmonary Function

Pulmonary function variables including respiratory rate, inspiratory and expiratory times, tidal volume, minute ventilation, respiratory system and lung compliance, and respiratory system and pulmonary resistance are given in figures 3, 4 and 5. Pulmonary function variables were obtained in intubated animals one hour pre operatively and during recovery, six and 24 hours post operatively.

Figure 3 depicts respiratory rate, inspiratory time and expiratory time for sham and vagally denervated animals. In the recovery period, vagally denervated animals exhibited a reduced respiratory rate compared to sham operated animals (52 vs. 30 breaths/min in sham operated and vagally denervated animals respectively), however, this difference was not significant. By six and 24 hours

post-operatively, however, a significantly lower respiratory rate was observed between the two groups of animals (Figure 3A).

Post-operatively, inspiratory (T_I) and expiratory (T_E) times were both elevated in vagally denervated animals compared to the sham operated animals (Figure 3B and 3C). Vagally denervated animals exhibited a significant increase in inspiratory time compared to sham operated animals during the recovery period, however, there were no significant differences in inspiratory time thereafter. Expiratory time was significantly increased in vagally denervated compared to sham operated animals all time periods post-operatively (Figure 3C).

Figure 4 illustrates tidal volume per kilogram and minute ventilation per kilogram in sham and vagally denervated animals. No significant difference was observed in tidal volume between the sham operated and vagally denervated animals at any of the time periods of pulmonary function testing post-operatively (Figure 4A). Minute ventilation was significantly decreased in vagally denervated compared to the sham operated lambs at six and 24 hours post-operatively (Figure 4B).

Studies of respiratory system (R_{RS}) and pulmonary resistance (R_{DYN}) using static and dynamic methods respectively exhibited no significant differences between sham operated and vagally denervated animals at any time periods post-operatively (Figure 5A and 5B). In addition, there were no significant differences

between the two methods of resistance measurement in either sham operated or vagally denervated animals.

Results of lung and respiratory system compliance measurements are illustrated in figures 5C and 5D. At 6 and 24 hours post-operatively, for both C_{RS} and C_L, compliance was significantly reduced in vagally denervated animals compared to sham operated animals. At the 6 hour post-operative period, C_{RS} was 2.98 vs. 1.54 ml/cmH_20/kg ($P<0.05$) and C_L was 3.00 vs. 1.30 ml/cmH_20/kg ($P<0.05$) in sham and vagally denervated animals respectively. By 24 hours post-operatively, C_{RS} values were 3.41 vs. 1.87 ml/cmH_2O/kg ($P<0.05$) and C_L values were 3.11 vs. 1.44 ml/cmH_2O/kg ($P<0.05$) in sham and denervated animals respectively (Figure 5C and 5D). Although comparisons between C_{RS} and C_L between both sham and vagally denervated animals showed that C_{RS} was slightly higher than C_L at the various time periods of pulmonary function testing, these differences were not significant.

3.4. Surfactant Large and Small Aggregate Phospholipid Content and Surface Tension

We observed no significant differences in phospholipid content between sham and denervated lambs in either the large or small aggregates (Figure 6). Phospholipid levels of large aggregates were 38.92 ± 21.03 mg/kg and 34.90 ± 5.5 mg/kg in sham and denervated lambs respectively (Figure 6). Small

aggregate phospholipid levels were 61.30 ± 36.06 mg/kg in sham operated and 57.60 ± 18.44 mg/kg in denervated animals.

Surface tension measurements were analyzed using the captive bubble technique as described by Schürch et al and again, no significant differences were seen between the two groups in surface tension lowering properties of the surfactant large aggregates (Figure 7). Together, this data shows that surfactant production, secretion and function was not disrupted by vagal denervation. In addition, lung light and electron microscopy showed no difference between vagally denervated and sham operated animals for the presence of pulmonary edema, alveolar type II cells, or tubular myelin (Figures 8 and 9).

3.5. Sleep State

Figure 10 shows the three sleep states, NREM, REM and Arousal, as a percentage of total time recorded in sham and vagally denervated animals. No significant differences existed in the incidence of NREM, and REM sleep or arousal states between the two groups. In both groups, NREM, REM, and arousal sleep was observed approximately 35%, 8%, and 57% of total time recorded respectively.

3.6. Cardiovascular Variables: Systolic, Diastolic and Mean Blood Pressures and Heart Rate

No significant differences were observed between sham operated and vagally

denervated animals in systolic, diastolic or mean blood pressures and heart rate (Figure 11 and 12).

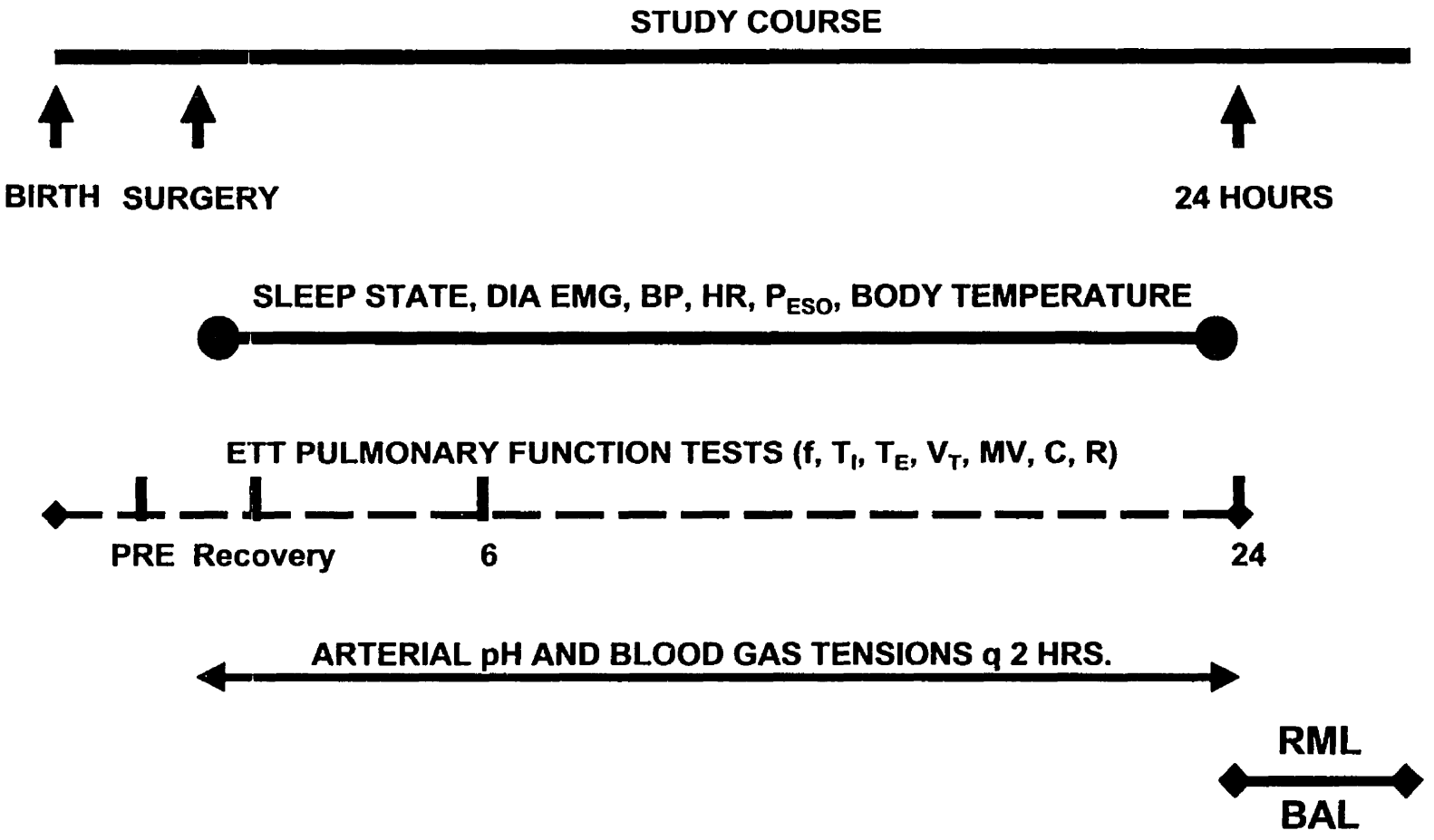


Figure 1 - Experimental Design

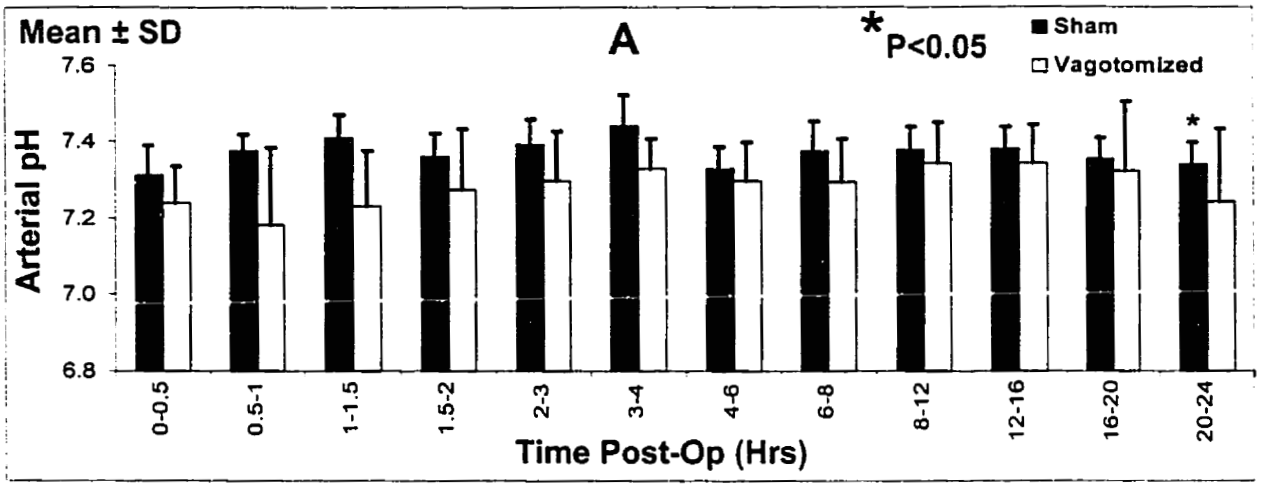
		Post-Op Course					
		Onset of Spontaneous Breathing (min.)	**Manual Positive Pressure Ventilation**	**Oxygen Requirement**	**Time of Extubation (min.)**	**Awake and Alert (min.)**	**Duration of Supplemental Heating (min.)**
SHAM	Mean	10.5	0	1	97.17	33.5	93
n=6	SD	11.79			49.65	24.46	66.78
VAGOT	Mean	70	5	7	165.13	356.3	481.89
n=8	SD	33.088			110.57	534.91	420.3
		P<0.05	P<0.05	P<0.05	NS	P<0.05	P<0.05

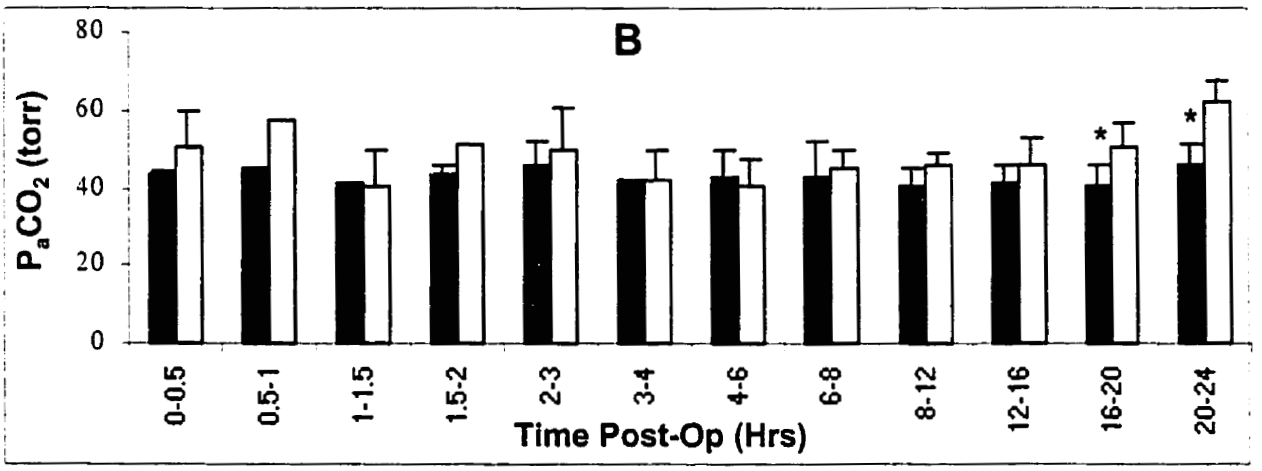
Table 1 - Post-Operative Course

Post-operative course of sham operated and vagally denervated animals.

Figure 2 - Arterial pH and Blood Gas Tensions

Arterial pH and blood gas tensions at various time bins up to 24 hours post-operatively. (A) There were no significant differences in pH between the sham operated (solid bar) and vagally denervated (hatched bar) animals until 20 hours post-operatively where vagally denervated lambs had a lower arterial pH ($P<0.05$) compared to the sham operated animals. (B) Arterial carbon dioxide tensions exhibited no significant differences between sham operated and vagally denervated animals until 16 hours post-operatively where denervated animals has a significantly higher P_aCO_2 compared to sham operated animals ($P<0.05$). (C) Arterial oxygen tensions in vagally denervated animals remained lower over the majority of the study duration ($P<0.05$).


