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Vagal Innervation and Surfactant System in Fetal Sheep

by

Luxmi Gahlot

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ABSTRACT

Inferential evidence suggests that vagal denervation compromises pulmonary surfactant system. To elucidate the effects of vagal denervation on surfactant system we performed experiments on time dated 12 fetal sheep; 8 were cervical vagally denervated, and 4 were sham–operated. Vagal denervation was performed at 110-113d gestation and caesarian section was done at 130-133 d (term-147 d). Lung wet-dry weight ratios, light and electron microscopy, surfactant proteins A and B in tissues as well as broncho-alveolar lavage and plasma cortisol concentrations determined were comparable in sham-operated and denervated animals. Vagal denervation at 110-113 day gestation had no influence on alveolar architecture and lamellar bodies in type II cells. Our data suggest that vagal input at mid gestation has no effect on surfactant system in fetal sheep.

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1.0 INTRODUCTION

1.1 ROLE OF SURFACTANT IN PULMONARY ADAPTATION

Adaptation to a nonaqueous respiratory environment was achieved in vertebrates by the development of lungs, which provide an extensive surface area for gas exchange. The unique physiochemical boundary between respiratory gases and the highly solvated molecules composing the surfaces of cells of the respiratory epithelium creates a region of high surface tension, which is generated by the unequal distribution of the molecular forces at alveolar surface. Pulmonary surfactant, which decreases the surface tension at the air- liquid interface, is therefore a pre-requisite for successful adaptation to air breathing. Once the lung is aerated, surfactant plays a critical role in maintaining the expansion at the alveolar level, and therefore, functional residual capacity in terms of overall lung volume (46). It is not surprising that pulmonary surfactant is found in all air breathing vertebrates (85). The preparation of fetal lung for air breathing requires both qualitative and quantitative changes in the ability of type II cells to synthesize and secrete pulmonary surfactant (98).

1.2 SURFACTANT COMPONENTS

Surfactant from bronchoalveolar lavage fluid *i.e.* an unresolved mixture of the material lining the alveolar lumen, is composed of 80-90% of lipids, about 10% proteins and 2% carbohydrates (49). The surfactant lipid composition is similar in various different mammalian species. The phospholipid, constituting 80-90% of the total lipid weight, consist of about 75% phosphatidylcholine (PC), 10% phosphatidylglycerol, 5% phosphatidylethanolamine, 5% phosphatidylserine plus phosphatidylinositol. Cholesterol is the dominant neutral lipid, and constitutes 6-8% of the total lipids. Nearly half the PC content is dipalmitoylphosphatidyl choline (DPPC), and this molecule, which is the single major component of surfactant, is also the principal surface tension reducing compound. The higher percentage of disaturated PC, especially DPPC, in surfactant is unique as compared to the composition of PC from other sources (79). The lipid content of isolated lamellar bodies is very similar to that of alveolar surfactant (79) but differs from plasma membranes (5).

In addition to the lipids, the surfactant contains four specific proteins, termed as surfactant proteins (SP); SP-A, SP-B, SP-C, and SP-D.

1.3 ROLE OF VARIOUS COMPONENTS

1.3a LIPIDS- DPPC, the most abundant lipid in pulmonary surfactant is also the major surface-active component. The physiological function of this compound is thought to be the provision of alveolar stability by lowering of the surface tension at the alveolar surface to values less than 10mN/m at the end of expiration. The role of other surfactant lipids is less certain. They may aid in rapid absorption and spreading of surfactant at the air-liquid interface.

Surface Tension: Surface tension arises from the differences in attractive forces on molecules at the air-liquid interface (4,22). If the molecules in the liquid were not attracted to each other, they would spontaneously form a vapor (gas). This occurs when the temperature of a liquid is raised above the boiling point (e.g., 100 degree C for water), so kinetic energy becomes high enough to overcome the attractive forces, resulting in vapor. Molecules at the surface possess excess potential energy relative to molecules in the bulk phase and also the molecules at the surface experience an attraction into bulk phase. The most stable situation arises with minimum surface area. Surface tension may be considered in terms of an elastic film of the surface molecules, whose attraction into the bulk phase resists expansion and works toward minimizing surface area. This force or tension in the surface film is due to attractive forces of the molecules at the surface that resist expansion and consequently acts to contract the surface area.

The surface tension reducing capacity of surfactant is due to its phospholipid constituents. Phospholipid contains hydrophilic polar head groups and hydrophobic fatty acyl groups. Such molecules are termed as amphipathic (literally means "feeling both ways"). The polar phosphotidylcholine group interacts with water, whereas the hydrophobic fatty acyl moieties extend toward the air. As in the case of ethanol, these lipids reduce surface tension by displacing water from the surface. The monolayer of phospholipids act at the air-liquid interface to lower surface tension by replacing water at the surface. The equilibrium surface tension of approximately 25mN is attained when the surface is saturated with lipid.

1.3b PROTEINS A great number of physiological roles have been proposed for surfactant proteins. SP-A is known to bind lipids, (39,40) particularly DPPC (54) and to cause aggregation of phospholipid vesicles in the presence of calcium. (39,40) These properties are essential for the formation of tubular myelin from secreted lamellar body material. *In vitro* reconstitution experiments showed that the formation of tubular myelin depended on the presence of surfactant lipids (DPPC and PG), SP-A, SP-B, and calcium (89). The preferential localization of SP-A in the corners of the tubular myelin lattice suggests that it provide the framework for this structure (96). Another physiological role of SP-A may be the regulation of surfactant homeostasis. Studies from *in vitro* experiments suggest that SP-A decreases the secretion of lamellar bodies from type II cells (24,83) and increases the uptake of DPPC into these cells (101,91). Finally, the SP-A

may play a role in pulmonary defense mechanisms as suggested by it being a C-type lectin.

SP-B combined with lipid mixtures reconstitutes most of the surface activity of natural surfactant *in vitro* and increases lung compliance *in vivo*. This may be related to involvement of SP-B in transformation of lamellar bodies into tubular myelin and subsequent formation of monolayer. The interactions of SP-B with DPPC have been proposed to result in removal of DPPC from the lipid monolayer in a complex with SP-B a process that may also require SP-C. Therefore its role may involve both in formation and removal of surfactant monolayer. In addition to its molecular effects on the lipid structure, SP-B produces an effect on vesicular structure. SP-B increases the size of small unilamellar vesicles and causes vesicle fusion. The ability of SP-B to facilitate lipid mixing among vesicles is enhanced by presence of negatively charged phospholipids and divalent ions.

The absorption of phospholipid at the air liquid interface is also promoted by SP-C, but less actively than by SP-B.

SP-D, like SP-A a C-type lectin, is also thought to be involved in defense mechanisms. It activates alveolar macrophages and binds to lipopolysaccharide.

1.4 INTRACELLULAR TRANSPORT AND STORAGE OF SURFACTANT

Pulmonary surfactant is stored in type II cells as lamellar bodies (14). However these organelles lack a number of enzymes needed for the *de novo* synthesis of PC and PG and for remodeling of PC to DPPC, which makes it unlikely that they contribute significantly to the synthesis of surfactant lipids. Therefore, lipids must somehow be transported to the nascent lamellar bodies. The details are unclear; it may involve vesicular transport or transport by phospholipid-transfer protein and / or fusion of small vesicles to lamellar bodies by phospholipid binding proteins. Studies by Chevalier and Collet involving [^{3}H]- choline injection into mice, indicated that the bulk of surfactant PC synthesized in endoplasmic reticulum of type II pneumocytes is transferred from the golgi system to the lamellar bodies by the small lamellar bodies (17). It is suggested that small lamellar bodies are intermediate between golgi body and mature lamellar bodies. The multivesicular bodies which are involved in the transport of surfactant proteins from golgi body into the lamellar bodies probably play no role in phospholipid transport.

1.5 SURFACTANT SECRETION

The surfactant lipids are secreted from type II cells by exocytosis of lamellar bodies (48,50,64). Microtubules and microfilaments appear to be involved in this process. Various studies have indicated a number of factors that affect he secretion of surfactant lipids; It is stimulated by hyperventilation, type II cell distortion and high intracellular pH (16).

In addition its secretion is stimulated by β- adrenergic agonists, purinergic and vasopressin receptors. These stimuli cause an increase in intracellular c-AMP levels and cytosolic Ca^{2+} concentrations and involves activation of c-AMP dependent protein kinase and protein kinase C pathways. Inhibitory mechanisms may also be involved in surfactant phospholipid secretion. A recent study has shown that pure DPPC formed in vivo may act as inhibitory regulators of surfactant. SP-A also inhibits PC release from isolated type II cells. The mechanism of SP-A secretion is unclear. Although a number of studies indicate that it is secreted via the lamellar bodies together with surfactant lipids, there are also observations that SP-A is secreted by a pathway independent of lamellar bodies. The observation that SP-B and SP-C are highly enriched in lamellar bodies suggests that these hydrophobic proteins are secreted with surfactant lipids. The apparent absence of SP-D from lamellar bodies indicates that this protein is secreted independently from lamellar bodies.

1.6 INTRA-ALVEOLAR METABOLISM OF SURFACTANT

After the secretion into the alveolus, the lamellar bodies expand and form the lattice like structure called tubular myelin. SP-A, SP-B and Ca^{2+} are involved in this rearrangement of phospholipids (28). Tubular myelin and tubular myelin structures are rapidly absorbed at the air-liquid interface with increase in surface area and SP-A, SP-B and SP-C may be involved in this process (45). The rapid absorption is accomplished by detachment of connections that stabilizes the tubular myelin structure. With decrease in surface area, reattachment of these connections lead to reformation of the tubular myelin like structures, which behave as large aggregates. SP-A in conjunction with SP-B is involved in the reformation process (93). Cleavage of the SP-B during adsorption process would prevent reassembly of large aggregates (103). It was hypothesized that in large aggregates SP-B was inaccessible to the serine proteases, but after the increase in surface area, when surfactant absorbs to the air-liquid interface, the SP-B could be degraded by the proteases. During subsequent decrease in surface area, the SP-B depleted surfactant would form small aggregates. Similarly, in the absence of SP-A large aggregates adsorb at the air liquid interface but cannot reform into large aggregates thus leading to formation of small aggregates (93).

