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The time course of responses to intratracheally instilled toxic *Stachybotrys chartarum* spores in rats

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Abstract

Stachybotrys chartarum is a fungal species that can produce mycotoxins, specifically trichothecenes. Exposures in the indoor environment have reportedly induced neurogenic symptoms in adults and hemosiderosis in infants. However, little evidence has linked measured exposures to any fungal agent with any health outcome. We present here a study that focuses on quantitatively assessing the health risks from fungal toxin exposure. Male, 10 week old Charles River-Dawley rats were intratracheally instilled with approximately 9.6 million *Stachybotrys chartarum* spores in a saline suspension. The lungs were lavaged 0 h (i.e., immediately post-instillation), 6, 24 or 72 h after instillation. Biochemical indicators (albumin, myeloperoxidase, lactic dehydrogenase, hemoglobin) and leukocyte differentials in the bronchoalveolar lavage fluid and weight change were measured. We have demonstrated that a single, acute pulmonary exposure to a large quantity of *Stachybotrys chartarum* spores by intratracheal instillation causes severe injury detectable by bronchoalveolar lavage. The primary effect appears to be cytotoxicity and inflammation with hemorrhage. There is a measurable effect as early as 6 h after instillation, which may be attributable to mycotoxins in the fungal spores. The time course of responses supports early release of some toxins, with the most severe effects occurring between 6 and 24 h following exposure. By 72 h, recovery has begun, although macrophage concentrations remained elevated.

Key words: *Stachybotrys chartarum*, bronchoalveolar lavage, fungi, mycotoxins, lactic dehydrogenase, hemorrhage

Introduction

Inhalation of fungal particles can cause allergic responses and pulmonary infections [1–3]. It has also been suggested that exposure to mycotoxin-producing fungi may cause severe effects independent of these more familiar illnesses [4–6]. In particular, *Stachybotrys chartarum* (= *Stachybotrys atra*) has been blamed for nonspecific symptoms such as muscle aches, headaches, cough, pulmonary hemorrhage, dermatitis, and interstitial lung disease following indoor inhalation exposures [7–10].

S. chartarum is a fungus that preferentially utilizes cellulose as a carbon source. It can grow at room temperatures under wet conditions. Under certain growth conditions (e.g., appropriate substrate, temperature, light, relative humidity), the fungus can produce macrocyclic trichothecenes [11–14]. Trichothecenes are potent inhibitors of protein and DNA synthesis resulting in disruption of cellular function as well as cellular injury [15].

Much of the research on *Stachybotrys chartarum* or the specific mycotoxins it produces has used ingestion, injection or dermal application of purified toxins as the route of exposure [16–22]. Animal studies have examined histological changes in lung tissue after intranasal exposure to *Stachybotrys chartarum* spores [23, 24]. Effects observed included severe inflammatory changes and interstitial inflammation with hemorrhagic exudate. The effects of inhaled spores in humans have been primarily reported through case studies [9, 25, 26].

Bronchoalveolar lavage (BAL) can serve as a rapid screening tool by providing estimates of relative toxicity of pulmonary toxicants, especially uncharacterized toxicants and complex mixtures [27, 28].

BAL yields quantitative estimates of inflammatory responses. The severity is measured by the release of cellular and serum constituents into the epithelial fluid following lung injury (e.g., albumin, lactic dehydrogenase [LDH], myeloperoxidase [MPO], hemoglobin) and by increases in the quantity of inflammatory cells (e.g., alveolar macrophages, polymorphonuclear leukocytes [PMNs], eosinophils, lymphocytes) [27]. The biochemical indicators allow evaluation of pulmonary edema (albumin), cytotoxicity (LDH), PMN enzyme secretion (MPO) and pulmonary hemorrhage (hemoglobin). BAL is more sensitive than histological examination [29, 30] and is used clinically to help diagnose pulmonary disease [31]. We have used BAL to assess the pulmonary effects of mycotoxin-containing fungal particles. We describe herein experiments designed to evaluate the time course of these effects.

