Title

Lung toxicity in mice of airborne particulate matter from a modern layer hen facility containing Proposition 2-compliant animal caging

Journal

Toxicology and Industrial Health, 33(3)

ISSN

0748-2337

Authors

Franzi, Lisa M
Linderholm, Angela L
Rabowsky, Michelle
et al.

Publication Date

2017-03-01

DOI

10.1177/0748233716630490

Peer reviewed

Page Proof Instructions and Queries

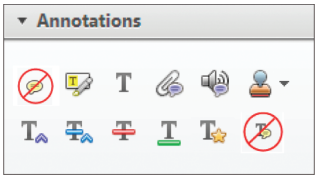
Journal Title: TIH

Article Number: 630490

Greetings, and thank you for publishing with SAGE. We have prepared this page proof for your review. Please respond to each of the below queries by digitally marking this PDF using Adobe Reader.

Click "Comment" in the upper right corner of Adobe Reader to access the mark-up tools as follows:

For textual edits, please use the "Annotations" tools. Please refrain from using the two tools crossed out below, as data loss can occur when using these tools.



For formatting requests, questions, or other complicated changes, please insert a comment using "Drawing Markups."

Detailed annotation guidelines can be viewed at: http://www.sagepub.com/repository/binaries/pdfs/AnnotationGuidelines.pdf
Adobe Reader can be downloaded (free) at: http://www.adobe.com/products/reader.html.

No.	Query
	Please confirm that all author information, including names, affiliations, sequence, and contact details, is correct.
	Please review the entire document for typographical errors, mathematical errors, and any other necessary corrections; check headings, tables, and figures.
	Please confirm that the Funding and Conflict of Interest statements are accurate.
	Please ensure that you have obtained and enclosed all necessary permissions for the reproduction of artistic works, (e.g. illustrations, photographs, charts, maps, other visual material, etc.) not owned by yourself. Please refer to your publishing agreement for further information.
	Please note that this proof represents your final opportunity to review your article prior to publication, so please do send all of your changes now.
AQ: 1	Mench et al. (2015 has been changed to Mench et al. 2011 to match the reference list. Please approve.
AQ: 2	Please provide the location details for manufacturer "Millipore."
AQ: 3	Please provide complete reference details for Zhao et al., 2015 or allow us to delete the citation.
AQ: 4	Please provide expansion for "BAL."
AQ: 5	Please provide expansion for "OSHA."
AQ: 6	Please provide complete reference details for Zhao et al. (2015) or allow us to delete the citation.
AQ: 7	Please provide accessed date for reference CDC, 2005.
AQ: 8	Please provide accessed date for reference OSHA, 2006.
AQ: 9	Zhao Y, Shepherd TA, Li H, et al. (2015b) is not cited in the text. Please indicate where a citation should appear or allow us to delete the reference.

Toxicology and Industrial Health
1–11

DOI: 10.1177/0748233716630490
tih.sagepub.com

Lung toxicity in mice of airborne particulate matter from a modern layer hen facility containing Proposition 2-compliant animal caging

Lisa M Franzi, Angela L Linderholm, Michelle Rabowsky and Jerold A Last

Abstract

Proposition 2, which requires that egg-laying hens be confined only in ways that allow these animals to lie down, stand up, fully extend their limbs and turn around freely, was passed by the voters of California in 2008. These new housing requirements were introduced in the United States and European Union without considering the potential impact of changes in layer hen housing on the health of poultry workers in the new facilities. Particles were collected from ambient air inside a large layer hen complex featuring separate barns with conventional battery caging, enriched caging, or "free range" (aviary) housing during winter, spring, and summer seasons over 1 year. Toxicity of the particles was evaluated by analysis of inflammatory cell influx into lung lavage fluid after intratracheal instillation into mice. Capacity of the particles to elicit oxidative stress was evaluated using a macrophage cell line engineered with a reporter gene sensitive to nuclear factor κB activation. We observed similar pro-inflammatory and pro-oxidant effects of the particles collected from different types of barns and over different seasons, suggesting that standard industrial hygiene techniques for evaluating respirable particles in ambient air can adequately monitor worker risk. Based on particle concentrations found in ambient air in the barns, we can rank the facilities for worker exposure to particles as conventional caging (now banned) approximately equal to enriched caging (permitted under Proposition 2). Aviary housing is associated with increased exposure of workers to particulate matter and, therefore, to greater risk of allergic reactions and/or decreased respiratory function.