Mean ± SD
A
*P<0.05
Sham
Vagotomized
Arterial pH
7.6
7.4
7.2
7.0
6.8
0-0.5
0.5-1
1-1.5
1.5-2
2-3
3-4
4-6
6-8
8-12
12-16
16-20
20-24
Time Post-Op (Hrs)


B
P_aCO_2 (torr)
80
60
40
20
0
0-0.5
0.5-1
1-1.5
1.5-2
2-3
3-4
4-6
6-8
8-12
12-16
16-20
20-24
Time Post-Op (Hrs)

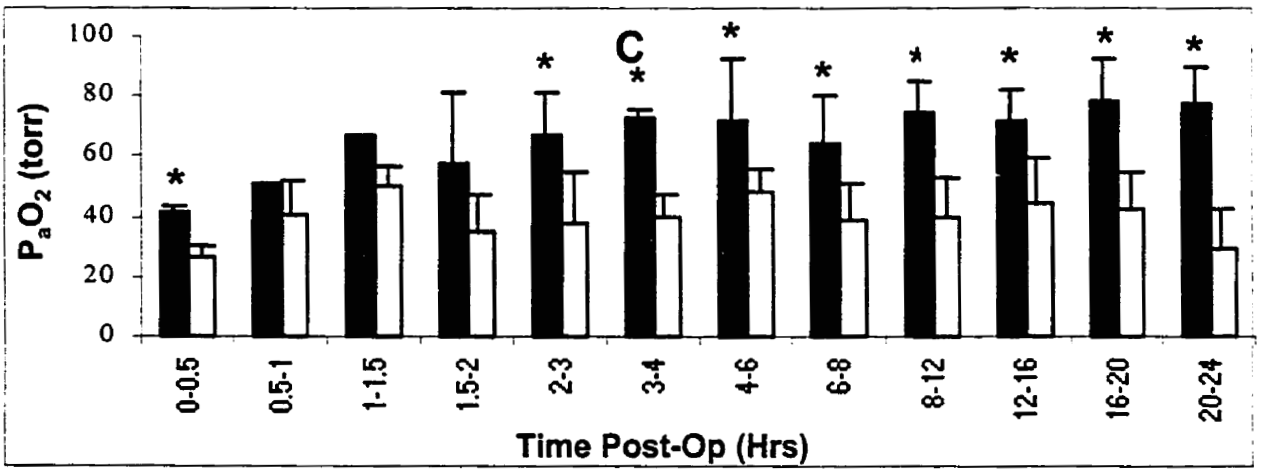
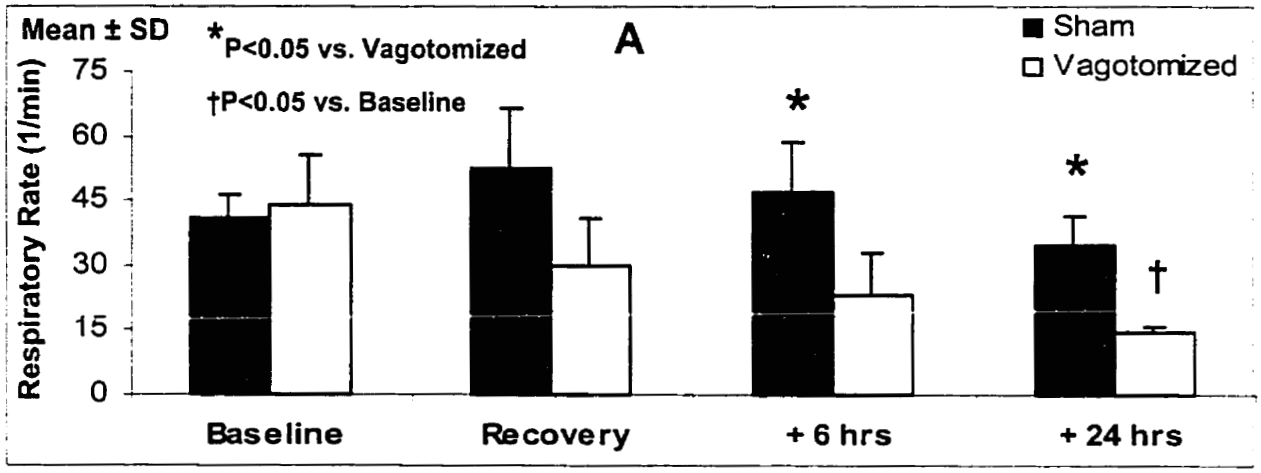
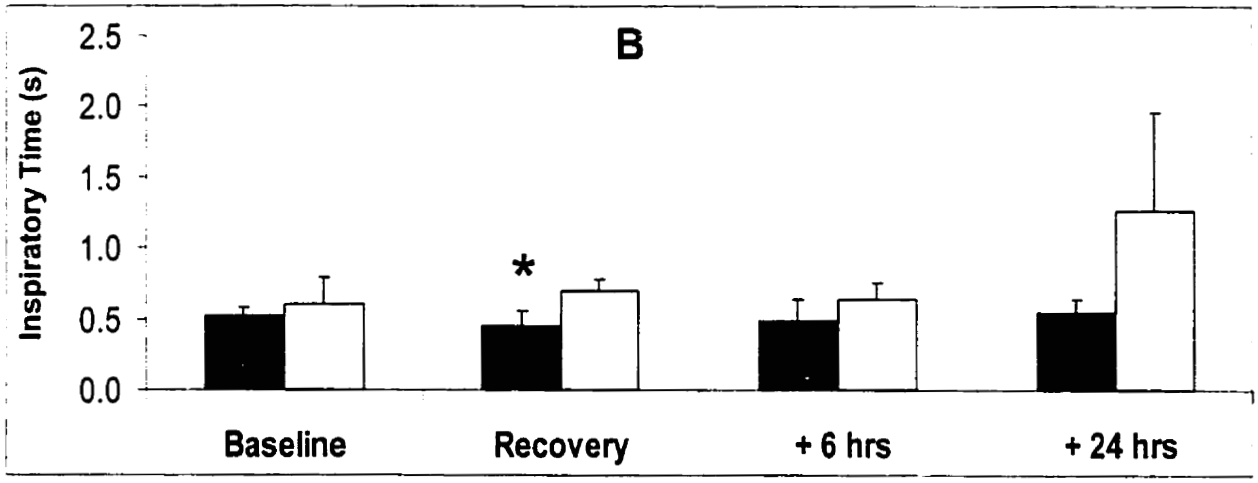

C
P_aO_2 (torr)
100
80
60
40
20
0
0-0.5
0.5-1
1-1.5
1.5-2
2-3
3-4
4-6
6-8
8-12
12-16
16-20
20-24
Time Post-Op (Hrs)

Figure 3 - Respiratory Rate, Inspiratory Time and Expiratory Time

(A) Respiratory rate per minute was similar in both groups prior to surgery (baseline). By 6 and 24 hours post-operatively, however, respiratory rate was significantly reduced in vagally denervated animals compared to sham operated animals. (B) Inspiratory time increased in vagally denervated animals during the recovery period compared to the sham operated group ($P<0.05$). (C) Expiratory time was significantly higher in vagally denervated animals during recovery, six and 24 hours post-operatively as compared to the sham operated group.


Mean ± SD
*P<0.05 vs. Vagotomized
†P<0.05 vs. Baseline
A
Sham
Vagotomized
Respiratory Rate (1/min)
75
60
45
30
15
0
*
*
†
Baseline
Recovery
+ 6 hrs
+ 24 hrs


B
Inspiratory Time (s)
2.5
2.0
1.5
1.0
0.5
0.0
*
Baseline
Recovery
+ 6 hrs
+ 24 hrs

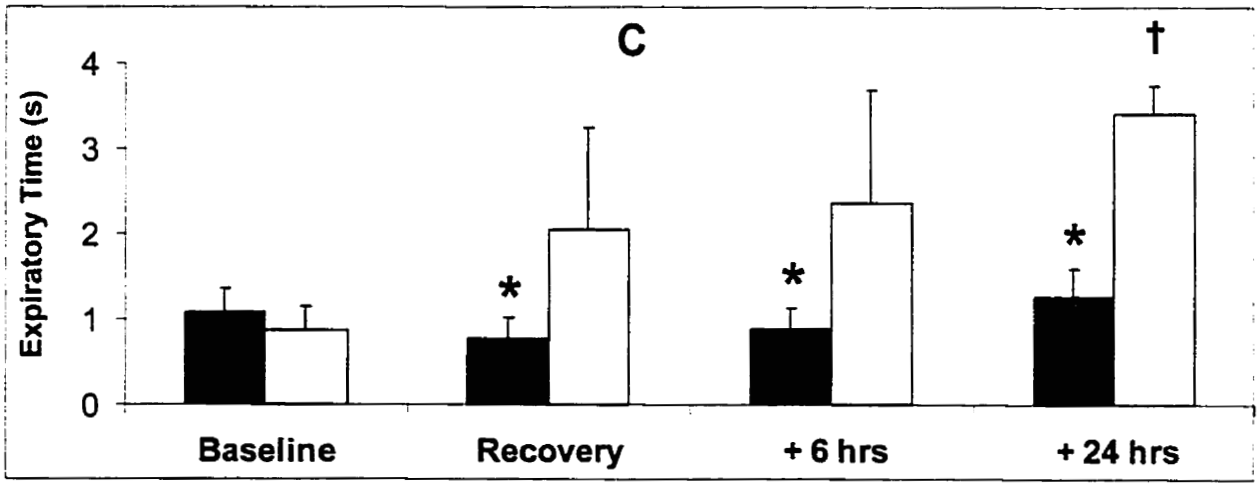
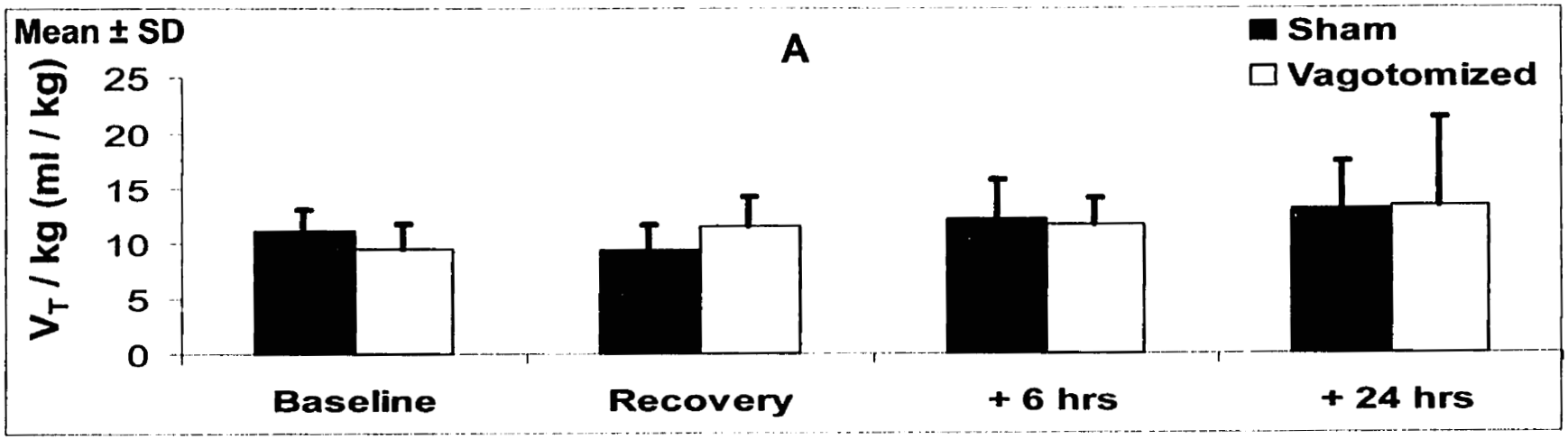

C
†
Expiratory Time (s)
4
3
2
1
0
*
*
*
Baseline
Recovery
+ 6 hrs
+ 24 hrs

Figure 4 - Tidal Volume per Kilogram and Minute Ventilation Per Kilogram

(A) There were no differences between sham and vagally denervated animals at any of the time periods of pulmonary function testing in tidal volume per kilogram (V_T/kg). (B) Minute ventilation per kilogram (ml/min/kg) was significantly lower in vagally denervated animals at 6 and 24 hours post-operatively compared to sham operated animals ($P<0.05$).


