Stachybotrys chartarum spore extracts and their effects on surfactant protein expression and surfactant quantity and quality in fetal rat lung epithelial cells

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Abstract

***Stachybotrys chartarum* (*S. chartarum*) spore extracts and their effects on surfactant protein expression and surfactant quantity and quality in fetal rat lung epithelial cells.**

Moulds are constant contributors to air pollution particularly to air quality in buildings. The spores themselves or their volatile organic products are present in variable amounts in almost all environments, particularly in buildings affected by flooding. These moulds and products can account for the sick building syndrome and have been tied to such occurrences as the outbreak of pulmonary hemosiderosis in infants in Cleveland, Ohio, several years ago.

Rationale: While past studies have focused on the effects of *S. chartarum* spores on surfactant in terms of composition and quantity, very little has been done to investigate the effects of *S. chartarum* extracts on surfactant protein expression in the developing lung. The results of these studies will establish an *in vitro* model for monitoring environmental toxins and provide a knowledge base to be utilized in future studies.

Methods: *S. chartarum* extracts obtained by agitating spores in saline were incubated with cultures of several cell types. Human lung A549 cells, a continuously growing cell line derived from surfactant producing type II alveolar cells, isolated fetal lung type II cells and fetal lung fibroblasts were used. MTT formazan assays were employed to test cell viability. The synthesis and release of the predominant surfactant protein A (SP-A), which is involved in the regulation of surfactant turnover and metabolism, and surfactant protein B (SP-B) involved in shuttling phospholipids between surfactant sub

compartments was also assessed. Antibodies to these proteins (available commercially) and western blotting results were used to assess the quantity of protein produced by the various cell types. Captive bubble surfactometry was employed to investigate the quality of surfactant in terms of surface tension and bubble volume measurements. Electron microscopy was used to examine changes in cellular structure of control and *S. chartarum*-treated cells.

Conclusions: Exposure to the *S. chartarum* extracts had negative effects on fetal lung epithelial cell viability and their ability to produce pulmonary surfactant. *S. chartarum* extracts induced deleterious changes to the developing fetal lung in terms of expression of SP-A and SP-B as well as to the surface tension reducing abilities of their produced pulmonary surfactant. Ultrastructurally, spore associated changes were apparent in the isolated lung cells most notably in the lamellar bodies of fetal rat lung fibroblasts, alveolar type II, and human A549 cells.

List of Abbreviations

MEM: Minimum Essential Medium

NCS: Newborn Calf Serum

SNCS: Stripped Newborn Calf Serum

HBSS: Hankôs Balanced Salt Solution

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

DPPC: Dipalmitoylphosphatidylcholine

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

SP: Surfactant protein

CBS: Captive Bubble Surfactometre

kDa: Kilodalton

ml: millilitre

ug: microgram

RPM: Revolutions Per Minute

Nm: Nanometre

w/v: Weight/Volume

M: Molar

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Chapter One

Introduction

1.0 Lung Development

1.0.1 Stages of Lung Development

There are 5 stages of lung development in humans as outlined in McGowan and Snyder, (2004). The first stage is that of lung bud formation at 4 weeks gestation where the lung begins as a diverticulum from the embryonic foregut. The diverticulum and the foregut are composed of epithelial cells derived from the endoderm. The diverticulum is also covered in mesoderm which gives rise to the connective tissue found in the lung (Burri 1991). The second stage, which is characteristic of early lung development, is that of branching morphogenesis. The buds undergo dichotomous branching to form the primary, secondary and tertiary bronchi and so forth (Shannon and Deterding 2004). Following embryonic development, there are 3 stages of lung development during the fetal period. The pseudoglandular phase occurs at 6-16 weeks and is comprised of repeated branching as well as differentiation of the epithelium in the conducting airways (Burri 1991). At the end of this stage, the most distal portions of the airways which will become the alveoli remain undifferentiated. The canalicular phase occurs from weeks 16-24 and is the stage where the distal portions are remodelled to begin forming the gas-exchange units or alveoli. The epithelium begins to differentiate, an increase in capillaries between terminal ducts is evident, and alveolar type I and type II cells form

(Mallampalli, Acarregui et al. 1997). The fifth stage is the saccular phase which occurs from approximately 24 weeks gestation to term. The air spaces undergo enlargement giving the lungs their characteristic spongy appearance. The terminal ducts undergo further branching to form the alveolar ducts and sacs during the alveolar stage which occurs late in gestation through to childhood. In humans, it is thought that approximately 15-18% of alveoli form in late gestation but that the majority form after birth with the final stage in lung development being the postnatal stage of microvascular maturation which is characterized by remodelling of the parenchymal septa and the capillary system (Burri 1991).

1.0.2 Surfactant and Lung Development

The pulmonary surfactant system is one of the last systems to develop prenatally and its development occurs between the 29th and 32nd week of gestation. The composition and amount of surfactant varies throughout the systemôs development. Towards the end of gestation the concentration and the saturation of the phospholipids in surfactant increase (Orgeig, Daniels et al. 2004). It is not until late in gestation that the surfactant proteins (SP) appear. SP-A is detectable in the human lung at week 30 (Mendelson C.R. 1991), whereas the other proteins can be detected earlier in gestation. SP-B and SP-C are present at week 15 (Khoor, Stahlman et al. 1994), and SP-D is present in the second trimester (Dulkerian, Gonzales et al. 1996). The actual mechanisms which control the development of the surfactant system are poorly understood. However,

various neuro-hormonal factors are known to influence its development, mainly glucocorticoids, thyroid hormones, autonomic neurotransmitters and expansion or stretch of the fetal and newborn lung (Orgeig, Daniels et al. 2004).

1.1 The Alveolus

The alveolus is the gas-exchange unit of the lung. Alveoli have a simple structure consisting of a connective tissue septa made up of fibroblasts, capillary endothelial cells, and extracellular membrane components, mainly elastin. The epithelium of the alveolus is comprised of two cells types, alveolar type I and type II cells (Crapo, Barry et al. 1982). The formation of the alveolus begins late in gestation and continues after birth. These small, thin walled structures facilitate the exchange of oxygen and carbon dioxide between the capillaries and the air brought into the lung during inspiration. The alveolar surface area available for gas exchange is approximately $100m^2$ in adults and is comprised of approximately 300×10^6 alveoli (Crapo, Barry et al. 1982; Burri 1991).

1.2 Fibroblasts

During alveolarization, the interstitium assumes the role of structural support. Fibroblasts are the main cells which provide such support for the gas exchange unit. Interstitial fibroblasts form the majority of the extracellular matrix comprised largely of collagen secreted by fibroblasts (McGowan and Snyder 2004). Fibroblasts are the largest

and most prevalent cell type found in the lung interstitium (Crapo, Barry et al. 1982). These cells are spindle-shaped with long extensions and a large cigar shaped nucleus (Kuhn 1978). Fibroblasts are mesenchymal cells which are involved in the maintenance of the integrity of lung tissue in the alveolus through deposition, degradation and remodelling of the components of the extracellular matrix (McGowan and Snyder 2004). In times of lung injury, interstitial fibroblasts are activated to form fibrotic tissue. Via interaction with phagocytic and immune cells, interstitial fibroblasts will both proliferate and migrate to the site of injury most notably in cases of inflammatory lung disease (McGowan and Torday 1997).

Based on ultrastructural characteristics, there are three subpopulations of fibroblast cells (Brody and Kaplan 1983; Maksvytis, Niles et al. 1984). Vaccaro and Brody (1978), classified two of the three types of fibroblasts based on the presence or absence of lipid droplets; non-lipid interstitial cells and lipid interstitial cells. Myofibroblasts, a third type, were described by Kapanci, Assimacopoulos et al. (1974), which are contractile cells exhibiting features of both smooth muscle and fibroblast cells. In times of lung injury, it has been shown that lipid interstitial cells can differentiate into myofibroblasts which can lead to altered pulmonary function. Myofibroblasts are the principle cell involved in pulmonary fibrosis (Rehan and Torday 2003). Non-lipid interstitial cells are rich in organelles for protein synthesis and secretion and are involved in the formation of extracellular membrane and lung interstitial fibres. They contain no lipid granules, unlike the lipid interstitial cells which accumulate lipid inclusions late

prenatally and early postnatally. Lipid interstitial cells decrease in number with lung maturity and are absent from the adult lung (Brody and Kaplan 1983).

1.3 Alveolar Epithelial Cells:

1.3.1 Type I Pneumocytes

Alveolar type I cells are considered terminally differentiated cells as they are unable to divide. The type I cell plays an important role in the air-blood barrier since it overlies the capillaries in the alveolar wall. In fact, type I cells comprise almost 90% of the alveolar surface area (McGowan and Snyder 2004). Alveolar type I cells function as a thin walled gas-permeable membrane and because they are terminally differentiated, they depend on the division and differentiation of type II cells for their replacement. They appear as large, flat, squamous epithelial cells and are also branched (Schneeberger 1991).

1.3.2 Type II alveolar cells

Alveolar type II cells are characterized by microvilli on their apical edge and the presence of lamellar bodies in their cytoplasm. Alveolar epithelial type II cells are able to divide and also able to differentiate into type I cells (McGowan and Snyder 2004). Type II cells only comprise approximately 7% of the alveolar surface area, however, they are about twice as prevalent as type I cells (Crapo, Young et al. 1983). These small,

cuboidal epithelial cells are also responsible for repair of injured alveolar epithelium (Mason and Williams 1991).

Pulmonary surfactant lipids and proteins are synthesized in type II cells and are stored in the lamellar bodies. The lamellar bodies are the storage organelle for surfactant and are composed of a protein core and parallel, stacked bilayers of lipid. Lamellar bodies are secreted from alveolar type II cells via exocytosis at the alveolar cell membrane and are adsorbed into the overlying liquid interface.
(Orgeig, Daniels et al. 2004).

1.4 A549 Lung Epithelial Tumour Cells

A continuous tumour cell line from human lung carcinoma, A549 cells, have many similarities to type II cells and can provide a source of human surfactant. It is suggested that human A549 cells originate from a neoplastic transformation of alveolar type II cells. These cells contain two types of lamellar cytoplasmic inclusions which are typical of alveolar type II cells. One type of lamellar body, loose lamellar body, has a loose concentric arrangement of lamellae whereas dense lamellar bodies have tightly compacted lamellae. It is unknown as to whether the different morphological lamellar bodies represent differences in cell maturity or functional types of lipoprotein synthesis. The A549 cell line permits *in vitro* investigations of surfactant synthesis from human cells (Lieber, Smith et al. 1976).

1.5 Pulmonary Surfactant: Composition and Function

Pulmonary surfactant is composed of lipids, phospholipids and surfactant associated proteins. The storage form of surfactant is the lamellar bodies found in type II alveolar cells where surfactant is present in densely packed bilayers. Surfactant proteins A-D (SP-A, SP-B, SP-C, SP-D) comprise approximately 10% of the composition of surfactant (Goerke 1998). Phosphatidylcholine makes up approximately 80% of the surfactant composition, dipalmitoylphosphatidylcholine (DPPC), in particular, is the most abundant. The acidic phospholipids, phosphatidylglycerol and phosphotidylinositol make up 10-15% of the total phospholipid composition. The remaining 5-10% of the composition of surfactant is comprised of cholesterol and free fatty acids mainly palmitate (Veldhuizen, Nag et al. 1998).

The primary role of surfactant is to reduce surface tension at the air-water interface in the alveoli which subsequently increases lung compliance which is carried out by the phospholipid portion in surfactant (Veldhuizen, Nag et al. 1998). DPPC, a saturated phospholipid, is accountable for most of the surface tension lowering abilities of surfactant (McCormack 1998). DPPC is a phospholipid with two 16-carbon saturated chains and a phosphate group with a quaternary amine group attached. It also has higher compaction capacity than the other phospholipids, due to its non-polar fatty acids whose conformation is more uniform since they are fully saturated . However, even with this compaction capacity, without the other substances of the pulmonary surfactant mixture, the adsorption kinetics of DPPC is very slow. This happens primarily because the phase

transition temperature between gel to liquid crystal of pure DPPC is 41°C, which is higher than the body temperature (Schurch, Bachofen et al. 1992).

Surface tension is extremely important in normal lung function. As the alveoli increase in size with inspiration, the surfactant spreads over the surface of the liquid interface. This increases surface tension which slows the rate of expansion of the alveoli according to Laplaceôs law (P = 2T/r), the pressure (P) in a bubble is equal to 4 times the surface tension (T) divided by the radius (r) (Prange 2003). This also helps all alveoli in the lungs expand at the same rate because, if one alveolus expands more quickly it will experience a large rise in surface tension slowing its rate of expansion. It also means the rate of compression is more regular, because if one reduces in size more quickly the surface tension will be further reduced so that the other alveoli can contract more easily than it can. Pulmonary surfactant reduces surface tension more readily when the alveoli are compressed because the surfactant is more concentrated. Surface tension forces also draw fluid from capillaries to the alveolar spaces hence, reducing fluid accumulation and keeping the airways dry. Pulmonary surfactant also plays an immunological role primarily due to the presence of SP-A and SP-D. These proteins can bind to carbohydrate recognition sites on the surface of pathogens and thereby optimize them for phagocytosis. Surfactant also regulates inflammatory responses and interacts with the adaptive immune response. The degradation of pulmonary surfactant, or the inactivation, may contribute to susceptibility to lung inflammation and infection (Wright 2004)

1.6 Surfactant Proteins (SP-A, SP-B, SP-C, SP-D)

Four surfactant associated proteins have been identified; SP-A, SP-B, SP-C and SP-D. SP-B and SP-C are hydrophobic and of low molecular weight. SP-A and SP-D are hydrophilic proteins. The surfactant proteins reduce the critical temperature of DPPC's phase transition from 41°C to lower than 37°C. This improves the adsorption and spreading velocity of pulmonary surfactant. Compression of the air-liquid interface causes a phase change of the surfactant molecules to liquid-solid. By lowering the phase transition temperature to a level more consistent with that of body temperature, pulmonary surfactant remains in the liquid phase and the adsorption velocity necessary to maintain the integrity of the gas exchange region of the lungs is maintained. (Schurch, Bachofen et al. 1992; Hills 1999)

1.6.1 Surfactant Protein-A

SP-A has a highly ordered structure, and is an octadecameric molecule resembling a bouquet of tulips with a molecular weight of 36 kDa (McCormack 1998). SP-A gene transcription is highest just prior to birth and is initiated at approximately 29 weeks gestation.(Mendelson C.R. 1991). SP-A has been shown to inhibit the secretion of surfactant which mediates the uptake of lipid and is hence important in the regulation of surfactant turnover and metabolism (Ikegami and Jobe 1998). In addition to the regulation of secretion and uptake of surfactant by alveolar type II cells, SP-A also plays

an immunological role against pathogens in the lung (Dobbs, Wright et al. 1987). Because of its presence in other organs, it is thought that SP-A has a more general immunologic role. It binds to carbohydrate structures on a wide range of viruses, bacteria and fungi and may also play a role in modulating allergic reactions (Haagsman 1998).