1.7 RECYCLING OF SURFACTANT

The turnover time of surfactant lipid removal from alveoli is 5-10 hr (101). It can be either degraded by alveolar macrophages, or lost by ciliary transport up the airways, or the movement through the epithelium and endothelium into blood or lymph. However it is now clear that a large part of the phospholipds are taken up and reutilized by the type II cells. The efficiency of this reutilization may be up to 85% and may vary with age. A part of the internalized phospholipids is degraded, possibly in the lysosomes, after which the degradation products are used for phospholipid formation in the endoplasmic reticulum. However another part is recycled intact into lamellar bodies. Both SP-A and SP-B are taken by type II cells by receptor-mediated endocytosis and deposited into lamellar bodies. Part of the SP-B is degraded in type II cells. For a steady state, the secretion of surfactant into alveoli and its removal have to be in balance. A regulatory link between these processes can be provided by the β-adrenergic agonists which stimulate surfactant secretion and reuptake. It is suggested that another such link is provided by SP-A but its role is still controversial.

1.8 ONTOGENY OF SURFACTANT PHOSPHOLIPIDS AND PROTEINS

1.8a PHOSPHOLIPIDS-Though the time of initiation may be different in different mammalian species, the rate of synthesis increases substantially during the last 15% of gestation in all mammalian species. In the human, surfactant synthesis begins at the end of second trimester and increases during the third trimester in preparation for the onset of breathing at birth. Type II cells containing lamellar bodies can be recognized at 20-24 weeks in the human and surfactant synthesis begins at 30 weeks (51). In fetal lambs and rodents lamellar bodies-containing type II cells are not seen until ~80 and 95 % of gestation (27). In addition to developmental increases in the content of surfactant phospholipids, precursor incorporation into PC and activities involved in fetal lung PC and DPPC biosynthesis increase near the end of gestation.

1.8b PROTEINS The developmental regulation of surfactant proteins is very species specific. In humans, SP-A gene expression and protein synthesis tend to parallel the synthesis of surfactant lipids. SP- A protein in amniotic fluid is generally not detectable until 28-30 weeks, after which its concentration increases exponentially to term (59,80). Fetal gender does not seem to alter SP-A content in human amniotic fluid (80,88). In rabbits, SP-A gene expression begins several days prior to the synthesis of surfactant phospholipids. SP-A gene expression is evident on day 21-22 (term 31days) and low levels of SP-A mRNA can be detected by days 24-26 just prior to augmented synthesis of surfactant

phospholipids (12,67). Subsequently, the rate of SP-A increases substantially and is at a maximum by 28 and 31 days. SP-A protein and mRNA levels are first found in rat lung on day 18 (term 22 Days). Levels increase to 50% of adult values by 21days, then decline during 1st week of life. The SP-A levels thereafter increase to near adult level by 28 days postnatal age.

Limited data is available on the ontogeny of SP-D. Studies show that SP-D was detected as early as 26 weeks in human and increased moderately by term. In rat lung very low levels of SP-D are present on day 18 and increases moderately at term but do not decrease after birth as do SP-A (23).

Studies in a number of species have demonstrated that SP-B and SP-C expressions begin at an earlier stage of lung development than does SP-A or SP-D. In human fetal lung tissue, SP-B and SP-C mRNA are present as early as 13 weeks and increase to 50 and 15% of the adult levels by 24 weeks (60). However, further studies have shown mature SP-B protein is not present prior to 31 weeks after which it increases exponentially. In rabbit fetal lung, SP-C and SP-B mRNAs are detected on day 19 and 26 of gestation, respectively. Levels of both mRNA increased at term and then declined somewhat in the adult. In the rat lung SP-C and SP-B mRNAs are present at day 17 and 18, respectively and both mRNA levels increase to adult level at day 21 (12,102).

1.9 HORMONAL FACTORS

The discovery by Liggins and Howie that administration of synthetic glucocorticoids (GC) to fetal lambs and rabbits resulted in accelerated lung maturation and appearance of alveolar type 2 cells led to numerous studies that supported the concept that GC are important in regulation of surfactant PL synthesis in fetal lung tissue (30,57). The result of numerous *in vitro* and *in vivo* studies using a number of species suggest that surfactant PL synthesis by fetal lung is indeed under multifactorial control. In addition to GCs, prolactin, thyroid hormones, estrogen, androgen, growth factors, insulin, catecholamines acting through β-adrenergic receptors and c-AMP are important in its regulation. Except for glucocorticoids and possibly thyroid hormones, the physiological and pathological role of multi- hormonal regulation is uncertain.

1.9a GLUCOCORTICOIDS

Key enzymes in the biosynthetic pathway of surfactant phospholipids have been proposed targets of glucocorticoid action. Although the catalytic activity of many of these enzymes is increased by glucocorticoids, many of these effects appear to be indirect and mediated by increased synthesis of cofactors or intracellular translocation of enzymes. Among enzymes of phospholipid biosynthesis, available data are strongest for the induction of the fatty acid synthetase (FAS)

protein (20,73). Glucocorticoid treatment of the fetal rat lung, either in vivo or explant culture has been shown to increase the rate of *de novo* fatty acid synthesis. Glucocorticoids act through a receptor mediated process to increase FAS gene expression via a pretranslational mechanism.

1.9b THYROID HORMONE

Evidence from both *in vivo* and *in vitro* studies show that thyroid hormone increase the synthesis of surfactant phospholipid in a number of species and act in an additive or synergistic fashion when combined with glucocorticoids (8,84). The role of thyroid hormone in enzymes of surfactant phospholipid synthesis is been investigated but current studies suggest that it acts to increase choline incorporation into PC (32).

1.9c cAMP AGONISTS

Although the principal effects of cAMP agonists have traditionally been considered to be stimulation of surfactant secretion and resorption of fetal lung fluid just prior to birth, recent evidence suggests that these agents also stimulate the synthesis of several elements of the surfactant system. In the studies by Gross et al., treatment of explants from fetal rat lung with methylxanthines (aminophylline and caffeine) increased the rate of choline and acetate

incorporation into PC (32). Additive stimulation occurred when these phosphodiesterase inhibitors were combined with dexamethasone (31).

It is becoming increasingly evident that other hormones and growth factors including Fibroblast Pneumocytic Factor (FPF), Epidermal Growth Factor (EGF), prolactin, estrogen, androgen and insulin can influence the expression of pulmonary surfactant, either stimulating or inhibiting its synthesis

1.10 MECHANICAL FACTORS

Physical factors are regulators of cell proliferation and differentiation in a variety of mammalian cells especially regulation of the structure, function and metabolism of the lung. The lung is subjected to complex physical factors including breathing, pulmonary blood flow, and surface tension. Abnormal physical forces exerted on lung tissues contribute to many pathological conditions e.g. excessive mechanical forces are associated with lung injury at the cellular level. Increased physical forces are also associated with the development of pulmonary hypertension (61).

The two main mechanical factors controlling surfactant system are discussed below:

1.10a LUNG EXPANSION

von Neergaard in 1929 observed that a greater pressure was required to keep the lungs inflated with air than with fluid (81). Work done against surface tension forces constitute the major part of effort required to inflate the lung. Filling the lung with saline eliminates the surface tension forces and the lung expands readily and uniformly (18). Therefore it seems logical that the lung expansion requires the release of surfactant to reduce the surface tension and work of breathing. There are also studies that indicate that immediately after birth, rate of surfactant secretion into alveoli increases over and above the fetal secretion rate. With the onset of breathing, augmented surfactant secretion that result from constant air-expansion lowers surface tension and increases saturated lecithin in lungs. In studies performed by Lawson et al, lack of lung expansion resulted in decrease in alveolar phospholipids (55). Similarly Oyrazon and Clements and Hildebran and Clements have demonstrated in different animal models that increased minute ventilation augments air space phospholipids (42;75). It is difficult to understand how air inflation effects surfactant flux. It is possible that inflation to a total lung capacity deforms the lateral or basilar membranes of the type II cell in such a way as to increase plasma membrane calcium permeability, thus initiating a cyclic-AMP related lamellar body release.

1.10b CHOLINERGIC INNERVATION

The role of autonomic nervous system in surfactant system is controversial (19). The possibility that cholinergically mediated mechanisms might influence the synthesis, secretion, and perhaps removal of pulmonary surfactant has interested physiologists for the past two decades. Both biochemical and morphological observations have indicated that cholinergic pathway might influence surfactant flux. The cholinergic (vagal) innervation of airways in the young has been reported by Fisher et al. (26). Meyrick and Reid during their experiments reported nerve bundles in the alveolar walls of rat (70). Another study has identified two different types of nerve endings in the alveoli of the mouse lung (43). One of them is in close contact with type II cell and its appearance is consistent with a motor function. Ultrastructurally, this nerve ending was neither typically adrenergic or cholinergic. This study provided some morphological evidence for a relationship between the nervous system and surfactant system. When Olsen administrated pilocarpine injections to rats there was an increase in release of phospholipids in lung lavages (74). Similar results were observed by Morgan et al (72). However in studies performed by Massaro et al. though pilocarpine caused a release of surfactant in alveoli this effect was not blocked by atropine, a pilocarpine antagonist (65). In summary, evidence though controversial, exists that cholinergic innervation plays a role in surfactant system.

1.10c OTHER MECHANICAL FACTORS INFLUENCING LUNG DEVELPOMENT

Experimental evidence indicates that the following physical factors are necessary for normal fetal lung growth-

a) ADEQUATE INTRA-THORACIC SPACE- Pulmonary hypoplasia of the lungs results from the lesions that encroach upon the space normally occupied by the lungs. In human infants with severe Rh-isoimmunisation, pulmonary hypoplasia may occur and is probably due to the presence of fetal hydrops, a condition associated with ascites and pleural effusions, limiting the space available for lung growth (15). The association of the hypoplastic lungs with congenital diaphragmatic hernia in human infants is well-known. In fetal sheep, bilateral section of the phrenic nerves results in hypoplastic lungs (38). Lung weight is decreased to about 80% of the normal 21 days after the experimental procedure; lung water concentrations and DNA/g of the lung tissue is unchanged from controls. Phrenic nerve section cause paralysis and atrophy of the diaphragm and is likely to result in decrease in intra-thoracic allowing abdominal contents to encroach upon the intra-thoracic space, thus limiting the space available for lung growth in a manner analogous to diaphragmatic hernia.

b) ADEQUATE INTRA-UTERINE SPACE- Thomas and Smith observed pulmonary hypoplasia in cases of renal agenesis and postulated it to be related to

oligohydramnios. They noted that lung growth is normal in infants with renal agenesis if there is a normal amount of amniotic fluid. They also noted pulmonary hypoplasia in other conditions associated with oligohydramnios (90). It is speculated that loss of amniotic fluid results in decreased space for lung growth because of the compression of the uterine wall upon the chest and the abdomen; this effect would be similar to the lesions that inhibit lung growth by decreasing intra-thoracic space.