Mean ± SD
A
Sham
Vagotomized
V_T / kg (ml / kg)
25
20
15
10
5
0
Baseline
Recovery
+ 6 hrs
+ 24 hrs

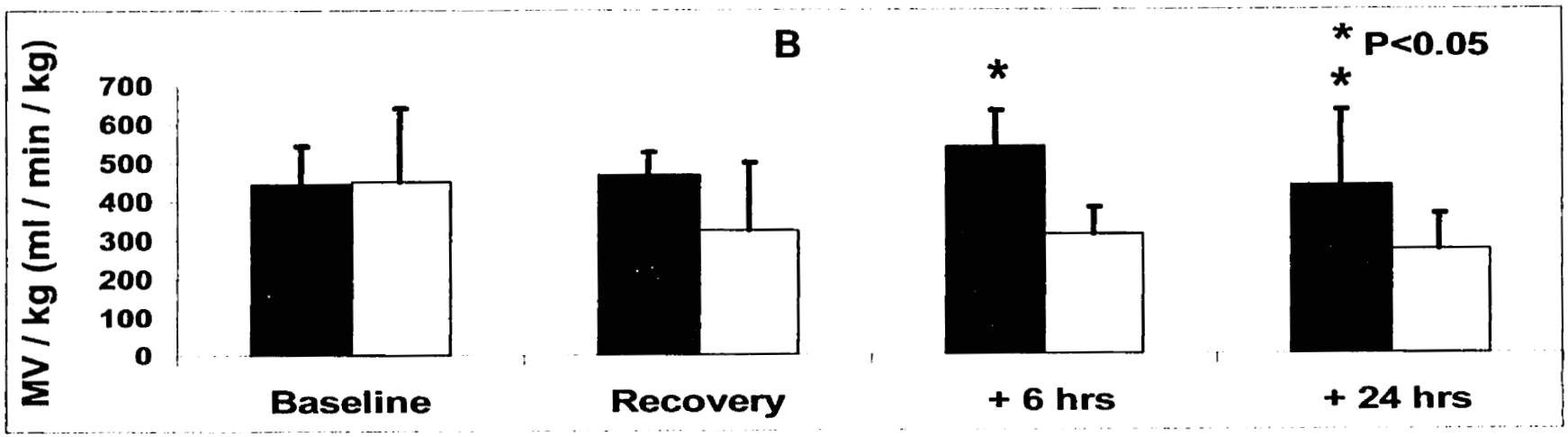
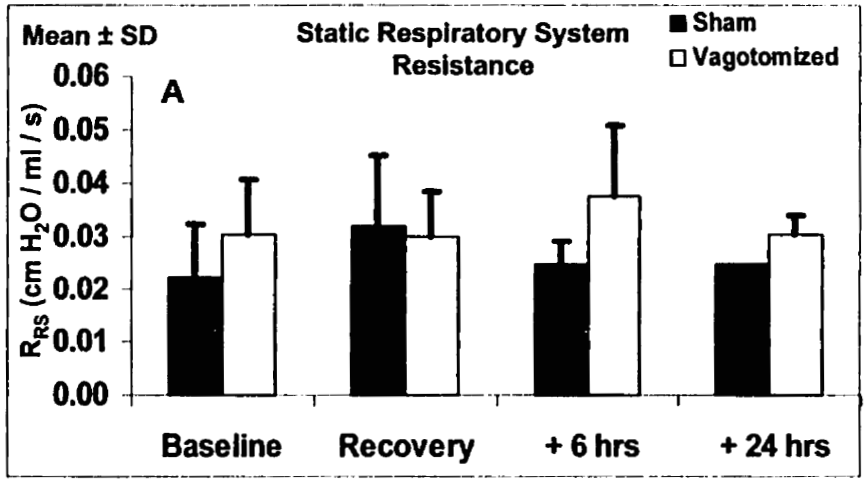
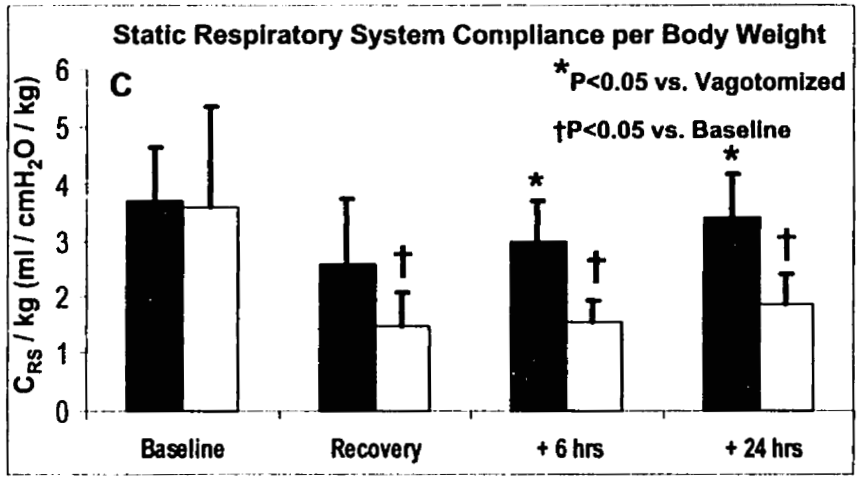
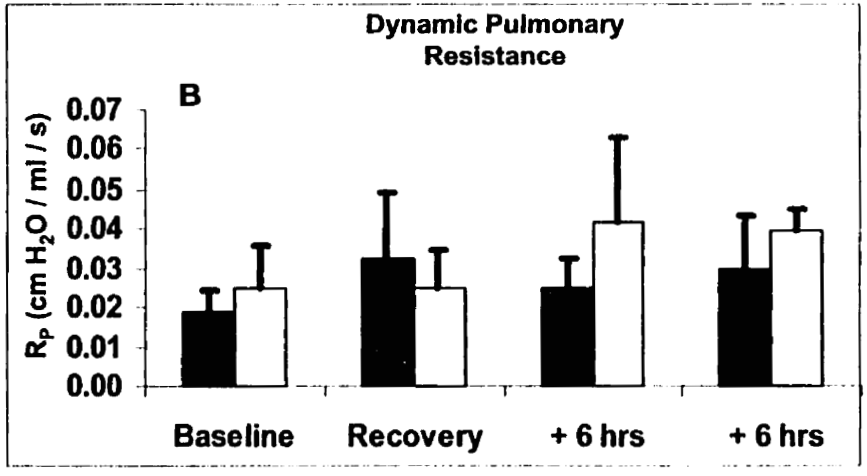

B
* P<0.05
*
*
MV / kg (ml / min / kg)
700
600
500
400
300
200
100
0
Baseline
Recovery
+ 6 hrs
+ 24 hrs

Figure 5 - Pulmonary Function Variables; Compliance and Resistance

(A,B) No differences existed between sham and vagally denervated animals at any of the time periods of pulmonary function testing in either dynamic pulmonary resistance (R_{DYN}) or static respiratory system resistance (R_{RS}). (C,D) In contrast, static respiratory system compliance per body weight (C_{RS}/kg) and dynamic lung compliance per body weight (C_{DYN}/kg) were significantly lower in vagally denervated animals compared to sham operated animals at 6 and 24 hours post-operatively.


Mean ± SD
Static Respiratory System Resistance
Sham
Vagotomized
A
R_{RS} (cm H_2O / ml / s)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Baseline
Recovery
+ 6 hrs
+ 24 hrs


Static Respiratory System Compliance per Body Weight
C
*P<0.05 vs. Vagotomized
†P<0.05 vs. Baseline
C_{RS} / kg (ml / cmH_2O / kg)
6
5
4
3
2
1
0
Baseline
Recovery
+ 6 hrs
+ 24 hrs


Dynamic Pulmonary Resistance
B
R_P (cm H_2O / ml / s)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Baseline
Recovery
+ 6 hrs
+ 6 hrs

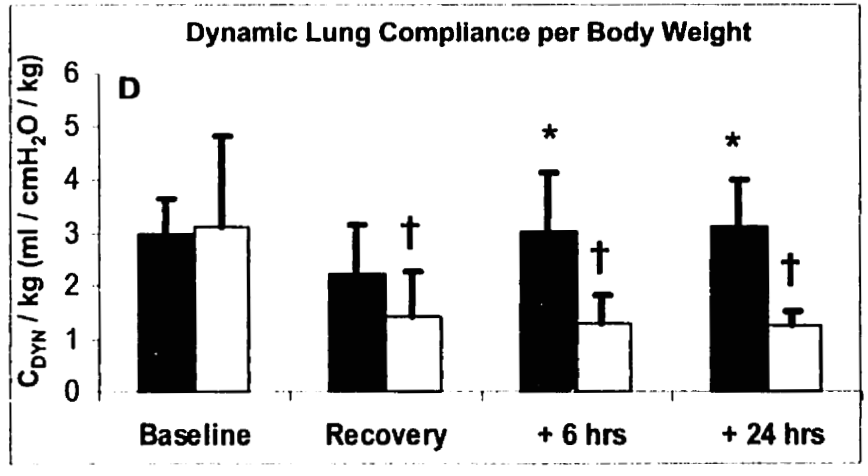
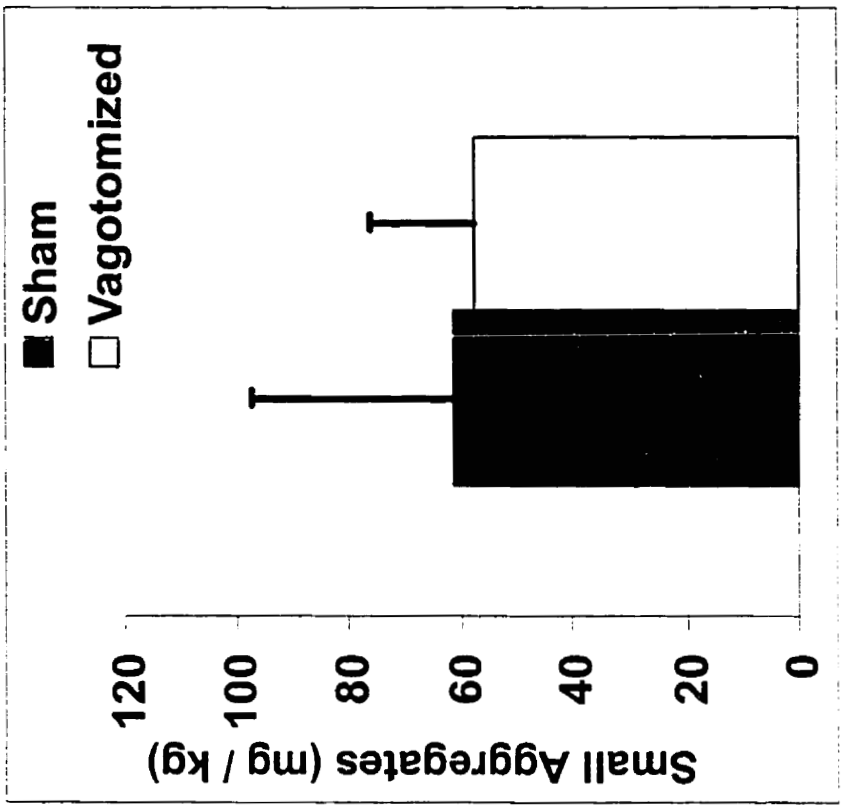

Dynamic Lung Compliance per Body Weight
D
C_{DYN} / kg (ml / cmH_2O / kg)
6
5
4
3
2
1
0
Baseline
Recovery
+ 6 hrs
+ 24 hrs

Figure 6 – Phospholipid Content of Surfactant Large and Small Aggregates

Analysis of surfactant phospholipid content in large aggregates (surface active proteins and phospholipids) and small aggregates (inactive surfactant) in mg/kg body weight showed no significant differences between the sham operated and vagally denervated animals in phospholipid content in either population of surfactant aggregates.


Sham
Vagotomized
Small Aggregates (mg / kg)
120
100
80
60
40
20
0

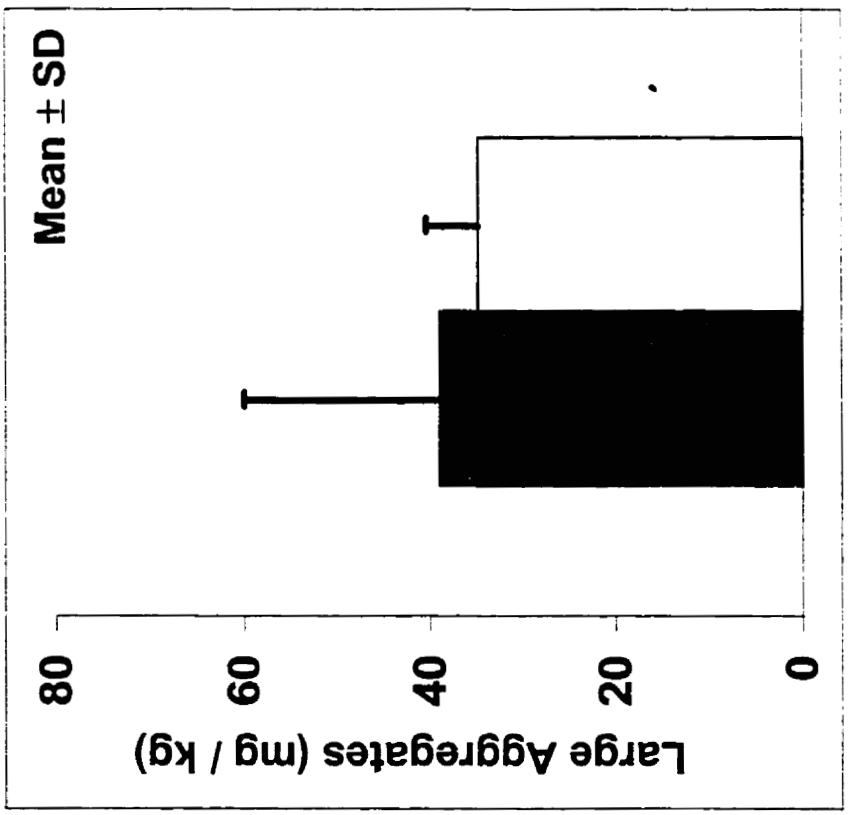

Mean ± SD
Large Aggregates (mg / kg)
80
60
40
20
0

Figure 7 - Analysis of Surface Tension

Surface tension analysis using the captive bubble technique. No differences in the ability of large aggregates from both sham operated and vagally denervated animals to reduce surface tension (mN / m) at an air-liquid interface over a period of 300 seconds was observed, indicating that surfactant large aggregates obtained from vagally denervated animals were functional in reducing alveolar surface tension.

