Open Journal of Pediatrics, 2013, 3, 243-256 **OJPed**
http://dx.doi.org/10.4236/ojped.2013.33043 Published Online September 2013 (http://www.scirp.org/journal/ojped/)

Stachybotrys chartarum (*atra*) spore extract alters surfactant protein expression and surfactant function in isolated fetal rat lung epithelial cells, fibroblasts and human A549 cells

Gail F. Pollard[1], Anthony Shaw[2], Michael Sowa[2], Thomas Rand[3], James A. Thliveris[1], James E. Scott[4,5]

[1]Department of Human Anatomy and Cell Science, University of Manitoba, Winnipeg, Canada
[2]National Research Council, Biodiagnostics Institute, Winnipeg, Canada
[3]Department of Biology, St. Mary's University, Halifax, Nova Scotia
[4]Department of Oral Biology, University of Manitoba, Winnipeg, Canada
[5]Biology of Breathing Group, University of Manitoba & Manitoba Institute for Child Health, Winnipeg, Canada
Email: jscott@cc.umanitoba.ca

Received 30 May 2013; revised 2 July 2013; accepted 9 July 2013

ABSTRACT

Moulds, notably *Stachybotrys chartarum* (*atra*), 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 and hemorrhage in infants in Cleveland, Ohio. The present study was designed to investigate the effects of *S. chartarum* extracts on surfactant protein expression, surfactant quality and cell survival in the developing lung. *S. chartarum* extracts were incubated with cultures of several cell types; isolated fetal lung type II cells and fetal lung fibroblasts, and human lung A549 cells, a continuously growing cell line derived from surfactant producing type II alveolar cells. 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 subcompartments was also assessed. Antibodies to these proteins and western blotting results were used to assess the quantity of protein produced by the various cell types. A novel approach utilizing 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. Results of the study showed that exposure to the *S. chartarum* extracts had deleterious effects on fetal lung epithelial cell viability and their ability to produce pulmonary surfactant. *S. chartarum* extracts also induced deleterious changes to the developing fetal lung cells in terms of expression of SP-A and SP-B as well as to the surface tension reducing abilities of the pulmonary surfactant. Ultrastructurally, spore toxin associated changes were apparent in the isolated lung cells most notably in the lamellar bodies of fetal rat lung alveolar type II and human A549 cells. This study has demonstrated the potential damage to surfactant production and function which may be induced by inhaling *S. chartarum* toxins.

Keywords: Fetal Lung; Surfactant; Surface Tension; Black Mould; Captive Bubble Surfactometer; Surfactant Proteins

1. INTRODUCTION

In 1993-1994, a 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 [1]. In a larger study 51 cases of acute pulmonary hemorrhage of which 16 infants died has been reported [2]. In both cases upon discharge many of the infants displayed a recurrence of symptoms. A case-control study was conducted identify-

ing an agent-host interaction associated with water damaged homes as an environmental risk factor and probable cause of the idiopathic pulmonary hemosiderosis [1,2]. Investigators found haemolysis in 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. The recognition of this risk has led to the attribution of *Stachybotrys chartarum* spore exposure in infants as a cause of Sick Building Syndrome [1]. Even though epidemiological and case studies have suggested an association between *S. chartarum* exposure and pulmonary hemorrhage in these infants [3], this relationship remains controversia l [4] and the precise etiology yet to be clarified. On the other hand, support has been provided in animal studies [5-8] that demonstrated *S. chartarum*'s ability to produce several classes of toxins that are deleterious to the lung. Among these are trichothecenes [9,10] which are potent inhibitors of protein synthesis [11] and hemolysin [12,13] which leads to destruction of the alveolar capillaries and subsequent alveolar hemorrhage.

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, inflammation and cytotoxicity in the upper and lower respiratory tracts and pulmonary hemosiderosis [14,15]. 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. 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, and the second produces atranones and simple trichothecenes [16].

Macrocyclic trichothecenes are the most potent small molecule protein synthesis inhibitors known and are considered the most acutely toxic mycotoxin [17]. Trichothecenes produced by *S. chartarum* isolates include satratoxins F, G and H, roridins and verrucarins [18]. Trichothecenes directly inhibit either initiation, elongation or termination of protein synthesis. 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 appears to be due to the atranones [19].

It has been documented, that the effects of exposure to *Stachybotrys chartarum* are dose dependant [20]. In fact, previous investigations have used a wide range of spore exposures from 30 to 800,000 spores/gram body weight [20,21]. 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 in a rodent model [22]. 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 [23]. 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 [6,20]. As suggested by Yike and Dearborn [22], studies which involve long term exposure to lower doses may be of more relevance in terms of human exposure. In addition, studies by Rand *et al*. (2002) [24] and McCrae *et al*. (2001) [25,26] demonstrated that mice exposed to *S. chartarum* or isosatratoxin F also affected changes in synthesis and secretion of surfactant, which is essential in maintenance of lung surface tension and compliance.

In light of the above, the present study was designed to analyze the effects of exposure to *Stachybotrys chartarum* extract on isolated fetal rat lung cells and human A549 cells, their expression of surfactant proteins SP-A and SP-B, cell survival and, using a novel approach of captive bubble surfactometry, assess both quality and quantity of surfactant produced. We hypothesized that exposure to extracts produced by spores of *S. chartarum* in addition to affecting pulmonary hemorrhage causes deleterious effects on fetal lung epithelial cells and their ability to produce sufficient surfactant of good quality. 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 intratracheal 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 investigating fetal lung cell response is both novel and opportunistic potentially developing an in vitro model for monitoring the effects of maternal exposure to environmental toxins and their effects on the developing fetal lung. In addition it will provide a knowledge base to be utilized in further fetal and subsequent newborn lung investigations. Furthermore our study makes no supposition that altered surfactant production leads to pulmonary hemorrhage as the study examines additional toxic effects specifically on surfactant production and quality. Thus the lung may be additionally compromised

both structurally and functionally in the fetus and ultimately in the newborn.

2. MATERIALS AND METHODS

2.1. Animals and Supplies

Random bred Sprague-Dawley rats of 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, Hank's Balanced Salt Solution (HBSS), Minimum Essential Medium (MEM), antibiotics, fungizone and newborn calf serum (NCS), 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 were 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, USA). 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, USA). *Stachybotrys chartarum* cultures (Cleveland strain #58-17) were obtained from Dr. Tom Rand (St. Mary's University, Halifax).

2.2. Primary Cell Cultures

Cultures of fetal rat lung fibroblasts and type II cells were done following the protocol currently active in the laboratory [27]. Timed pregnant Sprague Dawley rats were euthanized at gestational day 21 via an intraperitoneal injection of 1.5 ml Euthanyl (240 mg/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 10 ml trypsin-EDTA (0.05%) and 90 ml HBSS in a water-jacketed trypsinization flask at 37˚C for 45 minutes. Trypsinization was stopped by the addition of 30 ml 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 25 cm^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 4.5 ml 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 [26].