c) **FETAL BREATHING MOVEMENTS-** Several studies in chronically catheterized fetal sheep have indicated that fetal breathing movements of normal incidence and intensity are necessary for normal lung growth (21,68). In fetal rats treated with curare, a muscle relaxant that abolishes fetal breathing movements resulted in pulmonary hypoplasia. Several other experiments by Wigglesworth et al, and Liggins and associates have shown that FBM are critical for lung growth (97). It is now recognised that FBM associated with URT (upper respiratory tract) influence the movement and hence the volume of lung liquid and maintain the lung in an expanded state which is critical for lung tissue growth. The laryngeal dilator muscles (posterior cricoarytenoid) are active in association with FBM widening the glottis with each inspiratory effort and decreasing the resistance of the fetal URT (34,37). Laryngeal constrictors (thyroarytenoid) are essentially inactive during FBM. During periods of fetal apnea and prolonged pauses between FBM, the laryngeal constrictor muscles become tonically active leading to increased resistance of fetal URT to the efflux of tracheal fluid. The net efflux

of fluid therefore increases during FBM when the resistance is low and retarded during apnea when URT resistance is high (34,37). Thus laryngeal muscles especially the adductors play an important role in maintaining expansion of the lungs.

d) NORMAL BALANCE OF VOLUME AND PRESSURE IN THE LUNG- In the absence of FBM there is a positive intra-tracheal pressure in the lung, which is higher than the amniotic pressure (58). It was speculated from these studies that positive intra-tracheal pressure may be due to resistance to outflow of fluid produced by the lungs and may be important in regulating the volume of fluid within the future air-spaces (95). Furthermore, studies by Alcorn et al in fetal sheep clearly demonstrate that a normal balance of volume and pressure in the lung within the potential airways and airspaces are necessary for normal growth and differentiation. When tracheal ligation was performed, the lung became distended with fluid under increased pressure, lung growth was stimulated and the lungs appeared more mature with alveoli of large size and thinner walls. In contrast, with chronic tracheal drainage to atmospheric pressure, lung growth was inhibited and the lungs had small potential air spaces with thick walls (6). Similar findings have been made in human fetuses with atresia of the upper airway. The effects of alteration in lung expansion on lung growth are likely due to local changes in tissue stress rather than circulating systemic factors. Opposite growth responses can be obtained in the same animal; prolonged overdistension of one lung caused hyperplastic changes in that lung, such that

pulmonary DNA content was increased by 64% compared with control lungs while simultaneous drainage of liquid from opposite lung caused a virtual cessation of lung growth. These experiments demonstrate that lung growth is very sensitive to sustained changes in transluminal tissue stress during late gestation.

Over the past decade, various *in vitro* studies at cellular and molecular levels using organotypic culture have been conducted to simulate the effect of physical forces on various cells. In these studies mechanical strain induced proliferative activity was observed in a gestation dependant manner. Applying cyclic strain in primary cultures of the fetal rabbit epithelial cells in monolayer culture increased the synthesis and secretion of surfactant phospholipids (86). Separate studies also suggest that mechanical forces not only stimulate growth factor production but also regulate the responsiveness of the cells to mitogenic activity.

1.11 RATIONALE

Although the role of hormonal and physical factors contributing to lung development and surfactant maturation is well established, very few studies to date have investigated the precise role of lung innervation in lung growth especially with respect to surfactant system. Experiments have shown that this increased minute ventilation augments airspace phospholipds. Since atropine administration prevented the increase in extracellular phopholipid in response to increased ventilation, the authors summarized and that this increase might be mediated via cholinergic mechanisms. (75;76). There have been studies in literature that suggests that vagal denervation compromises surfactant synthesis, release, and may cause dysfunction (7;30;53). Alcorn et al demonstrated that bilateral cervical vagal denervation at 103-113 days resulted in absent secretory granules in type II cells (lamellar bodies) (7). However, studies done by Wong et al demonstrated that intrathoracic vagal denervation had no effect on lamellar bodies in type II cells (100). Moreover, Alcorn et al observed on electron microscopy empty vacuoles in type II cells indicative of absent lamellar bodies. However, these empty vacuoles could be artifactual, being the result of inadequate fixation techniques. On the other hand, Wong et al performed intrathoracic vagal denervation later in gestation (130 d) and the animals were studied close to term. Thus vagal denervation may have occurred after the surfactant system was mature.

To circumvent the limitations of the previous experiments, we investigated the role of cervical vagal denervation on the surfactant system in fetal sheep.

Additionally, as cervical vagal denervation performed will eliminate the motor innervation to larynx, therefore bypassing the upper airway will abolish the increased resistance to fluid efflux in absence of FBM observed in fetal sheep and is used to elucidate the role of upper airway in lung growth.

1.12 SPECIFIC AIMS AND HYPOTHESES

The primary aim of the proposed study is to investigate the effects of cervical vagal denervation on the surfactant system to test the HYPOTHESES that cervical vagal denervation performed at 103-113 d gestation impairs surfactant synthesis as evidenced by 1) an absence of lamellar bodies in type II pulmonary epithelial cells. and 2) decrease the total lung phospholipids and surfactant associated proteins

The secondary objective of this study is to investigate the role of cervical vagal denervation on lung growth and to test the HYPOTHESIS that lack of motor innervation of larynx will result in impaired lung growth as evidenced by 1) lung weights and 2) morphometric analysis of the airway features.

2.0 MATERIALS AND METHODS

2.1 SURGERY

Surgery was performed on time-dated 14 fetal sheep at 103-113 d gestation. The fetal head and neck was partially exteriorized through midline maternal abdominal and uterine incisions under general anesthesia using ketamine hydrochloride (8-9mg/kg; Rogarsetic, Rogar/STB, London, Ontario) and 4% halothane in oxygen for induction and 1.5-2.5% halothane for maintenance. Using sterile techniques a 4-cm ventral neck incision was made to expose the fetal carotid arteries, jugular veins and vagosympathetic trunk. Instrumentation of the fetus included placement of carotid arterial and jugular venous catheters (2.0mm OD and 1.0mm ID; Portex, Hythe, Kent, UK) to draw blood to analyze pH and blood gas tensions, and to infuse fluids and antibiotics respectively. In eight fetuses (group I) vagal denervation was performed in the cervical area. Approximately 4cm of the vagus nerve was cleared of the surrounding tissue and sectioned. The sectioned ends were treated with 4% phenol and further folded on it and tied with 2.0 silk suture. Fetuses in group II (n=4, sham) were those in whom the vagi were identified but not sectioned. After the completion of instrumentation, the fetuses were returned to the uterine cavity and all incisions sewn in layers. The fetal vascular catheters were exteriorized through an incision in the left maternal flank and stored in a cloth pouch secured to maternal abdominal wall. The arterial catheter was used to draw blood samples daily to analyse arterial pH and blood gas tensions that served as indicators of fetal well being. A polyvinyl catheter was placed in the maternal

jugular vein for infusion of antibiotics and fluids (3mm OD; Tygon) The ewes were housed in large (~1.8 X 2.4 m) custom made) individual cages with free access to food and water. Daily care included administration of 125mg (for fetuses) and 375 mg (for ewes) of cefazolin sodium in saline (Ancef, SmithKline Beecham Pharma, Oakville, Ontario), 20 mg (for fetuses) and 80 mg (for ewes) of gentamicin sulphate (Garamycin Injectable, Schering Canada, Ponta-Claire, Quebec) twice daily for five days, and heparinized flush for the patency of vascular catheters.

2.2 EXPERIMENTAL DESIGN

2.2a ARTERIAL PH, BLOOD GASES

Blood was drawn from the arterial line of the lamb daily for the first five post operative days and on alternate day till 130 day of gestation for measurement of arterial pH, blood gas tensions, and oxygen saturation. Furthermore, fetal blood was drawn on last day of experiment for analysis of plasma cortisol concentrations.

2.2b POST MORTEM COLLECTION OF SAMPLES

The ewes were monitored for 20-28 days after surgery days for any signs of infection or the onset of labor and a repeat cesarean section was performed as close to130-133 days (the gestational age used in previous studies) as possible. Thereafter, the ewes and fetuses were sacrificed using Euthanyl (pentobarbitone 240 mg/ml)) according to the Canadian Council on Animal Care guidelines.

The gender of the fetuses was recorded and thereby the fetuses were weighed. Complete sectioning of the nerves was verified by identifying the cut ends of the nerve and examining for signs of regrowth. Lungs were exposed through a midline thoracotomy. The right middle lobe was removed, weighed, sectioned into small pieces and flash frozen in liquid nitrogen and stored at -70°C for future surfactant protein and mRNA analyses. The right main bronchus was tied off and

the remaining right lung was removed. The upper and lower lobes were further sectioned into smaller pieces used for light and electron microscopy and wet: dry ratios, to investigate the type II pulmonary epithelial cells and trophic changes, respectively that might have occurred due to vagal denervation. The left main stem bronchus was cannulated and bronchoalveolar lavage (BAL) done using chilled normal saline solution (0.9%); 100 ml/kg in four aliquots was slowly infused using gravitational method to avoid fluid leaks. BAL was performed and used for the assays of total phospholipids, large and small surfactant aggregates. Thereafter, the left lung was perfused with 10% formalin at 25 cm H_2O for 4 hours for routine light microscopy.

2.2c BRONCHO-ALVEOLAR LAVAGE

Bronchoalveoalar lavage fluid was stored at 4°C and centrifuged at 150 x g for 10 minutes to remove cellular debris. The supernatant was centrifuged at 40,000 x g for 20 minutes (60 Ti rotor in Beckman centrifuge) to isolate large and small surfactant aggregates. The pellet containing the large aggregate was resuspended using 15-ml saline. Total phospholipids in the resuspended pellet and supernatant were assayed using the well-established methods of Bartlett (9). Briefly, for assays six phosphorus standards were prepared by adding from 25μ g of phosphorus to 200 μ g of phosphorus. Large and small aggregates samples to be assayed were taken in triplicates. To ensure equal volumes of sample and standards, distilled water was added to equilibrate volume to 800 μl. 300 μl of

sulfuric acid was added to all tubes and placed in an oven at 170°C for an hour and a half. The sulfuric acid was neutralized using three drops of hydrogen peroxide and tubes heated at 170°C for another hour. Color estimation was performed by adding ammonium molybdate solution and Fiske solution and boiling the tubes in water bath for 20 minutes. Thereafter the tubes were read for the phosphorus absorbance at 820λ in spectrophotometer.