Material and methods

Fungal strains and spore suspensions

Eight strains of *S. chartarum* isolated from a Southern California residence were screened for toxin production using a modified brine shrimp lethality assay according to Eppley [32]. A toxin-producing strain was selected for these experiments. The selected strain of *S. chartarum* was maintained on potato dextrose agar (PDA) slants at 15 °C. Spores were vacuumed from the surface of 14-day agar cultures using a modified filter cassette with a 37 mm, 0.4 μm polycarbonate membrane filter (Poretics Corp., Livermore, CA, USA) and suspended in 0.9% saline to concentrations of 2×10^7 spores per milliliter. The concentration and appearance of the spore suspensions were evaluated under light microscopy at 200X in a hemocytometer chamber. Minor hyphal fragment content and negligible spore clumping were observed and the remainder of the particles was ovoid spores with a mean spore size of 6.1×8.8 μm.

Bronchoalveolar lavage procedures

Male 10-week old Charles River-Dawley (CD) rats were supplied by Charles River Laboratories (Wilmington, MA, USA). The initial weight of the animals averaged 321 grams ($\pm$35.6 g). Rats were housed in isolation at 25 °C for a minimum one-week acclimation period and fed Purina Rat Chow and water *ad libitum.*

Delivery of the spore suspensions into the lungs of each animal was achieved by intratracheal instillation. Comparisons to the control treatments of sterile pyrogen-free saline carrier assessed the possible effects of the carrier and the instillation protocol. The rats were anesthetized by inhaling 5% halothane gas and then were placed on a slanted board. The freshly prepared spore suspension or saline carrier control was instilled into the lungs with a 1-ml disposable tuberculin syringe attached to a 3.5-inch, 19-gauge, bent, blunt-tipped needle inserted between the vocal folds. Tracheal insertion was verified by detection of the cartilaginous rings by the blunt needle. The volume of the carrier control (saline) or the spore suspension was 150 μl per 100 g body weight. Each rat received approximately 9.6 million spores. The animal remained in the slanted position for approximately 1 minute, and then was placed on its back in the cage where it regained consciousness within a few minutes. After instillation, the animals were kept in the laboratory under pre-instillation housing and maintenance conditions.

After a pre-determined time period (i.e., immediately following instillation, 6, 24 or 72 h later), the rats were re-weighed and then injected with 1 ml of sodium pentobarbital and exsanguinated by cutting the abdominal aorta. The trachea was exposed and cannulated with an 18-gauge, blunt-tipped needle fitted with a 1-inch, flared, 19-gauge, polypropylene tube and a 5-ml disposable syringe. The lungs were lavaged *in situ* twelve times by injecting 3 ml of phosphate buffered saline (PBS) into the lungs and then massaging the ribcage while withdrawing the lavage fluid. The first two lavages were combined (approximately 5 ml) and centrifuged under refrigeration at 350Xg for 10 min. The supernatant was analyzed for LDH, MPO, and albumin. The resulting cell pellet was combined with the cell pellet from the next ten lavages.

Analysis of bronchoalveolar lavage fluid

The total leukocyte cell count in the lavage fluid was estimated in a hemocytometer chamber at 100X magnification. Leukocytes were identified by size and granularity. The leukocytes were deposited onto a microscope slide by cytospin (72Xg for 5 min), fixed, stained, and mounted in Permount. Macrophages, PMNs, eosinophils and lymphocytes were differentiated by light microscopy at 200X.

The supernatant obtained from the first two lavages was recentrifuged under refrigeration at 14,500Xg for

30 min. The resultant supernatant was analyzed for lactic dehydrogenase, myeloperoxidase and albumin by spectrophotometry. Hemoglobin analysis was performed on the combined cell pellet from all 12 lavages. Biochemical analyses were performed according to Beck [33].