1.6.2 Surfactant Protein-B

Surfactant protein-B (SP-B), has been found to be a homodimer made up of 79 amino acids and having a molecular weight of 8 kDa (Warr, Hawgood et al. 1987). This surfactant protein is found to be tightly associated with lipids in the alveolar space and plays a role in the structural rearrangement of secreted surfactant into tubular myelin and subsequently the fast insertion of these tubular structures into the lipid surface film. SP-B has a critical role in intracellular assembly of surfactant and is involved in membrane binding, fusion and lysis, lipid adsorption to the air-water interface, stabilization of surface films and re-spreading of these films during collapse phases in the breathing cycle (Hawgood, Derrick et al. 1998). In a report on deficiencies in surfactant proteins by Nogee, de Mello et al. (1993), it was found that deficiencies in surfactant proteins, in particular SP-B, are lethal.

1.6.3 Surfactant Protein-C

Pulmonary surfactant has been shown to contain less than 1% by weight of surfactant protein-C. SP-C is made up of 35 amino acid residues and has a molecular weight of 4.2 kDa and plays a role in membrane packing (Johansson 1998). It was found that individuals with reduced SP-C levels suffered from interstitial pulmonary fibrosis (Nogee, Dunbar et al. 2001)

1.6.4 Surfactant Protein-D

Surfactant proteinï D differs from the other surfactant associated proteins in that it is not processed with the phospholipids comprising surfactant and is not found in lamellar bodies or in tubular myelin (Mason and Voelker 1998). SP-D is usually found as a dodecamer with a molecular weight of 43 kDa (Crouch 1998). This surfactant protein is thought to play an immunological role against pathogens as well as having a role in surfactant recycling (Johansson and Curstedt 1997). SP-D, similar, to SP-A, plays an important role in innate immunity by binding to carbohydrate domains on various bacteria, viruses and fungi and is also found in other organs. SP-D may also play a role in the modulation of allergic reactions by binding to certain glycosylated allergens (Reid 1998).

1.7 *Stachybotrys chartarum*

Stachybotrys chartarum is a saprophyte which grows on wet cellulose-containing building materials such as wall board and ceiling tiles and is often found in the flora of mould growing in water damaged buildings (Kuhn, Trimble et al. 2005). The capacity of this mould to produce potent mycotoxins and its previous association with animal mycotoxicosis has resulted in *Stachybotrys chartarum* being commonly referred to as the toxic black mould. Two chemotypes of *Stachybotrys chartarum* exist. One chemotype produces macrocyclic trichothecenes which are potent mycotoxins. A second, chemotype, produces less toxic atranones and simple trichothecenes (Andersen, Nielsen et al. 2002).

1.7.1 Brief History

The effects of *Stachybotrys chartarum* exposure can be traced back to events in 1931 where exposure effected horses in the Ukraine which were fed black mould contaminated hay (Drobotoko 1945). In 1993-1994 a disturbing cluster of ten infants in Cleveland, Ohio were admitted to hospital with unexplained pulmonary hemosiderosis, a rare occurrence in infants. One of the infants died from the illness. Upon discharge many of the infants displayed a re-submergence of symptoms. A case-control study was conducted identifying an agent-host interaction associated with water damaged homes as an environmental risk factor and probable cause of the idiopathic pulmonary

hemosiderosis (Montana, Etzel et al. 1997). Etzel, Montana et al. (1998), delved further into the Cleveland, Ohio cases. They found no differences between patients and controls with respect to exposures to household pesticides, infant care products, or cocaine and ruled out all other known causes of pulmonary haemorrhage including coagulopathies and abuse. Investigators found haemolysis on peripheral blood smears leading them to consider that the infants had been exposed to toxins produced by indoor moulds. Their hypothesis was that infants with pulmonary haemorrhage were more likely than controls to live in homes where *Stachybotrys chartarum* was growing due to water damage.

1.7.2 Toxins

Macrocyclic trichothecenes are the most potent small molecule protein synthesis inhibitors known and are considered the most acutely toxic mycotoxin (Jarvis 2003). Trichothecenes produced by *S. chartarum* isolates include satratoxins F, G and H, roridins and verrucarins (Miller, Rand et al. 2003). Trichothecenes directly inhibit either initiation, elongation or termination of protein synthesis. Satratoxin G, produced by *Stachybotrys chartarum*, was reported to be the most toxic of trichothecenes (Yang, Jarvis et al. 2000). The satratoxins are potent inhibitors of protein translation and initiate both inflammatory gene expression and apoptosis *in vitro* (Chung, Jarvis et al. 2003). They are water-soluble mycotoxins found in the outer plasmalemma surface and the inner wall layers of spores (Gregory, Pestka et al. 2004) as well as in nonviable airborne particulates (Brasel, Douglas et al. 2005). Pulmonary toxicity of the spores of *S. chartarum* and associated trichothecenes has been demonstrated in animal studies using

intranasally or intratracheally exposed laboratory rodents (Rand, Mahoney et al. 2002; Yike, Miller et al. 2002; Rand, White et al. 2003; Yike and Dearborn 2004).

Atranones are a secondary mycotoxin family which can contribute to adverse health effects. Seven atranones, A through G have been isolated from *Stachybotrys chartarum* isolates. Cytotoxity, due to fungus exposure, is associated with satratoxins whereas inflammation is due to atranone production (Nielsen, Huttunen et al. 2002).

The major class of secondary metabolites produced by *S. chartarum* are the spirocyclic drimanes (Jarvis, Salemme et al. 1995). This fungus produces up to forty different spirocyclic drimanes. These compounds can be produced in large quantities and have a broad spectrum of biological activities including inhibition of proteolytic enzymes, disruption of the complement system, inhibition of TNF-Ŭrelease, endothelin receptor antagonism, stimulation of plasminogen, fibrinolysis, thrombolysis as well as cytotoxic and neurotoxic effects. Their role in the pathology of *S. chartarum* exposure is yet to be determined (Andersen, Nielsen et al. 2002).

Stachylysin, a haemolysin which causes the lysis of red blood cells, has been isolated from the fungus as well. Stachylysin localizes within the inner cell wall of *Stachybotrys chartarum* spores (Pestka, Yike et al. 2008). Stachylysin may also serve as a biomarker for acute exposure to *S. chartarum* (Yike and Dearborn 2004).

Fungal proteinases induce inflammation. The types and quantities of proteinases vary in isolates of *S. chartarum.* The most common protease is stachyrase A, a serine protease which can hydrolyse several collagens, proteinase inhibitors, and several neuropeptides (Kordula, Banbula et al. 2002). *Stachybotrys chartarum* proteinases contribute to degradation of the extracellular matrix proteins which may play a role in the induction of pulmonary haemorrhage in young infants (Pestka, Yike et al. 2008).

(1-3)-ɓ-D-Glucan is a cell wall component common in fungi including *Stachybotrys chartarum*. It is linked to the development of inflammatory reactions and has been shown to exacerbate inflammatory effects in the upper respiratory tract (Beijer, Thorn et al. 2002).

1.7.3 Sick Building Syndrome

Sick Building Syndrome, also known as Damp building-related illness, is an illness comprised of various respiratory, immunologic and neurologic symptoms which are linked to the indoor growth of *Stachybotrys chartarum.* Most notably, symptoms include allergic sensitivity, inflammation and cytotoxicity in the upper and lower respiratory tracts (Pestka, Yike et al. 2008). Specific respiratory symptoms reported in patients due to exposure to *S. chartarum* include rhinitis, asthma, alveolitis, bronchiectasis, fibrosis and hemosiderosis (Hossain, Ahmed et al. 2004). Sick Building Syndrome is commonly seen in young infants and children.

Chapter Two

Experimental Procedures

2.0 Hypothesis

Exposure to the extracts produced by spores of *Stachybotrys chartarum* causes deleterious effects on fetal lung epithelial cells and their ability to produce sufficient surfactant of good quality.

2.1 Objective

The objective of this study was to analyze the effects of *Stachybotrys chartarum* extract exposure on fetal rat lung epithelial cells, their expression of surfactant proteins SP-A and SP-B and both the quality and quantity of surfactant produced.

2.2 Materials

Random bred Sprague-Dawley rats gestational age day 21, were obtained from Central Animal Care Services at the University of Manitoba. All animals were cared for and treated according to approved protocols through the Canadian Council of Animal Care and their local representative agencies. Reagents used in cell culture including media, Hankôs Balanced Salt Solution, antibiotics, fungizone and newborn calf serum, were obtained from Life Technologies-Gibco/BRL (Burlington, Ontario). MTT assay kit

and various chemicals used were obtained from Sigma-Aldrich (St. Louis, Missouri). Plastic tissue culture flasks were obtained from Fisher Scientific (Nepean, Ontario). Western blotting reagents, formazan assay kit, Snap I.D, and Mini-PROTEAN precast gels obtained from Biorad Laboratories (Mississauga, Ontario). Antibodies (primary; SP-A (c-20) and SP-B (m-19), secondary; donkey anti-goat IgG) were obtained from Santa Cruz (California, U.S.A.). Chemiluminescence reagents used in chemiluminescent detection of proteins on immunoblots as well as the hyperfilm used to develop blots were obtained from GE Lifescience (Oakville, Ontario). Human A549 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA, U.S.A). *Stachybotrys chartarum* cultures were obtained from Dr. Tom Rand (St. Maryôs university, Halifax).

2.3 Methods

2.3.1 Primary Cell Culture

Cultures of fetal rat lung fibroblasts and type II cells was completed following the protocol currently active in the laboratory. Pregnant Sprague Dawley rats were euthanized at gestational day 21 via an intraperitoneal injection of 1.5 ml Euthanyl (240mg/ml sodium pentobarbital). An abdominal incision was employed whereby fetuses were removed, decapitated and placed in cold, sterile HBSS. Lungs were removed, chopped using a razor blade and incubated and stirred on a metallic stirring plate in a solution of 10ml trypsin-EDTA (0.05%) and 90ml HBSS in a water-jacketed trypsinization flask at 37°C for 45 minutes. Trypsinization was stopped by the addition of

30ml MEM/NCS and the solution was filtered through three layers of nitex gauze. The filtered solution was centrifuged at 1000 RPM for 10 minutes. The pellet was collected and resuspended in MEM/SNCS and separated into five large culture flasks (75 cm^2) and incubated for 1 hour. Media from the flasks which now contains unattached type II cells was poured off and separated into twenty 25cm^2 culture flasks. Fresh MEM/NCS with 1% antibiotics and 1% antifungal was added to the 75 cm^2 flasks containing fibroblasts and MEM/SNCS with 1% antibiotics and 1% antifungal was added to the flasks containing the type II alveolar cells. Media was changed 24 hours later in all flasks and every 48 hours afterwards until confluence. After 5-7 days most cultures of fibroblasts reached confluence and were passaged at a ratio of 1:3 by first washing the cells with HBSS and incubating in a solution of 0.5 ml trypsin and 4.5ml HBSS until cells detached from the culture flask. After the third passage, fibroblasts were allowed to reach confluence and were treated with *Stachybotrys chartarum* spore extracts. After 7-10 days, type II alveolar cells reached confluence and were subsequently treated with *Stachybotrys chartarum* spore extracts. (McCrae, Rand et al. 2007)

2.3.2 A549 Cell Culture

Human A549 cells were obtained from American Type Culture Collection. Cells were thawed and resuspended in media/NCS in one 25cm^2 flask overnight. Media were changed after 24 hours and subsequently at 48 hour intervals. After 1-3 days cells reached confluence and were passaged in a ratio of 2:1.

2.3.3 Stachybotrys chartarum Culture

Stachybotrys chartarum spores were cultured for 4-6 weeks at room temperature on cornmeal agar extract as previously employed in the lab with the addition of Whatman filter paper to provide a better substrate for spore growth. Spores were collected following the procedure outlined in Mason, Rand et al. (2001) as well as, McCrae, Rand et al. (2007). Isolates were flooded with saline solution and agitated with a heat sterilized Pasteur pipette. Spores were collected and washed in saline solution three times in centrifuge tubes at 750g and re-suspended in saline at a concentration of 1×10^6 spores/ml. Spores were left in the solution overnight to secrete their toxins and were separated by centrifugation at 750g to collect the supernatant which was filtered with Whatman paper to remove any remaining spores. Supernatant containing *S. chartarum* extracts was used in experimental treatments.

2.3.4 Treatment with Stachybotrys chartarum Extracts

Cells were treated with spore extracts as outlined in McCrae, Rand et al. (2007). Spore supernatant was diluted with MEM in a ratio of 20 ml supernatant to 70 ml MEM without addition of antibiotics or antifungals. Controls were treated with a solution of HBSS diluted with MEM, in the same ratio, also without antibiotics or antifungals. Flasks were selected randomly for treatment with n=5 for each treatment as well as controls. Flasks were incubated for a 24 hour period with 4.5 ml of treatment MEM. Treatment was stopped by pouring off media and washing the cells with HBSS. Cells

were treated with 10^{-6} molar phorbol ester (phorbol 12-myristate 13-acetate) for 3-4 hours to stimulate surfactant secretion. Supernatant was collected and cells were scraped from the bottoms of flasks for surfactant protein analysis. Supernatant was centrifuged for 18 hours at 100,000g to separate surfactant (McCrae, Rand et al. 2001).

2.3.5 Protein Assay

Protein concentrations were determined from collected cell samples using the Bradford method (Bradford 1976). The assay was carried out using the BioRad DC Protein Assay kit which is based on the coomasie dye-binding protocol. Absorbances were measured at 595nm using a Beckman DU series 640 spectrophotometer. Bovine serum albumin was used as the protein standard for all assays. Results of protein assays were utilized to calculate dilutions of cell samples required for subsequent protein electrophoresis and Western Blotting analysis.