2.2d LIGHT MICROSCOPY

The airways and parenchyma were sampled using standardized procedures and processed for routine light microscopy (56). For light microscopy, random samples of tissues of approximately 1.0X1.0 X0.2 cm were taken from each lobe, with one from each lobe. Sections five μm thick of the airways and the parenchyma were stained by haematoxylin and eosin and elastic trichrome.

2.2d-i MORPHOMETRIC ANALYSIS

To investigate subtle differences in the airways and parenchyma of the sham operated and denervated animals morphometric analysis was performed on the airways and parenchyma in the study groups. Airways were analyzed if seen with cartilage and in true cross-section. The boundary of the airway was determined by surrounding lung parenchyma. When two airways were in close proximity *e.g.* close to bifurcation such as there was no lung parenchyma to separate them, an imaginary line was drawn between the two airways such that the dividing wall

was equally distributed between the airways. The inner border of the basement membrane defined the internal perimeter. The lumen was defined as points falling internal to the basement membrane.

The area fractions of selected features in the airway wall profile were determined by a point counting technique (Weibel et al 1962), using a Axioplan light microscope, (carl Zeiss model # 451888), drawing tube and square lattice grid containing 240 points, a Baxter Tally III electronic tabulator, model #008485. The point grid was superimposed onto a segment of the airway wall, and the number of points falling on each area of interest per grid were counted. The size of the airways varied, so it was necessary to adjust the magnification according to the airway size (the majority being counted at 10 or40X magnification) Under a magnification of 10, the distance between two points on the grid (Z) is .012mm and under 40, Z is .03mm. The features that were quantified were interstium, cartilage, mucous gland, nerve, smooth muscle, blood vessel, epithelium and lumen.

Calculations

Using sterologic principles the area proportion occupied by the structures counted was calculated, using the formula: 1) Area (μm^2)=Z^2 n, where Z is the distance between two points on the grid (magnification factor), and n is the number of points that land on the structure of interest.

Luminal surface length (internal perimeter) was determined by using the formula Luminal surface length (μm)=bmi*Z*π/4, where bmi is the number of times the grid

intersects with the basement membrane, and Z is the magnification factor (or the distance between two points on the grid). To determine the thickness (smooth muscle, cartilage) the following formula was used. Thickness (μm)= Area (μm^2)/ Luminal surface length (μm).

2.2e ELECTRON MICROSCOPY

For electron microscopy, lung samples from both upper and lower lobes were fixed using a 2.5% freshly prepared glutaraldehyde solution (320m osmol). In addition, other samples were fixed with 1%osmium tetroxide (wt./vol) in fluorocarbon FC-75 fluid for 90 minutes and further post-fixed in glutaraldehyde solution stored at 4°C until tissue preparation, normally within 48 hrs. The lung tissue was then embedded in epon, sectioned and stained with uranyl acetate/lead citrate and examined using Hitachi 7000 TEM and 450 SEM electron microscopes.

2.2f WET TO DRY RATIO

Lung wet-to-dry-weight ratios were determined by using methods described by Alcorn et al. Briefly, three pieces of lung tissue from upper and lower lobes were tied and dissected from sham operated and denervated animals, weighed and dried in preweighed aluminum containers for 3 days at 120° C (7).

2.2g SURFACTANT PROTEINS A AND B

The SP-A concentrations were determined as previously described (29). In brief, samples were added to microtitre plates coated with goat polyclonal antihuman SP-A antibody; bound antigens were then reacted with rabbit polyclonal antihuman SP-A antibody. After this, bound rabbit antibody was detected by reaction with antirabbit IgG conjugated with horseradish peroxidase; quantification was performed by the addition of peroxidase substrate and spectrophotometric measurement of absorbance at 490 nm. SP-A standards were isolated from patients with pulmonary alveolar proteinosis.

The SP-B was also measured by capture enzyme-linked immunosorbent assay. Samples were added to microtitre plates coated with rabbit antihuman synthetic SP-B antibody; bound antigen was then reacted with mouse monoclonal antihuman SP-B antibody, which is prepared against SP-B isolated from cadaver lung. After reaction with mouse antibody, bound was detected by reaction with antimouse IgG conjugated with horseradish peroxidase; quantification was performed by the addition of peroxidase substrate and spectrophotometric determination of absorbance at 490 nm. SP-B standards are isolated from bovine lungs.

2.2h STATISTICAL ANALYSIS

The effects of group I (cervical vagal denervation; n=8) and group II (sham–operated animals; n=4) on fetal cortisol levels were analyzed using unpaired student t-test. The arterial pH and blood gases between sham and denervated animals were analyzed using repeated measures of the analysis of variance (ANOVA). Body weights at birth, gestational ages, dry and wet weight of lungs, total phospholipids in BAL, morphometric analysis and surfactant proteins in tissues and BAL were also analyzed using ANOVA. All values are given as Mean +/- SE. The P value used in our statistics is 0.05. The study protocol was approved by the animal care committee of the University of Calgary.

3.0 RESULTS

3.1 ARTERIAL pH AND BLOOD GASES

The results of arterial pH, Pa_{o2} and Pa_{Co2} are given in figures 1.3. There were no statistically significant differences in the pre birth values across the gestation pH, Pa_{Co2} and Pa_{o2} between sham operated and denervated animals. The pH along with the blood gases decreased in sham-operated animals and denervated animals immediately after surgery and gradually returned to normal range.

3.2 GESTATIONAL AGES AND BODY WEIGHT

The gestational ages of the sham-operated and denervated animals are given in table 1.

The body weights of the fetuses between sham operated animals are shown in Figure 4. Control animals weighed 3.6 ± 0.26, sham-operated fetuses weighed 3.6kg ± 0.125 and the denervated animals were 3.5kg ± 0.21. There were no statistically significant difference observed in the two groups. Complete section of the vagal nerves was confirmed by identifying the cut ends and checking for any re-growth of nerve after sectioning at autopsy in all eight denervated animals whereas all four sham operated animals had bilaterally intact vagal nerves.

3.3 LUNG TISSUE DRY TO WET WEIGHT RATIO AND PLASMA CORTISOL LEVELS

The results for lung wet-to-dry -weight ratios are given in figures 5. Lung wet-to-dry -weight ratios were 7.8 ± 0.53 for controls, 7.533 ±1.4 in sham-operated and 7.846 ± 0.25 in denervated animals respectively (P>.05). Plasma cortisols at day 130-133 of gestation between sham operated and denervated animals are shown in figure 6. The sham operated animals had cortisol levels of 23ng/ml and denervated animals had cortisol levels of 51ng/ml. There were no differences statistically in the two study groups.

3.4 LIGHT MICROSCOPY

Gross examination of the lungs between sham operated and denervated animals did not show any signs of segmental or lobar atelectasis. The results of the light microscopy on lung parenchyma and airways of sham-operated and denervated animals are shown in plates 1-4. The histologic appearance of the lungs in both the groups are typical of 130-133 days ovine lungs. Lung parenchyma did not show any signs of pulmonary edema, hemorrhage or vascular congestion. The airways in the two study groups did not show any visible difference.

3.5 MORPHOMETRIC ANALYSIS

The result of the morphometric analysis on airway features is illustrated in figures 10 and 11. There were no significant differences in thickness in of cartilage, smooth muscle, mucous glands, nerves, blood vessel, interstium and lumen in the control, sham operated and denervated animals. These results verified the subjective assessment of light microscopy of the lung.

3.6 ELECTRON MICROSCOPY

Plates 5-6 shows the results of electron microscopy performed on lung samples from upper and lower lobes of right lung. The sham-operated and denervated animals showed mature lamellar bodies in the type II cells. The number of lamellar bodies in type II alveolar epithelial cells between sham-operated and denervated animals were comparable by subjective assesment. There were no empty vacuoles observed in type II cells of both sham-operated and denervated animals.

3.7 TOTAL PHOSPHOLIPIDS

The results of the total phospholipids measured in BAL and calculated as mg/kg body weight of the animal is shown in figure 7. Total phospholipids were 13.29 ± 2.15 in controls with sham-operated having 6.8 ± 0.638 and 9.2 ± 1.58 in denervated group. No statistically significant difference was observed between study groups

3.8 SURFACTANT PROTEINS A AND B

Surfactant associated proteins measured in lung tissues as well as BAL between control, sham-operated animals and denervated animals are shown in figures 8 and 9. The surfactant protein A (SP-A) in control, sham-operated and denervated animals in BAL were not different. Similarly, SP-B in BAL was same in the three groups. The surfactant protein A in tissues in sham-operated and control animals were very similar while denervated animals exhibited a trend to increase though it was not significant. In BAL the value of SP-B was similar in the three groups with no statistically significant differences observed between the study groups.