Statistical analyses

Statistical analyses were performed using SAS statistical software (Version 6.12; SAS Institute, Cary, NC). The effects of the saline carrier over the time course were examined using an analysis of variance with Dunnett's test to account for multiple comparisons against a control (i.e., saline carrier effects at time 0). The critical value was set at $p < 0.05$.

A non-parametric one-way analysis of variance (SAS PROC NPAR1WAY) was performed comparing the differences between responses of the carrier-instilled and the *Stachybotrys chartarum* spore-instilled animals within each time period. One-sided exact p-values were calculated and compared to a Bonferroni-adjusted critical value ($p < 0.0125$).

Results

Overall, 39 animals were instilled with either the spore suspension or the saline carrier alone. At least 4 animals for each exposure level (spore or carrier) were sacrificed and lavaged at each time point (time 0, 6, 24 or 72 h after instillation). All endpoints (concentrations of LDH, hemoglobin, albumin, MPO, total macrophage levels, total PMNs, total eosinophils, total lymphocytes and percent weight change) were measured for each animal.

Differences between carrier- and Stachybotrys chartarum spore-instilled animals

Physiological effects: weight change

The body weight of saline carrier-instilled animals did not change significantly during the first day. By seventy-two hours after saline instillation, an average 9% increase in weight was observed (Figure 1).

In comparison to the carrier-instilled animals, spore-instilled animals lost a significant amount of their starting body weight during the first 24 h post-instillation (average 10%, $p = 0.0006$). After 72 h some weight had been regained ($p = 0.008$, Figure 1), although average body weight remained 4% below starting values.

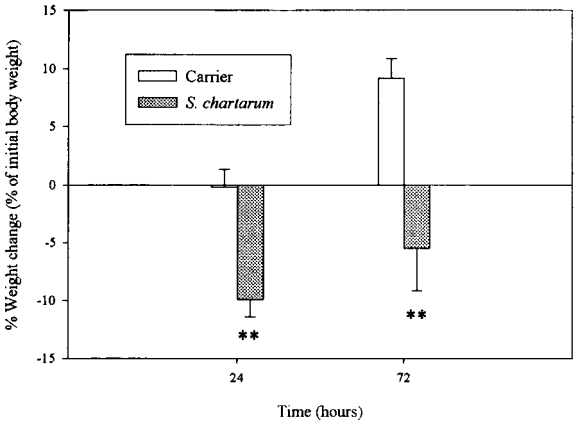

Figure 1. Mean % weight change ± S.E. over time. *S. chartarum* spore-instilled animals compared to carrier-instilled animals where ** denotes $p < 0.0125$ (Bonferroni-adjusted p-value).

Bronchoalveolar lavage fluid indicators

No significant differences were observed between the saline carrier and the *S. chartarum*-exposed animals in any of the BAL indicators immediately post-instillation (i.e., time 0).

The highest total inflammatory cell count occurred at 24 h and total leukocyte counts ($p = 0.0012$) and PMN counts were elevated ($p = 0.0013$, Figure 2a) in comparisons to carrier control animals at 24 h. However, concentrations of MPO, an extracellular measure of PMN degranulation, were not different at any of the experimental exposure time points (data not shown). There were no significant differences between the carrier control animals and the spore-instilled animals in macrophage (Figure 2b), lymphocyte (Figure 2c) or eosinophil (Figure 2d) counts over the whole time course. However, an obvious trend in macrophage and lymphocyte concentrations was observed in which an initial decrease at 6 h after instillation was followed by an increase to a maximum at 72 h ($p = 0.03$) after instillation (Figure 2b and 2c). At 72 h, macrophages were the dominant cell type.

At 24 h, albumin and LDH concentrations were significantly elevated ($p = 0.0003$ and $p = 0.0006$, Figures 3 and 4 respectively) in comparison to carrier control animals. Albumin concentration had decreased by 72 h, but remained significantly above carrier control values ($p = 0.008$, Figure 4). Although hemoglobin reached a peak at 24 h, levels were significantly different from carrier controls only at the 72 h time point (Figure 5).