Human A549 cells were obtained from American Type Culture Collection. This cell line was included due to the many similarities to type II cells and can provide a source of human surfactant. Cells were thawed and resuspended in media/NCS in one 25 cm^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. *Stachybotrys chartarum* Culture

Stachybotrys chartarum spores (Cleveland strain #58-17) 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 by Mason *et al*. (1998) [28] and McCrea *et al*. (2007) [26]. 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 750 g and re-suspended in saline at a concentration of 1×10^6 spores/ml. Spores were left in the solution overnight at room temperature to release their toxins and were separated by centrifugation at 750 g to collect the supernatant which was filtered with Whatman paper to remove any remaining spores. Supernatant containing *S. chartarum* extracts were used in experimental treatments.

2.4. Treatment with *Stachybotrys chartarum* Extracts

Cells were treated with spore extracts as outlined by McCrae *et al*. (2001) [26]. 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} M phorbol ester (phorbol 12-myristate 13-acetate) for four 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 100,000 g to separate surfacetant [25].

2.5. Protein Assay

Protein concentrations were determined from collected cell samples using the Bradford method [29]. The assay was carried out using the BioRad DC Protein Assay kit which is based on the coomassie dye-binding protocol. Absorbances were measured at 595 nm 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.

2.6. 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 (1.0 M 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 25 mM Tris, 192 mM Glycine and 0.1% (w/v) SDS.

2.7. 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 25 mM Tris, 192 mM Glycine, 0.05% SDS and 20% methanol. After transfer, nitrocellulose membranes were washed in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.1% Tween-20 (TBST) and immunostained using the Snap I.D. system (Biorad Laboratories, Mississauga, Ontario).

Western blotting 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 system's vacuum drawing the solution through the blot. 3 ml 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.

2.8. 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 then treated overnight with either saline or *S. chartarum* extracts. After incubation, MTT solution equal to 10% of original culture volume was added to each well and incubated for 3 - 4 hours allowing formation of the formazan reaction product, which was measured at an absorbance of 567 nm using a Flexstation 3 fluorescence plate reader.

2.9. Phosphorous Assay

Surfactant-like material concentrations were determined using the Bligh and Dyer chloroform methanol extraction method [30] 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} M was incubated with the cells in order to induce surfactant release. After a four hour incubation period, supernatant containing secreted surfactant-like material was collected extracted into chloroform/methanol and a sample of the organic phase removed and phosphorus was quantitated using the method described by Bartlett (1976) [31].

2.10. 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 eliminating surface tension changes due to phospholipid escape. 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 µg 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 0.15 ml. 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 [32].

2.11. 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 0.1 M phosphate buffer (pH 7.3), followed by post-fixation in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3). Cells were dehydrated and embedded in Epon 812 using standard techniques [33]. 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.

2.12. Statistical Analysis

Analysis of variance (ANOVA) was employed to compare the means of the control samples with the *S. chartarum* extract exposed samples. An alpha level $p < 0.05$ was used to determine statistical significance.

3. RESULTS

3.1. Cell Viability

Cell viability was measured using an MTT Formazan assay. Cells were incubated in 96 well plates until confluent. Spore extract exposed cells received media incubated with *Stachybotrys chartarum* in 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 570 nm and background absorbance at 690 nm was subtracted.

Figure 1(a) shows the difference in mean absorbance

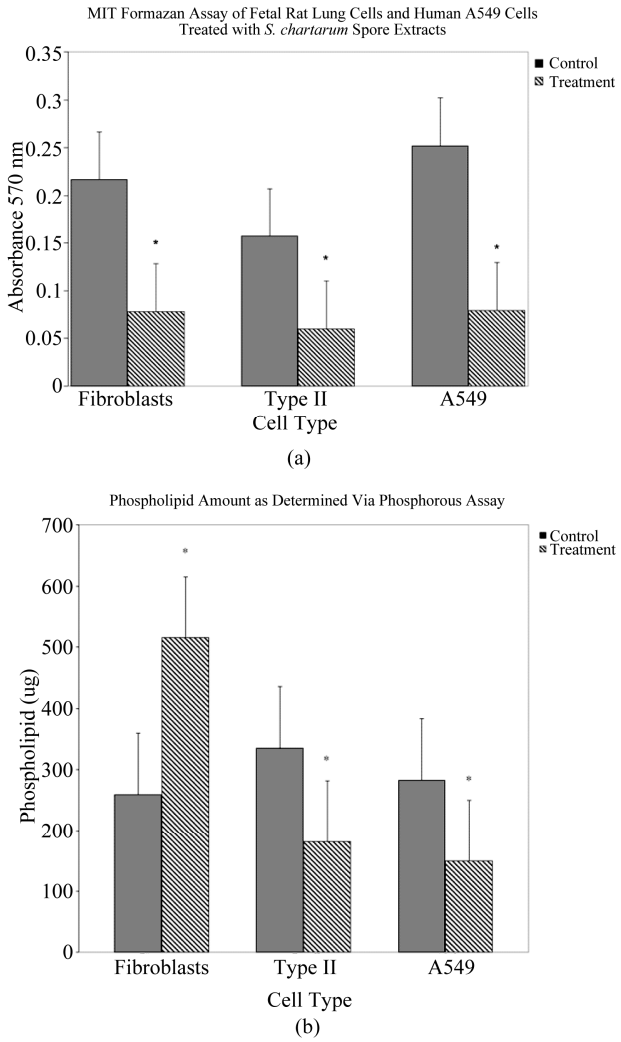


Figure 1. (a) Effect of *Stachybotrys chartarum* extracts on cell viability. MTT Formazan assays were done to determine cell viability when cells were exposed to media containing extract from *Stachybotrys chartarum* spores compared to controls. Cells not exposed to spore extracts were considered controls.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

at 570 nm between control and *S. chartarum* extract exposed sample of the three cell lines, fetal rat lung fibroblasts, fetal alveolar type II and human A549 cells. Significant decreases in cell viability were observed ($p < 0.05$) in the three cell lines exposed to *Stachybotrys chartarum* extract media compared to controls.

3.2. Surfactant-Like Production

Surfactant-like material was isolated using the Bligh and Dyer chloroform methanol extraction method followed by analysis and quantification via phosphorous assay.

Figure 1(b) demonstrates the significant differences observed between control and *S. chartarum* extract exposed samples in terms of release of surfactant-like material. Significant decreases in surfactant-like material release were observed in both the A549 and type II cell lines exposed to *Stachybotrys chartarum* extract medium compared to controls samples. In contrast the extract exposed fibroblasts showed a significant increase in surfactant secretion.

3.3. Surfactant Protein Expression

Expression of surfactant protein A (SP-A) and surfactant protein B (SP-B) was determined using SDS Page and Western Blot analysis. SP-A has been shown to be critical in synthesis and secretion of surfactant [34]. SP-B has a critical role in intracellular assembly of surfactant and spreading of surfactant onto the surface of the alveolar epithelium [35]. Antibody specific to each surfactant protein bound to bands corresponding to their respective molecular masses. Bands were measured using densitometric analysis. β-actin was used as loading control.

3.3.1. SP-A

Western Blot analysis of cell lysates using antibody specific to SP-A as well as the densitometric analysis of band intensity are shown in **Figures 2(a)** and **(b)**. Antibody specific to SP-A bound to 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 observed in the three extract exposed groups. There were also significant differences ($p < 0.05$) in SP-A expression 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 compared to the other cell lines (**Figure 2(b)**).