FIGURE 1: ARTERIAL pH IN TORR ACROSS THE GESTATION IN SHAM-OPERATED AND DENERVATED ANIMALS

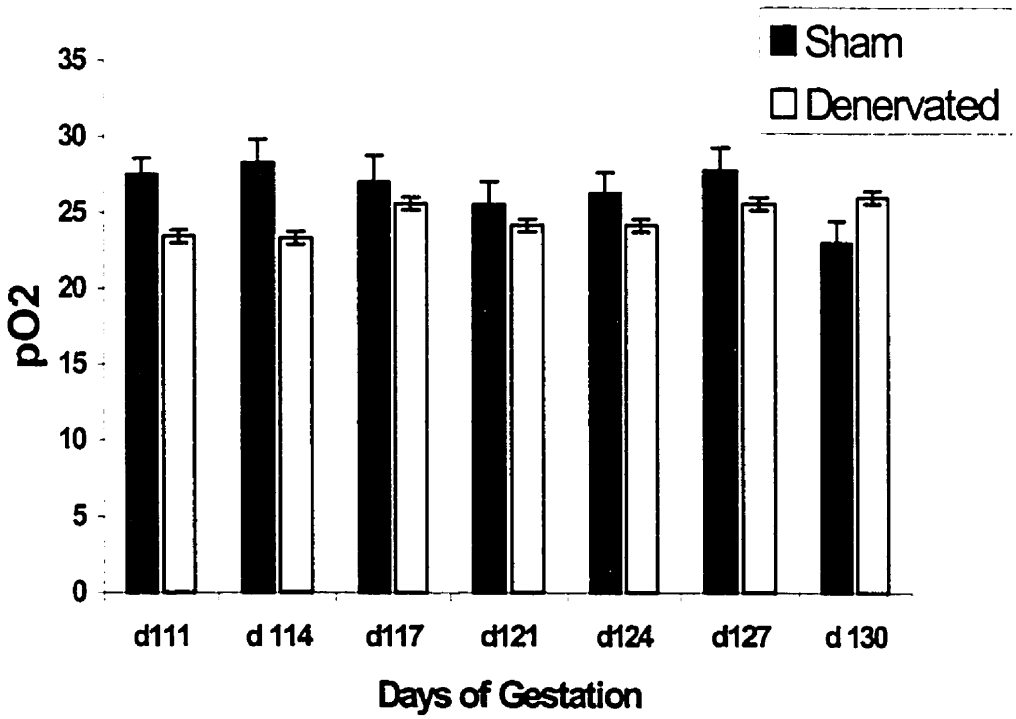


FIGURE 2: ARTERIAL PO2 IN TORR ACROSS THE GESTATION IN SHAM-OPERATED AND DENERVATED ANIMALS

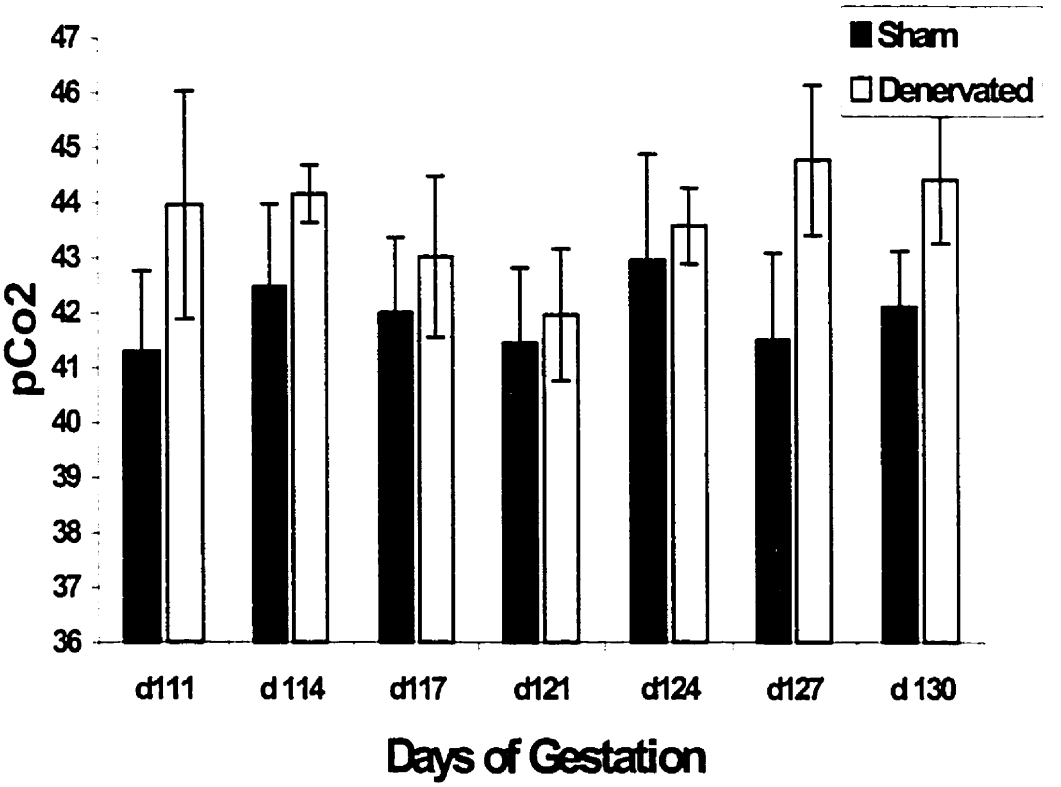


FIGURE 3: ARTERIAL PCO2 IN TORR ACROSS THE GESTATION IN SHAM-OPERATED AND DENERVATED ANIMALS

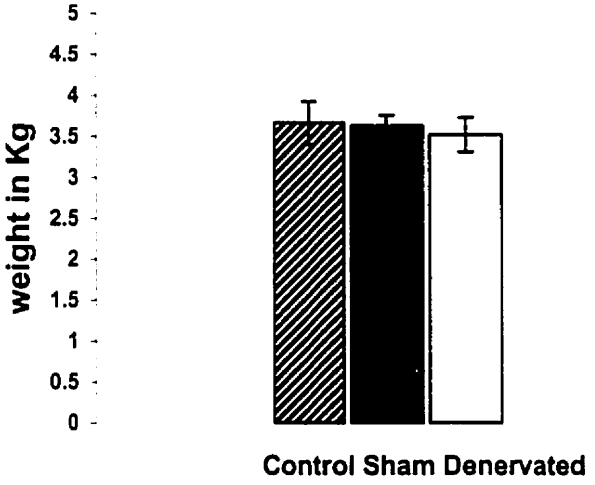


FIGURE 4: WEIGHT AT DAY 130-133 OF GESTATION BETWEEN CONTROL, SHAM AND DENERVETED ANIMALS

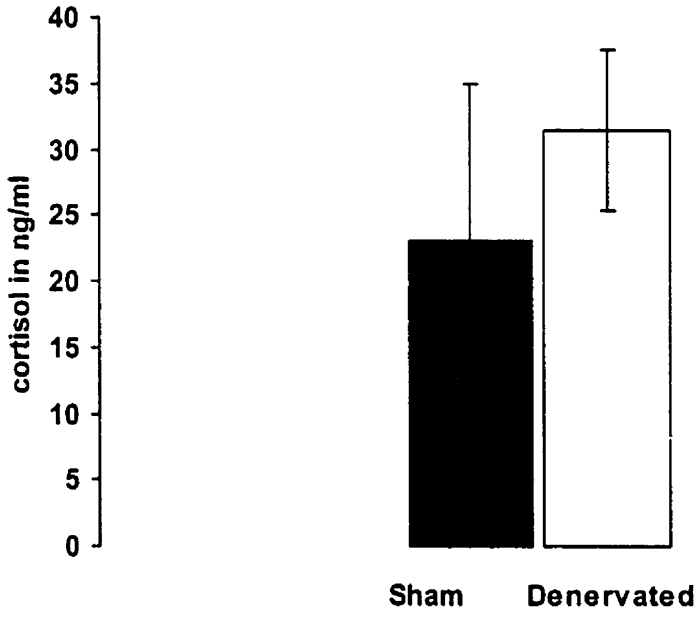


FIGURE 5: FETAL CORTISOL IN NG/ML AT DAY 130-133 OF GESTATION IN SHAM AND DENERVATED GROUPS

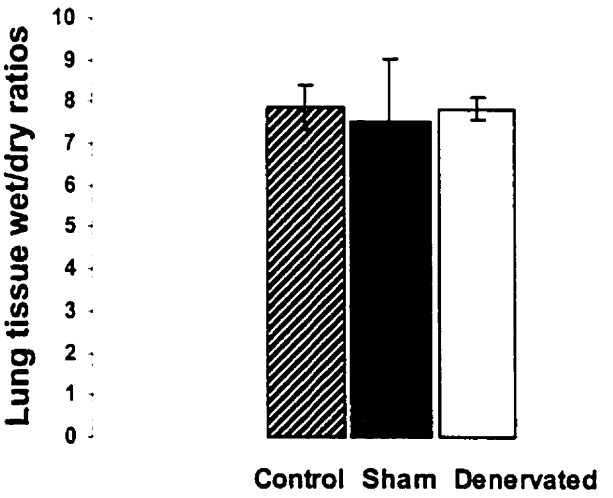


FIGURE 6: WET TO DRY RATIO OF LUNG TISSUE IN CONTROL, SHAM AND DENERVATED ANIMALS

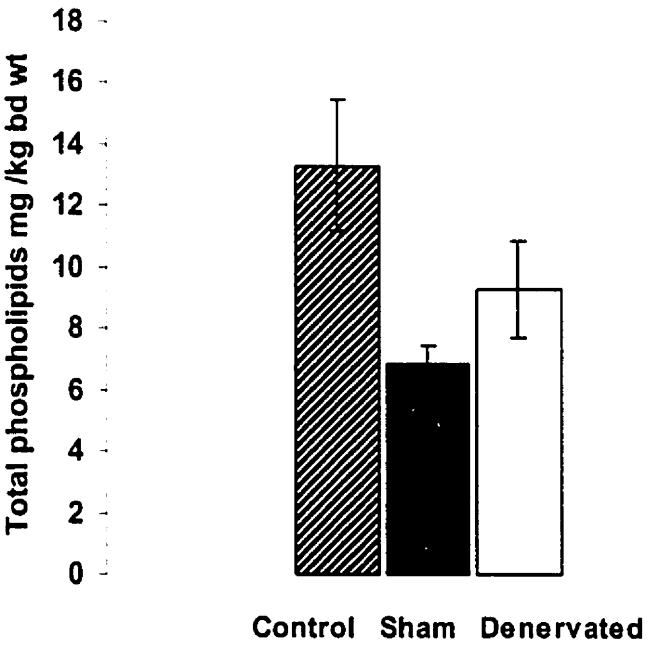