Keywords

Occupational health, egg production, oxidative stress, lung inflammation, endotoxin, macrophages

Introduction

Concerns about protection of animals being raised for farming purposes have been a major source of discussion and legislation in the European Union (EU) for at least half a century. With regard to humane requirements for layer hen facilities, the EU decided in 1999 to ban conventional battery cages in favor of alternative housing systems that allowed the hens greater freedom of movement. Landmark regulations include the 1976 Convention on the Protection of Animals Kept for Farming Purposes, which addresses freedom of movement for caged animals and the outright banning of conventional battery-type cages in 1999 with the ban to take effect in 2012. The fascinating history of these regulatory efforts is well described in a recent paper by Mench et al. (2011) and the many cited references therein. These concepts and regulations eventually surfaced in the United States, initially with

Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, School of Medicine, University of California, Davis, CA, USA

Corresponding author:
Jerold A Last, Department of Internal Medicine, Division of Pulmonary, Critical Care, and Sleep Medicine, School of Medicine, University of California, 6519 GBSF, 451 Health Sciences Drive, Davis, CA 95616, USA.
Email: jalast@ucdavis.edu

the 2008 passage of Proposition 2 in California. Proposition 2 was modeled after the EU legislation on this topic. Similar regulations were subsequently adopted by the State of Michigan in 2009.

More than 8 million California voters passed Proposition 2, which requires that egg-laying hens be confined only in ways that allow these animals to lie down, stand up, fully extend their limbs and turn around freely. The large quantity of concentrated animal waste produced in such facilities and the potential need for bedding materials, which can act as an additional source of particulate pollution, raises important issues with regard to worker health and safety (Mitloehner and Schenker, 2007; Mitloehner and Calvo, 2008). The existing industry had previously adopted methods to address these issues in conventional production of eggs. Proposition 2 effectively banned the use of conventional battery cages, which included fully automated conveyer belt systems directly under the cages for manure removal. Therefore, the egg production industry in California had to adopt the use of either larger cages or systems that allow laying hens to range freely over floors of enclosed facilities and an alternate waste removal strategy. In addition, eggs imported into California have to indicate the type of housing the hens were maintained in.

California is home to more than 12% of the total US population, is the largest egg consuming state, and produces about 14% of the eggs in the United States, so the impact of California's new caging laws has spread over much of the industry nationwide. The impact of these changes on worker health and safety received no consideration in the ballot proposition presented to the voters. Thus, California was presented with a novel situation where a US$650 million/year industry (20 million layer hens) had to rapidly update its operations without a well-defined strategy or optimal technology. Other states (and some European countries) have also followed California's lead and passed legal mandates via new propositions or legislative actions to change layer hen housing requirements.

Layer hens that range freely within enclosed barns, in compliance with Proposition 2, require bedding material (e.g., rice hulls) to be used on the floors. This material when mixed with manure, urine, feathers and other materials produced by the laying hens becomes a major source of indoor particulate and gaseous air pollution as it dries. This process creates a new source of exposure to toxic materials that presently does not exist for the workers. Bird feces contain uric acid, which is rapidly converted to ammonia (NH_3), the major noxious gas associated with poultry operations in the presence of appropriate microorganisms. The recommended maximum ammonia level for US poultry housing is 25 ppm, the same as the 8-h daily time-weighted average exposure limits for humans set by National Institute of Occupational Safety and Health (CDC, 2005). Ammonia emissions from the facility are also an issue because it can react with indoor air pollutants to form secondary particulate matter (PM; salts and aerosols) that may be toxic when inhaled.

The manure and bedding mixture is typically removed from facilities between flocks (or more often), resulting in additional worker exposure to gas and particulate pollutants. The frequency of removal of the bedding is a major determinant of the concentrations of ammonia, other noxious gases, PM, and endotoxin within the barns. The use of bedding materials causes dust concentrations and emissions to be much higher for non-cage barns than for the currently used battery cage systems with automated waste and egg removal systems (Martensson and Pehrson, 1997; Takai et al., 1998; Wathes et al., 1997).