3.3.2. SP-B

Western Blot analysis of cell lysates using SP-B specific antibody and densitometric analysis of band intensity is shown in **Figures 3(a)** and **(b)**.

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 observed in fetal rat lung alveolar type II and human A549 cell lines compared to the control samples. SP-B was not detected in fetal lung fibroblasts.

3.4. Captive Bubble Surfactometry

The 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 least amount of phospholipid detected in samples (73 µg) was injected into the chamber for each experimental sample after forming the bubble. Bubble volume was controlled by varying

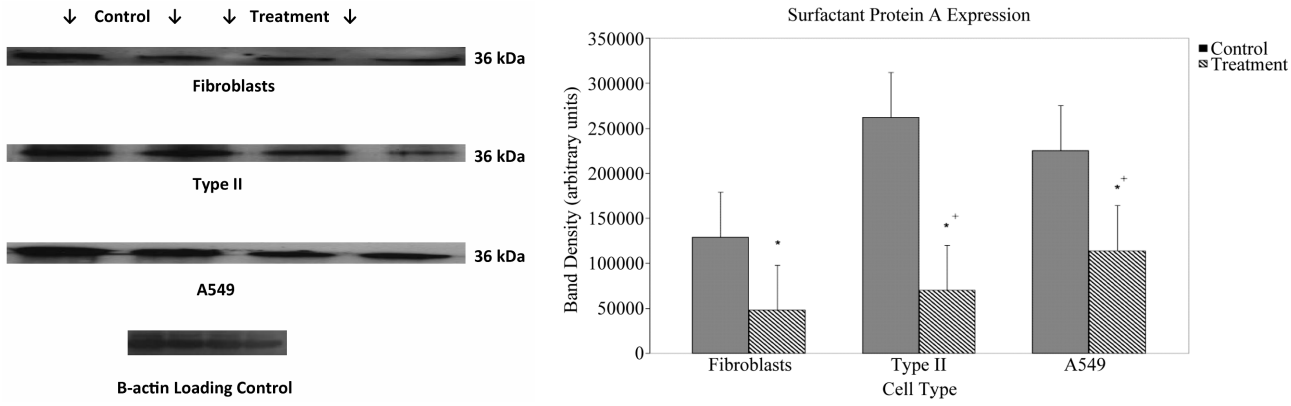


Figure 2. (a) and (b) Effect of *Stachybotrys chartarum* extracts on the expression of surfactant protein-A. SDS page, followed by Western Blotting produced visible bands corresponding to a molecular weight of 36 kDa. Bands were measured using densitometric analysis. Cells not exposed to spore extracts 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 compared to controls. (+) indicates a significant difference ($p < 0.05$) between fibroblasts and other cell lines. Each bar represents the mean ± SEM of five experiments.

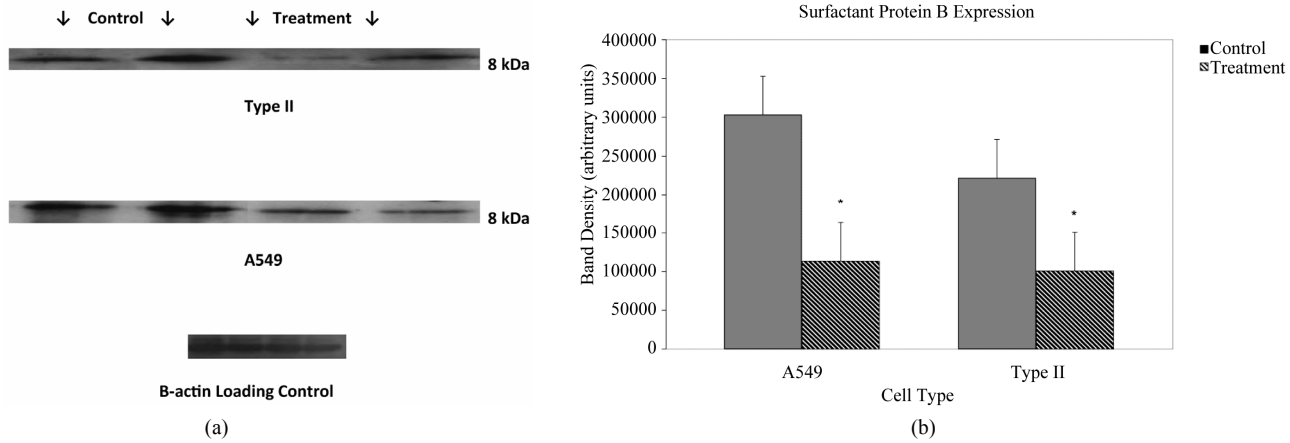


Figure 3. (a) and (b) Effect of *Stachybotrys chartarum* extracts on the expression of surfactant protein-B. SDS page, followed by Western Blotting produced visible bands corresponding to a molecular weight of 8 kDa. Bands were measured using densitometric analysis. Cells not exposed to spore extracts 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.

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.

Figure 4 shows typical captive bubble screen pressure-volume loops as surface tension measurements were obtained for surfactant from the three cells types with the graph constructed by the automated computer program. The various colored lines represent repetitive pressure-volume loops obtained from the same surfactant samples and are shown for demonstration purposes only. Quantification of these runs is shown in **Figures 5-8**.

3.4.1. Surface Tension Measurements

In quasi static cycles, maximum surface tension measurements were found to be very similar between control and *S. chartarum* extract exposed samples in surfactant isolated from the three cell types (**Figures 5(a)** and **(b)**). No significant differences in maximum surface tension were observed (**Figure 5(a)** and note scale of y axis). However minimum surface tension measurements were significantly increased ($p < 0.05$) in the *S. chartarum* extract exposed samples compared to control samples of surfactant released by isolated fibroblasts and the type II alveolar cells (**Figure 5(b)**).

In dynamic cycle measurements, a significant decrease of maximum surface tension in the *S. chartarum* extract exposed samples compared to the control samples ($p < 0.05$) was detected in surfactant from the fibroblasts (**Figure 6(a)**). Maximum surface tensions were similar in the *S. chartarum* extract exposed samples and control samples of both the alveolar type II cells and A549 cells. In contrast minimum surface tensions were significantly increased ($p < 0.05$) in the *S. chartarum* extract exposed samples from the fetal rat lung alveolar II cells and human A549 cells (**Figure 6(b)**).