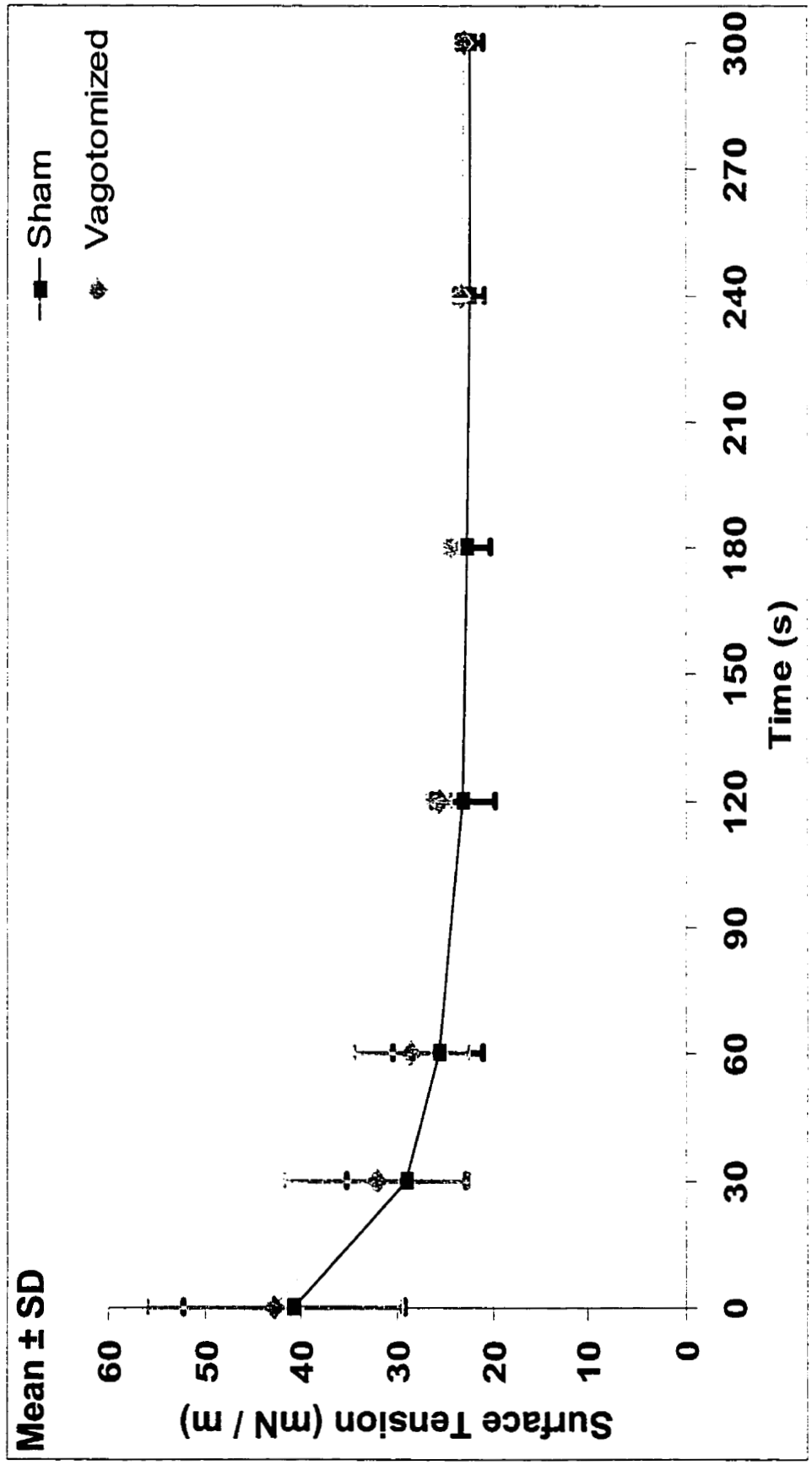
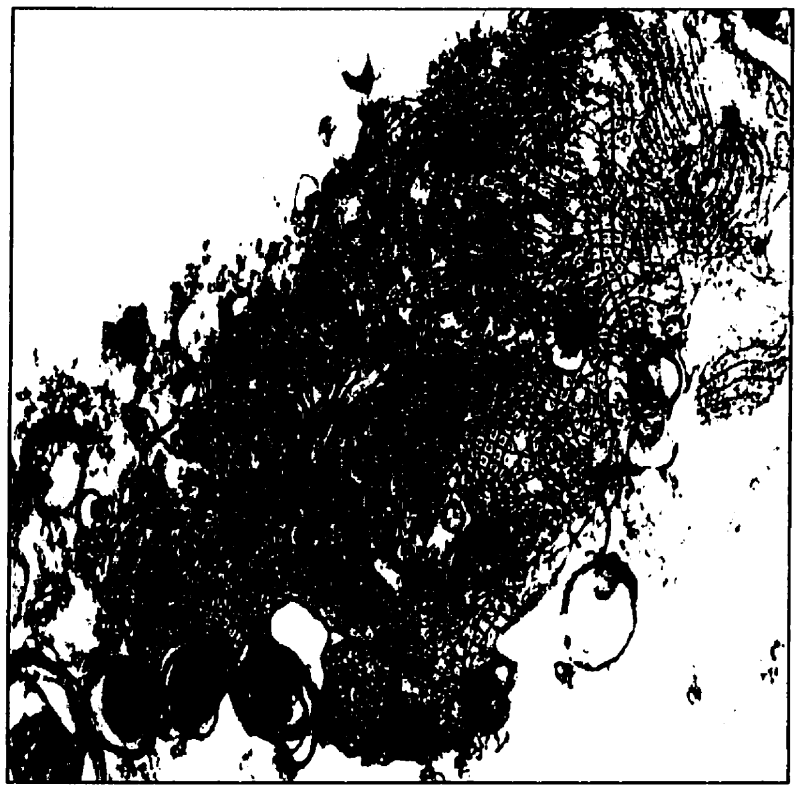

Mean ± SD
Sham
Vagotomized
Surface Tension (mN / m)
60
50
40
30
20
10
0
0
30
60
90
120
150
180
210
240
270
300
Time (s)

Figure 8 – Lung Electron Micrographs From Sham Operated and Vagally Denervated Animals

Electron microscopy from the right middle lobe of sham and vagally denervated animals shows no difference between the two groups. Electron micrographs from both groups show the secretion of tubular myelin from lamellar bodies indicating that surfactant production and secretion functions were intact in both groups.

Sham Operated



Vagally Denervated

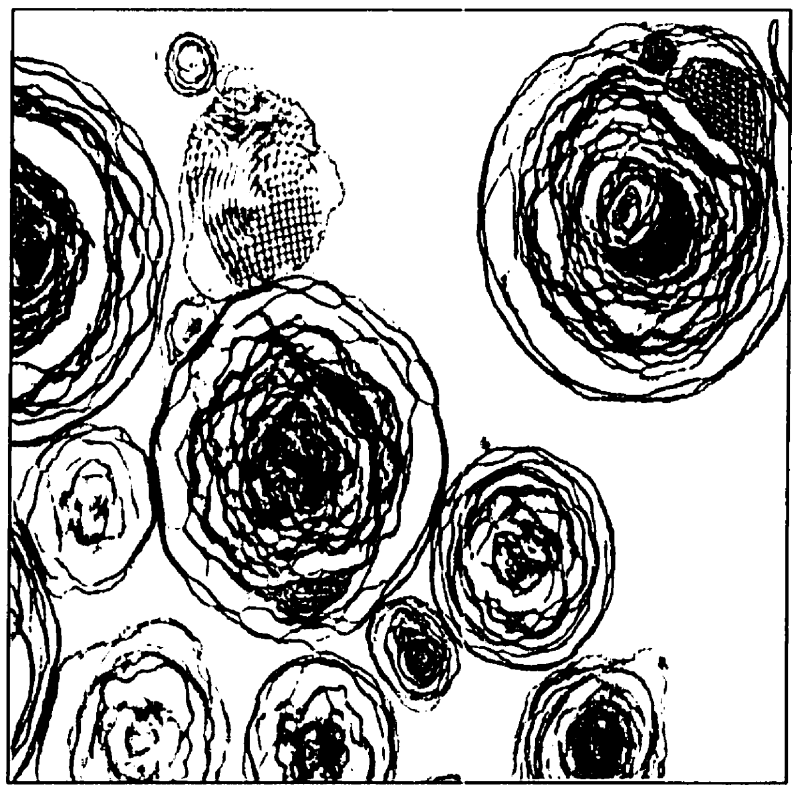
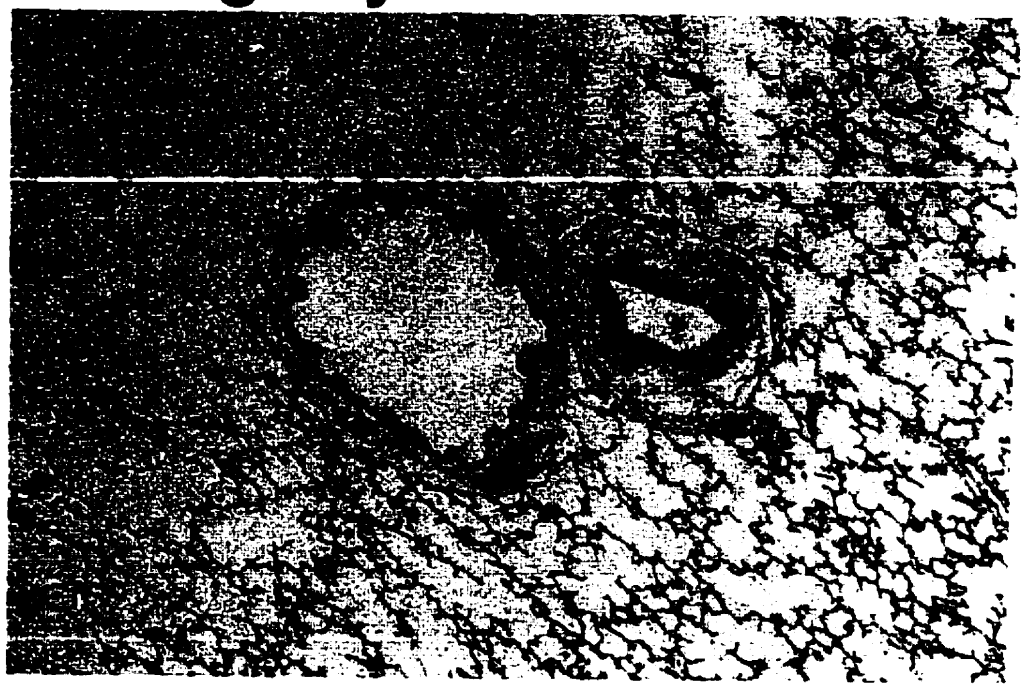


Figure 9 – Light Micrographs of Sham Operated and Vagally Denervated Animals

Light microscopy of lung parenchyma and airways of sham and vagally denervated animals show no differences between the two groups and no signs of pulmonary edema or vascular congestion in either group.

Vagally Denervated



Sham Operated

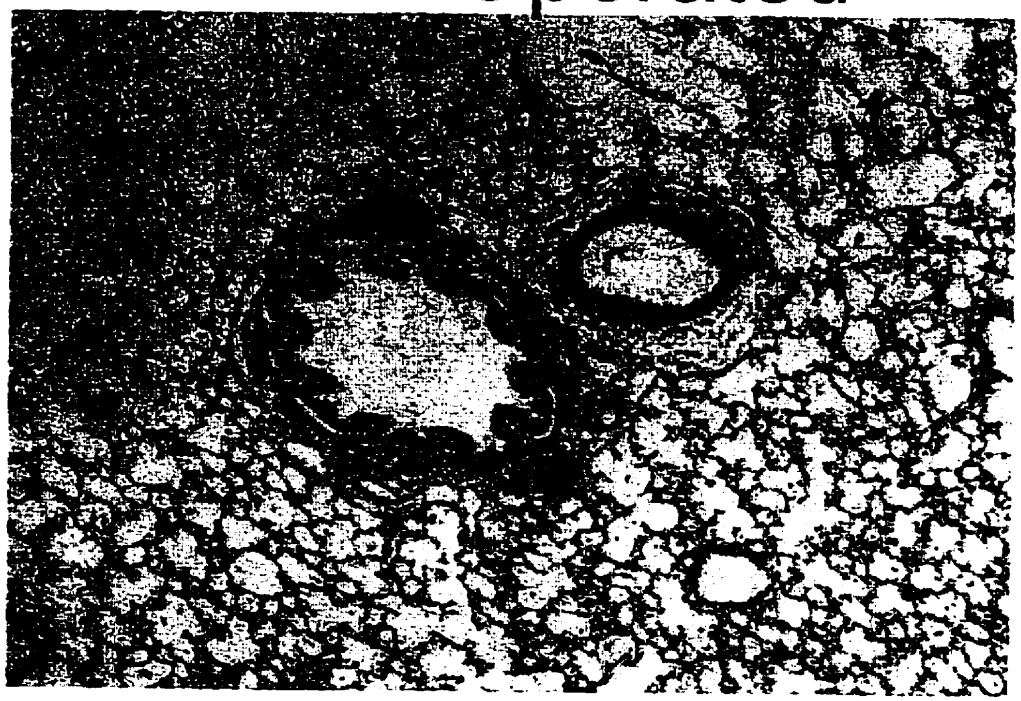


Figure 10 - Sleep States

The three sleep states, NREM, REM and Arousal are shown as a percentage of total experimental time in sham and vagally denervated animals. No differences exist between the two groups in any of the three sleep states.