2.3.6 SDS-Page: Protein Electrophoresis

Sample proteins from lyophilized cell fractions were size fractioned via Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The Mini-PROTEAN tetra cell electrophoresis apparatus and 10-12% mini-PROTEAN pre-cast gels, both obtained from Biorad Laboratories (Mississauga, Ontario), were used for all applications. Kaleidoscope polypeptide standards (Biorad Laboratories, Mississauga,

Ontario), were run in the first well of the pre-cast gels. Protein samples were prepared in a ratio of 1 part sample to 4 parts sample buffer (1.0M Tris-HCL, pH 6.8, 8% (w/v) SDS, 45% (w/v) glycerol, 2.86 M ϐ-mercaptoethanol, 0.02% (w/v) bromophenol blue). Sample buffer interacts with the proteins forming a complex which results in a similar charge to mass ratio. Samples were heated at 95°C for 5-10 minutes prior to electrophoresis to eliminate secondary and tertiary protein structures so that proteins were only separated based on size. Gels were run for 30-45 minutes at 200 volts until the dye line reached the green line at the bottom of the pre-cast gels. Electrode running buffer utilized was composed of 25mM Tris, 192mM Glycine and 0.1%(w/v) SDS.

2.3.7 Western Blotting

Sample proteins fractioned via SDS-Page, were transferred to a nitrocellulose membrane at 4°C at 25 volts overnight or at 100 volts for 1 hour. Transfer buffer utilized was composed of 25mM Tris, 192mM Glycine, 0.05% SDS and 20% methanol. After transfer, nitrocellulose membranes were washed in Tris-buffered saline (20mM Tris, 500mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBST) and immunostained using the Snap I.D. system (Biorad Laboratories, Mississauga, Ontario).

2.3.8 Immunostaining

Blot immunostaining procedures were outlined in the Snap I.D. system protocol. Blots were blocked with TBST including 3% skim milk and the solution was immediately removed via the systemôs vacuum drawing the solution through the blot. 3ml of primary antibody diluted in TBST wash buffer in a concentration of 1:200 was spread over the blot. After a 20 minute incubation, blots were washed with TBST three times without incubation. Washes were drawn through the blot via the snap I.D. system vacuum. Three ml of secondary antibody diluted in TBST wash buffer at a concentration of 1:1000 was spread over the blot. After a 20 minute incubation, blots were washed with TBST three times without incubation utilizing the vacuum system. To detect the proteins of interest, chemiluminescence techniques were used with ECL reagents as specified by the manufacturer. Pictures were captured on Kodak Hyperfilm ECL High Performance Chemiluminescence film which was exposed for 30 seconds to 5 minutes.

2.3.9 MTT Formazan Cell Proliferation Assay

MTT formazan assay kit was obtained from Sigma-Aldrich and assay was carried out according to the instructions provided. Cells were incubated in 96 well plates until confluent. The first three columns of the 96 well plate were blanks and contained only media/NCS. Columns 4-6 were controls and were treated with media containing saline. Columns 7-12 were treated with media containing *Stachybotrys chartarum* extracts.

Cells were treated over night. Media was removed and each well was washed with HBSS. MTT solution equal to 10% of original culture volume was added to each well and incubated for 3-4 hours allowing the solution to form purple crystals via interactions with mitochondrial dehydrogenase in active cells. After incubation, MTT solvent in an amount equal to the original culture volume was added to the MTT solution, and agitated to dissolve any crystals which formed. Absorbances were read with in 1 hour of application of solvent solution at 570nm and background absorbance at 690nm was subtracted.

2.3.10 Transmission Electron Microscopy

For electron microscopy, cultured cells were treated with trypsin and collected by centrifugation into pellets. The cell pellets were fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.3), followed by post-fixation in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.3). Cells were dehydrated and embedded in Epon 812 using standard techniques (Luft 1961). Thin sections were stained with uranyl acetate and lead citrate, viewed and photographed in a Philips CM 10 electron microscope. In order to eliminate observer bias, sections were examined without foreknowledge of their source.

2.3.11 Phosphorous Assay

Surfactant amount was determined using the Bligh and Dyer Chloroform Methanol Extraction method (Bligh and Dyer 1959) followed by analysis via phosphorous assay. After treatment with either control or *Stachybotrys chartarum* extract media, cells were washed with HBSS. Phorbol ester (phorbol 12-myristate 13-acetate) 10^{-6} molar was applied to surviving cells in order to induce surfactant secretion. After a four hour incubation period, supernatant containing secreted surfactant was collected.

Collected surfactant was centrifuged 16-18 hours at 27,200 rpm using a 60 Ti rotor. The pellet was resuspended in one ml double distilled water and underwent chloroform/methanol extraction to separate surfactant phospholipids from cellular membrane components. Using three mls of a solution of chloroform/methanol (1/2), the sample was brought to a single phase system. The addition of 0.8mls of 1% KCL ensures the sample was split into two phases with water soluble material in the top phase and organic material and phospholipids in the bottom phase. The bottom phase was collected and dried under air at 60ŭC. The sample was resuspended in a small amount of chloroform/methanol (20/1) solution to be used in phosphorous assay.

Lipid samples were transferred into clean phosphorous free glass tubes and the solvent completely evaporated. Perchloric acid (0.65ml) was added and the tubes placed in a heated block for 30 minutes until the yellow color had disappeared. When cool, 3.3mls of water was added to each tube as well as 0.5ml of ammonium molybdate

solution and 0.5ml of ascorbic acid solution. Tubes were agitated on a vortex after each addition. The tubes were placed in a boiling water bath for 5 min to develop a blue colour. The absorbance of samples including the standards, were read at 800 nm. Standards (1 to 5 µg P/tube) were diluted in 3.3mls water and 0.65 mls perchloric acid. Classically, 5 µg P gives an absorbance of 0.9. The amount of phospholipids were calculated directly on a molar basis from the amount of Phosphorous and on a weight basis after multiplying the amount of Phosphorous by 25 (Bartlett 1959).

2.3.12 Captive Bubble Surfactometry

The Captive Bubble Surfactometer (CBS) was used to analyze the quality of collected surfactant samples based on surface tension measurements. This system offers a leak proof environment as there are no interruptions by barriers or outlets. An air bubble was created in the chamber via the CBS crank mechanism. This bubble floats against a hydrophilic roof of 1% agarose gel. At the air-agarose interface, a thin layer of water prevents adhesion of the bubble. Bubble volume is controlled by a pressure tight piston which varies the pressure within the bubble chamber. 74 ug of surfactant (the lowest amount of surfactant detected in all samples) was injected into the chamber for each experiment. Using quasistatic cycles, the bubble undergoes compression (reducing bubble size and surface tension) and expansion (increasing bubble size and surface tension). The bubble undergoes a series of 20 dynamic cycles which are rapid expansion and compression cycles. Prior to cycling, the bubble was expanded to a volume of 0.15ml. From video images, surface tension, area and volume were calculated from the

ratio of the height and diameter of the bubble and dynamic surface tension area plots were obtained. (Schoel, Schurch et al. 1994)

2.3.13 Statistical Analysis

Analysis of variance (ANOVA) was employed to test treatment means against control means. An alpha level $p<0.05$ was used to determine statistical significance.

Chapter Three

Results

3.1 Cell Viability

Cell Viability was measured using an MTT Formazan assay. Cells were incubated in 96 well plates until confluent. Controls were treated with media containing saline. Treatment cells received media containing *Stachybotrys chartarum* extracts secreted into a saline suspension of 1×10^6 spores/ml. Each well received MTT solution equal to 10% of original culture volume and were incubated for 3-4 hours. After incubation, MTT solvent was added in an amount equal to the original culture volume and agitated to dissolve any crystals which formed. Absorbances were read within 1 hour of application of the solvent solution at 570nm and background absorbance at 690nm was subtracted.

Figure 1 shows the difference in mean absorbance at 570nm between control and treatment groups of all three cell lines; fetal rat lung fibroblasts, alveolar type II and human A549 cells. Significant decreases in cell viability were noted ($p<0.05$) in all three cell lines; human A549, fetal rat lung alveolar type II and fibroblasts, treated with *Stachybotrys chartarum* extract media as compared to controls.

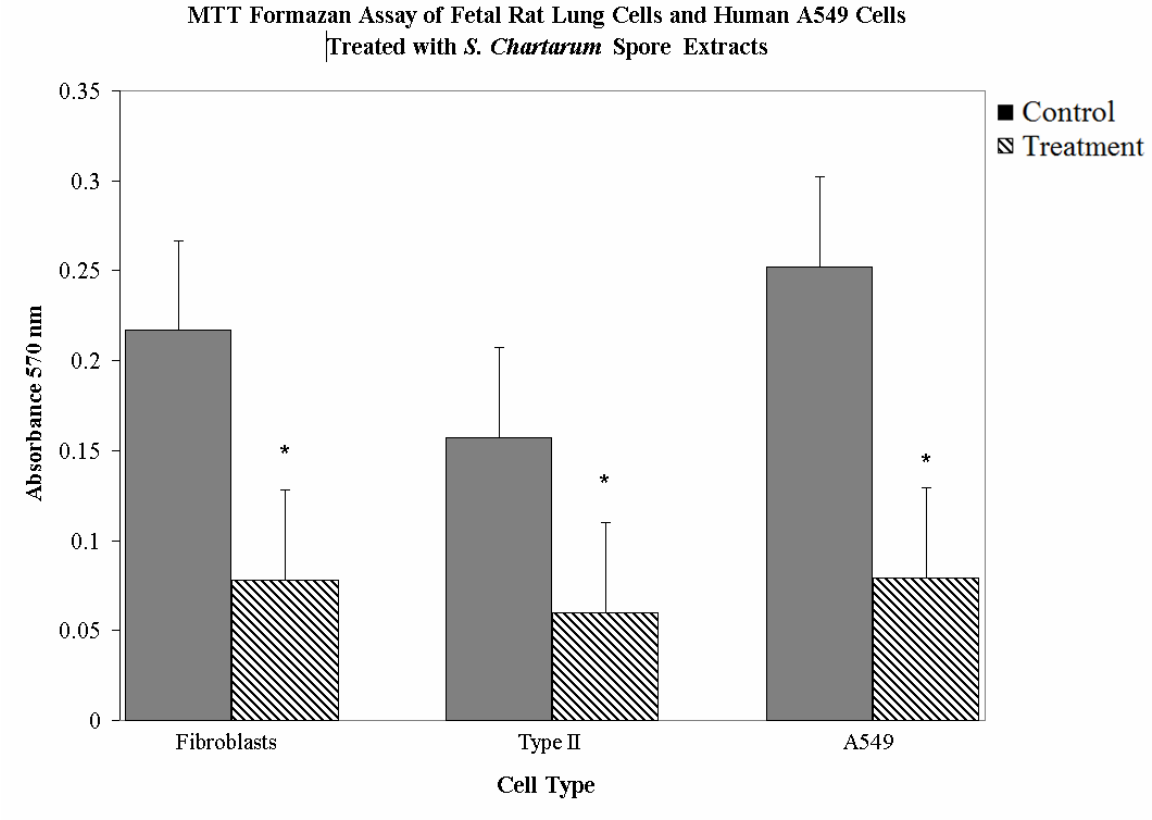


Figure 1: Effect of *Stachybotrys chartarum* extracts on cell viability
MTT Formazan assays were undertaken to determine cell viability when cells were exposed to media containing extract from *Stachybotrys Chartarum* spores as compared to controls. Cells not exposed to spore extracts were considered controls. (*) indicates a significant difference ($p<0.05$) in treatment results as compared to controls. Each bar represents the mean ± SEM of five experiments.

3.2 Surfactant Production

Surfactant amount was determined using the Bligh and Dyer Chloroform Methanol Extraction method followed by analysis via phosphorous assay.

Figure 2 demonstrates the significant differences observed between control and treatment groups in terms of secretion of surfactant. Significant decreases in surfactant secretion were observed in both the A549 and type II cell lines treated with *Stachybotrys chartarum* extract media when compared to controls. The treatment fibroblasts by contrast, showed a significant increase in surfactant secretion. In fact, the treatment group of fetal rat lung fibroblasts produced more than double the amount of phospholipids as detected via phosphorous assay.

Tables 1-3 show the comparisons of expected surfactant secretion based on cell counts for fetal rat lung fibroblasts, type II alveolar and human a549 cells respectively When cell counts were taken into consideration, treatment groups showed a significant increase ($p<0.05$) in surfactant secretion as compared to expected values in all three cell lines. .