The failure to detect statistically significant elevations at earlier time points may be due to small

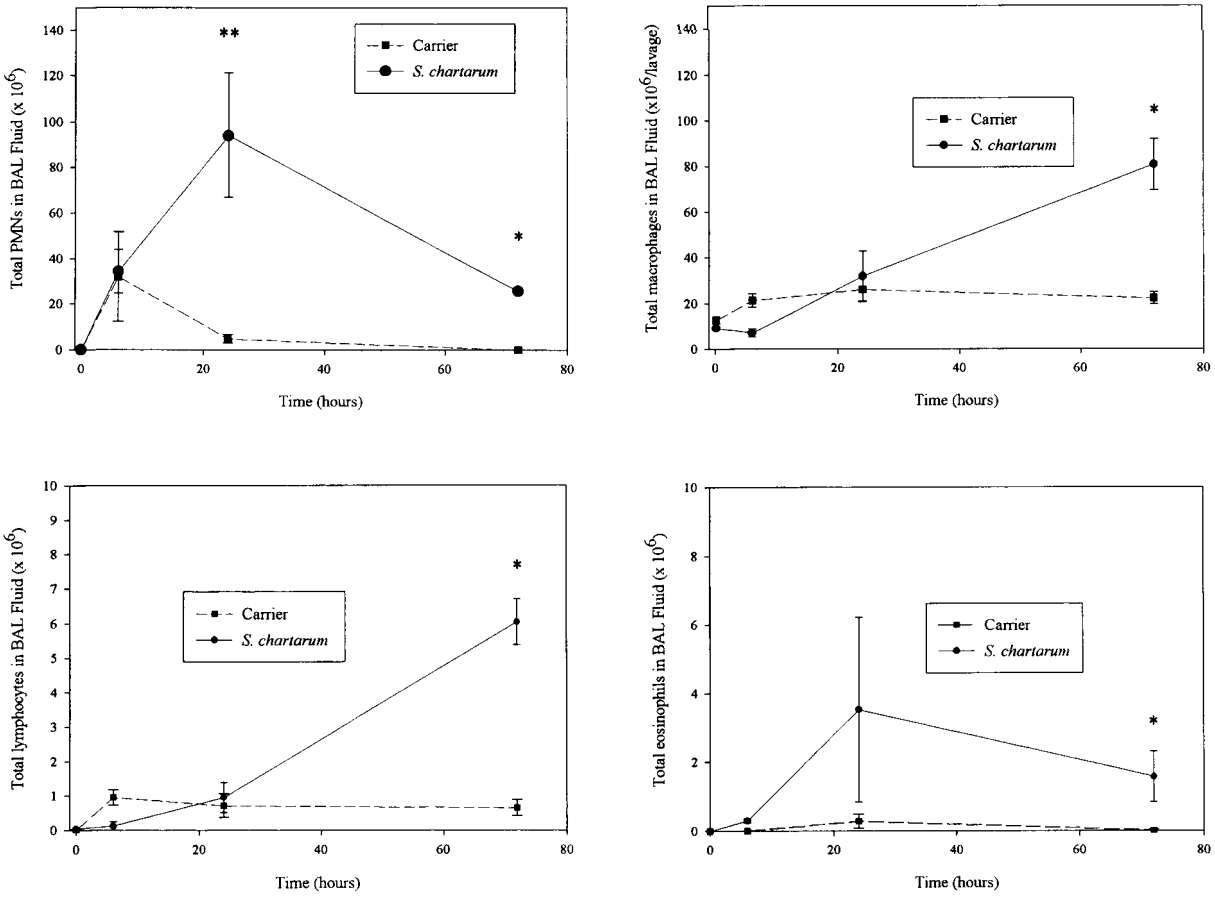

Figure 2. (a) Mean levels of PMNs, (b) macrophages, (c) lymphocytes, (d) eosinophils in bronchoalveolar lavage fluid ± S.E. over time. *S. chartarum* spore-instilled animals compared to carrier-instilled animals where * denotes $p < 0.05$ and ** denotes $p < 0.0125$ (Bonferroni-adjusted).