PM that can be inhaled into the lungs by humans is classified for regulatory purposes according to the size of the particles: coarse PM with mass median aerodynamic diameter (MMAD) between 2.5 and 10 μm ($PM_{2.5–10}$, also called PM_{10}) and fine PM with MMAD between 1.0 and 2.5 μm ($PM_{1.0–2.5}$, also called $PM_{2.5}$). The 8-h daily time-weighted average exposure limits for workers are 15 mg/m^3 for total dust and 5 mg/m^3 for respirable dust (OSHA, 2006). PM concentrations in animal housing are greatly influenced by the level of animal activities, feeding events, litter moisture content, and environmental conditions (e.g. temperature and humidity levels). Higher activity levels stir up more dust, especially with the presence of dry bedding materials and dry air as is typical for California, generating respirable dust (Li et al., 2008). These conditions also favor generation of bioaerosols containing endotoxin and other biologically active materials arising from microbial growth on the manure and other animal waste products collected on the bedding (Nimmermark et al., 2009). In this study, we examine the toxicity of PM produced with different strategies for animal housing and during different seasons of the year. Our results should provide improved guidance for waste handling in these facilities and indicate which strategies are the most effective at minimizing

worker exposures to the toxic components in PM within the production operation.

Thus, major changes in layer hen caging have occurred in the United States and the EU. This study addresses the following questions. (1) Is exposure of workers to PM the same in all three types of caging used—conventional (battery), enriched, and free range (aviary)? (2) Is toxicity of air emissions proportional to mass of PM pollutants emitted? Can conventional industrial hygiene monitoring of airborne PM concentration in the facility answer this question? (3) What is the role of endotoxin in layer hen facility PM toxicity?

Methods

The PM in this study was collected during three seasons, summer of 2011 and winter and spring of 2012. Total suspended particles (TSPs) were collected from a layer hen facility in the Midwestern United States (Zhao et al., 2015a). The facility consisted of three different types of barns for the hens: the conventional barn with the battery cages, the aviary with free-range ability for the hens, and the enriched barn with larger cages. The conventional barn housed 200,000 birds, while the aviary and enriched barns housed 50,000 birds each.

PM collection

TSPs were collected as described previously by Garcia et al. (2012) and Arteaga et al. (2015). Briefly, a SKC button sampler (225-360, SKC Inc., Eighty Four, Pennsylvania, USA) was attached to a high-flow Leland legacy personal sampling pump (SKC Inc.), placed on a shelf in the middle of the building. Samplers were calibrated for flow rate at the beginning and end of 48 h. A 2-day period was used to ensure collection of an adequate amount of PM for subsequent analyses. Millipore PTFE filters (FSLW02500, Millipore) with a diameter of 25 mm and pore size of 3.0 μm were used in the sampler. The sampler had a cut point of 100 μm under the conditions specified.

Filters were removed and weighed after sampling and stored at −20°C. The gross mass was recorded and the filter was scraped to remove as much material off the filter as possible. A cleaned and sterilized Hayman style microspatula was used to gently scrape across the filter to separate the TSP from the filter. A 2.0-mL sterilized microcentrifuge tube was weighed, then the scraped particles were placed inside and the final tube weight was recorded. Similar studies by Arteaga et al., 2015 have demonstrated similar recoveries of PM with 95% confidence intervals such that the differences between Aviary housing and conventional or enriched were significant at p values of less than 0.001 (their Figure 1(a)). Particles were then stored dry at −20°C until needed.

We examined particles from three seasons: spring, summer, and winter, and from the three types of barns: aviary, enriched, and conventional. Details of the three types of barns are given in Arteaga et al., 2015. Average conditions of temperature, relative humidity, ventilation rate, and concentrations of ammonia, carbon dioxide, methane, and coarse and fine PM in the various barns sampled are described in detail elsewhere (Zhao et al., 2015).

Animals

Male BALB/c mice, 8 weeks old, 20–25 g, were purchased from Jackson Laboratories, West Sacramento, California, USA. Animals were housed in an AALAC-approved facility under an IACUC-approved protocol. Animals were given food and water ad lib on a 12-h light and dark cycle, with a 1-week acclimation period before the start of any procedure.