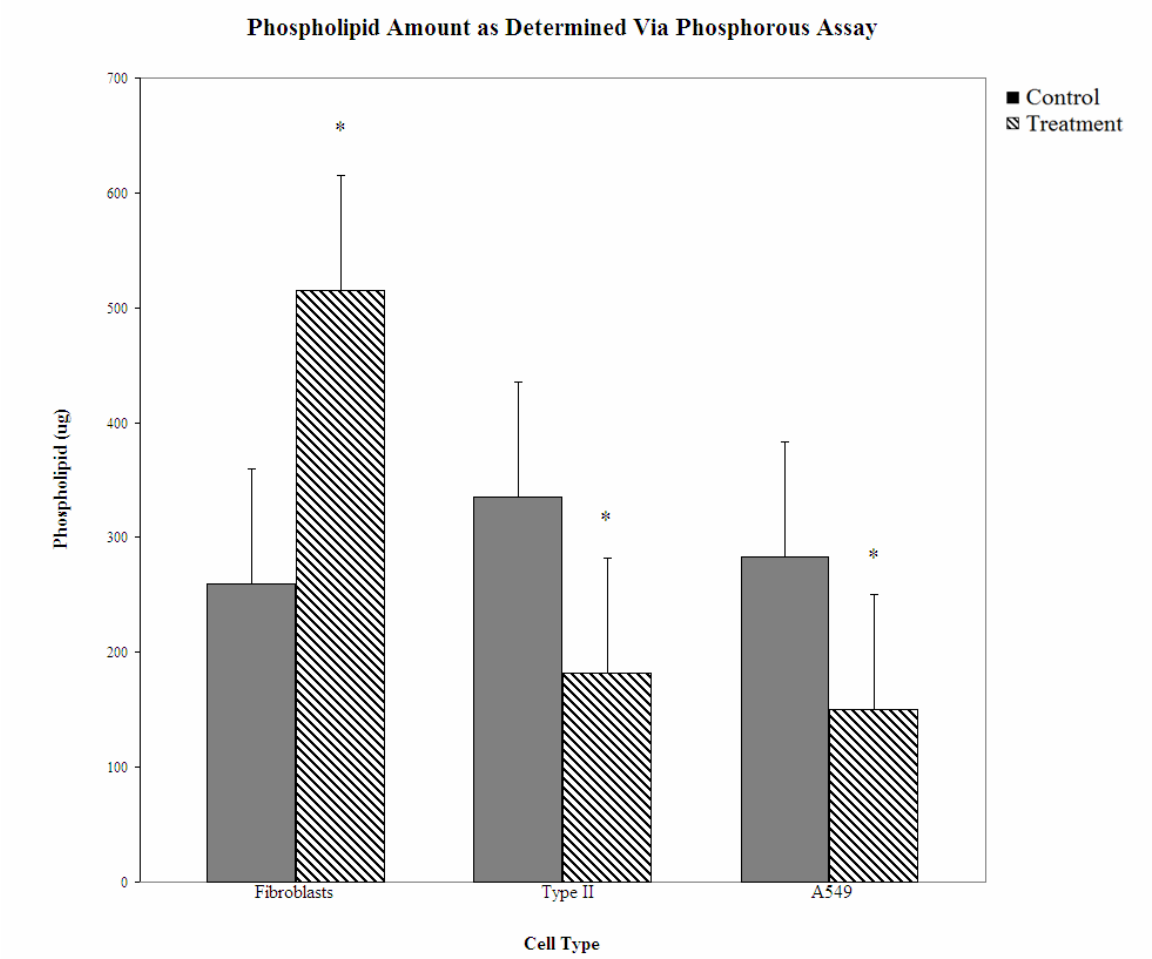


Figure 2: Effect of *Stachybotrys chartarum* Extracts on Surfactant Production
Phosphorous assays were employed after extraction of pulmonary surfactant via Bligh and Dyer chloroform-methanol extraction method to determine the amount of surfactant secreted by cell lines. Cells not exposed to spore extracts were considered controls. (*) indicates a significant difference ($p<0.05$) in treatment results as compared to controls. Type II and A549 cell lines showed significant decreases in surfactant production while the fibroblast treatment groups showed a significant increase in secretion of surfactant. Each bar represents the mean ± SEM of five experiments.

Phospholipid Secreted by Fetal Rat Lung Fibroblast Cells (ug)			
Sample	**Control**	**Treatment**	**Expected Secretion based on Treatment Cell Counts**
1	253.11	369.13	84.37
2	281.63	424.09	93.88
3	182.14	645.99	60.71
4	319.93	622.78	106.64
5	259.20	515.50	86.40
Average phospholipid secreted (ug)	259.20	515.49	86.4
Standard Deviation	50.4147	120.7442	16.80554
Cell Count / flask (avg.)	1.5×10^7	6×10^6	n/a
Standard Deviation	5.5×10^5	4.0×10^5	n/a

Table 1: Phospholipid amounts detected as compared to projected amounts for fetal rat lung fibroblast cells. Phospholipids collected and analysed from samples of fetal rat lung fibroblast cells (n=5) showed increased phospholipid secretion in treatment cells as compared to controls. When average cell counts were used to determine expected phospholipid secretion values, treatment cells showed a significant increase ($p<0.05$) in phospholipids secretion as compared to expected values. Formula used in calculations:

$$\frac{\text{Average Cells in Control Sample (Cells/flask x 5 flasks/sample)}}{\text{Phospholipids produced By Control Sample}} = \frac{\text{Average Surviving Cells in Treatment Sample (Cells/flask x 5 flasks/sample)}}{\text{Expected amount of phospholipids secreted by surviving cells.}}$$

Phospholipid Secreted by Fetal Rat Lung Alveolar Type II Cells (ug)			
Sample	**Control**	**Treatment**	**Expected Secretion based on Treatment Cell Counts**
1	313.47	189.75	94.04
2	296.09	164.02	88.827
3	549.03	297.56	164.71
4	218.205	141.54	65.46
5	301.28	118.25	60.472
Average phospholipid secreted (ug)	335.615	182.224	94.7018
Standard Deviation	155.72	52.95	46.72
Cell Count / flask (avg.)	3.9×10^6	1.2×10^6	n/a
Standard Deviation	2.4×10^6	9.5×10^5	n/a

Table 2: Phospholipid amounts detected as compared to projected amounts for fetal rat lung alveolar type II cells. Phospholipids collected and analysed from samples of fetal rat lung alveolar type II cells (n=5) showed increased phospholipid secretion in treatment cells as compared to controls. When average cell counts were used to determine expected phospholipid secretion values, treatment cells showed a significant increase ($p<0.05$) in phospholipids secretion as compared to expected values. Formula used in calculations:

$$\frac{\text{Average Cells in Control Sample (Cells/flask x 5 flasks/sample)}}{\text{Phospholipids produced By Control Sample}} = \frac{\text{Average Surviving Cells in Treatment Sample (Cells/flask x 5 flasks/sample)}}{\text{Expected amount of phospholipids secreted by surviving cells.}}$$

Phospholipid Secreted by Human A549 Cells (ug)			
Sample	**Control**	**Treatment**	**Expected Secretion based on Treatment Cell Counts**
1	142.445	73.44	39.57
2	267.51	132.56	74.31
3	272.53	138.84	75.703
4	378.56	220.11	105.16
5	356.22	188.22	98.95
Average phospholipid secreted (ug)	283.453	150.634	78.7386
Standard Deviation	93.0015	60.8482	25.8341
Cell Count / flask (avg.)	7.1×10^6	2.0×10^6	n/a
Standard Deviation	9.1×10^5	2.9×10^5	n/a

Table 3: Phospholipid amounts detected as compared to projected amounts for human A549 cells. Phospholipids collected and analysed from samples of human A549 cells (n=5) showed increased phospholipid secretion in treatment cells as compared to controls. When average cell counts were used to determine expected phospholipid secretion values, treatment cells showed a significant increase ($p<0.05$) in phospholipids secretion as compared to expected values. Formula used in calculations:

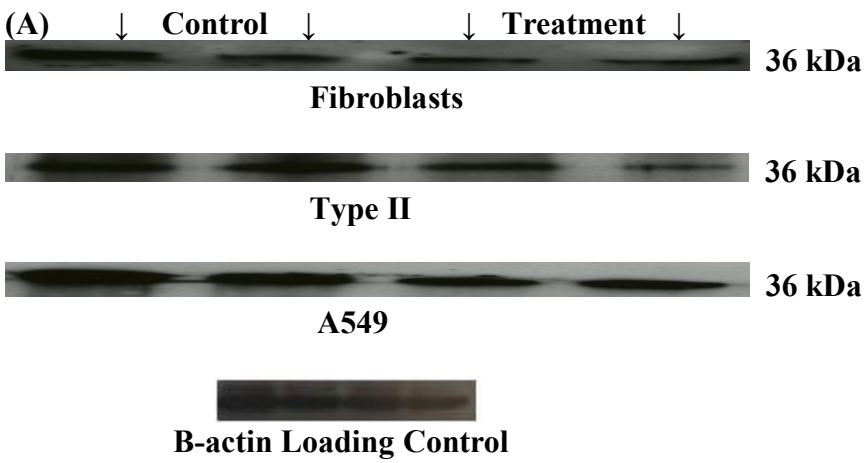
$$\frac{\text{Average Cells in Control Sample (Cells/flask x 5 flasks/sample)}}{\text{Phospholipids produced By Control Sample}} = \frac{\text{Average Surviving Cells in Treatment Sample (Cells/flask x 5 flasks/sample)}}{\text{Expected amount of phospholipids secreted by surviving cells.}}$$

3.3 Surfactant Protein Expression

Expression of surfactant protein A and surfactant protein B was determined using SDS Page and Western Blot analysis. Antibody specific to each surfactant protein produced bands corresponding to their respective molecular mass. Bands were measured using densitometric analysis. ϐ-actin was used as loading control.

3.3.1 SP-A

Figure 3 demonstrates resulting bands from Western Blot analysis of cell lysates using antibody specific to SP-A as well as the densitometric analysis of band intensity. Antibody specific to SP-A bound to protein forming bands corresponding to the accepted molecular mass for SP-A of 36 kDa. Significant decreases ($p<0.05$), in the expression of SP-A were seen in all three treatment groups; fetal rat lung fibroblasts, alveolar type II and human A549 cells. There were also significant differences ($p<0.05$) in the amount of SP-A expressed by fibroblast control samples compared to fetal rat lung alveolar type II cells and human A549 control cells. Fibroblast cells expressed far less SP-A as compared to the other cell lines.



(B)

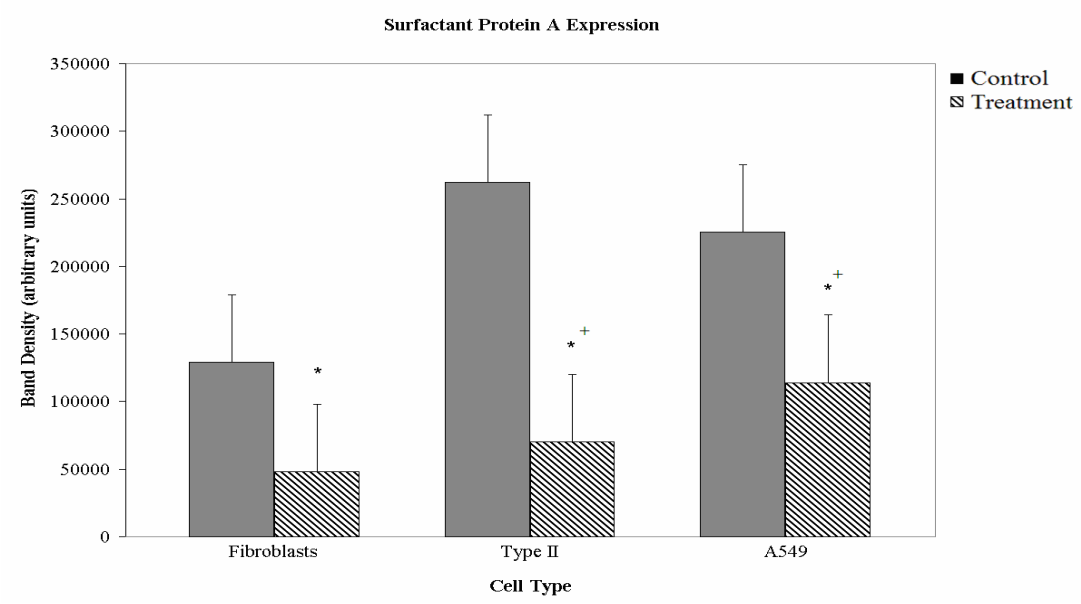
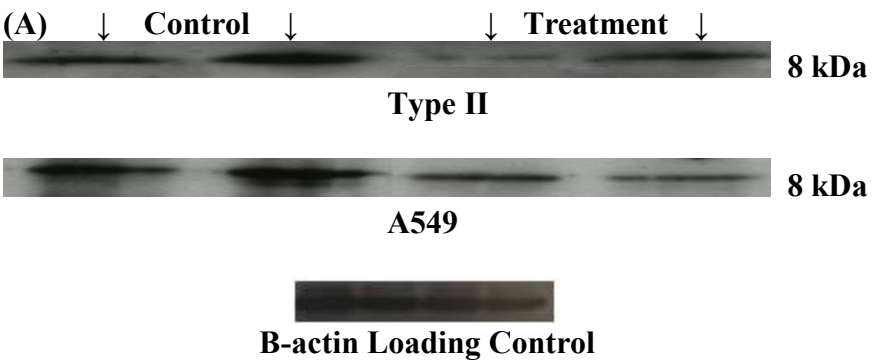


Figure 3: Effect of *Stachybotrys chartarum* extracts on the expression of surfactant protein-A. SDS page, followed by Western Blotting produced visible bands corresponding to a molecular weight of 36 kDa. Bands were measured using densitometric analysis. Cells not exposed to spore extracts were considered controls. (A) Western Blot Analysis: Lanes 1 and 2 are control cell lysates. Lanes 3 and 4 represent cell Lysates exposed to spore extracts. (B) Densitometric analysis of band intensity following Western Blotting. (*) indicates a significant difference ($p<0.05$) in treatment results as compared to controls. (+) indicates a significant difference ($p<0.05$) between fibroblasts other cell lines. Each bar represents the mean ± SEM of five experiments.

3.3.2 SP-B

Figure 4 demonstrates bands obtained from Western Blot analysis of cell lysates using antibody specific to SP-B as well as the densitometric analysis of band intensity.

Antibody specific to SP-B bound to protein bands corresponding to the accepted molecular mass for SP-B of 8 kDa. Significant decreases in the expression of SP-B were seen in fetal rat lung alveolar type II and human A549 cell lines. SP-B did not appear to be expressed in fibroblast cell lines.