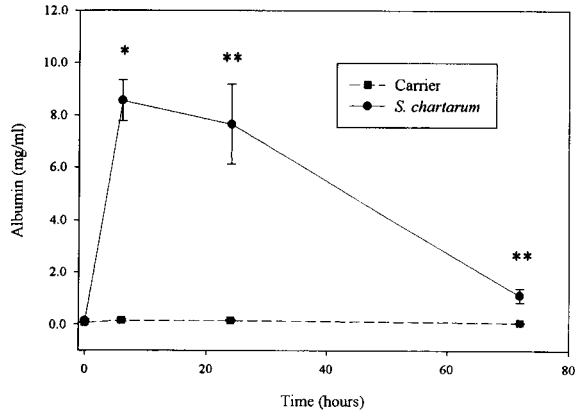

Figure 3. Mean concentrations of albumin in bronchoalveolar lavage fluid ± S.E. over time. *S. chartarum* spore instilled animals compared to carrier-instilled animals where * denotes $p < 0.05$ and ** denotes $p < 0.0125$ (Bonferroni-adjusted p-value).

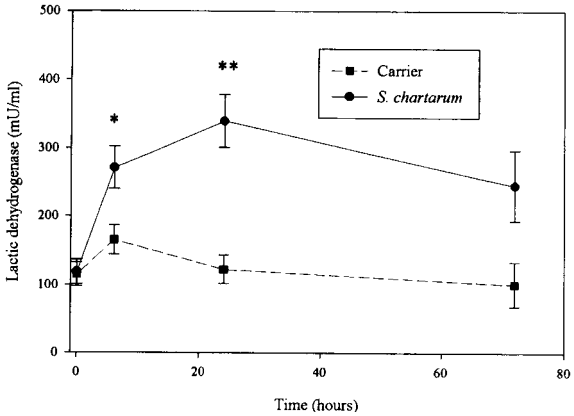

Figure 4. Mean concentrations of LD in bronchoalveolar lavage fluid ± S.E. over time. *S. chartarum* spore instilled animals compared to carrier-instilled animals where * denoted $p < 0.05$ and ** denotes $p < 0.0125$ (Bonferroni-adjusted p-value).

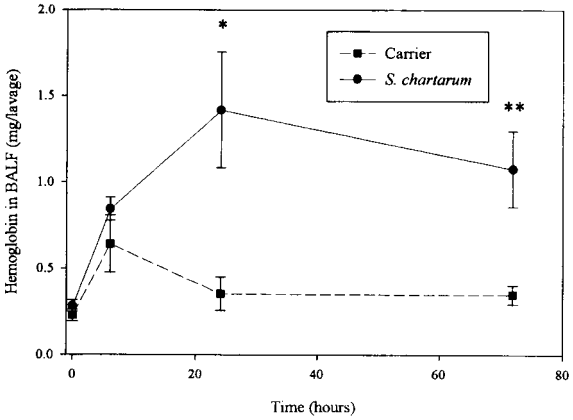

Figure 5. Mean concentrations of hemoglobin in bronchoalveolar lavage fluid ± S.E. over time. *S. chartarum* spore-instilled animals compared to carrier-instilled animals where * denotes $p < 0.05$ and ** denotes $p < 0.0125$ (Bonferroni-adjusted p-value).