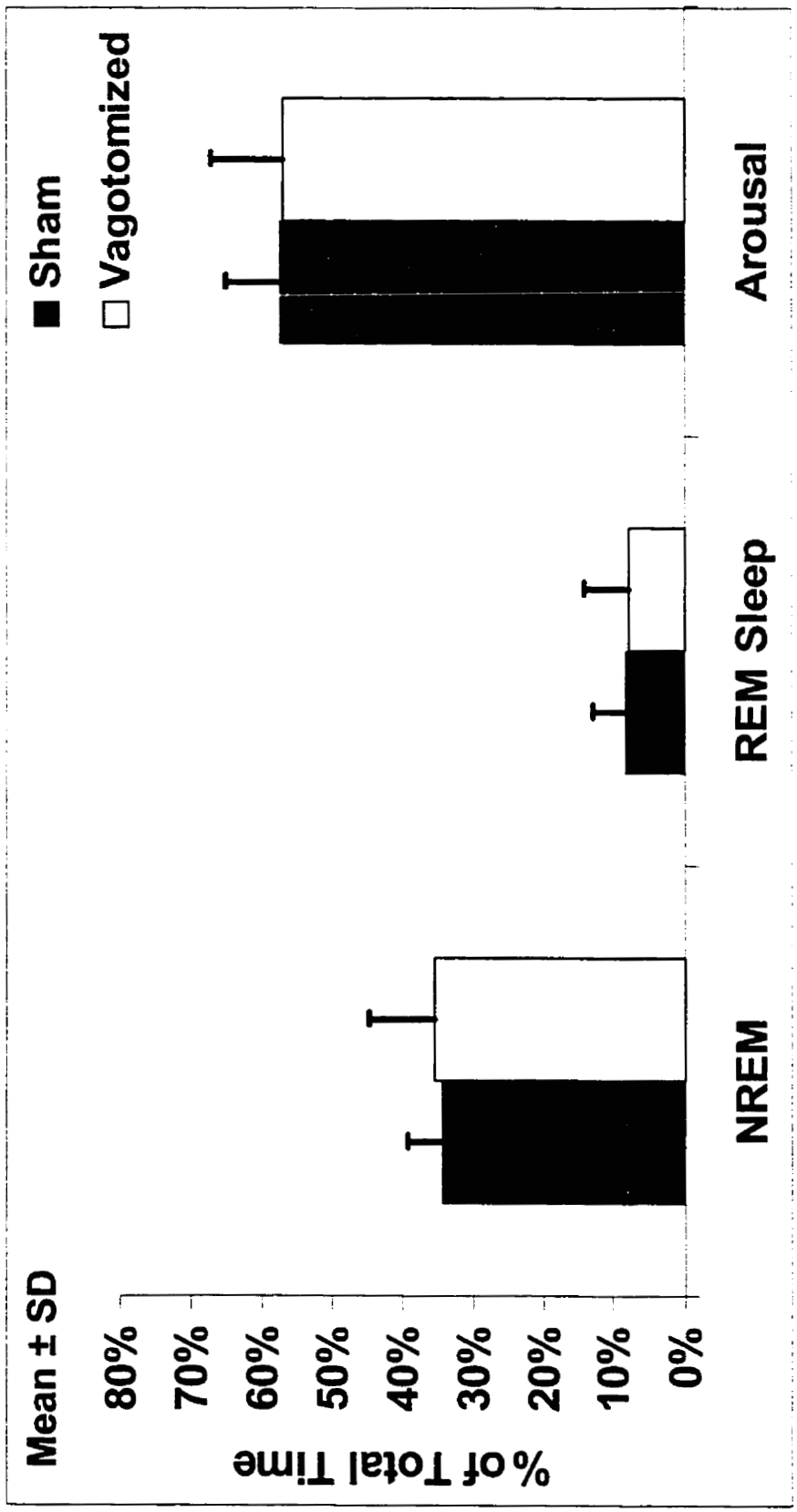
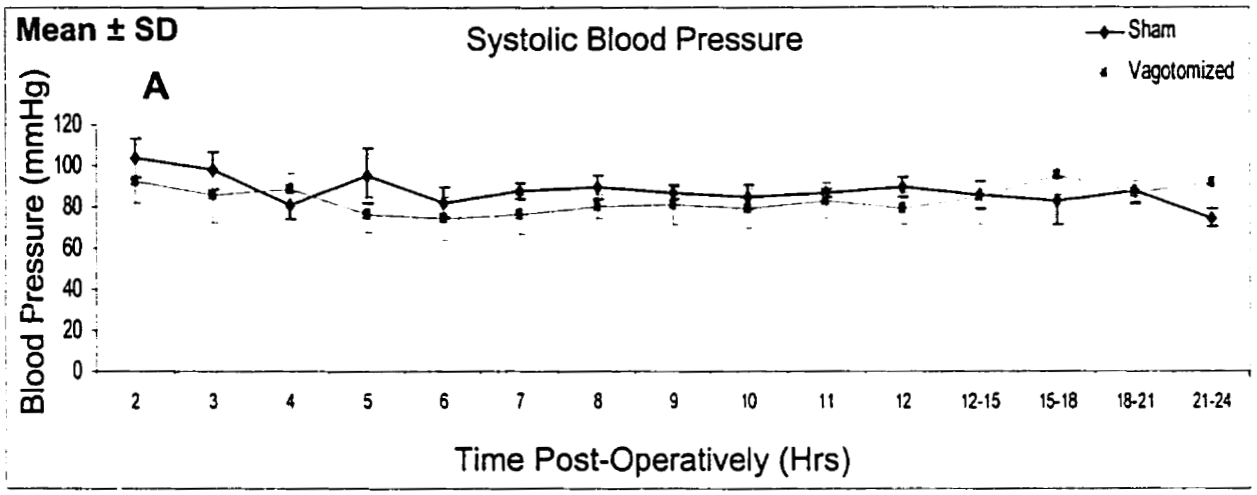
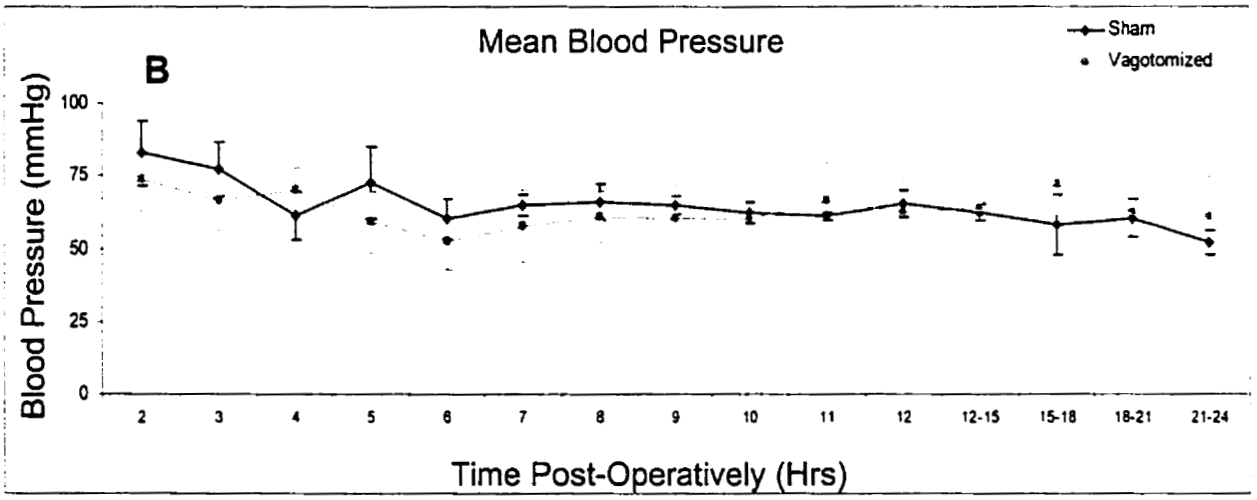

Mean ± SD
Sham
Vagotomized
% of Total Time
80%
70%
60%
50%
40%
30%
20%
10%
0%
NREM
REM Sleep
Arousal

Figure 11 – Systolic, Mean and Diastolic Blood Pressure in Sham and Vagally Denervated Animals

Arterial blood pressure data was obtained at various time bins post-operatively up to 24 hours. There were no significant differences in systolic, mean or diastolic blood pressures between sham (solid line with squares) and vagally denervated (solid line with diamonds) animals.


Mean ± SD
Systolic Blood Pressure
Sham
Vagotomized
A
Blood Pressure (mmHg)
120
100
80
60
40
20
0
2
3
4
5
6
7
8
9
10
11
12
12-15
15-18
18-21
21-24
Time Post-Operatively (Hrs)


Mean Blood Pressure
Sham
Vagotomized
B
Blood Pressure (mmHg)
100
75
50
25
0
2
3
4
5
6
7
8
9
10
11
12
12-15
15-18
18-21
21-24
Time Post-Operatively (Hrs)

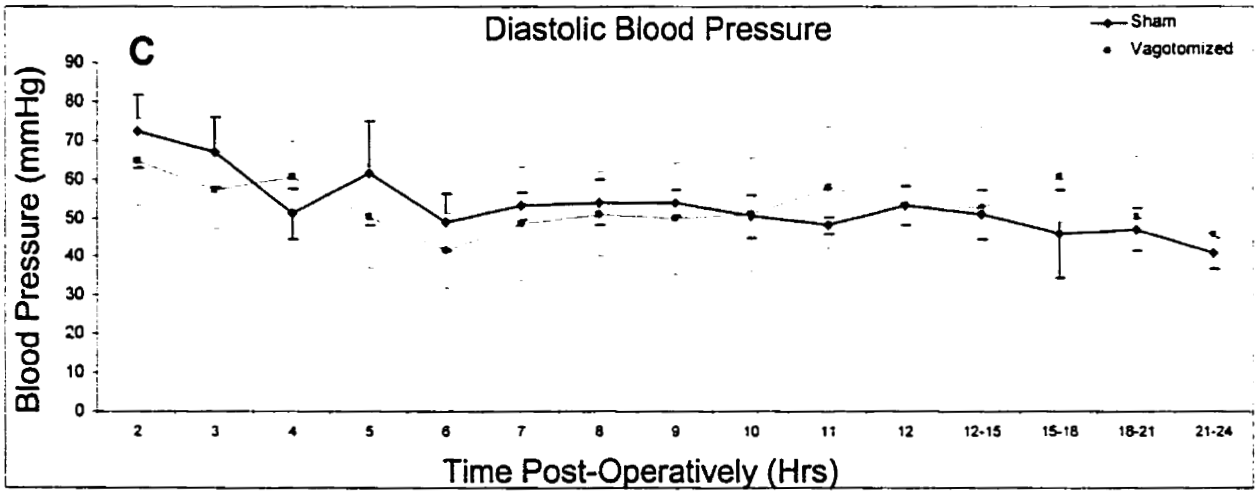
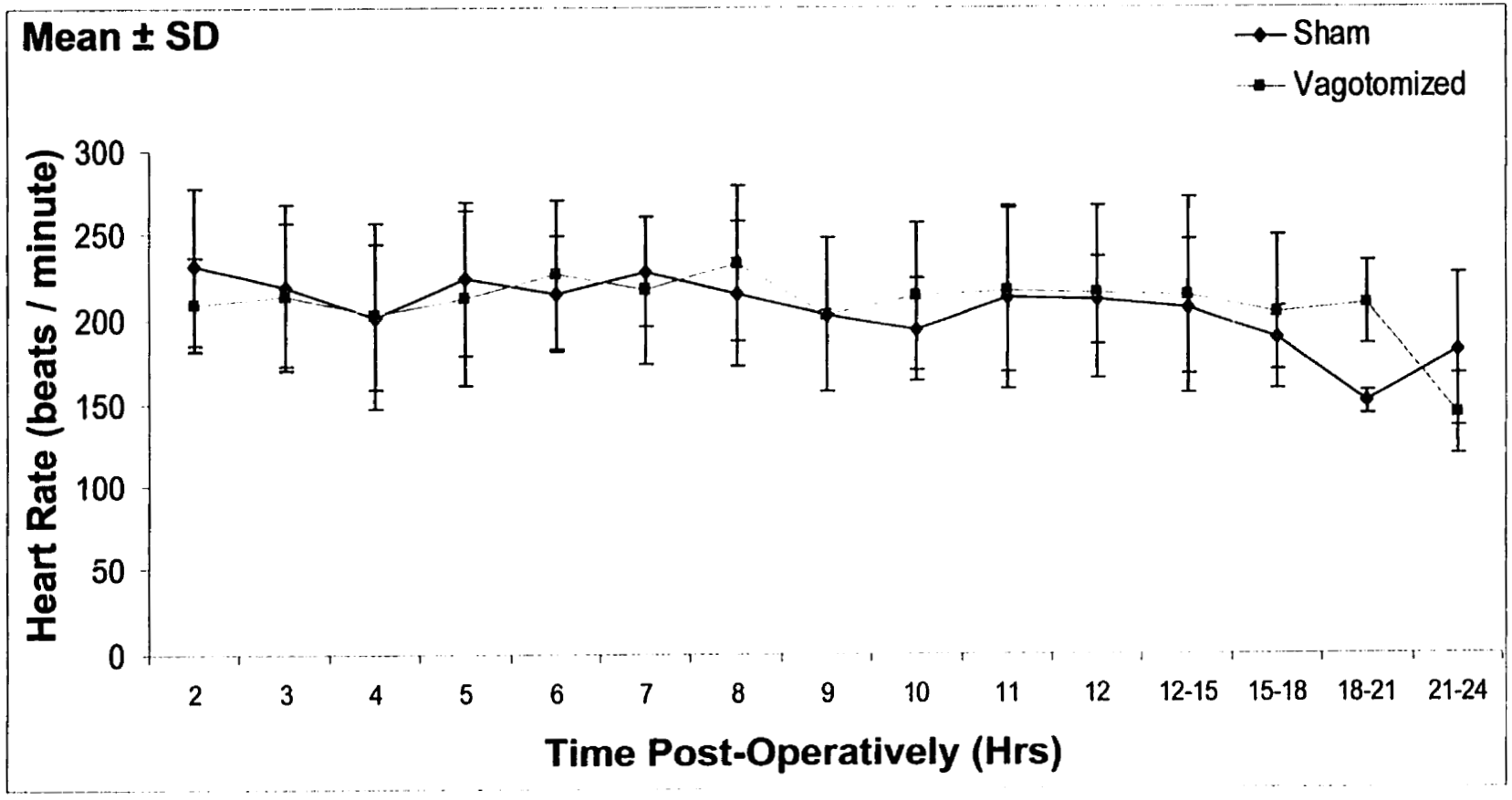

Diastolic Blood Pressure
Sham
Vagotomized
C
Blood Pressure (mmHg)
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
11
12
12-15
15-18
18-21
21-24
Time Post-Operatively (Hrs)

Figure 12 – Heart Rate in Sham and Vagally Denervated Animals

Heart rate analysis of sham (solid line with squares) and vagally denervated animals (solid line with diamonds) at various time bins post operatively shows no significant differences between the two groups at any of the time bins post-operatively.