(B)

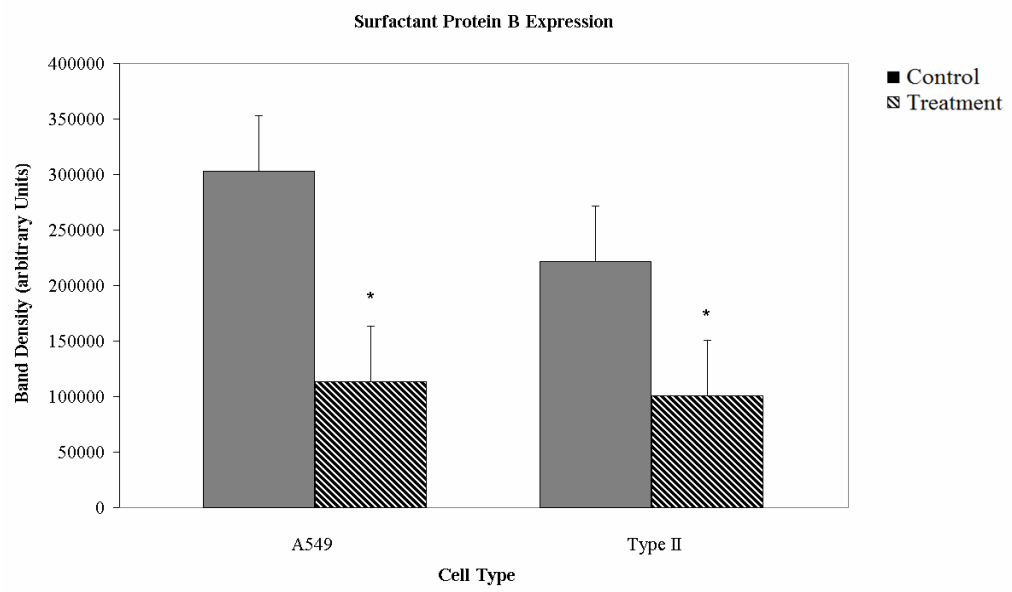


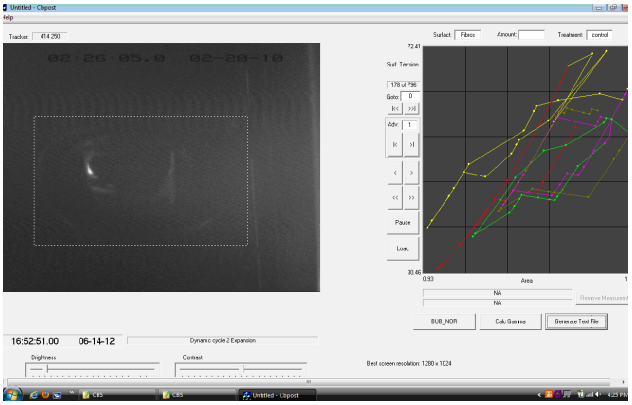
Figure 4: Effect of *Stachybotrys chartarum* extracts on the expression of surfactant protein-B. SDS page, followed by Western Blotting produced visible bands corresponding to a molecular weight of 8 kDa. Bands were measured using densitometric analysis. Cells not exposed to spore extracts were considered controls. (A) Western Blot Analysis: Lanes 1 and 2 are control cell lysates. Lanes 3 and 4 represent cell Lysates exposed to spore extracts. (B) Densitometric analysis of band intensity following Western Blotting (*) indicates a significant difference ($p<0.05$) in treatment results as compared to controls. Each bar represents the mean ± SEM of five experiments.

3.4 Captive Bubble Surfactometry

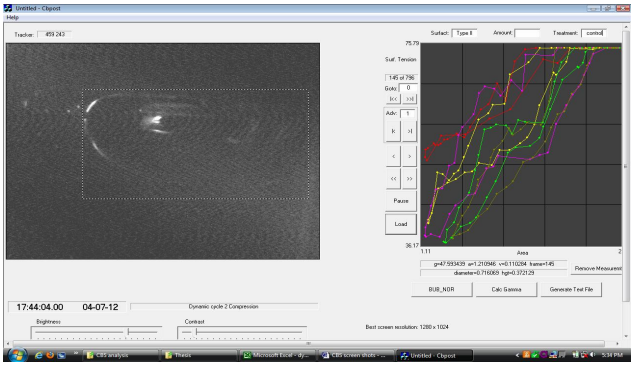
The captive bubble surfactometer (CBS) was used to analyze the quality of collected surfactant samples based on surface tension measurements. In the CBS, an air bubble floats against a hydrophilic roof of 1% agarose gel. The lowest amount of phospholipid detected in all samples (73 ug) was injected into the chamber for each experiment after forming the bubble. Bubble volume was controlled by varying pressure within the chamber. Using quasi static cycles, the bubble undergoes compression (reducing bubble size and surface tension) and expansion (increasing bubble size and surface tension). The bubble undergoes a series of 20 dynamic cycles which are rapid expansion and compression cycles. From video images, surface tension, area and volume were calculated from the ratio of height and diameter of the bubble and dynamic surface tension area plots were obtained.

Figures 5 and 6 show the captive bubble screen display as surface tension measurements were obtained for surfactant from all three cells lines with the automatic graph constructed by the automated computer program.

(A)



(B)



(C)

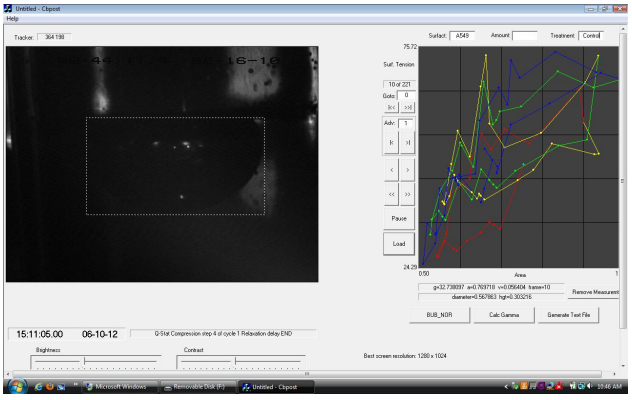
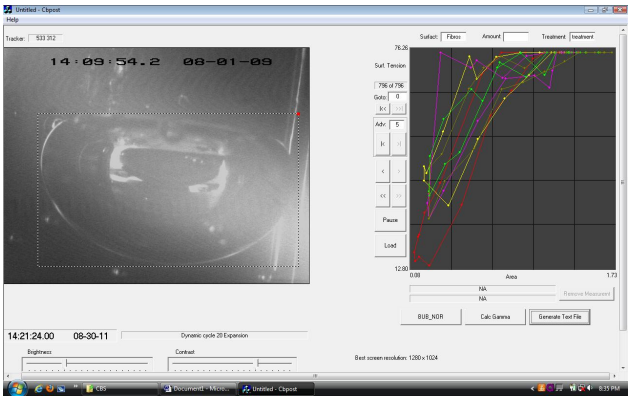


Figure 5: Captive Bubble Surfactometry: Control Groups
Screen shots of captive bubble surfactometry showing typical loops of compression and expansion cycles during control group surfactant analysis. (A) Fetal rat lung fibroblast cell line (B) Fetal rat lung alveolar type II cell line (C) Human A549 cell line

(A)



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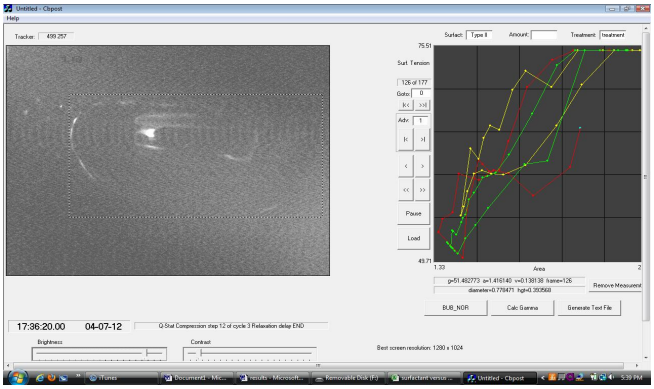


(C)

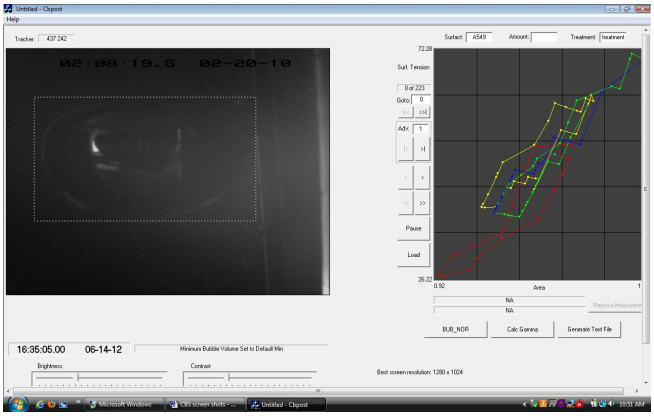


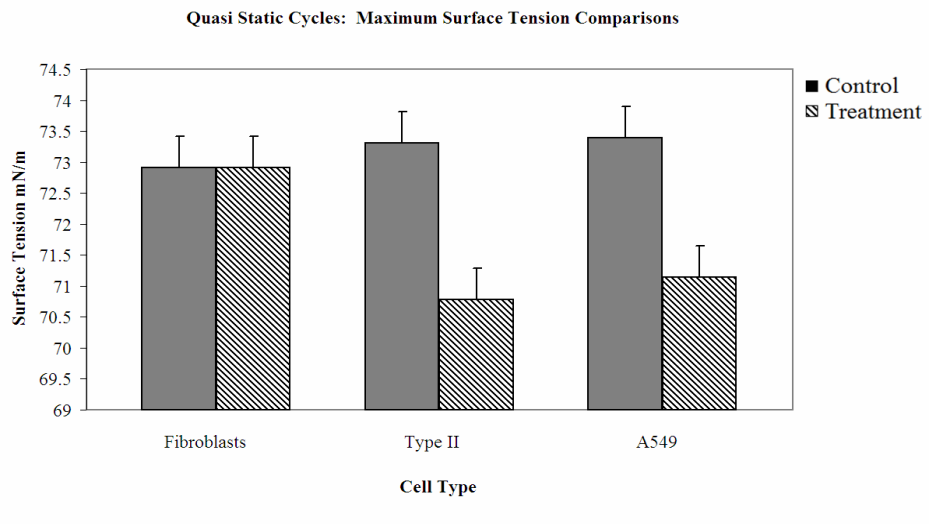
Figure 6: Captive Bubble Surfactometry: Treatment Groups
Screen shots of captive bubble surfactometry showing typical loops of compression and expansion cycles during surfactant analysisfrom cells exposed to *S. chartarum* extracts. (A) Fetal rat lung fibroblast cell line (B) Fetal rat lung alveolar type II cell line (C) Human A549 cell line.

3.4.1 Surface Tension Measurements

In quasi static cycles, maximum surface tension measurements were found to be very similar between control and treatment groups in surfactant isolated from all three cell lines. In fact, there were no significant differences in maximum surface tension. However, minimum surface tension measurements were increased in the *S. chartarum* treatment groups as compared to controls in all three cell lines. Significant increases ($p<0.05$) of minimum surface tension measurements were noted in surfactant from the fetal rat lung fibroblasts and alveolar type II cells. (Figure 7)

In dynamic cycle measurements, a significant decrease in the treatment group as compared to controls ($p<0.05$) was found in surfactant from the fibroblast cell line (Figure 8). Maximum surface tension was found to be very similar between treatment and control groups for both the alveolar type II cells and A549 cell lines with no significant differences noted. Minimum surface tension measurements were increased in the treatment groups for all three cell lines however, a significant increase ($p<0.05$) was only noted in the fetal rat lung alveolar II cells and human A549 cells. (Figure 8)

(A)



(B)

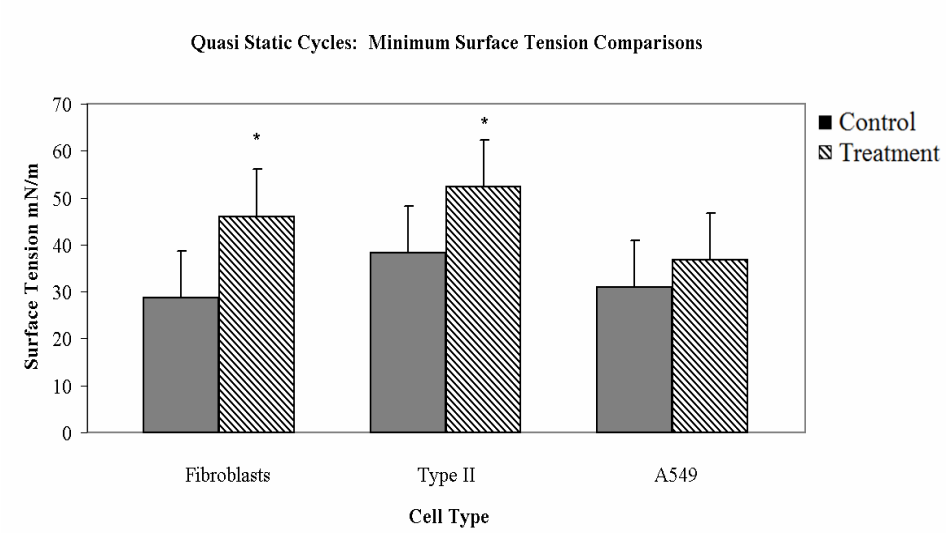
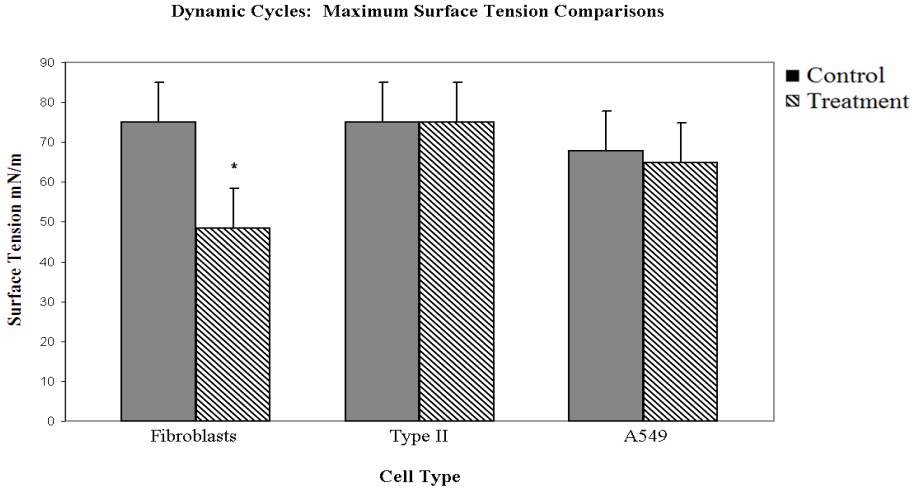


Figure 7: Effect of *Stachybotrys chartarum* Extracts on Pulmonary Surfactant and the Control of Bubble Surface Tension: Quasi Static Cycles. Captive Bubble Surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, type II and human A549 cells. (A) Shows the maximum surface tension measured during quasi static cycles. (B)Shows the minimum surface tension measured during quasi static cycles. (*) indicates a significant ($p<0.05$) difference between treatment and controls. Each bar represents the mean ± SEM of five experiments.