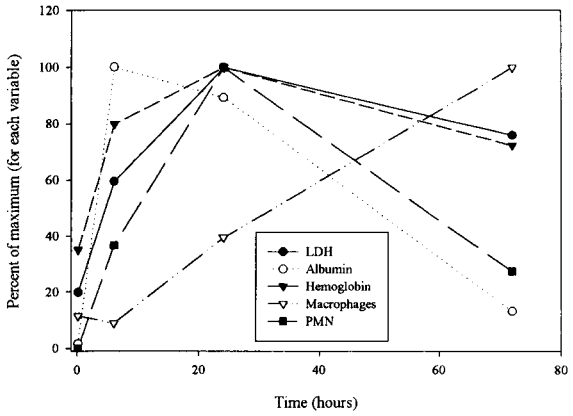

Figure 6. Time course of different bronchoalveolar lavage indicators expressed as percent of peak levels. The point at each time was derived by dividing the concentration at each time point by the maximum concentration measured over the time course.

sample sizes (we only tested 4 animals at time 0 and 6 h) or to increases in carrier control responses (e.g., hemoglobin and total PMNs). For most endpoints, differences between spore and carrier instilled animals become statistically significant at 24 h ($n = 7$) or 72 h ($n = 5$) after instillation.

Figure 6 depicts the time-course of these BAL indicators where the percent of the maximum for each indicator is plotted against time. Although some of the indicators were not statistically significantly different than the carrier control animals at their peak time point, the figure illustrates the overall time-dependence of the pulmonary effects of *S. chartarum* spores in the lungs.

Discussion

Acute pulmonary injury manifests itself as inflammation. When epithelial cells and alveolar macrophages encounter a toxicant, a series of responses occurs: increases in capillary permeability, chemotaxis of leukocytes, diapedesis of leukocytes through endothelial and epithelial barriers (sometimes accompanied by erythrocytes), mediator release, fibrin deposition in the area and disposal of necrotic debris by macrophages [34]. Our data generally demonstrate these events. We observed a peak albumin level at 6 h followed by maxima in LDH, PMNs, and hemoglobin (a major constituent of erythrocytes) at 24 h and macrophages at 72 h (Figure 6). In addition, initial weight loss followed by slow subsequent weight gain in the spore-exposed animals provides evidence that these exposures resulted in physiologically important changes (Figure 1).

Albumin is an abundant plasma protein with a molecular weight of 68,000 daltons that can pass through the air-blood barrier, especially during injury. It is the principal protein constituent in normal BAL fluid. Albumin levels vary with age, chronic pulmonary disease and smoking status [35]. Increased albumin can be a sensitive and early indicator of pulmonary inflammation. Immediate increases have been observed in response to ozone exposure (2 ppm) [36]. We observed significant increases in albumin levels 24 h after instillation of *S. chartarum* spores and also observed a clear peak in albumin level 6 h after *S. chartarum* spore instillation (Figure 3, $p = 0.014$). Although other factors may play a role, this early effect may represent a response to the *S. chartarum* toxins that are readily soluble in biological fluids [37].

Lactic dehydrogenase is a cytoplasmic metabolic enzyme present in essentially all cells. It is released from cells when damage or death and lysis have occurred. Intracellular concentrations are very high (e.g., lung cells contain 9500 U/g wet weight), and even localized damage can increase extracellular LDH levels in BAL fluid. Damage to phagocytic cells and epithelial cells following *S. chartarum* instillation may have contributed to the increased levels of LDH that we observed.

Hemoglobin is a surrogate indicator of erythrocyte infiltration into alveolar spaces. A marker of major changes in capillary permeability and localized injury, hemoglobin is not as sensitive to limited damage as albumin. Hemorrhaging of mucosal surfaces has been consistently reported as a major consequence of in-

tense exposures to *S. chartarum* [9, 24]; our data support these observations.