FIGURE 7: TOTAL PHOSPHOLIPIDS MEASURED IN BAL AT 130-133 DAYS IN CONTROL, SHAM AND DENERVATED ANIMALS

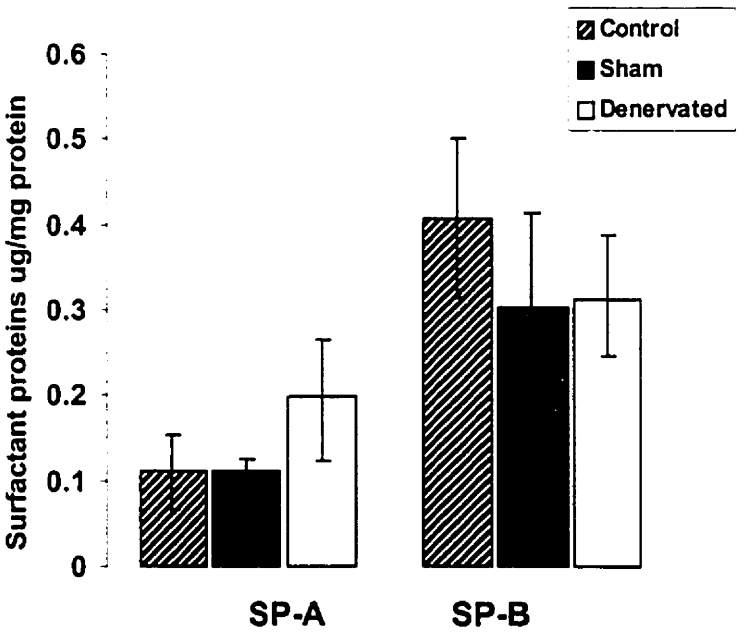


FIGURE 8: SURFACTANT PROTEINS A AND B MEASURED IN LUNG TISSUES

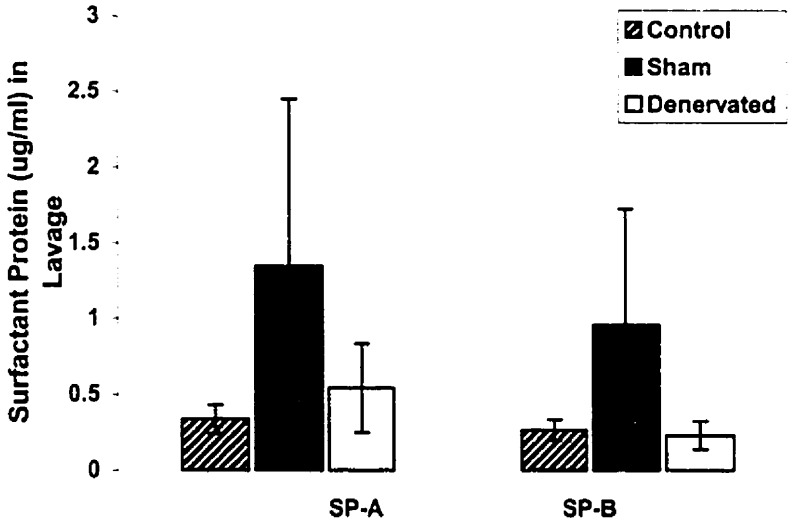


FIGURE 9: SURFACTANT PROTEINS A AND B MEASURED IN BAL

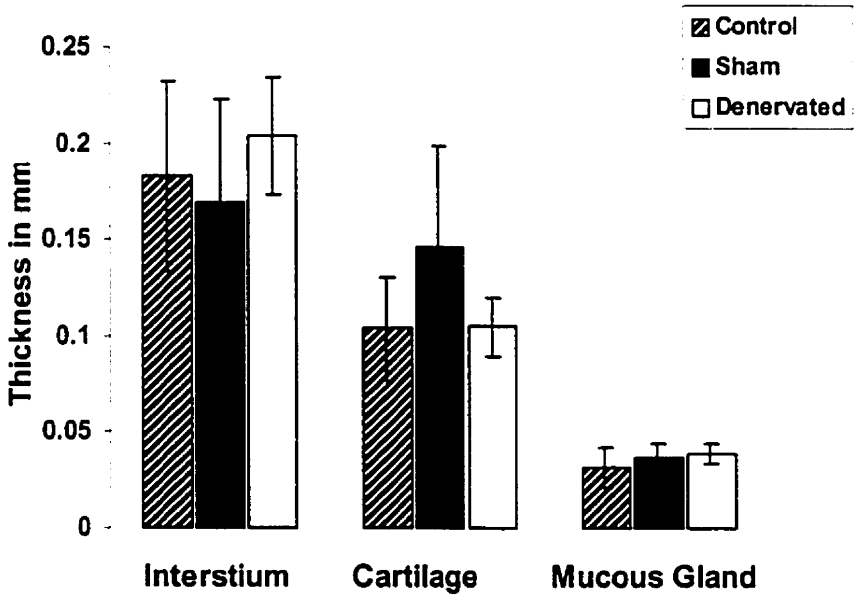


FIGURE 10: AIRWAY FEATURES IN CONTROL, SHAM AND DENERVATED ANIMALS

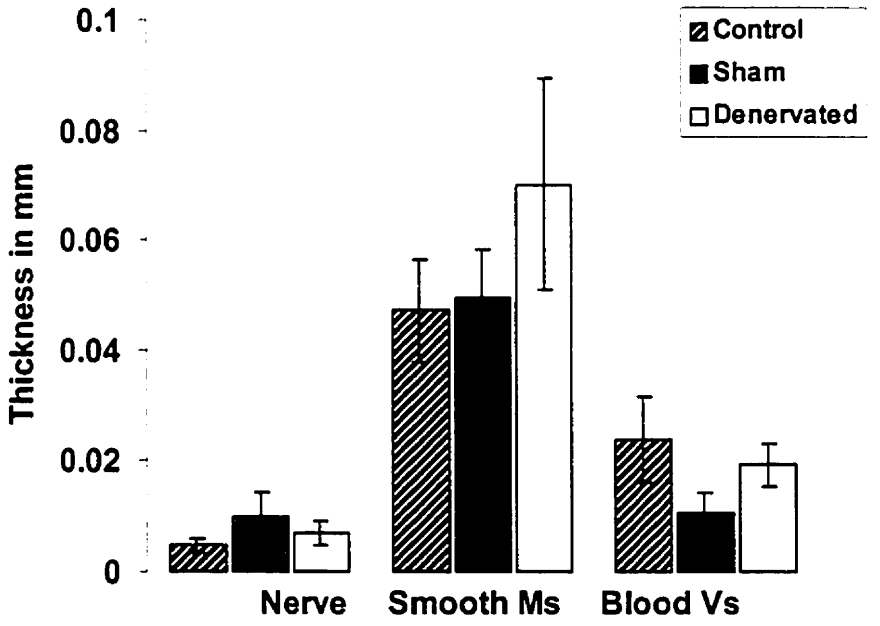
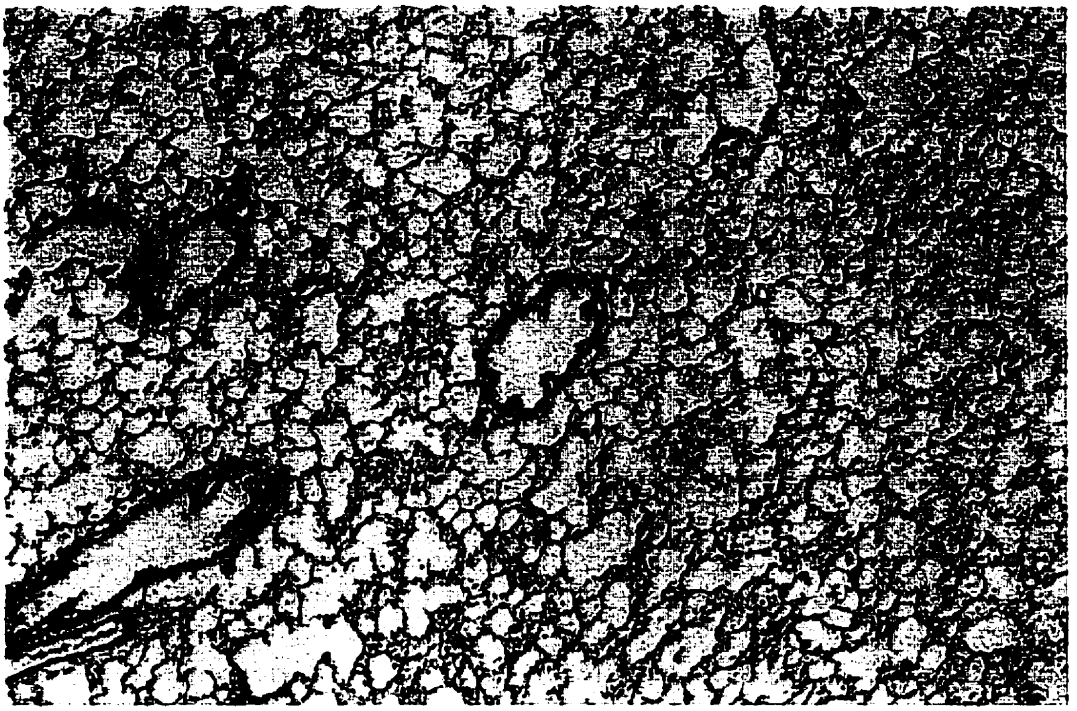


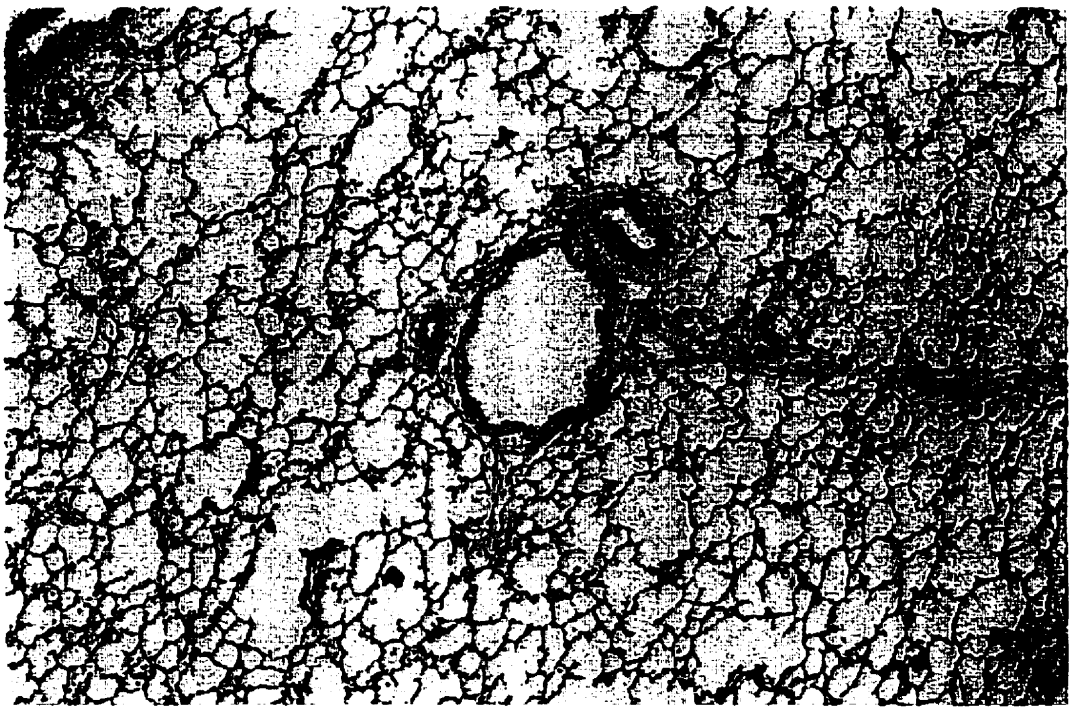
FIGURE: 11 AIRWAY FEATURES IN CONTROL, SHAM AND DENERVATED ANIMALS

Table 1. **Gestational ages in sham-operated and denervated animals on day of surgery and day of c-section**

Animals		Day of Surgery	Day of C-Section	Mortality
Sham	Mean	110	131	50%
	SE	0	0.29	
Denervated	Mean	110	131	61%
	SE	0.30	0.36	