(A)



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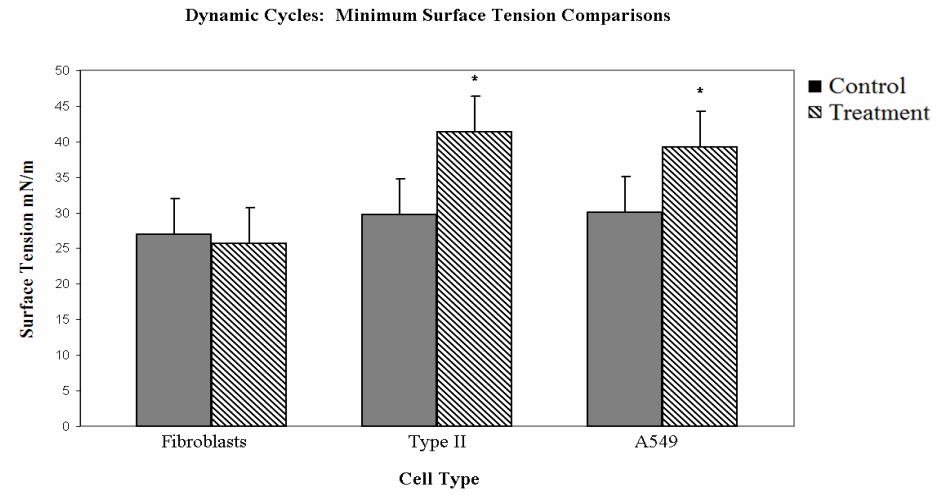


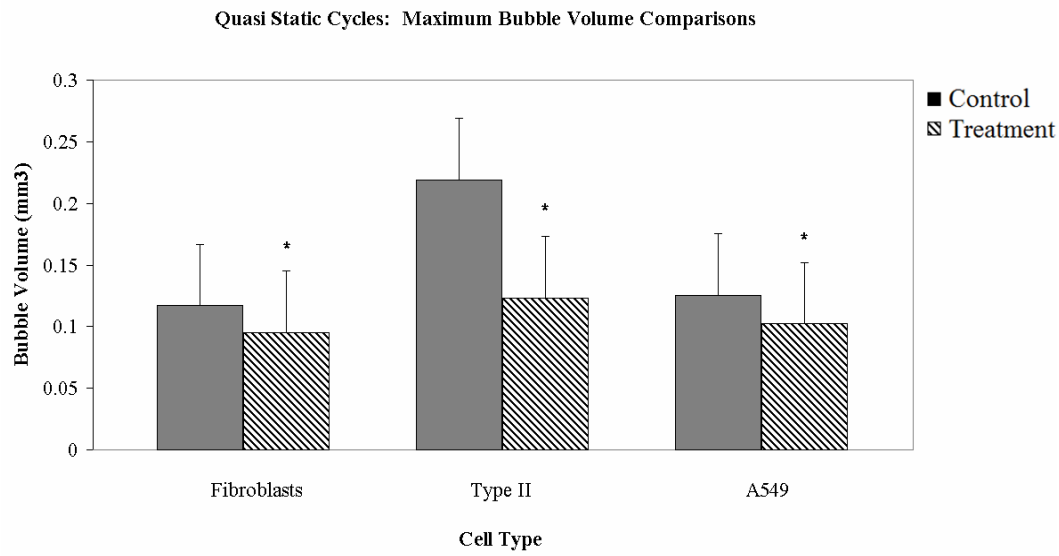
Figure 8: Effect of *Stachybotrys chartarum* Extracts on Pulmonary Surfactant and the Control of Bubble Surface Tension: Dynamic Cycles. Captive Bubble Surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, alveolar type II and human A549 cells. (A)Shows the maximum surface tension attained during dynamic cycles. (B) Shows the minimum surface tension achieved during dynamic cycles. (*) indicates a significant ($p<0.05$) difference between treatment and controls. Each bar represents the mean ± SEM of five experiments.

3.4.2 Bubble Volume Measurements

Quasi static cycles showed significant decreases ($p<0.05$) in maximum volume measurements for treatment groups as compared to controls in all three cell lines (Figure 9). A significant increase ($p<0.05$) in minimum bubble volume was noted for treatment groups as compared to controls for all three cell lines. There was also a significant difference noted in the minimum bubble volume attained in fetal rat lung alveolar type II cells as compared to the fibroblast cell lines for *S. chartarum* treated groups.

In dynamic cycles all three cell lines showed a significant decrease ($p<0.05$) in maximum bubble volume in treatment groups as compared to controls (Figure 10). Minimum bubble volume was also increased in treatment cells as compared to control cells ($p<0.05$), in fetal rat lung alveolar type II and human A549 cell lines. (Figure 10)

(A)



(B)

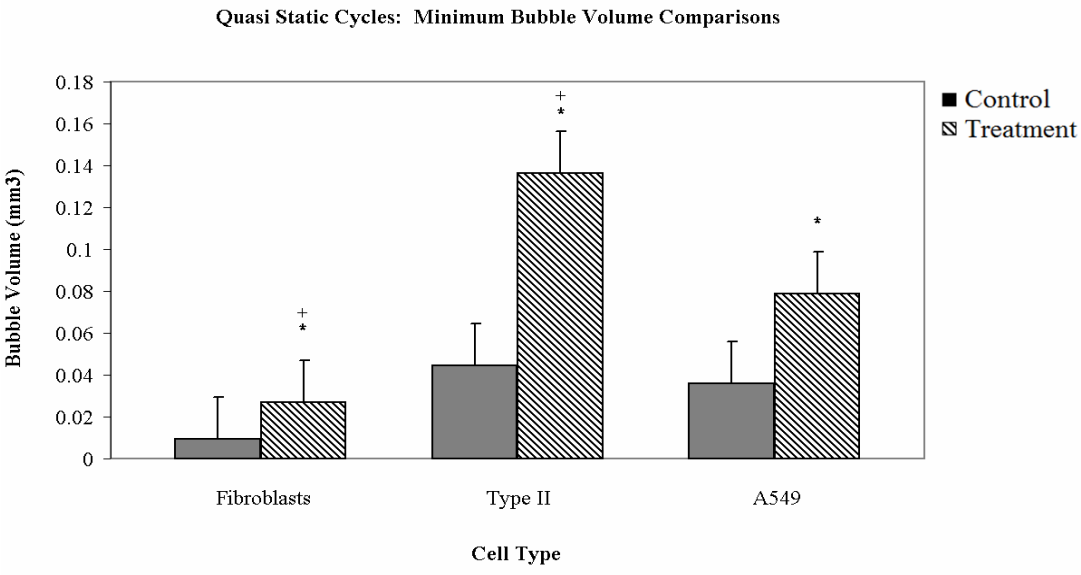
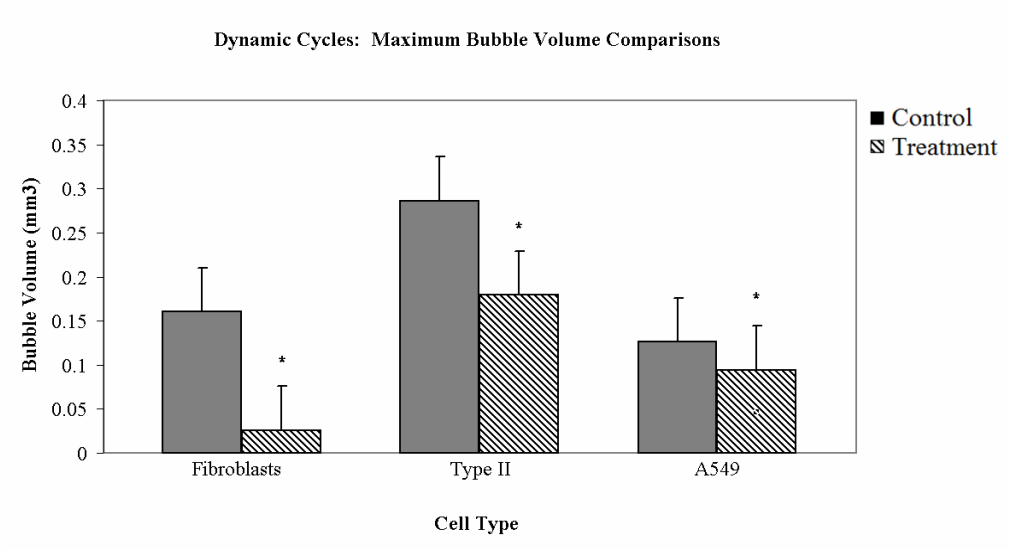


Figure 9: Effect of *Stachybotrys chartarum* Extracts on Pulmonary Surfactant and the Control of Bubble Volume: Quasi Static Cycles. Captive Bubble Surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, type II and human A549 cells. (A) Shows the maximum bubble volume attained during quasi static cycles. (B) Shows the minimum bubble volume achieved during quasi static cycles. (*) indicates a significant ($p<0.05$) difference between treatment and control groups. (+) indicates significant difference between treatment groups of fetal rat lung fibroblasts and alveolar type II cells.

(A)



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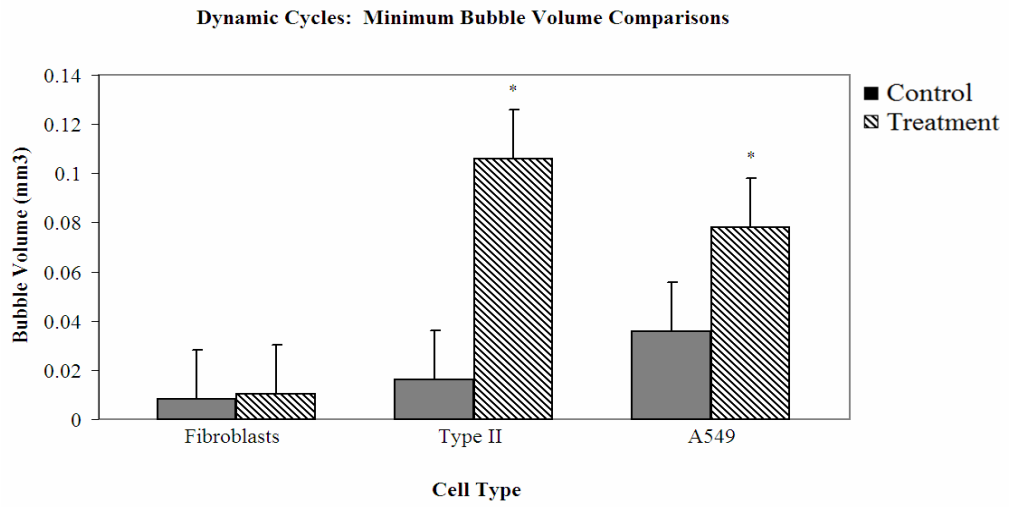

Figure 10: Effect of *Stachybotrys chartarum* Extracts on Pulmonary Surfactant and the Control of Bubble Volume: Dynamic Cycles. Captive Bubble Surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, type II and human A549 cells. (A) Shows the maximum bubble volume attained during dynamic cycles. (B) Shows the minimum bubble volume achieved during dynamic cycles. (*) indicates a significant ($p<0.05$) difference between treatment and control groups. Each bar represents the mean ± SEM of five experiments.

3.5 Transmission Electron Microscopy

Figures 11-16 show images obtained by transmission electron microscopy which demonstrate the differences observed in cell integrity between control and treatment groups for fetal rat lung fibroblasts, alveolar type II, and human A549 cells. In all three cell types, vacuoles containing fragments of lamellar bodies were the most notable changes in terms of cellular integrity. All other organelles and inclusions remained unaltered in all three cell types. Figure 17 is a transmission electron microscopy image demonstrating the cell debris found in the pellet collected from the supernatant of fetal rat lung alveolar type II cells following treatment with *S. chartarum* extracts. The image obtained from treatment supernatant was very similar for all three cell types.

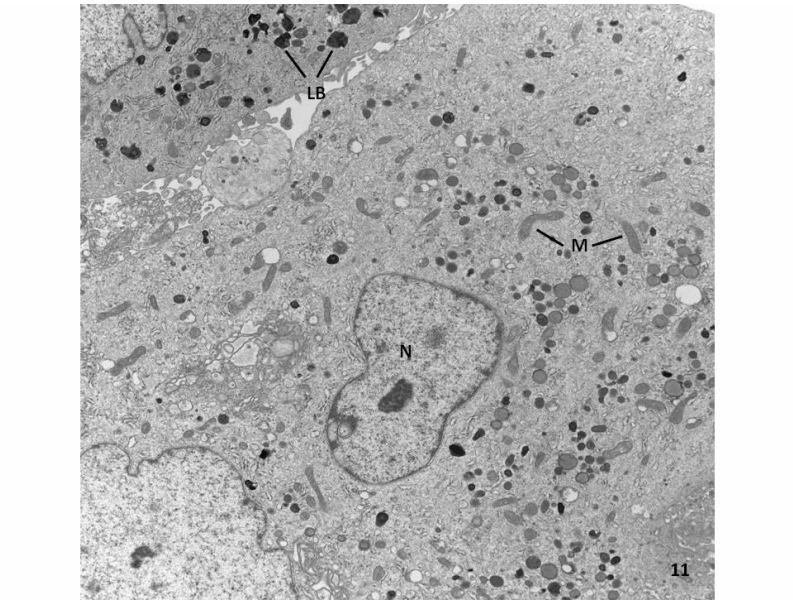

Figure 11: Transmission Electron Microscopy of Control Fetal Rat Lung Fibroblast Cells. Electron micrograph of control fetal fibroblasts showing well developed organelles and inclusions. N (nucleus), LB (lamellar bodies), M (mitochondria). Original magnification X 4,000

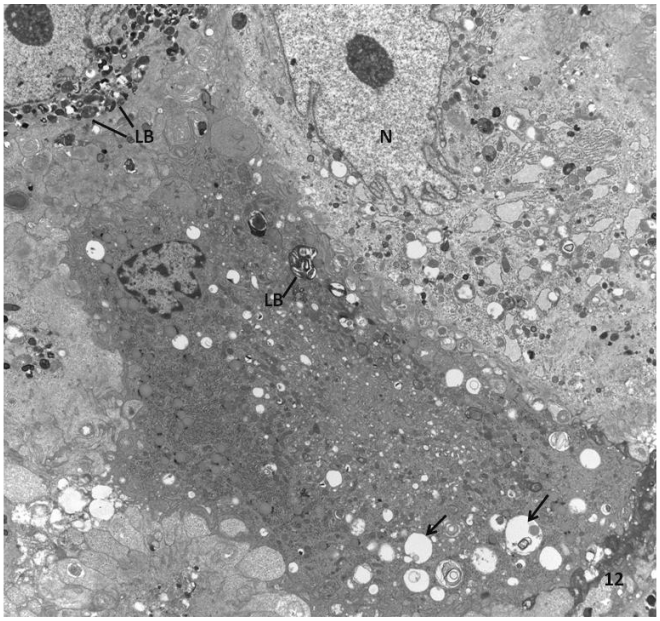

Figure 12: Transmission Electron Microscopy of *Stachybotrys chartarum* Extract Exposed Fetal Rat Lung Fibroblast Cells. Electron micrograph of fetal fibroblasts treated with S chartarum. Note numerous vacuoles, some with remnants of lamellae (arrows). LB (lamellar bodies), N (nucleus). Original magnification X 3,400