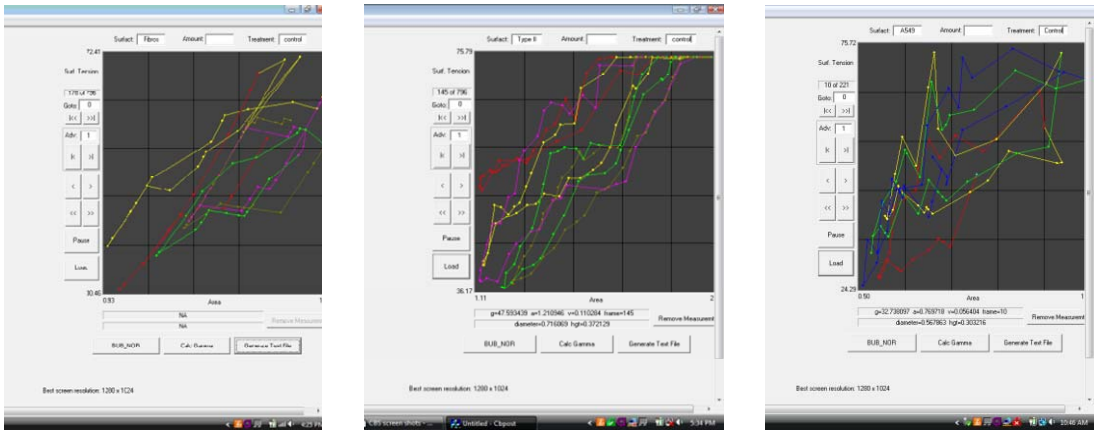
3.4.2. Bubble Volume Measurements

Quasi static cycles showed significant decreases ($p < 0.05$) in maximum volume measurements for *S. chartarum* extract exposed samples compared to controls in the three cell lines **(Figure 7(a))**. A significant increase ($p < 0.05$) in minimum bubble volume was observed in *S. chartarum* extract exposed samples compared to controls samples of the three cell lines. There was also a significant difference detected in the minimum bubble volume measured from fetal rat lung alveolar type II cells compared to the fibroblasts of *S. chartarum* treated samples (**Figure 7(b)**).

In dynamic cycles the three cell types showed a significant decrease ($p < 0.05$) in maximum bubble volume in *S. chartarum* extract exposed samples compared to the controls (**Figure 8(a)**). Minimum bubble volume was also significantly increased ($p < 0.05$) in surfactant from spore extract exposed cells compared to control samples ($p < 0.05$) from fetal rat lung alveolar type II cells and human A549 cells (**Figure 8(b)**).

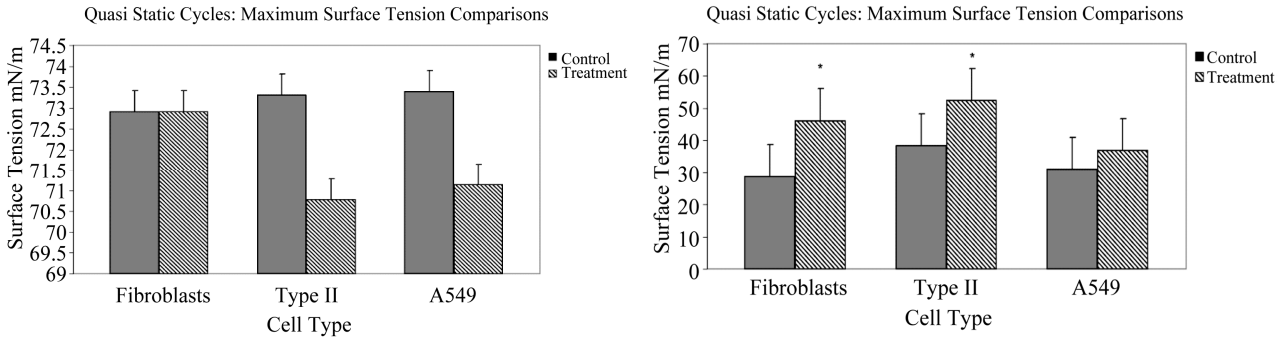
3.4.3. Transmission Electron Microscopy

Figures 9-12 show images obtained by transmission electron microscopy which demonstrate the differences observed in cell integrity between control and spore extract exposed groups of fetal rat lung fibroblasts, fetal



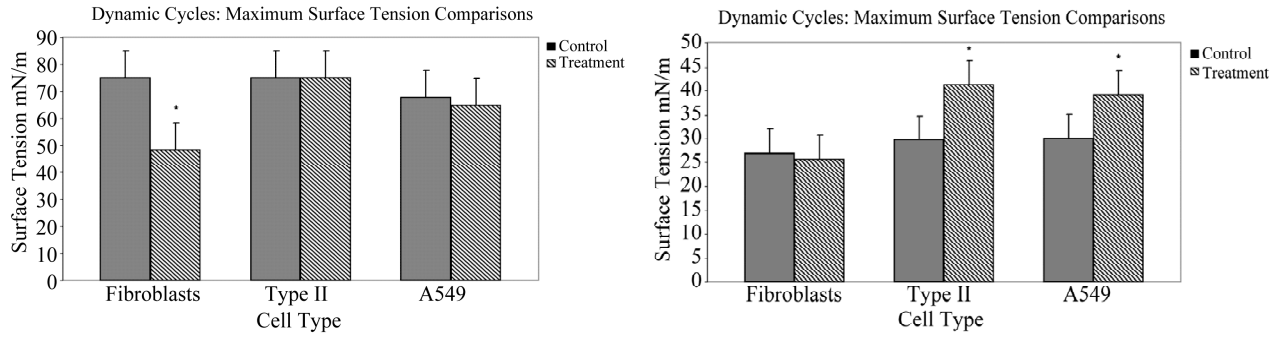
(a) (b) (c)

Figure 4. Captive bubble surfactometry: Typical pressure-volume loops from control groups. Screen shots of captive bubble surfactometry showing typical loops of compression and expansion cycles during surfactant analysis. (a) Fetal rat lung fibroblasts (left); (b) Fetal rat lung alveolar type II cells (middle); (c) Human A549 cell line (right).



(a) (b)

Figure 5. (a) and (b) 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 quasistatic cycles; (b) shows the minimum surface tension measured during quasistatic cycles. (*) indicates a significant difference ($p < 0.05$) between *S. chartarum* extract exposed samples and controls. Each bar represents the mean ± SEM of five experiments.



(a) (b)

Figure 6. (a) and (b) 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 difference ($p < 0.05$) between treatment and controls. Each bar represents the mean ± SEM of five experiments.

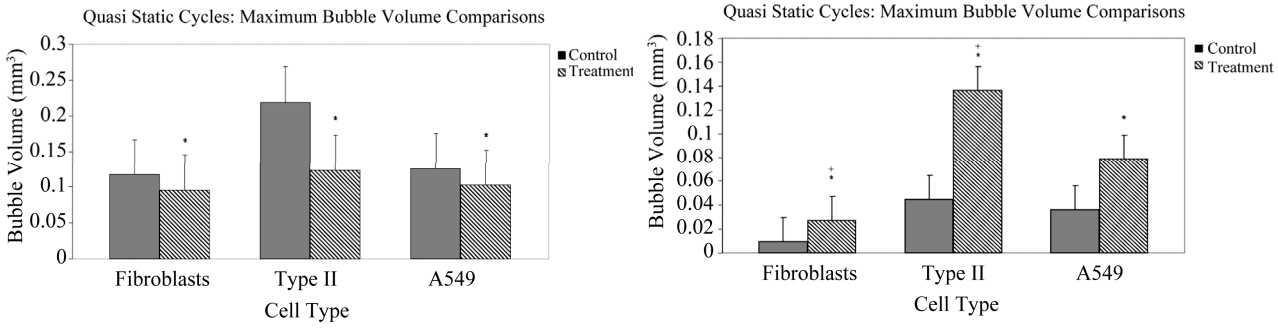


Figure 7. (a) and (b) Effect of *Stachybotrys chartarum* extracts on pulmonary surfactant and the control of bubble volume: Quasi static cycles. Captive bubble surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, type II and human A549 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.