Intratracheal instillations

Prior to PM instillation, mice were anesthetized with Attane (isoflurane) using an enclosed box chamber. The mice were placed in the supine position and the mouth/lower jaw was opened using forceps, with the tongue and lower jaw held open during the instillation. A 22-gauge Hamilton blunt-end needle attached to a glass syringe was inserted into the trachea. Tracheal insertion was confirmed by palpating the tracheal rings with the tip of the syringe. TSP suspensions were vortex mixed immediately before instillation. The mice were instilled with a single injection of 50 μL of saline (control) or with 50 μg of TSP (unless indicated otherwise) suspended in PBS. The 50 μg dose was chosen based upon previous dose–response experiments evaluating lung inflammation by lung lavage with PM isolated from ambient air collected from similar facilities (Wegesser and Last, 2008, 2009). Mice were allowed to fully recover ambulation before placement back into their cages. The animals were studied 24 h after PM instillation, the time interval at which we observe the peak

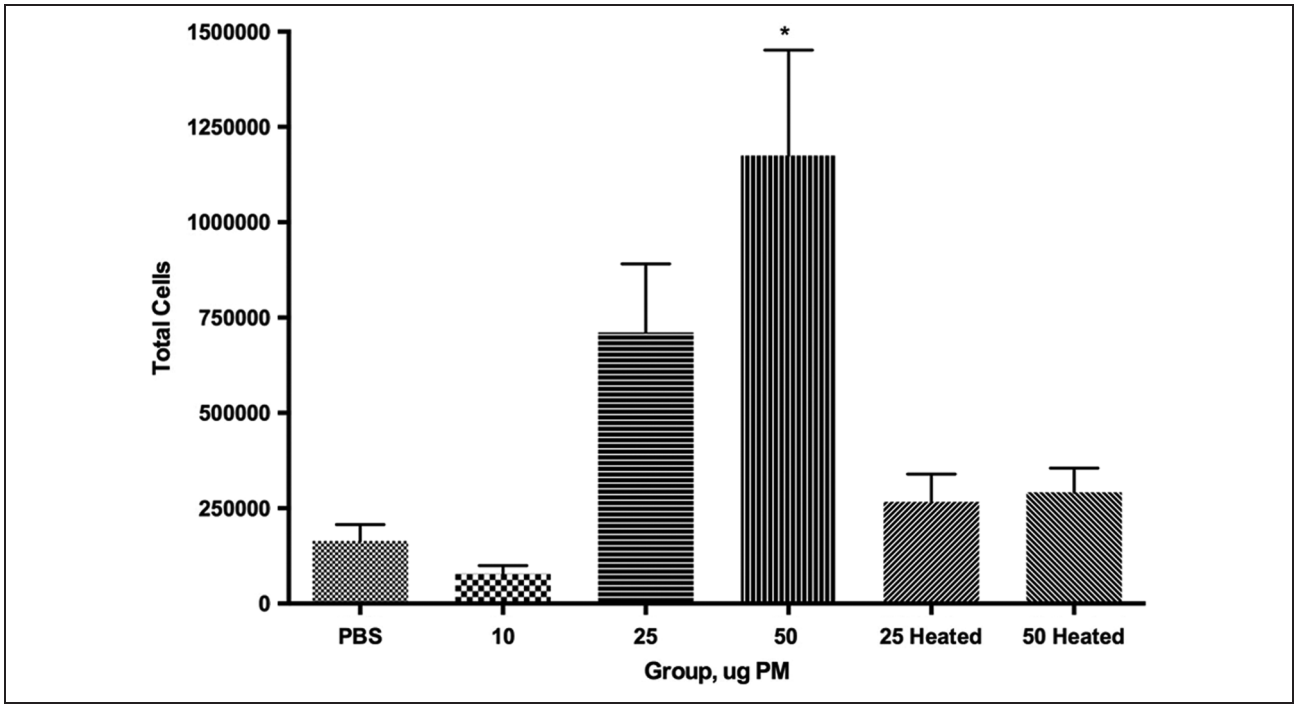


Figure 1. Dose–response of the total cells recovered by lung lavage from mice instilled intratracheally with the indicated amount (μg) of PM. The PM shown was collected during the winter season from a layer hen barn containing enriched caging. The asterisk indicates significant difference from the PBS control ($p = 0.0004$; 1-way ANOVA with Tukey's posttest for individual groups). $N = 6$ mice per group; data are means ± SEM. PBS (vehicle) instilled control animals. Heated: heat treatment at 130°C, sufficient to inactivate endotoxin present in the PM sample. ANOVA: analysis of variance; PM: particulate matter; PBS: phosphate-buffered saline.

neutrophil influx into the lungs of treated animals (Wegesser and Last, 2009).