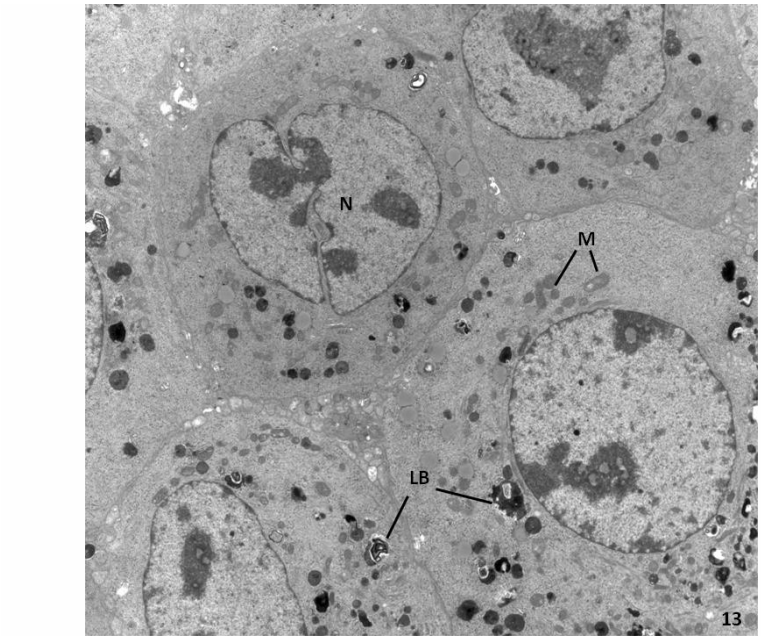

Figure 13: Transmission Electron Microscopy of Control Fetal Rat Lung Alveolar Type II Cells. Several control fetal rat lung type 2 alveolar cells contain well developed organelles and inclusions. The most conspicuous structures are numerous lamellar bodies (LB). N (nucleus), M (mitochondria). Original magnification X 3,400

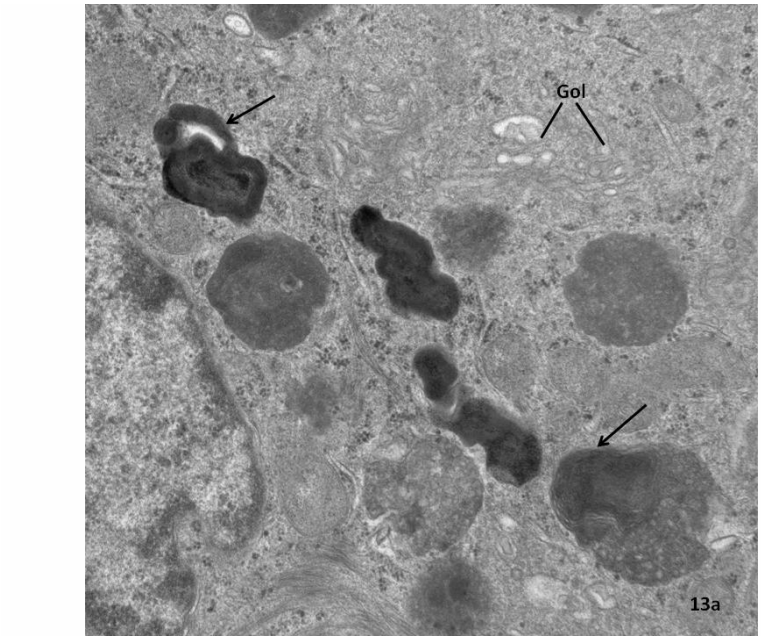

Figure 13a: Transmission Electron Microscopy of Control Fetal Rat Lung Alveolar Type II Cells (Higher Power). High power view of lamellar bodies from a control fetal type 2 pneumocyte. Tightly packed lamellae are seen (arrows). Gol (Golgi membranes). Original magnification X 25,000

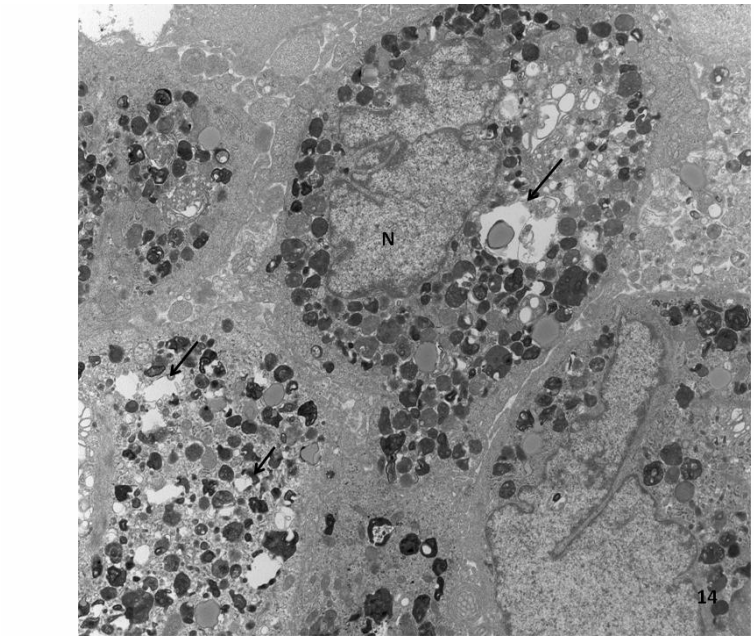

Figure 14: Transmission Electron Microscopy of *Stachybotrys chartarum* Extract Exposed Fetal Rat Lung Alveolar Type II Cells. Note numerous vacuoles and varying degree of loss of lamellar bodies (arrows). N (nucleus). Original magnification X 5,800

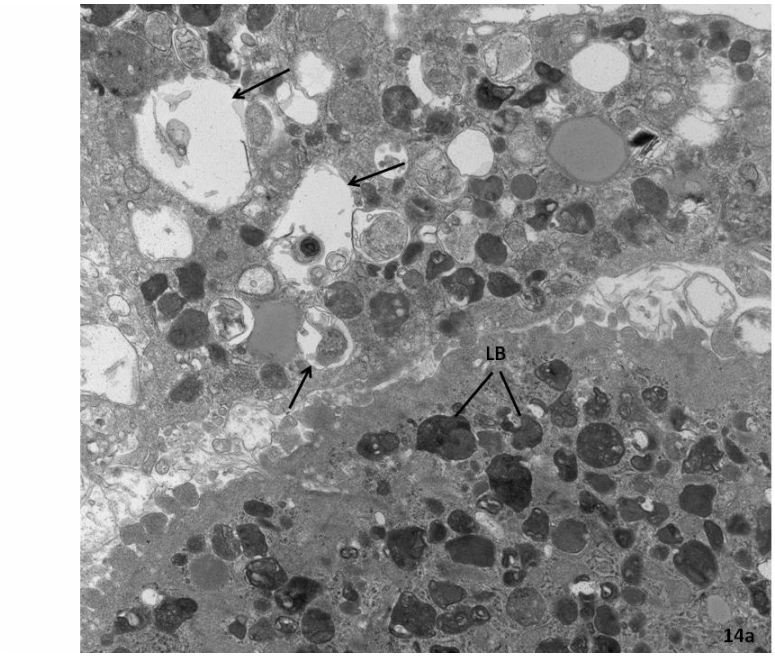

Figure 14a: Transmission Electron Microscopy of *Stachybotrys chartarum* Extract Exposed Fetal Rat Lung Alveolar Type II Cells (Higher Power). High power view of vacuoles from a fetal type 2 pneumocyte treated with S. chartarum. Several vacuoles contain remnants of lamellar bodies (arrows). LB (typical lamellar bodies). Original magnification X 10,500

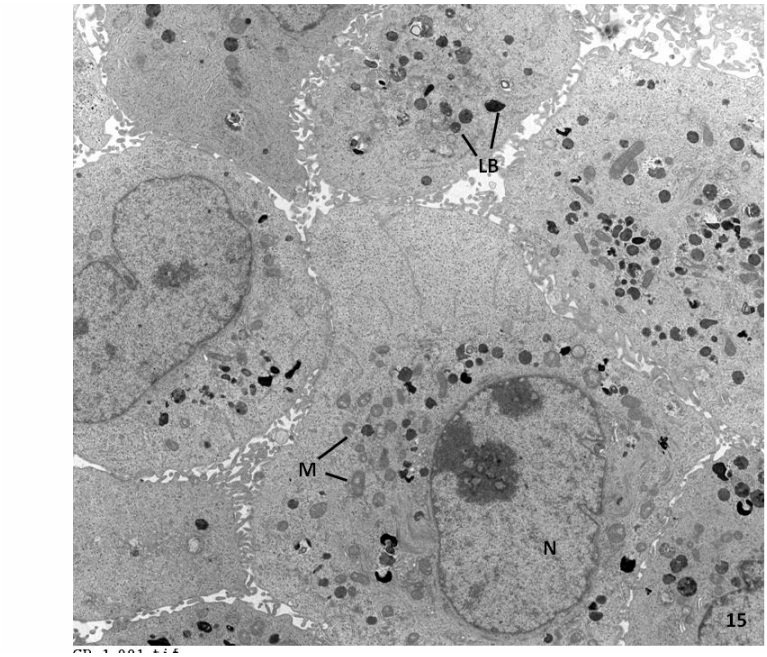

Figure 15: Transmission Electron Microscopy of Control Human A549 Cells. Low power of several control adult A549 cells showing numerous lamellar bodies (LB). N (nucleus), M (mitochondria). Original magnification X 3,400

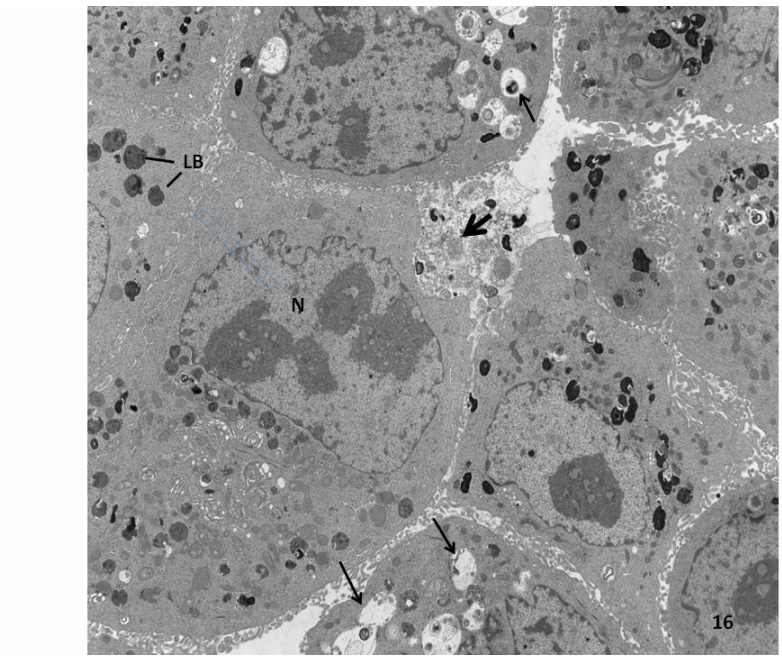

Figure 16: Transmission Electron Microscopy of *Stachybotrys chartarum* Extract Exposed Human A549 Cells. Low power view of adult A549 cells treated with *S. chartarum* showing several vacuoles with residual lamellar bodies (arrows). Cell debris (large arrow), LB (lamellar bodies). Original magnification X 3,400

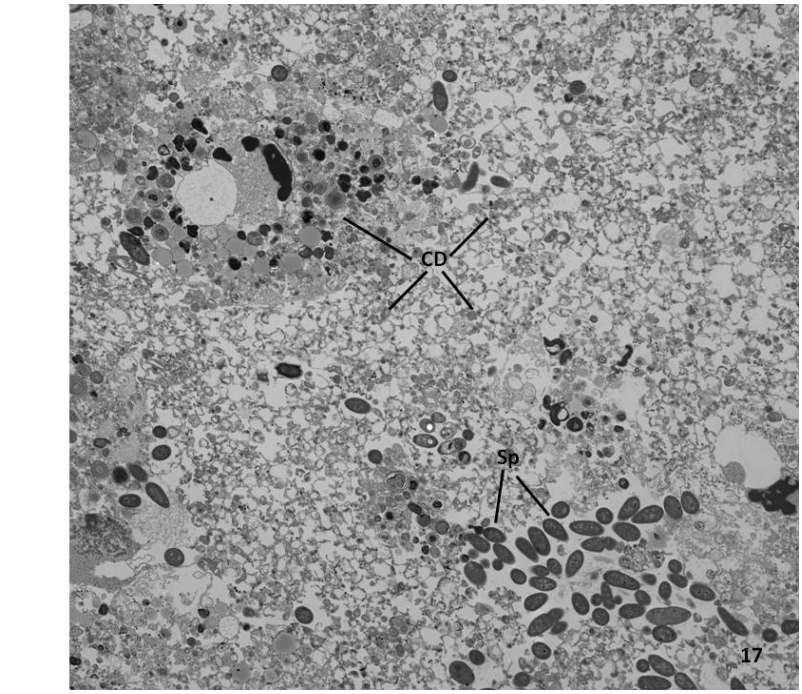

Figure 17: Transmission Electron Microscopy Image of Supernatant Collected from Stachybotrys chartarum Treated Fetal Rat Lung Alveolar Type II Cells. Seen here are numerous mould spores (Sp) and cell debris (CD). Original magnification X 2,600

Chapter Four
Discussion

4.1 Relevance

Stachybotrys chartarum is a common household mould which is often found in water damaged homes. It has been implicated in various respiratory disorders including asthma, allergies and idiopathic pulmonary hemosiderosis. Most notable in terms of disease implication, is the prevalence of *S. chartarum* growth. This fungus grows often in large quantities, on the floors, ceilings and walls of water damaged buildings. *S. chartarum* thrives on wet cellulose containing materials such as drywall and gypsum board. Providing a substrate for growth, water damaged buildings can be a potent environmental risk factor for the development of various pulmonary symptoms including pulmonary hemosiderosis as seen in the cases of the Cleveland, Ohio infants. The recognition of this risk has led to the attribution of *Stachybotrys chartarum* spore exposure in infants as a cause of Sick Building Syndrome (Montana, Etzel et al. 1997).