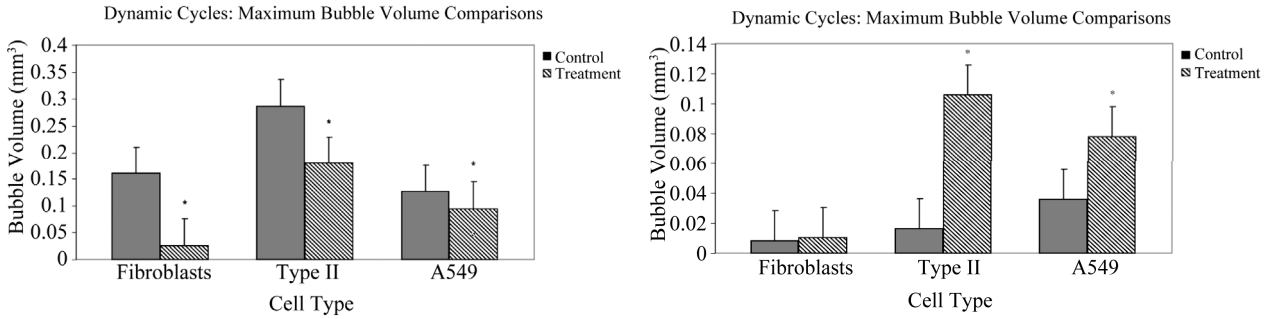
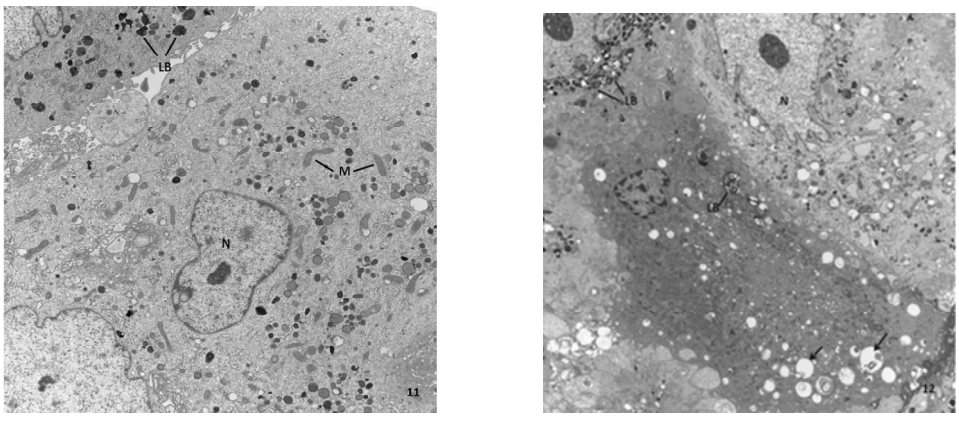
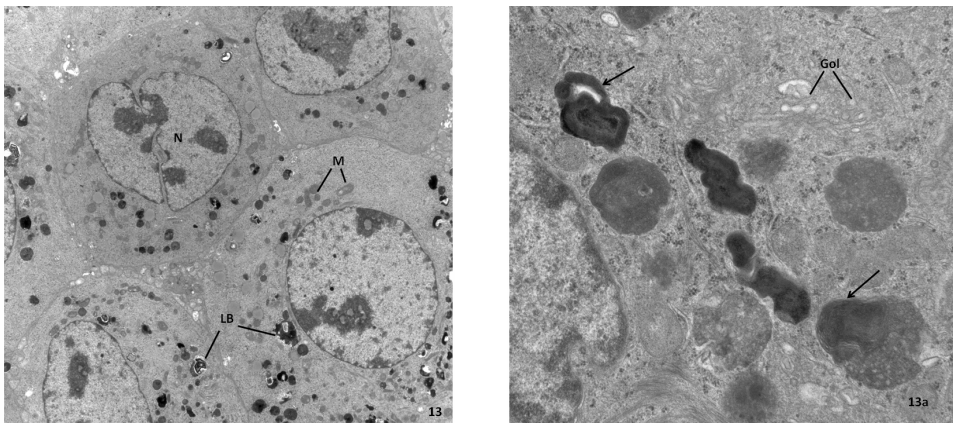


Figure 8. (a) and (b) Effect of *Stachybotrys chartarum* extracts on pulmonary surfactant and the control of bubble volume: Dynamic cycles. Captive bubble surfactometry was used to assess the quality of surfactant secreted by all three cell lines; fetal rat lung fibroblasts, type II and human A549 cells. (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.



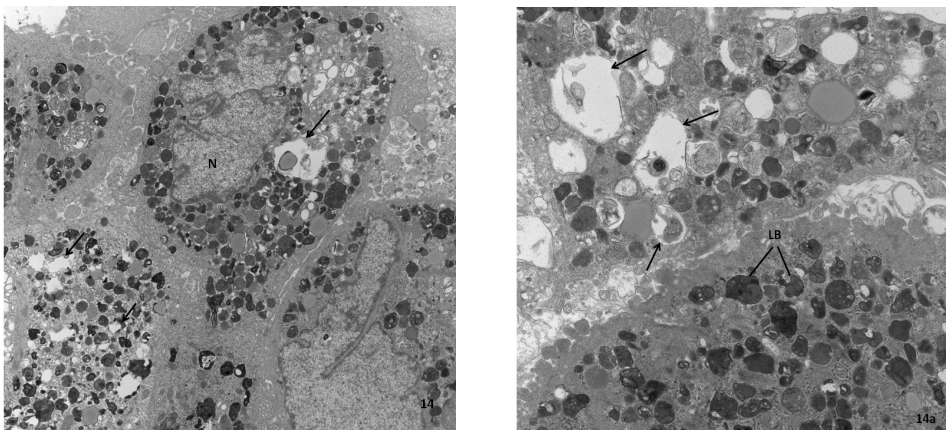
(a) (b)

Figure 9. (a) (left): Transmission electron microscopy of control fetal rat lung fibroblasts. Electron micrograph of control fetal fibroblasts showing well developed organelles and inclusions. N (nucleus), LB (lamellar bodies), M (mitochondria). Original magnification ×4000; (b) (right): Transmission electron microscopy of *Stachybotrys chartarum* extract exposed fetal rat lung fibroblasts. 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 ×3400.



(a) (b)

Figure 10. (a) (left): Transmission electron microscopy of control fetal rat lung alveolar type II cells. Several control fetal rat lung type II alveolar cells contain well developed organelles and inclusions. The most conspicuous structures are numerous lamellar bodies (LB). N (nucleus), M (mitochondria). Original magnification ×3400; (b) (right): Transmission electron microscopy of control fetal rat lung alveolar type II cells. High power view of lamellar bodies from a control fetal type II alveolar cell. Tightly packed lamellae are seen (arrows). Gol (Golgi membranes). Original magnification ×25,000.