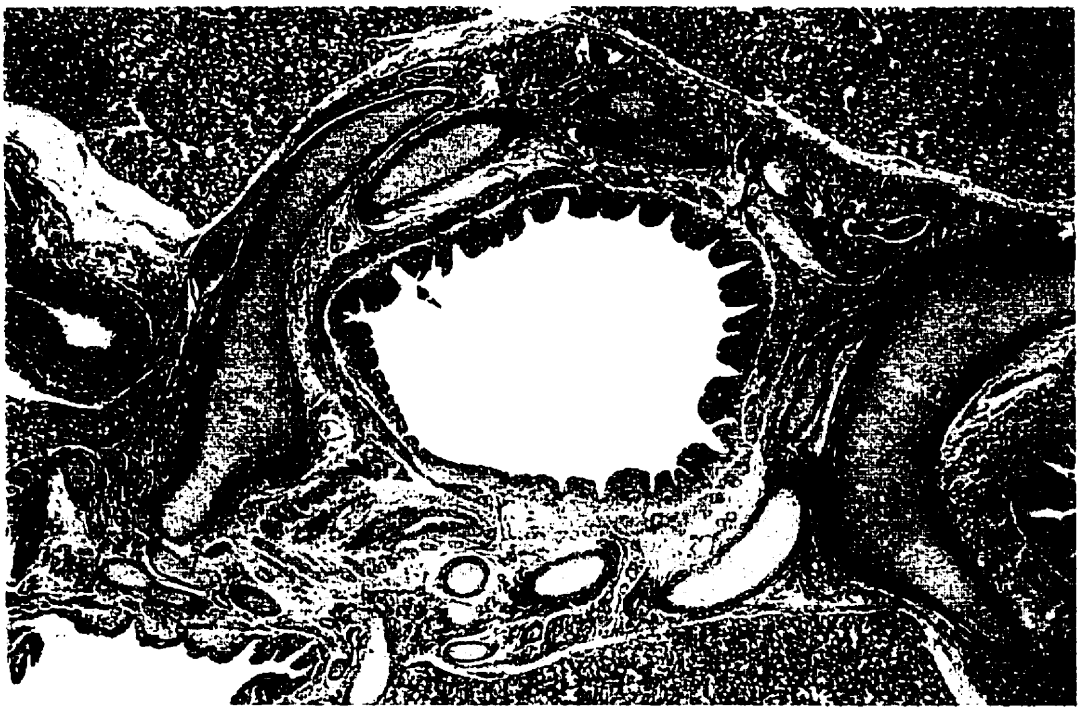


Sham

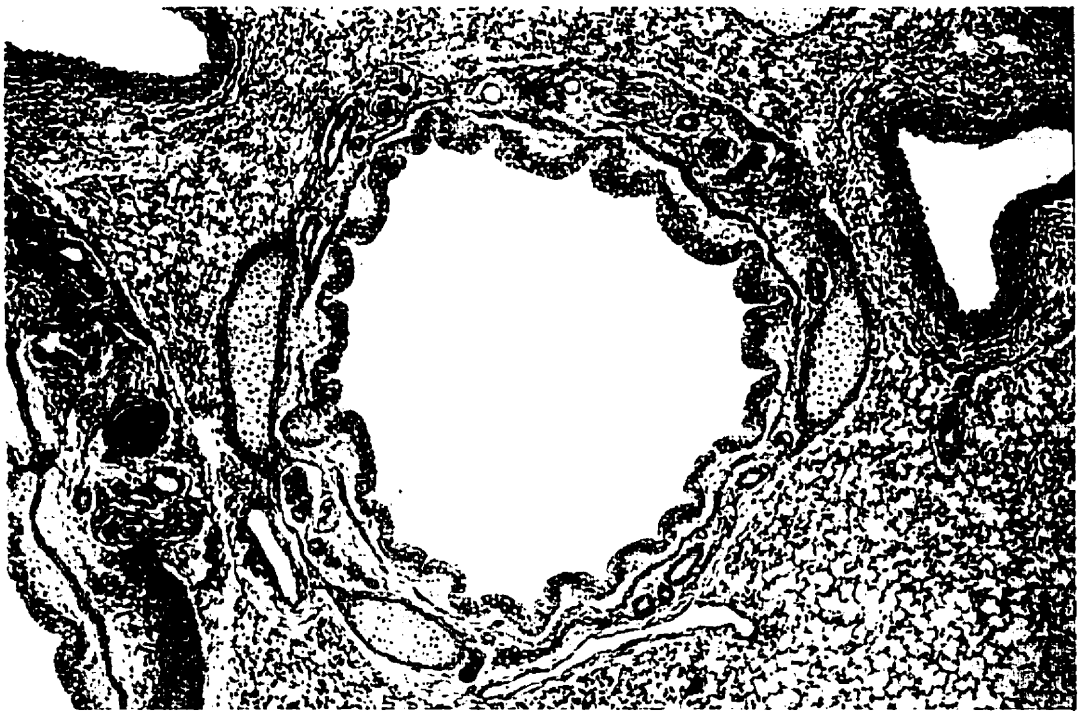


Denervated

Light Microscopy of Lung Parenchyma in Sham and Cervically Denervated Animals



Sham



Denervated

Light Microscopy of Airways in Sham and Cervically Denervated Animals

A. SHAM

Plate: 5

B. CERVICAL VAGAL DENERVATION

Plate: 6

4.0 DISCUSSION

The data in this study provide new evidence that vagal innervation of the lung at mid gestation is not necessary for surfactant synthesis. This study does not support the observations reported by other investigators following vagal denervation on lamellar bodies in type II alveolar epithelial cells in fetal sheep. Bilateral cervically vagal denervation in our studies did not cause any difference in lamellar bodies in type II cells. Alveolar architecture was maintained with no signs of visible alveolar edema in either sham-operated or denervated animals. The absence of alveolar edema was further supported by no differences in lung tissue dry to wet ratio between the two study groups. Our data also showed no changes in total lung phospholipids between sham and denervated animals. The denervated animals had similar surfactant proteins A and B in lung tissues and BAL as compared to sham-operated animals. The plasma cortisol concentrations measured between sham-operated animals and denervated animals were similar with no statistically significant difference between the two groups.

A number of morphological and biochemical studies have implicated cholinergic mediated mechanisms in influencing surfactant synthesis and secretion. The cholinergic (vagal) innervation in the airways of the young has been reported by Fisher et al (26). In studies done by Meyrick and Reid electron microscopy showed nerves in the alveolar walls of rats (70). Similarly Hung et al identified two types of nerve endings in the alveoli of mouse in close contact to the type II

cell, one of which had motor functions (43). These observations give some credence to the concept of the nervous control of the surfactant system.

Oyrazon and Clements found that increased minute ventilation augments air space phospholipids and such release might be mediated via cholinergic mechanisms. Similarly in other studies by the same authors it was shown that lack of lung expansion caused the lung phospholipid profile to change to fetal pattern (75,76). Lawson et al also observed that surfactant concentrations increased after 30 minutes of air and nitrogen breathing, whereas, in the animals with occluded trachea, little or no increase was observed. They speculated that the mechanism for surfactant release was mediated by vagal nerves (55).

However, in support of the role of vagal innervation in surfactant flux, there is evidence, that vagal denervation compromises surfactant synthesis, release or may cause dysfunction. Alcorn et al investigated the role of bilateral cervical vagal denervation on type II cells in fetal sheep of 103-113 days gestation and animals were studied prenatally (130d). Their experiments reported no differences in lung wet-to-dry-weight ratio, following vagal denervation. The alveolar counts in lung parenchyma were increased in sham–operated and denervated animals as compared to the test group (bilateral phrenectomy). Light microscopy did not reveal any histological differences between the sham-operated and denervated animals. At higher resolution the differentiation of type I and type II cells appear as normal, as did the components of alveolar wall.

However the inclusion bodies in type two cells displayed little osmophilic staining activity, appearing as empty vacuole-like, membrane bound intra-cytoplasmic spaces (7). In our experiments neither sham-operated nor denervated animals showed any difference in alveolar architecture; there were no signs of alveolar or interstitial edema, hemorrhage, or vascular congestion. The absence of edema was further confirmed by the lack of change observed in lung tissue wet-to-dry- weight ratios. The electron microscopy of the lung tissues also showed that the type II cells in sham-operated and denervated animals had mature and comparable number of lamellar bodies suggesting that the synthesis of inclusion bodies of type II cells (lamellar bodies) was unaffected by vagal denervation.

Similar results were observed by Wong et al. They reported the presence of lamellar bodies in type II cells following intra-thoracic vagal denervation below the recurrent laryngeal nerve at 130 days in fetal lambs studied close to term. However, in experiments done by Wong et al vagal denervation was performed late in gestation and the animals were studied close to term (100). It is possible that by the time experiments were conducted the processes for surfactant synthesis might already be mature. Furthermore, It is likely that in the experiments performed by Alcorn et al the presence of vacuole-like, membrane bound intra-cytoplasmic spaces were due to washing out of lamellar bodies at the time of fixation. To ensure proper fixation of lamellar bodies the primary fixation should involve gluteraldehyde along with 1% osmium tetraoxide (OsO_4)

which introduces both inter and intramolecular crosslinks thereby binding the lipids in the lamellar bodies. Fixation with OsO_4 has an additional advantage that it imparts a modest conductive property to the fixed tissue (41). This critical step for lamellar bodies fixation was lacking in the techniques used in experiments by Alcorn et al. Gluteraldehyde fixed specimens remain osmotically active unless the duration of fixation is prolonged or the tissues are postfixed immediately with OsO_4. Thus gluteraldehde fixed specimens remain vulnerable to distortion in immediate postfixation treatment. Furthermore, gluteraldehydes do not preserve most of the lipids and substantial amounts of phospholipids are extracted during dehydration (41). The experiments performed by Alcorn et al was done using only gluteraldehyde and were not optimal for preserving lipids in the tissues.

In studies performed by Goldenberg et al. and Kunc et al. on adult rats, cervical vagal denervation was performed in postnatal spontaneously breathing animals. In these studies the concentration and the total amount of phospholipid remained unchanged while there was an elevation of total protein. There was a rise in minimum surface tension in denervated animals. They also reported absence of lamellar bodies in type II cells and atelectasis and interstitial edema in lung parenchyma (30,53) As the total amount of phospholipids in these experiments was not affected it is possible that pulmonary edema influenced surfactant function. Following vagal denervation, there is a sympathetic predominance and sympathetic stimulation leads to enhancement of intra-alveolar content of proteins (10,87). Increased protein content inactivates surfactant that could be a

possible explanation for the increase in surface tension in these experiments. It is also likely that increased surface tension could have increased the hydrostatic pressure in the interstitial space and increased intra-alveolar protein content led to loss of colloidal pressure in the capillaries allowing fluid to leak into the interstium and alveoli.