Research to date has focused on the effects of spore exposure directly, whereas this current research looks at the implications of exposure solely to the potent extracts produced by this common mould. To date, most studies have focused on intratrachial exposure to the mould in adult and juvenile animal models as well as the determination of mycotoxin quantity and type. The fact that this study investigates fetal lung cell response is both novel and opportunistic potentially leading to further fetal lung investigations.

Considering that *Stachybotrys chartarum* is implicated in lung infections in an infant population, it makes sense that research should be conducted on immature lungs.

It has been documented, that the effects of exposure to *Stachybotrys chartarum* are dose dependant (Flemming, Hudson et al. 2004). In fact, previous investigations have used a wide range of spore exposures from 30 to 800,000 spores/gram body weight (Yike, Vesper et al. 2003; Flemming, Hudson et al. 2004). In order to attempt to reproduce the human infant pulmonary haemorrhage disorder, spore exposures ranging from 1×10^5 to 8×10^5 spores/gram body weight have been used (Yike and Dearborn 2004). It is difficult to determine the actual levels of indoor exposure to air-carrying spores as environmental studies do not provide accurate assessments since the spore counts are based on cultures which can lead to as much as a ten-fold underestimate of total spores (Shelton, Kirkland et al. 2002). Most animal studies have focused on acute exposure to spores. Strain, dosage and duration of exposure are all factors which contribute to the severity and nature of lung injury upon *S. chartarum* exposure. Many studies have shown, however, that some degree of inflammatory response results after spore exposure regardless of dose or duration (Nikulin, Reijula et al. 1997; Flemming, Hudson et al. 2004). As suggested by Yike and Dearborn (2004), studies which involve long term exposure to lower doses may be of more relevance in terms of human exposure.

4.2 Cell Viability

Exposure to the extracts produced by *Stachybotrys chartarum* showed extreme deleterious effects on all three cell lines; fetal rat lung fibroblasts, alveolar type II and human A549 cells. Cells exposed to the extracts demonstrated a reduction in cell viability as shown in MTT formazan assay results. Cell numbers showed an approximate 70% reduction in cell number in all three cell lines. Transmission electron microscopy showed an abundance of cell debris in the collected supernatant after treatment with spore extract media which corresponds to the high degree of cell death noted in treatment samples. A loss of cell integrity was also noted in electron microscopy images of surviving cells. This was most notable in the lamellar bodies of all three cell lines. The fact that the lamellar bodies seem to be affected whereas all other organelles remain intact, suggests that the lamellar bodies have a high sensitivity to the extracts collected from *S. chartarum* spores. This may also be related to the differences noted in surfactant production and quality. Gregory, Pestka et al. (2004) isolated satratoxin G to the cytoplasm and rough endoplasmic reticulum in type II alveolar cells of adult male mice. This differs from the results of this study where the lamellar bodies are affected, demonstrating that the immature fetal lung responds differently to the toxins within *S. chartarum* spore extracts.

4.3 Surfactant Production

The main role of surfactant is reduce the effort needed to expand the lungs during inspiration, allowing gas exchange to take place and to preventing collapse of the alveoli at maximum expiration. Surfactant basically allows breathing to be relatively effortless. During expiration, the lungs have a tendency to collapse. If they are allowed to do so, a much greater inspiratory effort is required to open them with the next breath. Pulmonary surfactant prevents this by reducing surface tension, the force causing the alveoli to collapse, throughout the lung. Surfactant forms a very thin film which covers the surface of the alveolar cells. The components of surfactant work together in order to reduce surface tension in the alveoli, and therefore reduce the tendency of the alveoli to collapse (Wright 2004).

As expected with low viability, there appeared to be a decrease in the amount of surfactant secreted by human A549 and fetal rat lung alveolar type II cell lines exposed to *S. chartarum* extract as compared to controls. However, when compared with the expected amount of secreted surfactant based on cell numbers, both fetal rat lung alveolar type II and human A549 cell lines actually showed an increase in surfactant phospholipid secretion which is consistent with past studies (Mason, Rand et al. 1998; Mason, Rand et al. 2001). What was also surprising, was the fact that the fibroblast cell line secreted almost double the amount of surfactant-like material in treatment groups as compared to controls. This increase in surfactant secretion based on cell numbers suggests overcompensation by cells for an overall decrease in surfactant availability. The Bligh

and Dyer chloroform-methanol extraction and the fact that cells are washed of debris after treatment ensure that the phospholipid being measured is that of secreted material and not of cellular membrane origin.

This overcompensation is of particular interest as it relates to other studies which have also shown an increase in secreted surfactant in times of lung injury (Mason, Rand et al. 1998; Mason, Rand et al. 2001). The role of lipofibroblasts is also a possible explanation for the increased secretion of surfactant-like material by the fibroblast treatment groups. Lipofibroblasts have been shown to secrete surfactant-like material in times of lung injury. Also, in the fetal lung, fibroblasts are a surfactant secreting cell, assisting alveolar type II cells. Fibroblasts quickly lose his ability once exposed to oxygen after birth. The role of lipofibroblasts after birth is not well established, but it is thought that they continue to play a role in the secretion of some lipid components of surfactant (Brody and Kaplan 1983).

4.4 Surfactant Protein Expression

The *in vivo* growth of fetal lung tissue is quite vulnerable due to cellular hypersensitivity, immature defence systems and high rates of DNA and protein synthesis. Exposure to environmental extracts can drastically effect lung development (Suk and Collman 1998).

Surfactant protein-A is the most abundant of the surfactant proteins. This protein regulates the reuptake of surfactant by type II cells. It is often found in other tissues of the body possibly due to its immunological role, hence its presence in all three experimental cell lines. Fibroblast cells play an immunological role in that they are involved in the immune response to injury via migration, proliferation and the formation of fibrotic tissue as well as interactions with immune cells (Fries, Blieden et al. 1994). Hence, expression of SP-A is not surprising.

Surfactant protein B is of utmost importance in the lung. SP-B has a critical role in the assembly of surfactant and is also involved in membrane binding, fusion and lysis, lipid adsorption to the air-water interface, stabilization of surface films and re-spreading of these films during collapse phases in the breathing cycle (Hawgood, Derrick et al. 1998). An absence of this protein is fatal. Because it is found exclusively in alveolar type II cells, it makes sense that the fibroblast cell lines did not express SP-B. SP-B has been noted in lung Clara cells; found in the bronchioles and involved in protection of the bronchiolar epithelium (Miranda, Gommers et al. 2007). However, it has not been shown to be expressed in such cells *in vivo*. SP-B is synthesized in pulmonary type II and Clara cells as a 40-kD precursor peptide. In alveolar type II cells, this protein undergoes several processing steps to produce mature SP-B of 8 kDa. This processing in Clara cells is incomplete and mature SP-B is not produced (Whitsett and Weaver 2002). Being lung epithelial tumour cells, the A549 cells are very similar in structure and function when compared to type II cells and hence production of SP-B was seen as would be expected.

Since the mycotoxins produced by *S. chartarum* have a particular role in inhibition of protein synthesis, our results support the fact that potent mycotoxins are present in the extracts collected experimentally which decreased the expression of SP-A and SP-B in all three cell lines. Because of its immune role, it would be expected that SP-A would be involved in the inflammatory response when *S. chartarum* extracts are introduced *in vivo*. A decrease in expression of this protein would have further negative effects in terms of cell protection which corresponds to the high rate of cell mortality demonstrated in this study.

4.5 Surfactant Quality

Some interesting trends were observed in comparing surface tension to bubble volume measurements. In general, pulmonary surfactant plays an important role in lowering surface tension so that the lungs reach a point of stability at maximum expiration. An interesting trend was noted in treatment groups where maximum surface tensions were achieved at smaller bubble volumes, and often, the ability of a bubble to compress was limited with higher surface tensions noted. A phenomenon, known as bubble clicking often occurred with treatment surfactant samples. The bubble would cease to expand or compress any further causing the bubble to appear to bounce in the chamber and the surface tension would increase without a change in bubble volume.

In quasi static cycles of CBS no significant differences were noted in terms of maximum surface tension. There were however, significant differences noted in maximum bubble volume for all three cell lines with treatment groups attaining significantly smaller maximum bubble volumes when compared to control groups. This shows that the same maximum surface tensions are attained at lower bubble volume in treatment surfactant experiments meaning that quality-wise, the surfactant is unable to lower the surface tension sufficiently to allow bubble expansion. In the same cycles, significant increases in minimum surface tension measurements were noted in fetal rat lung fibroblast and alveolar type II cell treatment groups when compared to controls, but not in the human A549 cell line. Significant increases in minimum bubble volume were noted in all three treatment groups as compared to controls. These results further demonstrate the reduced ability of treatment surfactant to control surface tension. There is a noted decrease in the ability of the bubble to compress indicating that the higher minimum surface tension measurements are once again attained at a higher bubble volume, alluding to reduced surfactant quality.

In dynamic cycles of CBS, there was only a significant decrease in maximum surface tension measurements between control and treatment groups for the fetal rat lung fibroblast cell line. The maximum surface tension measurements are however, once again noted at lower bubble volumes for treatment groups in all three cell lines. This demonstrates the fact that the surfactant of cells exposed to *S. chartarum* extracts, is unable to sufficiently lower the surface tension to allow for bubble expansion. In terms of minimum surface tension, significant differences were noted in all three cell lines with

treatment groups demonstrating an increase in minimum surface tension attained. In fetal rat lung alveolar type II and human A549 cells, there is once again an increase in minimum bubble volume. The dynamic cycle results demonstrate, similar to the quasi-static cycles, that minimum surface tensions are higher and attained at increased bubble volumes in surfactant collected from cells exposed to *S. chartarum* extracts.

The trend that maximum surface tension measurements are seen at lower bubble volumes for treatment groups in all three cell lines is a very noteworthy result. This alludes to the fact that the surfactant of cells exposed to *S. chartarum* extracts, is unable to sufficiently lower the surface tension to allow for bubble expansion which is a necessary function in the lung in which surfactant lowers the surface tension to allow for alveolar expansion. The more important trend clinically, is the fact that minimum surface tensions are increased with an increase in minimum bubble volume as well. The importance of the surface tension lowering abilities of surfactant are most important at maximum expiration where lower surface tensions allow greater compression of the alveolus and prevent alveolar collapse as well as increase pulmonary compliance. The results of this study indicate a significant deficit in this surface tension lowering ability.

In a study by Rand, Mahoney et al. (2002), alveolar type II cells exposed to *S. chartarum* extracts were altered morphologically including damage to lamellar bodies. This is consistent with the transmission electron microscopy images obtained in this study. This study differs from the current study in that it is an *in vivo* study on juvenile mice versus the current study which is an *in vitro* model on fetal rat lung cells. With

differences between studies, it is interesting to note that similar morphological differences are still seen in lamellar bodies suggesting that these organelles are quite susceptible to the extracts secreted by *S. chartarum* spores. Because the lamellar bodies are the storage organelles for pulmonary surfactant, it follows that damage to them equates to issues with the pulmonary surfactant system.

Another study showed that exposure to the mycotoxins of *S. chartarum* changes the composition of surfactant collected via lung lavage most notably in the dipalmitoylphosphatidylcholine (DPPC) content but not overall phosphatidylcholine content (Hastings, Rand et al. 2005). Since DPPC is the primary phospholipid component of surfactant involved in the ability of pulmonary surfactant to compact and achieve high levels of surface pressure on alveolar compression (Bi, Taneva et al. 2001), it makes sense that collected surfactant from *S. chartarum* treated cells would show differences in quality as compared to controls. This is consistent with results from the current study where surfactant collected from *S. chartarum* extract treated cells demonstrated a reduced ability to sufficiently lower surface tension to allow bubble volume to compress to a sufficiently small volume.

4.6 Summary and Conclusions

In general, *Stachybotrys chartarum* has a negative effect on cells cultured from fetal rat lungs. Deleterious effects were observed in terms of cell structure, cell viability, surfactant production and quality as well as surfactant protein expression.

Conclusion 1: There was a significant reduction in cell number in all three cells lines exposed to spore extracts as compared to control groups. Loss of cell integrity was observed in all three cell types.

Conclusion 2: The rate of expression of surfactant protein-A is decreased in fetal rat lung fibroblast, alveolar type II and human A549 cells exposed to *S. chartarum* extracts.

Conclusion 3: The rate of expression of surfactant protein-B is decreased is fetal rat lung alveolar type II and human A549 cells exposed to *S. chartarum* extracts.

Conclusion 4: In terms of cell number after treatment with spore extracts, the amount of surfactant secreted by fetal rat lung fibroblast, alveolar type II and human A549 cells is greater than expected but is of lower quality when compared to cells not exposed to spore extracts.

4.7 Future Directions

The current study served as a baseline for the changes that occur within fetal rat lung cells in terms of viability, cell integrity, protein expression and surfactant secretion. Future studies will involve intratrachial instillation of spores into the pregnant rat with a number of potential investigations to follow. Questions have arisen as to whether or not the mycotoxins within the extracts produced by *Stachybotrys chartarum* spores will cross the placenta. If they are able to cross the placenta, what effect will they have on the fetal rat lungs? Will the same effects be observed? Which extract components are able to cross the placenta and will they be absorbed by the foetuses? If absorption does occur, will it effect the lungs solely or will other tissues be involved? Future studies may also investigate cell recovery after exposure. It is not known whether the cells exposed to the potent mycotoxins are permanently damaged or able to recover in some manner. No follow-up studies were completed on the Cleveland, Ohio infants to determine if they recovered or suffered long-term effects and it would be interesting to see what their long-term future looked like.

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