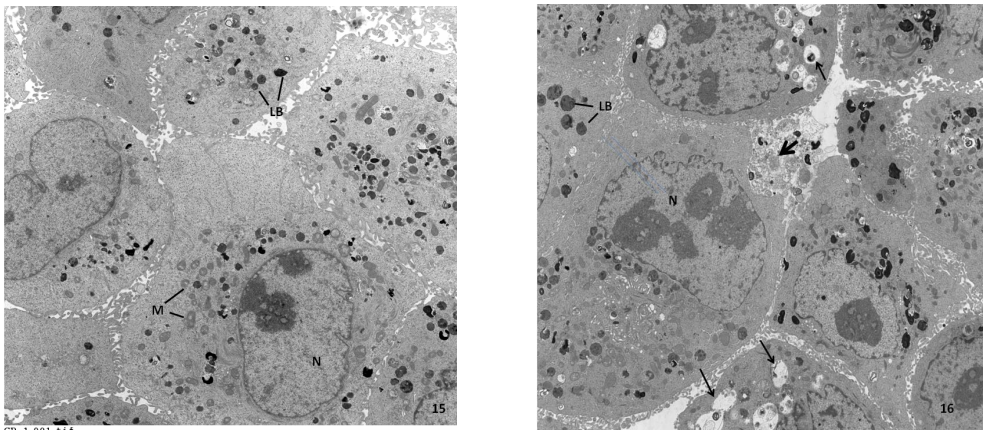
(a) (b)

Figure 11. (a) (left): Transmission electron microscopy of *Stachybotrys chartarum* extract exposed fetal rat lung alveolar type II cells. Note numerous vacuoles and varying degrees of disruption of lamellar bodies (arrows). N (nucleus). Original magnification ×5800; (b) (right): Transmission electron microscopy of *Stachybotrys chartarum* extract exposed fetal rat lung alveolar type II cells. High power view of vacuoles from a fetal type II alveolar cell exposed to extract from *S. chartarum*. Several vacuoles contain remnants of lamellar bodies (arrows). LB (typical lamellar bodies). Original magnification ×10,500.

alveolar type II cells 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 appeared to be unaltered.

4. DISCUSSION

Exposure to the *Stachybotrys chartarum* extracts induced deleterious effects in fetal rat lung fibroblasts, alveolar type II cells and human A549 cells. Cells exposed to the extracts had significantly reduced survival rates as shown in the MTT formazan assay. This observation is supported by the transmission electron microscopy which showed that exposure to spore extract induced vacuolization of organelles, in particular the lamellar bodies. Nevertheless it is not possible to exclude damage to other organelles as the MTT formazan assay measures mitochondrial dehydrogenases [36]. Indeed loss of cellular integrity as noted in electron microscopy images of cells is probably a later effect of spore exposure while biochemical alterations would culminate in the observed



(a) (b)

Figure 12. (a) (left): 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 ×3400; (b) (right): Transmission electron microscopy of *Stachybotrys chartarum* extract exposed human A549 cells. Low power view of adult A549 cells treated with *S. chartarum* extract showing several vacuoles with residual lamellar bodies (arrows). Cell debris (large arrow), LB (lamellar bodies). Original magnification ×3400.

morphological changes. In fact previous observations support the contention that lamellar bodies seem to be affected and may have a particular sensitivity to the extracts from *S. chartarum* spores [24]. This may also have an impact on surfactant production and quality. Gregory *et al*. (2004) [37] reported localization of spore satratoxin G in lysosomes, rough endoplasmic reticulum and the nucleus of type II alveolar cells of juvenile male mice. However labelling was not detected in the lamellar bodies. This latter finding differs from the results of the present study in which the lamellar bodies were markedly affected. This result may reflect the different nature or metabolism of the fetal lung cells in their response to the toxins from *S. chartarum* spore extracts. In the study noted above by Rand *et al*. (2002) [24], alveolar type II cells from juvenile mice exposed to *S. chartarum* extracts were altered morphologically including distended lamellar bodies with irregularly arranged lamellae. Even with these differing results, it is important to note that similar morphological differences are still seen in lamellar bodies suggesting that these organelles are quite susceptible to the extracts released *by S. chartarum* spores. Possibility these observations may be related to the lipid solubility of some of the toxins [24].

Unexpectedly the fibroblasts released almost double the amount of surfactant-like material in *S. chartarum* extract exposed samples compared to controls. As the total supernate from the *in vitro* experiments was used for the chloroform-methanol extraction and subsequent quantification, the released material may in fact not be pulmonary surfactant typical of that produced by type II alveolar cells. Importantly the observation that SP-B, the one surfactant protein that is critical for surfactant function [35] was not detectable in material from the fibroblasts supports this contention. In addition lipofibroblasts have also been identified in the alveolar septa which is also a possible explanation for the release of surfactant-like material by the fibroblast in *S. chartarum* extract exposed samples. Lipofibroblasts have been shown to secrete surfactant-like material in times of lung injury [38]. Also, in the fetal lung, some evidence suggests fibroblasts to contribute to the secreted surfactant pool and thus assisting alveolar type II cells [39]. The role of lipofibroblasts after birth is not well established, but it is thought that they may continue to play a role in the secretion of some lipid components of surfactant [40]

Previous studies suggest 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 [41]. This action may be attributable to spore induced changes in CTP: cholinephosphate cytidylyltransferase activity which regulates DPPC synthesis [41] or changes in convertase activity at the air-liquid interface [42]. DPPC is the primary phospholipid component of surfactant involved in the ability of pulmonary surfactant to compact and achieve high levels of surface pressure upon alveolar compression [43]. It therefore follows that changes in surfactant DPPC in *S. chartarum* treated cells in which surfactant collected from *S. chartarum* extract treated cells demonstrated a reduced ability to lower surface tension in the CBS. This finding may also be related to the decreased expression of SP-A and/or SP-B observed after spore exposure thus affecting both the quality and quantity of surfactant. An interesting trend was noted in

S. chartarum extract exposed samples 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 spore exposed 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. However significant differences exist in maximum bubble volume for all three sample types in which *S. chartarum* extract exposed samples showed significantly smaller maximum bubble volumes in comparison to control groups. This suggests that the same maximum surface tensions were attained at lower bubble volume in surfactant from spore exposed samples indicating that in terms of quality, this surfactant was 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 surfactant from fetal rat lung fibroblasts and alveolar type II cell *S. chartarum* extract exposed samples but not in the human A549 cell line. Significant increases in minimum bubble volume were noted in all three *S. chartarum* extract exposed samples as compared to controls. These results demonstrate the reduced ability of surfactant from spore exposed cells 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 supporting the contention that the quality of the surfactant is reduced after spore exposure.

In dynamic cycles of the CBS, there was only a significant decrease in maximum surface tension measurements between control and *S. chartarum* extract exposed samples for the fetal rat lung fibroblasts once again supporting the idea that material released by the fibroblasts is not typical and/or complete surfactant. Similarly observations of the dynamic minimum surface tension and minimum bubble volumes generated by surfactant from the type II cells and the A549 cells showed a different reaction to spore exposure than the material released by the fibroblasts. Furthermore the maximum surface tension measurements were observed at lower bubble volumes for *S. chartarum* extract exposed samples 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 the three cell types with *S. chartarum* extract exposed samples demonstrating an increase in minimum surface tension attained. 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 observed at lower bubble volumes for *S. chartarum* extract exposed samples in all three cell types is a noteworthy result. This indicates that the surfactants released from cells exposed to *S. chartarum* extracts are unable to sufficiently lower the surface tension to enable bubble expansion. This is a critical function in the lung in which alveolar expansion and stabilization is dependent on the ability of surfactant to lower the surface tension [44]. 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 relevant at maximum expiration where lower surface tensions allow greater compression of the alveolus and prevent alveolar collapse as well as increase pulmonary compliance [45]. The results of this study indicate a significant deficit in this surface tension lowering ability following exposure to *S. chartarum* spore products.