Preparation and analysis of lung lavage fluid (from Wegesser and Last, 2009)

The total lavage fluid from each mouse lung was combined and centrifuged at 1800–2000 rpm for 10 min using a bench top unit (Centrifuge #5415C; Eppendorf, New York City, New York, USA). The supernatant was removed and the resulting pellet was resuspended in ACK Lysis Buffer (0.15 M ammonium chloride, 1.0 M potassium bicarbonate, 0.1 mM disodium ethylenediaminetetraacetate, and water) and centrifuged for another 10 min to remove lysed red blood cells. The supernatant was discarded and the pellet was suspended in 1 mL of PBS.

Total cell counts

The trypan blue exclusion method (Moshell et al., 1981) was used to count the total number of viable cells in each BAL sample using a hemocytometer.

AQ4

Differential cell counts

Additional cell cytospin preparations were performed with 100 μL of BAL fluid using a cytocentrifuge (StatSpin Cytofuge 2, Iris and Westwood, Massachusetts, USA). Prepared cytospin slides were stained with DiffQuick® (International Reagent Corp, Kobe, Japan). Cell differentials were counted as described previously (Kenyon et al. 2006). Briefly, cells were counted and classified by counting 10 fields (~400 Å) from each stained cytospin slide using a light microscope. Differential cell counts were expressed as percentages of total cell counts.

Heat treatment of PM to inactivate endotoxin

We have previously reported total cell counts in lung lavage fluid from mice instilled intratracheally with PM and the differential cell counts (predominantly neutrophils found after instillation of PM from a dairy barn) (Wegesser and Last, 2008; figure 11; neutrophils in BAL; Wegesser and Last, 2009; figure 6C) and the effects of heat treatment of the PM on these parameters. We have also demonstrated a strong

correlation between loss of endotoxin units from heated PM2.5-10, and from pure lipopolysaccharide (LPS) (Wegesser and Last, 2009; figure 8).

Determination of NF-κB activation

RAW-Blue™ cells (InvivoGen, San Diego, California, USA), a cell line derived from RAW 264.7 macrophages containing a nuclear factor (NF)-κB-driven reporter gene (Bruschi et al., 2010), were used to determine activation of NF-κB by measurement of the expression of secreted embryonic alkaline phosphatase (SEAP). In the presence of specific agonists (in this case the layer hen facility particles), these cells are induced to activate signaling pathways that lead to NF-κB activation. Once activated these cells secrete alkaline phosphatase, which is detectable and measurable by the use of QUANTI-Blue™ (InvivoGen) SEAP detection medium (Bruschi et al., 2010). To detect NF-κB activation, RAW-Blue™ cells were grown in semi-suspension in 100 mm nontissue culture treated Petri dishes until they reached approximately 80% confluence, then the cells were counted and resuspended in growth medium (Dulbecco's modified Eagle medium, 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum, 100 µg/mL Normocin™, and 2 mM L-glutamine). Twenty microliters of PM at various doses (prepared by dilution into PBS of 1–2 mg/mL stock solutions of frozen PM) were added to each well of a flat-bottomed 96-well plate. Endotoxin-free water, PBS, and DMSO (0.1 ng) were tested as negative controls. Then 100,000 cells in a total volume of 1 mL of medium were added per well and incubated at 37°C for 24 h. The following day, QUANTI-Blue™ was added to the supernatants prepared from the induced RAW-Blue™ cells. The plate was finally incubated for 30 min at 37°C, and the alkaline phosphatase levels were determined using a spectrophotometer at a wavelength of 650 nm (Franzi et al., 2011).

Polymyxin B experiments

We determined the role of LPS, the soluble fraction of endotoxin arising from the cell walls of gram-negative bacteria, in the responses of RAW 264.7 macrophages to the layer hen barn PM. To do this, we treated RAW-Blue™ cells with polymyxin B, an antibiotic that specifically binds LPS and prevents its binding to toll-like receptor (TLR)-4 receptors on the macrophages, using the methods described by Shoenfelt et al. (2009). Briefly, polymyxin B was added to the PM suspension at a concentration of 10 µg/mL and the resulting mixture was preincubated for 30 min with the LPS-binding agent prior to addition of PM to the cells during their 24 h of incubation with the particles (Franzi et al., 2011).