Alveolar macrophages are resident phagocytic cells present on the alveolar epithelial surface and are capable of elaborating a vast array of inflammatory mediators. Macrophages phagocytose foreign bodies, process and present antigens to lymphocytes, secrete protein messengers involved in inflammation, and activate helper T cells. We observed spores inside alveolar macrophages. In normal rodent BAL fluid, macrophages comprise over 95% of the leukocytes recovered. In our experiments, at time 0, over 98% of the leukocytes recovered were macrophages. Although the macrophage percentage of the total leukocyte population decreased over time as total leukocyte counts increased, the total number of macrophages increased over the time course of our experiments, reaching a maximum at 72 h. This increase in macrophages would probably help eliminate cellular debris and spores. Lymphocyte counts followed a similar pattern as macrophage counts (Figures 2b, c). In humans, lymphocytes make up about 8–10% of the recovered BAL cells with 60–70% of them being T lymphocytes [34]. A possible function of the alveolar macrophage is to present novel antigens to T lymphocytes, which may explain the concordance of macrophage and lymphocyte count trends.

PMNs (synonym neutrophils) are a second line of defense (after alveolar macrophages) involved with phagocytosis and release of chemicals involved in inflammation (vasodilators). They are a hallmark of acute inflammation. PMNs normally constitute less than 2% of human BAL cells. During inflammation, the PMN proportion may rise to over 90% [34]. PMNs enter lung parenchyma after recruitment from the systemic circulation by chemokines secreted by alveolar macrophages and other pulmonary cells. Some particle constituents that have chemotactic activity also recruit PMNs. In our experiments, PMN counts peaked at 24 h but had not yet returned to time 0 concentrations at 72 h. Although MPO is an enzyme mediator of oxidant production and an indicator of PMN degranulation, we did not observe increases in MPO levels. The *Stachybotrys chartarum* spores could have inhibited MPO release or failed to induce production of MPO. Alternatively, substances in BAL may have altered or inhibited MPO activity.

Our animals were exposed to a relatively large number of spores in one bolus (an average of 9.3×10^6 spores/rat). Realistic exposures are probably chronic and at low concentrations. In a study of multiple intranasal exposures (six, 1×10^5 spore doses over three weeks) to *Stachybotrys chartarum* in a murine model, histopathology showed severe inflammatory changes and hemorrhagic exudate in the bronchioles and alveoli [24]. We also observed increases in hemoglobin, an indicator of pulmonary hemorrhage, and acute inflammatory changes.

Although it is unlikely that an animal could inhale the very large number of spores in the few seconds that an intratracheal instillation requires, the method does allow delivery of a relatively precise dose directly into the lungs [38]. The deposited dose can only be estimated for inhalation protocols. The distribution of particles within the pulmonary system resulting from instillation will also be more centralized and less diffuse than for exposures by inhalation [38, 39]. However, if the total dose into the lung is equivalent, the pulmonary effects from intratracheal instillations and from inhalation exposures are comparable [40].

Our data indicate that direct pulmonary exposure to *S. chartarum* spores can cause severe inflammatory effects in the lungs. Inflammation in the respiratory tract can be caused by immune responses, infection, or injury [34]. Increases in eosinophils and lymphocytes, the common markers of allergic response, were not observed. Previous research found that IgG antibodies for *S. chartarum* were produced in mice when exposed intraperitoneally but not when intranasally instilled with *S. chartarum* spores [24]. *S. chartarum* is a cellulose-decaying saprophyte with no evidence of infectiveness [41]. These factors lead us to believe that the inflammatory effects of *S. chartarum* in the rat lungs were due to pulmonary injury caused by chemical constituents of the spores rather than to fungal infection. We have previously shown that reduction of toxin in *S. chartarum* spores by methanol extraction reduces the inflammatory pulmonary effects [42].

With the data obtained from the described experiments, we can begin to estimate risks of *S. chartarum* in the indoor environment. This method can provide a model to estimate risk for other airborne mycotoxins, and possibly other airborne biological agents. To assess the risks to humans from fungal exposures, investigations into the dose-effect relationship for these acute effects and pulmonary deposition characteristics via inhalation are essential, as are chronic exposure studies.

Acknowledgements

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