Overall this study highlights potential pathological damage that toxins from *S. chartarum* can have on pulmonary cells and their ability to produce pulmonary surfactant. Two important aspects have been highlighted. Specifically, toxins from this mould have the potential to reduce the levels of production of surfactant phospholipids by type II alveolar cells. Toxins also reduced the levels of two of the four surfactant proteins, SP-A and SP-B. Secondly, functional dynamics related to the ability of pulmonary surfactant to reduce surface tension were altered in samples exposed to mould toxins. These changes suggest that *S. chartarum* exposure has serious implications for pulmonary function.

5. ACKNOWLEDGEMENTS

Funding source: Natural Sciences and Engineering Research Council of Canada, and the Manitoba Institute of Child Health.

REFERENCES

[1] Montana, E., Etzel, R.A., Allan, T., Horgan, T.E. and Dearborn, D.G. (1997) Environmental risk factors associated with pediatric idiopathic pulmonary hemorrhage and hemosiderosis in a Cleveland community. *Pediatrics*, **99**, E5. doi:10.1542/peds.99.1.e5

[2] Etzel, R.A., Montana, E., Sorenson, W.G., Kullman, G.J., Allan, T.M., Dearborn, D.G., Olson, D.R., Jarvis, B.B. and Miller, J.D. (1998) Acute pulmonary hemorrhage in infants associated with exposure to *Stachybotrys atra* and other fungi. *Archives of Pediatrics & Adolescent Medicine*, **152**, 757-762. doi:10.1001/archpedi.152.8.757

[3] Weiss, A. and Chidekel, A.S. (2002) Acute pulmonary

hemorrhage in a Delaware infant after exposure to *Stachybotrys atra*. *Delaware Medical Journal*, **74**, 363-368.

[4] CDC Update (2000) Pulmonary hemorrhage/hemosiderosis among infants—Cleveland, Ohio, 1993-1996. *Morbidity and Mortality Weekly Report* (*MMWR*), **49**, 180-184.

[5] Nikulin, M., Reijula, K., Jarvis, B.B. and Hintikka, E.L. (1996) Experimental lung mycotoxicosis in mice induced by *Stachybotrys atra*. *International Journal of Experimental Pathology*, **77**, 213-218. doi:10.1046/j.1365-2613.1996.9250323.x

[6] Nikulin, M., Reijula, K., Jarvis, B.B., Veijalainen, P. and Hintikka, E.L. (1997) Effects of intranasal exposure to spores of *Stachybotrys atra* in mice. *Fundamental and Applied Toxicology*, **35**, 182-188. doi:10.1006/faat.1996.2274

[7] Yike, I., Miller, M.J., Sorenson, W.G., Walenga, R., Tomashefski Jr., J.F. and Dearborn, D.G. (2002) Infant animal model of pulmonary mycotoxicosis induced by *Stachybotrys chartarum*. *Mycopathologia*, **154**, 139-152. doi:10.1023/A:1016098331428

[8] Rao, C.Y., Brain, J.D. and Burge, H.A. (2000) Reduction of pulmonary toxicity of *Stachybotrys chartarum* spores by methanol extraction of mycotoxins. *Applied and Environmental Microbiology*, **66**, 2817-2821. doi:10.1128/AEM.66.7.2817-2821.2000

[9] Jarvis, B.B., Salemme, J. and Morais, A. (1995) *Stachybotrys toxins*. *Natural Toxins*, **3**, 10-16. doi:10.1002/nt.2620030104

[10] Jarvis, B.B., Sorenson, W.G., Hintikka, E.L., Nikulin, M., Zhou, Y., Jiang, J., Wang, S., Hinkley, S., Etzel, R.A. and Dearborn, D. (1998) Study of toxin production by isolates of *Stachybotrys chartarum* and *Memnoniella echinata* isolated during a study of pulmonary hemosiderosis in infants. *Applied and Environmental Microbiology*, **64**, 3620-3625.

[11] Feinburg, B. and Maclaughlin, C.S. (1989) Biochemical mechanisms of action of trichothecenes mycotoxins. In: Beasley, V.R., Ed., *Trichothecene Mycotoxins*: *Pathophysiological Effects*, Boca Raton, CRC Press, 27-36.

[12] Vesper, S., Dearborn, D.G., Yike, I., Allan, T., Sobolewski, J., Hinkley, S.F., Jarvis, B.B. and Haugland, R.A. (2000) Evaluation of *Stachybotrys chartarum* in the house of an infant with pulmonary hemorrhage: Quantitative assessment before, during, and after remediation. *Journal of Urban Health*, **77**, 68-85. doi:10.1007/BF02350963

[13] Vesper, S.J., Magnuson, M.L., Dearborn, D.G., Yike, I. and Haugland, R.A. (2001) Initial characterization of the hemolysin stachylysin from *Stachybotrys chartarum*. *Infection and Immunity*, **69**, 912-916.

[14] Hossain, M.A., Ahmed, M.S. and Ghannoum, M.A. (2004) Attributes of *Stachybotrys chartarum* and its association with human disease. *Journal of Allergy and Clinical Immunology*, **113**, 200-208. doi:10.1016/j.jaci.2003.12.018

[15] Pestka, J.J., Yike, I., Dearborn, D.G., Ward, M.D. and Harkema, J.R. (2008) *Stachybotrys chartarum*, trichothecene mycotoxins, and damp building-related illness: New insights into a public health enigma. *Toxicological Sciences*, **104**, 4-26. doi:10.1093/toxsci/kfm284

[16] Andersen, B., Nielsen, K.F. and Jarvis, B.B. (2002) Characterization of *Stachybotrys* from water-damaged buildings based on morphology, growth, and metabolite production. *Mycologia*, **94**, 392-403. doi:10.2307/3761773

[17] Jarvis, B.B. (2003) *Stachybotrys chartarum*: A fungus for our time. *Phytochemistry*, **64**, 53-60. doi:10.1016/S0031-9422(03)00275-9

[18] Miller, J.D., Rand, T.G. and Jarvis, B.B. (2003) *Stachybotrys chartarum*: Cause of human disease or media darling? *Medical Mycology*, **41**, 271-291. doi:10.1080/1369378031000137350

[19] Nielsen, K.F., Huttunen, K., Hyvarinen, A., Andersen, B., Jarvis, B.B. and Hirvonen, M.R. (2002) Metabolite profiles of *Stachybotrys* isolates from water-damaged buildings and their induction of inflammatory mediators and cytotoxicity in macrophages. *Mycopathologia*, **154**, 201-205. doi:10.1023/A:1016383402963