Mean ± SD
Sham
Vagotomized
Heart Rate (beats / minute)
300
250
200
150
100
50
0
2
3
4
5
6
7
8
9
10
11
12
12-15
15-18
18-21
21-24
Time Post-Operatively (Hrs)

CHAPTER 4: DISCUSSION

4.1. General

This is the first study that has investigated the effects of intrathoracic vagal denervation in unanaesthetized and spontaneously breathing animals during the early postnatal life. The effects of vagal denervation on breathing patterns and gas exchange have previously described by Coombs and Pike, Schwieler, Duron and Marlot, Fedorko et al, Delacourt et al and Wong et al (29,35,37,40,118,133). These studies have one or more of the following limitations. Vagotomies were performed in the cervical location resulting in vocal chord paralysis and compromise of upper airway function. Animals were examined while under the effects of anaesthesia and tracheotomized, and the immediate newborn period was not monitored. It is known that anaesthesia results in respiratory depression and tracheotomy bypasses the upper airway which plays an important role in breathing. Additionally, all previous studies looked at vagotomy at least one day after birth. Studies investigating the role of the vagus on breathing patterns and gas exchange should be performed as close to birth as possible since the role of the vagus changes with development (29,118). Finally effects seen in previous studies may be influenced by variables present antenatally or at birth such as lung liquid absorption, surfactant secretion, or changes in pulmonary blood flow (133). Thus the role of the vagi in maintaining breathing and pulmonary gas exchange in the immediate newborn period has not yet been elucidated.

We have shown that vagal innervation is critical for the maintenance of

continuous breathing and gas exchange in the early neonatal period. Vagal denervation during this time leads to hypoxemia and significant effects on breathing patterns. In contrast to prenatal vagal denervation, postnatal denervation does not cause aberrations in the surfactant system as evidenced by absence of changes in biochemical, and physical properties of the broncho-alveolar lavage, or presence of normal type II cells. Additionally, vagal denervation does not result in any pulmonary edema as evidenced by the absence of differences between sham and denervated lambs in light and electron microscopy and surfactant variables. Therefore, vagally mediated pulmonary feedback is critical for the maintenance of gas exchange and normal breathing patterns during the immediate newborn period.

4.2. Post-Operative Course

In the immediate post-operative period, denervated animals required manual ventilation, oxygen, heat and took longer times to be extubated compared to sham operated animals. This difference is most likely due to lack of afferent information from the vagus nerves which relay information of lung volume centrally causing a disruptive effect on breathing pattern and ventilatory timing (40). Vagus nerves have also been shown to augment phrenic nerve output and the volume related feedback to be excitatory to the inspiratory activity (32).

4.3. Sleep States

We observed no significant differences in sleep states between the vagally

denervated and sham operated lambs over the 24 hour study course. Sleep states were determined by analysis of electrocortical, electro-ocular, and nuchal electromyograms. Based on these results, the observed decreases in breathing frequency, arterial gas tensions, and pH can not be explained on the basis of deficiency or excess of one particular sleep state.

During wakefulness, respiration is thought to be facilitated by a wakefulness stimulus, a voluntary component, or of reticular activation (42). Therefore, in the awake state, mechanisms including and in addition to vagal afferent information are responsible for the termination of inspiration allowing the maintenance of a high respiratory rate and termination of inflation produced apnea in the absence of vagal influence (45). Similar mechanisms are present in REM sleep which account for the maintenance of a high respiratory rate and variability despite vagal denervation (90). In contrast, NREM sleep lacks the compensatory mechanisms present during both wakefulness and REM sleep resulting in inability to maintain respiratory rate in the absence of vagal influence (45).

In neonates, Praud et al. observed that NREM sleep is characterized by increased vagal influence (unpublished observations). Thus although previous studies on the role of vagal afferents and sleep state were mainly performed in adult animals, the role of the vagus on respiration appears similar in neonates during the different sleep states. These results therefore strengthen the suggestion that the oscillation of the automatic control centers involved with

breathing is critically dependent on the level of incoming sensory stimuli as removal of the stimuli results in decreased respiratory rates and apnea (99).

4.4. Arterial Blood Gas Tension and pH

With respect to arterial blood gas tensions and pH, we observed no significant differences in either arterial pH or P_aCO_2 until the end of the study when vagally denervated lambs became acidotic and hypercapneic compared to sham operated animals. However, a marked difference was seen in arterial oxygen tensions between denervated and sham operated animals as denervated lambs remained hypoxic over the entire study duration. Together, the arterial pH and blood gas tension results suggest that vagally denervated animals developed profound respiratory failure post-operatively.

Respiratory failure, defined as arterial hypoxemia, occurs via three main categories; respiratory failure due to an intrinsic gas exchange problem, alveolar hypoventilation, or a combination of these two. An intrinsic pulmonary gas exchange problem is characterized by a normal P_aCO_2, and an increased alveolar-arterial difference in oxygen content (A-a DO_2). In vagally denervated animals, this "type" of respiratory failure was observed over the duration of the study. The second category of respiratory failure is alveolar hypoventilation which is characterized by an increased P_aCO_2, and a normal A-a DO_2. Although alveolar hypoventilation was not independently observed in vagally denervated animals, it did occur in conjunction with an intrinsic gas exchange problem

towards the end of the study.

Six pathologic mechanisms exist for the development of hypoxemia including, hypoventilation, right to left shunt, low ventilation/perfusion ratio, diffusion impairment, low barometric pressure, and a low inspired oxygen content (F_iO_2). Over the duration of the study respiratory failure was mainly due to an intrinsic pulmonary gas exchange problem and not alveolar hypoventilation. Additionally, there was no change in either the barometric pressure or the F_iO_2 over the study course. Thus, although hypoxemia may arise from a number of different pathologic mechanisms, the three possible mechanisms relevant to this study are hypoxemia arising from a low ventilation/perfusion ratio, a right to left intrapulmonary shunt, or a diffusion impairment.

Occurrence of these mechanisms are supported by the changes observed in the pulmonary mechanics, namely the reduction in both lung and respiratory system compliance. We speculate that the hypoxemia may arise from either pulmonary atelectasis, or pulmonary edema which would both result in a low ventilation/perfusion ratio and possibly right to left intrapulmonary shunt. Additionally, pulmonary edema would also result in a diffusion impairment since respiratory gases must diffuse not only through the membrane but also through the edema fluid. These mechanisms are further discussed in section 4.6.

Mammals respond to hypoxemia with alveolar hyperventilation as determined by

the ratio of minute ventilation/oxygen consumption, an index of alveolar ventilation. While the primary strategy adopted by adults to increase alveolar hyperventilation is hyperpnea, many newborn species respond to hypoxemia predominantly with hypometabolism as opposed to hyperpnea. As animals mature, hyperpnea gradually substitutes hypometabolism as the primary response to hypoxemia (87). Hypometabolism would reduce both oxygen consumption and carbon dioxide production, effectively increasing the index of alveolar ventilation and suggesting a reduction in alveolar carbon dioxide content as determined by the carbon dioxide production/alveolar ventilation ratio. Since chemoreception in newborns is immature, it is plausible that disruption of the carbon dioxide production/alveolar ventilation ratio may not result in reduced ventilation as observed in older animals. This has been shown previously where the chronic hypoxic state of the vagally denervated animals without a subsequent increase in P_aCO_2 was associated with a decrease in metabolic rate (51,121).

Hypoxic reduction in metabolic rate is also supported by the observation that denervated animals were unable to maintain normal body temperatures over the post-operative period and had to be warmed using a heat lamp; an intervention that may have affected the natural course of thermoregulation (133). We did not use any other methods to rewarm the lambs such as wrapping them in blankets or placing them in an incubator. Previous studies have also indicated that a hypoxic state results in decreased body temperature in a number of animal species (46,89,98). Hypoxia may decrease metabolic rate via a decrease in

thermogenesis although metabolic and ventilatory responses vary depending on species and postnatal age and weight (46). Results similar to our own were seen by Wong et al. in postnatal lambs that underwent prenatal intrathoracic vagotomy (133). They observed that after 10 minutes of birth, rectal temperature continuously declined despite attempts to rewarm the lambs (133). The decrease in rectal temperature was also concomitant with both hypoxemia and hypercapnia (133).

Toward the end of the study (approximately 16 hours post-operatively) vagally denervated lambs did show an increase in P_aCO_2 and a decrease in pH relative to sham operated animals indicating respiratory acidosis due to hypoventilation which did not give rise to any respiratory stimulation. This time period was also associated with a lowered respiratory rate and high expiratory times superimposed with periods of apnea in vagally denervated animals. These changes in respiratory parameters would account for the subsequent changes in arterial gas tensions and pH during this time period. Similar results were seen by Schweiler who showed that vagotomy in the newborn leads to decreased pH and increased P_aCO_2 without any effective stimulation of respiration (118). Another possible factor that could contribute to the observed apnea is diaphragmatic muscle fatigue as a result of increased anaerobic respiration towards the end of the study, however examination of blood lactic acid content was not performed, thus this theory can not be confirmed (52).

4.5. Cardiovascular Variables

The vagus is responsible for regulation of heart rate due to parasympathetic innervation of the heart. In the heart, the vagus alters rate via innervation of the sinoatrial and atrioventricular nodes and, when stimulated, the vagus decreases both rate and force of contraction. In our experiments we did not sever the vagal fibers leading to the cardiac plexus thus avoiding any cardiac effects of vagal denervation and observed no significant differences between sham operated and denervated animals in heart rate, systolic, or diastolic pressures.

In the adult, cardiac output can be increased during hypoxemia by increasing heart rate and/or venous return (via sympathetic nervous system stimulation or increasing levels of circulating catecholamines) in an effort to improve oxygen delivery to tissues. In the immediate newborn period, however, the stress of birth and the new arrangement of the circulation causes a large increase in left ventricular output. This response utilizes the reserves that the newborns have for increasing cardiac output such as the ability to increase sympathetic stimulation and circulating catecholamines. Thus with the additional stress of hypoxemia, newborns can not increase cardiac output any further. With increasing postnatal age cardiac output and heart rate decrease, re establishing functional reserves of the heart to respond to stress (103).

Absence of cardiovascular responses to hypoxemia were observed by Haddad where, in response to moderate hypoxemia (P_aO_2 of 43-48 mmHg), newborn

puppies failed to increase heart rate or cardiac output (52). Absence of changes in postnatal pulmonary blood flow with antenatal intrathoracic vagal denervation has also been previously established by Wong et al. (133). Additionally, absence of change in blood pressure following vagotomy was also demonstrated by Schweiler (118).

4.6. Pulmonary Function Tests

Pulmonary function tests and examination of lung mechanics were performed with an endotracheal tube to examine breathing patterns in sham and vagally denervated lambs. We observed a significant reduction in respiratory rate and increase in expiratory time in vagally denervated compared to sham operated animals. Furthermore, no difference was observed in inspiratory time between vagally denervated and sham operated animals.

Similar results were seen by Schweiler et al. in 1968 where cervical vagal denervation in newborns produced a reduction in breathing rate which became periodic or gasp like and apneic (118). Cervical vagotomy and vagal cooling experiments in rabbits, cats, dogs and newborn rats have shown that vagal denervation is associated with increased expiratory and inspiratory times (33,35,40,102,118,125).

In his study, Schweiler concludes that respiratory control systems which are active in the adult, such as the thoracic dorsal root afferents, are not fully

developed in the newborn cat and rabbit, thus the negative effects of vagotomy are more pronounced in newborns than in adults (118). Respiratory effects of vagotomy in newborns may also be due to a greater susceptibility to hypoxemia and hypercapnea which are consequences of the decreased ventilation after vagotomy (118). This is plausible since although newborns can survive oxygen deprivation for longer time periods than adults due to an increased ability to maintain anaerobic metabolism, the respiratory activity is more sensitive to oxygen deprivation and ceases more rapidly (118).