Experimental design

Experiments were separated by barn and season. For dose–response experiments, mice were instilled with a single IT dose of 10, 25, or 50 µg of TSP suspended in 50 µL PBS. TSP groups were divided into the following categories: Winter Aviary, Winter Enriched, Winter Conventional, Summer Aviary, Summer Conventional, Spring Enriched, Spring Aviary, and Spring Conventional. Not enough PM was collected from the Summer Enriched barn to perform a set of experiments. When enough TSP sample was available, each treatment was repeated to confirm results. Lung lavage fluid (BAL) was collected 24 h after PM instillation. For the preparation of heat-treated PM, a portion of each TSP sample was heated at 130°C for 24 h (Wegesser and Last, 2009).

We have reported the time course (3–72 h) of the response (including cell differentials in BAL) to particles isolated from a working dairy barn in other studies with similar mice (Wegesser and Last, 2009; figure 4). We chose 24 h duration for the current experiments to catch the peak for neutrophilic inflammation in this model.

Additional details of individual experiments are presented in the appropriate figure legends.

Statistical analysis of data

The statistical design of these experiments was designed to allow for comparisons between each facility tested and its respective control group as well as between different facilities. Initial comparisons were made using analysis of variance (ANOVA). Significant ANOVA effects were sub-tested with multiple comparison-corrected *t*-tests using Tukey's test (Prism 5.0 or 6.0; GraphPad Software, San Diego, California, USA). A two-tailed p value <0.05 was considered significant.

Results

Inhalation of airborne PM, especially PM rich in bioaerosol components such as endotoxin, causes lung inflammation in experimental animals (and in human subjects). In previous studies with

endotoxin-rich PM isolated from a dairy barn, we found that a dose of 50 μg of PM administered intratracheally into a mouse increased the total cells recovered by lung lavage significantly, with most of the additional cells being neutrophils (Wegesser and Last, 2009). Heat treatment sufficient to inactivate endotoxin in the samples reduced the recovery of total cells in the lavage fluid by approximately 60%, with the decrease being predominantly neutrophils. The 50-μg dose chosen for these studies was based on previous dose–response experiments with PM isolated from ambient air. In those experiments, we found a significant increase in total cells (predominantly neutrophils) recovered in lung lavage fluid at the 50 μg dose (Wegesser and Last, 2008).

Dose–response of whole PM

We confirmed that the 50-μg dose chosen for this study was appropriate with a dose–response experiment. In Figure 1, different doses of total PM (as isolated or heat treated) collected from a barn with enriched caging for layer hens were instilled. We observed an apparent dose–response relationship as measured by total cells recovered by lung lavage over a range of 0–50 μg. There was a significant response difference between 0 and 50 μg of PM. Comparable results were observed with PM collected from an aviary (''free range'') type barn, also with a dose–response. However, there were significant differences in total cells between 0 μg and both the 25 and 50 μg doses (data not shown).

Seasonal variation of PM

Previous studies have shown seasonal differences in the concentration of PM, and/or endotoxin emissions, in ambient air in layer hen facilities. We anticipated seasonal differences in PM and/or endotoxin levels in the layer hen barns featuring different types of animal housing. Therefore, we examined all of these variables in the series of experiments described below.

PM samples from all three seasons of collection and all three types of layer hen barns (Conventional Caging, Enriched Caging, and Aviary housing) were instilled into mouse lungs. We measured total cells in the lung lavage fluid for each condition except Summer Enriched, because there was not enough PM available to perform this experiment. When PM collected from any of the caging conditions was compared for each of the three seasons (two caging conditions for the summer season), we found no significant differences in the total cells in the lung lavage fluid. The results from the winter PM samples are shown in Figure 2. Similar results (no significant differences in the responses between caging modalities, albeit with lower numbers of total cells in the lung lavage fluid) were found for the spring and summer PM samples.

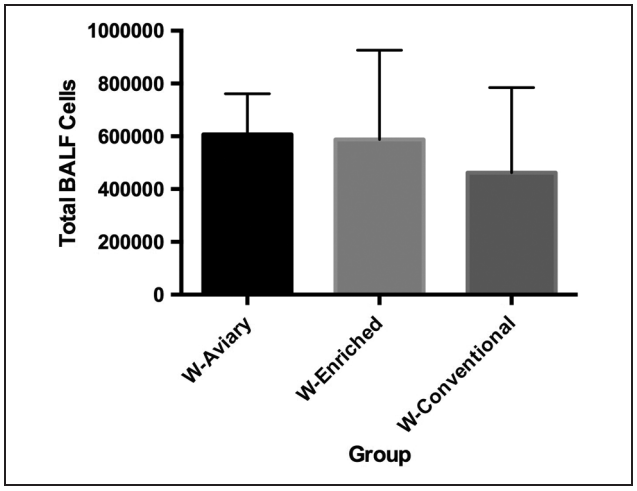


Figure 2. Total cells recovered by lung lavage from mice instilled intratracheally with PM, isolated in the winter season from barns containing each of the three different types of animal housing. $N = 6$ mice per group; data are means $\pm$ SEM. PM: particulate matter; SEM: standard error of the mean.