[20] Flemming, J., Hudson, B. and Rand, T.G. (2004) Comparison of inflammatory and cytotoxic lung responses in mice after intratracheal exposure to spores of two different *Stachybotrys chartarum* strains. *Toxicological Sciences*, **78**, 267-275. doi:10.1093/toxsci/kfh064

[21] Yike, I., Vesper, S., Tomashefski Jr., J.F. and Dearborn, D.G. (2003) Germination, viability and clearance of *Stachybotrys chartarum* in the lungs of infant rats. *Mycopathologia*, **156**, 67-75. doi:10.1023/A:1022924306264

[22] Yike, I. and Dearborn, D.G. (2004) Pulmonary effects of *Stachybotrys chartarum* in animal studies. *Advances in Applied Microbiology*, **55**, 241-273. doi:10.1016/S0065-2164(04)55009-8

[23] Shelton, B.G., Kirkland, K.H., Flanders, W.D. and Morris, G.K. (2002) Profiles of airborne fungi in buildings and outdoor environments in the United States. *Applied and Environmental Microbiology*, **68**, 1743-1753. doi:10.1128/AEM.68.4.1743-1753.2002

[24] Rand, T.G., Mahoney, M., White, K. and Oulton, M. (2002) Microanatomical changes in alveolar type II cells in juvenile mice intratracheally exposed to *Stachybotrys chartarum* spores and toxin. *Toxicological Sciences*, **65**, 239-245. doi:10.1093/toxsci/65.2.239

[25] McCrae, K.C., Rand, T., Shaw, R.A., Mason, C., Oulton, M.R., Hastings, C., Cherlet, T., Thliveris, J.A., Mantsch, H.H., MacDonald, J. and Scott, J.E. (2001) Analysis of pulmonary surfactant by Fourier-transform infrared spectroscopy following exposure to *Stachybotrys chartarum* (*atra*) spores. *Chemistry and Physics of Lipids*, **110**, 1-10. doi:10.1016/S0009-3084(00)00199-7

[26] McCrae, K.C., Rand, T.G., Shaw, R.A., Mantsch, H.H., Sowa, M.G., Thliveris, J.A. and Scott, J.E. (2007) DNA fragmentation in developing lung fibroblasts exposed to *Stachybotrys chartarum* (*atra*) toxins. *Pediatric Pulmonology*, **42**, 592-599. doi:10.1002/ppul.20608

[27] Scott, J.E., Possmayer, F., Quirie, M.A., Tanswell, A.K. and Harding, P.G. (1986) Alveolar pre-type II cells from the fetal rabbit lung: Effect of confluence on the production of disaturated phosphatidylcholine. *Biochimica et Biophysica Acta*, **879**, 292-300. doi:10.1016/0005-2760(86)90218-3

[28] Mason, C.D., Rand, T.G., Oulton, M., MacDonald, J.M. and Scott, J.E. (1998) Effects of *Stachybotrys chartarum* (atra) conidia and isolated toxin on lung surfactant production and homeostasis. *Natural Toxins*, **6**, 27-33. doi:10.1002/(SICI)1522-7189(199802)6:1<27::AID-NT6>3.0.CO;2-B

[29] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254. doi:10.1016/0003-2697(76)90527-3

[30] Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, **37**, 911-917. doi:10.1139/o59-099

[31] Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *The Journal of Biological Chemistry*, **234**, 466-468.

[32] Schoel, W.M., Schurch, S. and Goerke, J. (1994) The captive bubble method for the evaluation of pulmonary surfactant: Surface tension, area, and volume calculations. *Biochimica et Biophysica Acta*, **1200**, 281-290. doi:10.1016/0304-4165(94)90169-4

[33] Luft, J.H. (1961) Improvements in epoxy resin embedding methods. *Journal of Biophysical and Biochemical Cytology*, **9**, 409-414. doi:10.1083/jcb.9.2.409

[34] Ikegami, M. and Jobe, A.H. (1998) Surfactant protein-C in ventilated premature lamb lung. *Pediatric Research*, **44**, 860-864. doi:10.1203/00006450-199812000-00006

[35] Hawgood, S., Derrick, M. and Poulain, F. (1998) Structure and properties of surfactant protein B. *Biochimica et Biophysica Acta*, **1408**, 150-160. doi:10.1016/S0925-4439(98)00064-7

[36] Liu, Y., Peterson, D.A., Kimura, H. and Schubert, D. (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry*, **69**, 581-593. doi:10.1046/j.1471-4159.1997.69020581.x

[37] Gregory, L., Pestka, J.J., Dearborn, D.G. and Rand, T.G. (2004) Localization of satratoxin-G in *Stachybotrys chartarum* spores and spore-impacted mouse lung using immunocytochemistry. *Journal of Toxicologic Pathology*, **32**, 26-34. doi:10.1080/01926230490260790

[38] Rehan, V.K., Sugano, S., Wang, Y., Santos, J., Romero, S., Dasgupta, C., Keane, M.P., Stahlman, M.T. and Torday, J.S. (2006) Evidence for the presence of lipofibroblasts in human lung. *Experimental Lung Research*, **32**, 379-393. doi:10.1080/01902140600880257

[39] McGowan, S.E. and Torday, J.S. (1997) The pulmonary lipofibroblast (lipid interstitial cell) and its contributions to alveolar development. *Annual Review of Physiology*, **59**, 43-62. doi:10.1146/annurev.physiol.59.1.43

[40] Brody, J.S. and Kaplan, N.B. (1983) Proliferation of alveolar interstitial cells during postnatal lung growth. Evidence for two distinct populations of pulmonary fibroblasts. *American Review of Respiratory Disease*, **127**, 763-770.

[41] Hastings, C., Rand, T., Bergen, H.T., Thliveris, J.A., Shaw, A.R., Lombaert, G.A., Mantsch, H.H., Giles, B.L., Dakshinamurti, S. and Scott, J.E. (2005) *Stachybotrys chartarum* alters surfactant-related phospholipid synthesis and CTP: cholinephosphate cytidylyltransferase activity in isolated fetal rat type II cells. *Toxicological Sciences*, **84**, 186-194. doi:10.1093/toxsci/kfi045

[42] Mason, C.D., Rand, T.G., Oulton, M., MacDonald, J. and Anthes, M. (2001) Effects of *Stachybotrys chartarum* on surfactant convertase activity in juvenile mice. *Toxicology and Applied Pharmacology*, **172**, 21-28. doi:10.1006/taap.2001.9127

[43] Bi, X., Taneva, S., Keough, K.M., Mendelsohn, R. and Flach, C.R. (2001) Thermal stability and DPPC/Ca^{2+} interactions of pulmonary surfactant SP-A from bulk-phase and monolayer IR spectroscopy. *Biochemistry*, **40**, 13659-13669. doi:10.1021/bi011188h

[44] Andreassen, S., Steimle, K.L., Mogensen, M.L., Bernardino de la Serna, J., Rees, S. and Karbing, D.S. (2010) The effect of tissue elastic properties and surfactant on alveolar stability. *Journal of Applied Physiology*, **109**, 1369-1377. doi:10.1152/japplphysiol.00844.2009

[45] Curstedt, T. and Johansson, J. (2005) New synthetic surfactants—Basic science. *Biology of the Neonate*, **87**, 332-337. doi:10.1159/000084881