No changes were observed in either tidal volume or static or dynamic respiratory system resistance, and both static respiratory system and dynamic lung compliance and minute ventilation were significantly lower in vagally denervated lambs by six hours post operatively compared to sham operated animals. Absence of changes in respiratory system resistance were also shown in adult rabbits by Mortola et al. (88). Many studies have shown that vagal afferent feedback is essential in control of upper airway resistance through the abduction of the vocal cords during inspiration and adduction of the vocal cords during expiration (105). Thus, bypassing the upper airway with an endotracheal tube will not account for the role of the upper airway in breathing strategies of vagally denervated and sham operated animals. In further studies then, a well sealed mask should be used to examine breathing patterns in lambs with intact upper airways.

Minute ventilation is a product of respiratory frequency and tidal volume. Since tidal volume did not change in vagally denervated animals, the decrease in minute ventilation is due to the decrease in respiratory rate. Furthermore, since respiratory rate is determined by inspiratory and expiratory times, and expiratory times were increased to a greater degree than inspiratory time in vagally denervated lambs, the ventilatory depression is likely due to the prominent increase in expiratory time. The same conclusion was drawn by Fedorko et al., who suggested that the major influence on the central respiratory control system which is eliminated after vagotomy was one that promotes the initiation of inspiration and/or inhibits the expiratory phase (40).

Decreased minute ventilation has also been observed in cervically vagally denervated rabbits, lambs and rats and may be responsible for the resulting hypoxemia in our vagally denervated lambs (40,79,88). Previous studies have shown up to a three-fold increase in tidal volume of vagally denervated rabbits, and rats (newborn) compared to control animals (40,127). However, similar changes were not observed in our study. In fact, tidal volume showed no significant changes compared to sham operated animals over the course of the study. There are two possible reasons for the lack of increase in tidal volume as observed in previous studies. First, the small increase in inspiratory time would limit the volume of air going into the lungs. Since previous studies showed large increases in T_I, this could lead to an increase in tidal volume. Second, the low lung compliance in vagally denervated animals would also limit the volume of air

that can be inspired for any given driving pressure. These changes in pulmonary function data and mechanics support the hypothesis that vagal afferent input provides the positive feedback in newborns required for the maintenance of breathing patterns, ventilation and pulmonary mechanics.

4.6.1. Respiratory Drive

Respiratory drive reflects the excitability of the inspiratory neurons and can be quantified by examining the relationship between inspiratory volume and time, or the inspiratory flow given by the V_T/T_I ratio (108). The inspiratory drive thus determines the duration of the respiratory phases and the rate of airflow based on stimulation of inspiratory neurons as long as mechanics of the respiratory system are normal (108). Respiratory drive signals from the central pattern generator are transmitted to respiratory muscles which increase P_{TP} effectively increasing lung volume in normal subjects, such as in the sham operated group.

Since vagally denervated animals had "stiffer" lungs and a similar V_T compared to the sham group, this implies that denervated animals produced a higher P_{TP} compared to the sham operated group. Because of the force-length relationship of muscles, with a lower end expiratory lung volume in denervated animals, there will be an increased force generated by the respiratory muscles for the same neural output (50). Since the degree of muscle output is determined by respiratory drive via the CPG, increased muscle output suggests an increased respiratory drive in denervated compared to the sham operated animals.

Alternatively, there may be an increased neural output by the central pattern generator to the inspiratory muscles without a change in the length of the muscles. Thus, although the V_T/T_I ratio was similar in both sham operated and denervated animals, denervated animals had a significantly lower lung and respiratory system compliance suggesting an increased respiratory drive.

4.6.2. Pulmonary Atelectasis

Newborns have a chest wall compliance (C_W) five times higher than compliance of the lung (C_L). Since $1/C_{RS} = 1/C_L + 1/C_W$, if C_W is $5C_L$ than 1/1 + 1/5 = 1.2, hence respiratory system compliance (C_{RS}) is about 83% of C_L (103). Thus, in infants, respiratory system compliance (C_{RS}) is a good indicator of C_L. Our of experiments showed that both C_{RS} and C_L were lower in vagally denervated animals than in the sham operated animals. A number of factors could cause this decrease in pulmonary lung compliance including disturbances in surfactant function, alveolar derecruitment (atelectasis), connective tissue abnormalities, and/or pulmonary edema (28,49,63).

At low lung volumes, many airways and some alveoli are unstable and close (derecruit). These structures tend to remain shut due to surface tension forces, and work must be done to reopen them. On the inflation pressure-volume (PV) curve, this work is manifested as the high airway pressure required to overcome the critical pressures holding the atelectic units closed (64). The phenomena of recruitment will thus result in an elongation of the initial flattened portion of the

inflation curve causing the curve to shift right and adding to the area of hystereis. Once the pressure holding alveoli closed is overcome, the inflation curve will move into the steep portion which represents sequential "popping open" of airways and alveoli (63). On deflation, the opposite sequence of events will occur leading to derecruitment or collapse of airways and alveoli at low lung volumes, especially in smaller airways of the lung where airways are not very rigid (49).

This derecruitment will lead to a change in lung volume history over time depending on the breathing pattern exhibited by the subject. In our study, vagally denervated animals exhibited increased expiratory times with slightly elevated inspiratory times. Together with the loss of lower airway afferent input to the upper airway, this pattern of breathing could result in substantial derecruitment of alveoli which, as mentioned, would change lung volume history over time and explain the lower lung compliance values obtained from vagally denervated animals compared to sham operated animals. Atelectasis could occur via the following mechanism:

a. Vagal denervation would extinguish lower airway mediated braking activity of the upper airway and/or diaphragm which is crucial in the maintenance of a dynamic end expiratory volume above passive FRC.
b. Denervation would also disrupt vagally mediated modulation of respiratory timing leading to the observed increase in expiratory times compared to sham operated animals.

Together the effects of a large expiratory time through upper airway providing low resistance to airflow would lead to a reduction in the dynamic end expiratory volume of denervated animals towards FRC. Further, the reduction in lung volume would lead to severe atelectasis and respiratory failure characterized by hypoxemia and an increased A-a DO_2, as we have observed.

In 1963 Colebach and Halmagyi investigated the effects of cervical vagotomy in sheep and found that C_L was significantly lower in vagally denervated compared to an intact group of sheep (25). After correcting for lung volume history however, vagal denervation showed no significant effect on C_L indicating that atelectasis is a possible cause of respiratory distress in vagally denervated animals. Other studies of lung compliance in vagally denervated animals have yielded varying results ranging from a decrease in sheep from 3.53 vs. 2.16 ml/cmH_2O in sham vs. vagally denervated animals (25) an increase in rabbits from 3.67 to 4.82 ml/cmH_2O in control and vagally denervated animals respectively (88), and no effects in dog (91), pig (22) or rabbit (72). In newborns it has been shown that lung compliance increases with cervical vagotomy in rabbits from 0.126 ml/cmH_2O in control to 0.161 ml/cmH_2O in vagally denervated animals (88) and does not change in piglets (22). One possible explanation for the increase in compliance observed after vagotomy is self-inflation due to higher V_T and transpulmonary pressure seen post denervation in these spontaneously breathing animals.

In 1990, Pissari showed that in spontaneously breathing adult dogs, rapidly adapting receptors provide afferent feedback inversely proportional to changes in dynamic lung compliance indicating a role of vagal afferents in maintenance of lung compliance (101). This observation is supported by Mills et al. and Sellick et al. who have shown that background discharge of rapidly adapting receptors increases when the lungs become stiffer and is abolished when an augmented breath is induced (83,119). Since our lambs showed decreased respiratory system compliance with vagal denervation, it is likely that induction of augmented breaths, which function to open any collapsed alveoli and increase lung compliance, may have been reduced due to absence of rapidly adapting receptor input. It was shown recently by Wong et al. that the number of augmented breaths was significantly lower in vagally denervated animals compared to sham operated animals which may have resulted in pulmonary atelectasis (133). This information suggests that one possible and highly likely mechanism of reduced lung compliance in our vagally denervated lambs is pulmonary atelectasis.

Another possible mechanism of a reduction in lung compliance secondary to pulmonary atelectasis is the loss of afferent vagal information to the upper airways. There are two main functions of the upper airway in neonates, respiratory and non-respiratory. Non-respiratory functions include protective or defense reflexes when foreign materials enter the upper airway through inhalation, during feeding or regurgitation, or through the clearance mechanisms

of the lower airways (92). The strategies utilized to perform the defense or protective functions are to avoid further penetration in the tracheobronchial tree via control of breathing pattern and airway resistance and to trigger reflexes oriented to expel the material (cough or sneeze) or to swallow it (92). These reflexes are gradually developed over the postnatal period however, as the primary apneic response to upper airway (laryngeal) stimulation is replaced by the other defense mechanisms with maturation.

Afferent input from the upper airways are also able to elicit changes in respiratory rhythm. These respiratory rhythm alterations can be elicited from numerous upper airway stimuli which can be physical, chemical, or arise from the lower airways. One type of physical stimulation for modulation of respiratory rhythm are due to flow receptors which, when stimulated, result in a reduced respiratory frequency, decreased tidal volume, and occasionally apnea in newborns. Response of these receptors is dependent on the thermosensitivity of the endings, indicating that flow-induced reflexes are due to temperature sensitive receptors (92). Another stimulus which may result in apnea or reduced respiratory frequency and tidal volume is collapsing or subatmospheric pressures delivered to the isolated upper airways. These effects are believed to be mediated by the superior laryngeal nerve (SLN) which inhibits CNS respiratory output. Since this response is absent by one month of age, this maturational alteration may reflect increases in CNS integration or reduced susceptibility to inhibitory input (92). SLN section, anaesthesia and bypassing of the larynx have

been shown to abolish the inhibitory ventilatory reflexes of airflow and pressure stimuli on respiration indicating that the larynx is the major source of reflex respiratory inhibition.

Chemical stimuli introduced into the larynx induces a reflex characterized by apnea, bradycardia and hypertension of newborn lambs, as well as other animals (70,74,92). This response, which may become fatal if the apnea overrides normal respiratory drive, is abolished by sectioning the recurrent laryngeal nerve (RLN) and results from either reduced [Cl-], increased [K+], and extremes of pH (<4.5 or >8.7). Again, this response is believed to occur due to susceptibility of CNS to afferent input associated with immaturity since the ability to elicit this response is reduced with maturation until only brief apneas or swallowing are induced (16,77). Although this response is protective in that it prevents aspiration of liquid, it is unclear as to why laryngeal exposure to some liquids result in apnea, but not others that are potentially equally dangerous (92).

Of current interest to the project at hand is the influence of lower airways on the upper airway response. In the newborn, chest wall compliance/lung compliance is higher than in the adult. This means that chest wall opposes very little pressure to the inward recoil of the lung implying that passive functional residual capacity (FRC) of the newborn is less than the adult. Thus, after birth, an adequate end expiratory lung volume (EELV) above the passive FRC is essential for a number of reasons including the presence of an oxygen reserve, to minimize the

energetic losses during lung expansion and limit the cyclic oscillations in alveolar and blood gas. A beneficial role of the upper airway is to regulate lung expansion and control lung deflation.

In the newborn period, infants exhibit the strategy of expiratory braking, involving single or repeated interruptions in the expiratory phase, which functions to prolong the expiratory time constant resulting in airway pressures more positive than during normal respiration (109). This back pressure then would serve to maintain a sufficiently large EELV above passive FRC, and aid in the reabsorption of fetal pulmonary fluid (19,85). During expiratory braking, lung volume is above the end expiratory volume and expiratory flow approaches the zero baseline (43). Studies showed that this function is, in fact, due to two separate mechanisms. One mechanism involves the upper airway and particularly the larynx which increases resistance of airflow in expiration by contraction of the thyroarytenoid (TA) muscles resulting in adduction of the vocal cords (55,86,109). The second mechanism of expiratory braking involves retardation of expiratory flow by post-inspiratory contraction of the diaphragm and other inspiratory muscles (47,109). Both these mechanisms result in a prolonged expiratory duration. In lambs, Andrews et al showed that breaths with laryngeal expiratory activity decreases from 23% to 6% within the first six days of birth (4). Occurrence of expiratory braking is also dependent on sleep state where, as associated with vagal activity, activity of laryngeal muscles decrease during REM sleep.

In the lamb and puppy, it has been observed that deflation of the lung whether by opening a tracheal window thus bypassing the upper airway or by exposing the airway to an end expiratory subatmospheric pressure causes an increase in TA activity resulting in the adduction of the vocal cords. This response reflects an attempt by the animal to maintain lung volume elevated (92). Additionally, when upper airway is bypassed, post-inspiratory diaphragmatic activity will increase in an attempt to maintain lung volume (53). Harding showed that this effect is due to afferent feedback from the lung since it is eliminated by intrathoracic vagotomy, but whether the rapidly adapting or slowly adapting receptors are responsible is unknown (53). RAR' s respond to lung deflation, and increasing their activity is one possible mechanism of increasing TA activity (38). When a positive end expiratory pressure is maintained during a tracheal opening procedure, no recruitment of TA activity is seen. This effect of positive end expiratory pressure on the inhibition of TA activity thus suggests a role for vagal SAR activity in laryngeal modulation.

In adults, termination of phrenic activity is related to vagal volume feedback from the lungs (21). This afferent lung input is additionally responsible for inhibiting upper airway muscles such as the genioglossus and alae nasi (92). In contrast to the diaphragm though, decline in the upper airway muscle activity occurs at a lower lung volume and a greater extent (128).