In studies by Berry et al bilateral cervical vagal denervation was performed in adult rabbits and percent-saturated phosphotidylcholine and soluble proteins were measured in the alveolar wash. They reported that vagally denervated animals developed proteinaceous pulmonary edema. The proteins were of plasma origin as there was a marked increase in ^{125}I-albumin from denervated lungs. Surfactant pools in both denervated and sham groups were similar. Since the saturated phosphotidylcholine remained similar in the two groups and heart rate, cardiac outputs and pulmonary arterial pressures did not change following vagal denervation the mechanisms for pulmonary edema following vagotomy is not clear (11).

Pulmonary edema following vagal denervation is likely of neurogenic origin. A review by Reichsman concluded that the edema was the result of either disturbance a in pulmonary hemodynamics or laryngeal and airway spasms secondary to vagal denervation (82). Luisda and Sarnoff demonstrated that parasympatholytic drugs and atropine enhanced the pulmonary edema resulting from volume overloading, possibly because pulmonary hemodynamic alterations such as greater increase in pulmonary blood volume induced by blocking the

vagal activities (62). However in most of the above studies with vagal denervation which measured pulmonary vascular pressures did not observe any change in these parameters following vagal denervation. On the other hand, Lorber observed that lung edema following bilateral vagal denervation in rats, guinea pigs and rabbits were associated with intense respiratory obstruction due to laryngeal and airway spasms. Vagal denervation below the recurrent laryngeal nerve did not cause pulmonary edema or these airway changes. When respiratory obstruction was added in a group of rats, the degree and rapidity of edema was comparable to that occurring after vagal denervation suggesting that airway obstruction was responsible for vagally induced pulmonary edema (63). Severe airway obstruction may result in hyperinflation and markedly negative interstitial pressure in adjacent unobstructed lung regions due to an independent or teethering effect between adjacent segments (77). This may increase the transcapillary filtration pressure in the hyperinflated regions; it is possible that edema is the result of severe bronchomotor changes associated with vagal denervation.

The degree of distension of the fetal lungs is thought to have a major influence on pulmonary development (35). The volume of liquid in the airways is determined by its rate of secretion across pulmonary epithelium and by movement of liquid in trachea, which communicates with pharynx and amniotic sac (34). Though the rate of production of fetal lung fluid is uniform, it is now recognized that upper respiratory tract (URT) resistance associated with fetal

breathing movement (FBM) plays a major role in controlling the movement of tracheal fluid, and hence lung expansion. Although individual FBM have little effect on tracheal flow, episodes of FBM and apnea can exert a strong influence, such that the net efflux of fluid is accelerated during episodes of FBM (or low-voltage electrocortical activity) and retarded during apnea (or high–voltage electrocortical activity) (35). Changes in the efflux of the liquid can be attributed to changes in URT resistance in the presence of pulmonary elastic recoil. The resistance of the upper airway to efflux of liquid is lowered when FBM are present probably as a consequence of rhythmic activity in laryngeal dilator muscles (posterior cricoarytenoid). Laryngeal constrictor muscles (principally thyroarytenoid) are essentially inactive during episodes of FBM. Thus there is no active glottic narrowing during the expiratory phase of FBM. During episodes of fetal apnea, the laryngeal constrictors muscles become tonically active leading to increased resistance of fetal URT to the efflux of tracheal liquid (36). Since a laryngeal mechanism is implicated in control of efflux of lung fluid, if the braking effect of fetal larynx is reduced or eliminated, for example by the section of the recurrent laryngeal nerves (37) or by the formation of tracheoamniotic shunt, the rate of efflux of fluid during periods of fetal apnea is greatly increased (34). As it is has been observed that sustained expansion of lung is potent determinant for their growth, loss of lung liquid fluid leads to profound hypoplastic changes, together with structural immaturity. Studies done by Alcorn et al in fetal sheep demonstrated that creation of tracheal fistula causing chronic tracheal drainage inhibits lung growth as evidenced by small potential air spaces with thick walls.

In contrast when tracheal ligation was performed, lungs were distended and growth stimulated (6). Similar findings were observed in other studies and demonstrate that lung growth is very sensitive to sustained changes in transluminal tissue stress (47,71). In our experiments we did not observe any significant histologic differences in lung parenchyma of sham and denervated animals. Morphometric analysis performed on the airways of the two study groups for any possible differences did not reveal any statistically significant changes in the thickness in cartilage, interstium, smooth muscles, nerves, and blood vessels. The body weight and the lung tissue dry to wet weight within the two study groups were similar suggesting that the lung growth in our experiments were not influenced by vagal denervation. The cervical vagal denervation resulted in loss of motor innervation to the larynx causing paralysis of the adductive and abductive component of vocal cords. In paralysis, vocal cords assume a cadaevric position (midway between adduction and abduction) which may not decrease the resistance during apnea as much as observed when tracheal fistula is created thereby maintaining a supra-amniotic pressure in apneic periods and preventing efflux of fluid during episodes of fetal apnea.

Administration of glucocorticoids to fetal animals of several species accelerates both morphological development of lung and the appearance of surfactant in alveoli. This suggest that fetal lung, like certain other fetal tissues, is a target tissue for glucocorticoids. This observation is supported by findings of Liggins and Howie on fetal rabbits, lamb and the human, all contain the specific

glucocorticoid receptor thought to be necessary for hormone responsiveness (30). Although it has not been shown that lung development is influenced by endogenous glucocorticoids during fetal life, such an effect has been inferred. Mescher reported that in fetal lambs plasma cortisol levels were constant till about day 136 of gestation. Values increased slowly until day 141 of gestation after which there was a rapid increase at term (69). The plasma cortisol levels measured on day 130-133 in denervated animals in our experiments were higher as compared to sham-operated animals but this increase was not statistically significant. It is likely that denervation of vagosympathetic trunk in cervical area could have resulted in an exaggerated activity of thoracic sympathetic trunk resulting in baseline increase in catecholamines (10). But since in our studies serial measures were not done it is difficult to say whether the increase trend observed in denervated animals was due to the maturing fetus or whether this difference in the two groups was present since surgery.

Lamellar bodies, which are the site for surfactant storage, appear in humans at 20-24 weeks and in rabbits at around day 26 of gestation (term 31 days). Though it has not been investigated as to when lamellar bodies appear in fetal sheep low levels of surfactant are demonstrable by day 122-125. These levels increase gradually till day 135 after which they rise rapidly until term. The total phospholipid measured in our experiments in denervated animals is higher than in sham-operated animals but was not statistically significant. It is possible that increased thoracic sympathetic activity following cervical denervation could have

led to an increased release of catecholamines which are known to act on β - adrenergic receptors (52) in type II alveolar epithelial cells and cause of release of lamellar bodies in alveoli increasing the total lung phospholipids.

Surfactant protein A (SP-A) is responsible for aggregating phospholipids and forming a monolayer at the air-liquid interface. Surfactant protein B (SP-B) helps in this process and also prevents conversion of active form of surfactant i.e. large aggregates (LA) into inactive form of surfactant i.e. small aggregates (SA). Conversion of LA to SA has been demonstrated *in vitro* using the technique of surface area cycling. These experiments have shown that dissociation of SP-B from LA results in SA that is devoid of SP-B (44). Lack of SP-B is associated with a severe form of respiratory failure in term infants soon after birth (99). These studies and many others demonstrate the importance of SP-B in maintaining the active form of surfactant. The lack of change in surfactant proteins A and B in lung tissues as well BAL in our experiments between sham-operated and denervated animals suggest that neither their synthesis nor release was compromised by vagal denervation.

In summary, we provide unequivocal evidence that lamellar bodies are intact in cervical vagally denervated animals and that the total phospholipids and the surfactant associated proteins synthesis process is not affected by vagal

compromise at gestation of 103-113 days in fetal sheep. Additionally, motor innervation of larynx had no effect on lung growth in utero as evidenced by morphometric analysis and lung weights.

CONCLUSIONS

We provide new evidence that cervical vagal denervation in mid gestation has no effect on lamellar bodies in type II cells. The total phospholipids in BAL and SP-A and SP-B in tissues as well as BAL were not influenced by vagal denervation. Additionally, we provide evidence that motor innervation of larynx is not essential for lung growth in utero as evidenced by no difference in lung weights in sham-operated and denervated animals and morphometric analysis performed on airway features.

SIGNIFICANCE AND RELEVANCE

Respiratory distress syndrome is the most common cause of respiratory distress in neonates (66). It is defined as respiratory distress that occurs in a newborn infant after the onset of breathing, within the first few hours of life (94). In addition, pulmonary hypoplasia has been reported to occur in upto 25% of infants with RDS (78). The incidence of RDS is inversely proportional to gestational age; 80% for infants<28 weeks of gestation, 60% in infants born at 29 weeks of gestation and < 1% in term infants (37-41 weeks) (25;92).

Since Avery and Mead demonstrated that saline extracts of lung tissue from premature infants who died from respiratory distress syndrome had higher minimal surface tension, surfactant deficiency has been considered as the primary cause of respiratory distress syndrome. Functional surfactant deficiency could mean less surfactant, adequate surfactant with abnormal composition and function, an inactivation of normal surfactant or a combination of these possibilities.

For more then a decade, surfactant has been widely available for the prevention and treatment of RDS (13), improving survival and incidence of complications such as pnemothorax and severity of all grades of intraventricular hemorrhage (1,3). There is a consensus that because of surfactant therapy, the improvement of survival in VLBW (very low birth weight) infant has been specially striking.

This is primarily because survival of these babies has been remarkably low. Before 1960, survival in < 1500g weight category was less then 20% and for < 1000g less then 10% (2). For babies born in 1989-1990, based on data from the national institute of child health and human development neonatal network, overall survival rate for infants< 1500g was 78% ranging from less then 39% for <751g infants, of 77% for 751-1000g, to 93% for >1250g (33). These finding are supported by data from centers across United States as well as other countries.

Understanding the factors regulating surfactant system will provide crucial information on the improvements of available form of surfactant and aid in synthesis of artificial surfactant free of side effects associated with presently available surfactants. This will contribute to improved pediatric health care as RDS as well other mentioned conditions are associated with increased mortality and morbidity among preterm as well as full term infant.

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