In our study, vagally denervated animals would have lost the ability to provide afferent information to the upper airways, although this does not necessarily mean that expiratory braking activity was absent in the denervated animals. It has been shown in the newborn puppy and lamb that changes on chemical drive may exert an influence on upper airway muscles (15,69). Blum and McCaffrey observed that when animals were exposed to hypoxia, inspiratory resistance decreased approximately 25% in puppies corresponding to an increase in posterior crycoarytenoid (PCA) activity. As for expiratory activity during hypoxia, Johnson and Fewell recorded TA and diaphragmatic activity during inhalation of hypoxic gas mixtures in newborn lambs and observed an increase in the amplitude and duration of TA adductor activity during expiration. These two strategies of reduced inspiratory resistance and increased expiratory resistance would serve three functions. First, decreased inspiratory resistance would allow more air to enter the lungs. Second and thirdly, increased expiratory resistance would serve to elevate lung volume above FRC and enhance O_2 extraction by increasing alveolar gas reserves and maintain effective surface area in order to maintain effective gas exchange.

Thus, although the lengthening of expiratory time is due to absence of pulmonary vagal feedback initiating inspiration, expiratory braking resulting from hypoxemia may have also contributed to the increase in expiratory time of our vagally denervated animals (103). However, since tests of airway resistance were performed using an endotracheal tube thus bypassing the upper airways, and no

analysis was performed on TA, PCA or diaphragmatic EMG, we do not know whether this hypoxic braking mechanism is in fact present in our animals.

In addition to increased TA activity with an open tracheal window, Johnson also observed a decreased breathing frequency and respiratory arrhythmia in one month old lambs. He concluded from these observations that expiratory airflow retardation through vagally mediated pulmonary mechanoreceptor feedback is crucial for sustaining rhythmogenicity in the young animal and during tracheal breathing, the lamb is unable to maintain sufficient vagal feedback since expiratory airflow is high through the low resistance circuit and end expiratory volume falls.

This logic may also be applied to our study since it is possible that the lack of vagally mediated pulmonary mechanoreceptor feedback due to vagal denervation may compromise expiratory airflow retardation resulting in expiratory flow through a low-resistance circuit. The result of this would be a reduction of end expiratory lung volume towards FRC and atelectasis. Thus although there may have been a degree of expiratory adduction due to hypoxia which may have slightly benefited the animal for reasons previously mentioned, expiratory braking due to a hypoxic response was probably insufficient in providing the degree of expiratory braking required to prevent the loss of lung volume (atelectasis) and dysarrhythmias in the vagally denervated animals.

4.6.3. Pulmonary Edema

Another possible cause for the observed changes in arterial blood gas tensions and pH, as well as the lowered lung compliance in vagally denervated animals is pulmonary (alveolar) edema. This condition would lead to hampering of ventilation, impairment of gas exchange, low ventilation/perfusion ratios, increased A-a DO_2 values and hypoxemia (81,131). Numerous investigators have shown that cervical vagal denervation in rats, rabbits and lambs leads to interstitial and alveolar edema, hemorrhage, atelectasis, and respiratory failure evidenced by decreased lung compliance (11,48,75,124,133). In these studies surfactant function was also compromised by lower levels of lamellar and multivesicular bodies in alveolar type II cells (48), increased minimum surface tension (11,124,133), and changes in levels of phospholipids (Hasan, unpublished observations). Thus, in these studies, respiratory failure was accredited to either decreased levels of surfactant, removal of surfactant from the surface of the alveolar epithelium, or inactivation of surfactant apoproteins by pulmonary edema fluid (11).

Pulmonary surfactant is a lipoprotein comprised of approximately 80% glycerolphospholipids, 10% cholesterol, and 10% proteins and plays a crucial role in reducing surface tension across the air-liquid interface of alveoli. (60,68). Surfactant is composed of three main surfactant-associated proteins; SP-A, SP-B, SP-C. A fourth protein, SP-D, has also been identified, which is believed to be associated with host defense (129). Broncho-alveolar lavage obtained from our

lambs were divided by differential centrifugation into two subfractions called large and small aggregates. The larger (denser) subtype is composed of tubular myelin and lamellar bodies and contains all surfactant-associated proteins (78). This subtype has also been shown to reduce surface tension in the alveoli to low values (129). Small aggregates, on the other hand, are composed of phospholipids, and contain few to no surfactant proteins. Additionally, small aggregates are only slightly able to reduce surface tension at the air-liquid interface, and are metabolic products of the large aggregate fraction (129).

Vagally denervated animals showed no differences in phospholipid content of surfactant large or small aggregates compared to sham operated animals indicating that total phospholipid levels remained intact in vagally denervated animals. Additionally, electron micrographs of tissue samples taken from the right middle lobes of vagally denervated and sham operated animals depict the presence of tubular myelin formation and secretion of surfactant from lamellar bodies. This indicates that secretion of surfactant phospholipids and assembly of tubular myelin from phospholipids and surfactant associated proteins is preserved in vagally denervated animals. This also suggests that surface tension lowering properties of surfactant is intact in vagally denervated animals since studies indicate that tubular myelin migrates to the surface of the alveolar epithelium where it forms the surface tension reducing monolayer (10). However, right middle lobe micrographs of alveolar type II cells and lamellar bodies do not show the presence or formation of tubular myelin at the level of the

alveolar epithelium. Thus, although surfactant was being produced, the functioning of the surfactant was not ascertained since the inability of secreted surfactant to lower alveolar surface tension would compromise lung function and compliance.

To account for this possibility we measured surface tension lowering properties of the broncho-alveolar lavage using the captive bubble technique of Schürch et al. (115,116). We saw no difference in large aggregate surface tension lowering properties between the sham operated and vagally denervated lambs indicating that the surfactant produced was fully capable of reducing surface tension and promoting stability at the alveolar interface.

Two other plausible explanations for the observed results other than aberrations in the surfactant system are that fluid within the lungs is hampering with either the incorporation of tubular myelin at the surface of the alveolar epithelium or the "squeezing-out" of unsaturated phospholipids from tubular myelin at the air-liquid interface (111). These two events would also reduce surface tension lowering properties of surfactant and account for the lowered lung compliance in the vagally denervated animals since it has been shown that addition of albumin into sheep and rabbit large aggregates significantly increases large aggregate conversion to small aggregates (78). To account for this possibility we need to directly measure both interstitial and alveolar edema levels in the lungs.

When sections of right middle lobe were examined by light microscopy to observe distribution of fluid, no differences were seen between the sham and vagally denervated animals with respect to edema formation indicating that vagal denervation was not consistent with either interstitial or alveolar edema formation. However, further studies should be performed to quantify edema levels.

The mechanism of pulmonary edema from vagal denervation is unclear. In our previous set of experiments as in other studies (11,133), there were no significant differences between vagally denervated and sham operated animals with respect to heart rates (11,133), cardiac outputs (11), and pulmonary arterial pressure (11,133). Therefore it is improbable that any observed pulmonary edema is cardiogenic in origin (11). We can speculate that one possible cause of pulmonary edema following vagal denervation could be neurogenic in origin.

4.7. Role of Vagal Afferents in Pulmonary Atelectasis/Edema

4.7.1. Slowly Adapting Receptors (SAR)

Recall that pulmonary SAR's are located along the smooth muscle of lung parenchyma and mainly respond to changes in lung volume which determine respiratory timings (T_I and T_E). In addition, afferent information from SAR's determine the degree of expiratory braking by upper airway muscles which serves to: (a) elevate lung volume above passive FRC thus enhancing O_2

extraction by increasing alveolar gas reserves and (b) maintain an effective surface area in order to preserve effective gas exchange. Loss of lower airway SAR mechanoreception in denervated animals would initiate a series of events resulting in respiratory failure of the animals due to atelectasis via the following mechanism. First, vagal denervation would extinguish lower airway mediated braking activity of the upper airway which is crucial in the maintenance of a dynamic end expiratory volume above passive FRC. Additionally, due to the inability to modulate respiratory timing in the absence of the SAR's, a significantly higher T_E was observed in vagally denervated animals relative to the sham group. Together the effects of a large expiratory time through an upper airway providing low resistance to airflow would lead to a reduction in the dynamic end expiratory volume of denervated animals towards FRC. The reduction in lung volume would eventually lead to severe atelectasis and respiratory failure characterized by hypoxemia and an increased A-a DO_2.

4.7.2. Rapidly Adapting Receptors (RAR)

In normal eupnic breathing, rapidly adapting receptors function primarily as mechanoreceptors that respond to reductions in lung compliance arising from collapsed alveolar units. Upon stimulation by low lung compliance, RAR's respond by initiating an augmented breath or sigh that would inflate collapsed alveoli effectively increasing lung compliance to normal values. Absence of these pulmonary receptors would thus abolish vagally induced augmented breaths (83). In this study, reduction in lung volume due to loss of pulmonary SAR's

would reduce lung compliance secondary to pulmonary atelectasis. This low compliance would normally be resolved by the induction of augmented breaths, however, with loss of RAR's normal induction of sighs are not possible. Thus, unlike SAR's, loss of RAR activity does not directly lead to respiratory failure. Rather, loss of the RAR's protective function in inflating collapsed alveolar units, together with reductions in lung volume, would result in a low ventilation/perfusion ratio and eventual respiratory failure of the denervated group characterized by hypoxemia and an increased A-a DO_2.

Studies have shown that RAR's are also stimulated by both cardiogenic and non-cardiogenic pulmonary edema inducing rapid shallow breathing (107). Additionally, stimulation of RAR's results in increased expiratory resistance as evidenced by an augmentation of laryngeal adductors which would not only work to increase end expiratory lung volume above passive FRC, but also create positive intrapulmonary pressures upon expiration which would aid in resolution of pulmonary edema (122). Thus, besides pulmonary atelectasis, detrimental changes in pulmonary gas exchange observed in the denervated animals may be due to absence of the RAR response to edema resulting in low lung volumes and accumulation of fluid.

4.7.3. C-Fiber Receptors

In contrast to the RAR and SAR receptors, C-fiber receptors do not exhibit strong response to changes in lung volume. Rather, C-fibers primarily respond to

pulmonary congestion caused by pulmonary embolism, edema, or inflammation resulting in a rapid shallow breathing response and an augmentation of expiratory braking (59). This combination of rapid shallow breathing and increased expiratory braking would serve two purposes; to increase the end expiratory volume above the passive functional residual capacity, and to create a positive pressure in the lungs with expiration which would work to absolve the pulmonary edema (59). In denervated animals, absence of pulmonary C-fibers would extinguish the normal response to pulmonary edema resulting in fluid filled lungs and reduced lung volumes approaching the passive FRC resulting in the observed hypoxemia and increase in A-a DO_2.

In summary, our data shows that by approximately 20 hours post vagal denervation, lambs developed respiratory failure as evidenced by severe hypoxemia. Vagally denervated animals also had a lower minute ventilation, respiratory rate and respiratory system and lung compliance compared to sham operated animals and increased inspiratory and expiratory times. No significant difference existed in sleep states between the two groups. Further studies need to be performed to examine the cause of the reduced respiratory system compliance, two possibilities include either pulmonary atelectasis or pulmonary edema. Also, tests of pulmonary function and lung compliance were performed with an endotracheal tube inserted. Since the upper airways play an important role in modulation of respiratory frequency and maintenance of lung volume,

bypassing the upper airway with an endotracheal tube will not account for the role of the upper airway in breathing strategies of vagally denervated and sham operated animals. Thus studies need to be repeated using a well sealed mask to examine breathing patterns in lambs with intact upper airways. From this examination of vagally denervated and sham operated lambs, we conclude that vagally mediated pulmonary feedback is critical for the maintenance of normal breathing patterns and gas exchange during the immediate newborn period.

CHAPTER 5: FUTURE DIRECTIONS

From our study, we can speculate that the observed reduction in lung compliance may be due to either absence of respiratory drive resulting in atelectasis and/or pulmonary edema due to changes in breathing patterns. To deduce which mechanism was responsible for the decreased lung compliance a second set of experiments should be performed on vagally denervated and sham operated animals.

To determine if the reduction in lung compliance results from progressive atelectasis, pulmonary function tests should be performed before and after lung inflation to examine alterations in lung compliance due to changes in lung volume history, and intact pressure-volume curves should be constructed to examine the lungs for presence of alveolar derecruitment. One possible mechanism of lung volume reduction may be through the loss of afferent rapidly adapting receptor feedback resulting in the failure of induction of augmented breaths. Thus the number of augmented breaths should be quantified and compared between sham operated and vagally denervated animals to determine if this may have played a role in the reduction of lung compliance.

Loss of lung volume may also be due to loss of communication between the lower and upper airways, in particular, loss of afferent information regarding lung volume from lungs to the upper airway. To assess the role of the upper airway in

respiratory failure following intrathoracic vagal denervation, besides repeating pulmonary function tests using a well sealed mask to obtain pulmonary resistance measurements, EMG activity of the TA, PCA, and diaphragm should be quantified to ascertain the extent of expiratory braking in sham and denervated animals.

In addition, lung wet/dry ratios, total plasma protein quantification, and quantification of leak of intravenously administered fluorescently labeled albumin into pulmonary alveoli or interstitium to quantify the levels of pulmonary edema should be performed in sham operated and vagally denervated animals.

Finally, although we have determined that vagal integrity is essential for the maintenance of alveolar ventilation and gas exchange in the immediate newborn period, we do not know which pulmonary vagal fibers are responsible for our observations. Thus, experiments should be performed by denervating or blocking myelinated and unmyelinated pulmonary vagal fibers and observing the effects on breathing patterns and gas exchange in the immediate newborn period.

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