Inflammation response to instilled PM

Endotoxin (LPS), a component of the cell wall of gram-negative bacteria, elicits a lung inflammatory response initiated by its binding to TLR-4 receptors on cell surfaces. Other active components of bioaerosols derived from fungi or other types of microorganisms initiate the lung response by binding to other TLRs on cell surfaces. The observed response of the mouse lungs to exposure to bioaerosol components is known to include a rapid influx of neutrophils into the lung.

Cell differentials for the lung lavage fluid obtained from mice instilled with 50 μg of the winter season PM collected from all three types of barns studied are shown in Figure 3. Mice instilled with PM from all three types of barns showed a strong neutrophil-dominated inflammatory response. We also saw similar results, for all three types of barns, with the PM samples collected over spring or summer (data not shown).

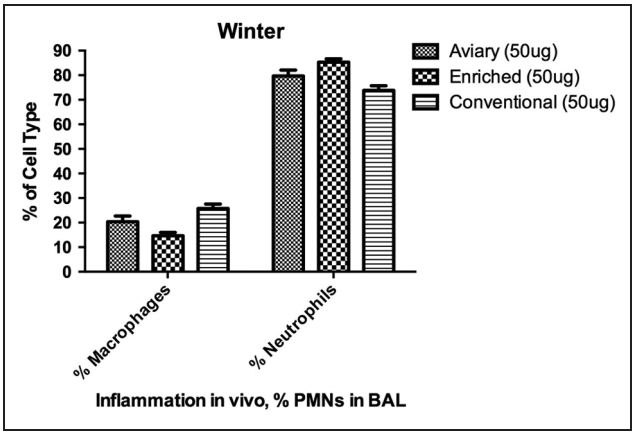


Figure 3. Cell differentials determined from bronchoalveolar lavage fluid prepared from mice instilled intratracheally with PM collected in the winter season from three different types of barns. $N = 6$ mice per group; data are means $\pm$ SEM. PM: particulate matter; SEM: standard error of the mean.

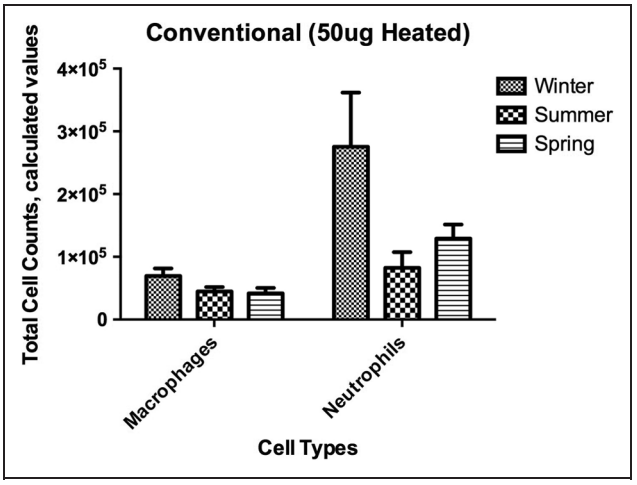


Figure 4. Cell differentials determined from bronchoalveolar lavage fluid prepared from mice instilled intratracheally with PM collected in all three seasons from conventional caging barns. $N = 4$ or 5 mice per group; data are means $\pm$ SEM. PM: particulate matter; SEM: standard error of the mean.

The neutrophil-dominated inflammatory response suggested that endotoxin in the PM was a major contributor to the observed lung inflammation. To confirm the role of endotoxin as a driver of lung inflammation with the various PM tested, we compared the response of mice to intratracheally instilled PM before and after heat treatment (Wegesser and Last, 2009). In a previous study with endotoxin-rich PM from a dairy barn, we demonstrated (Wegesser and Last, 2009) the rate of heat inactivation of endotoxin (measured by Limulus bioassay over a 2-h period) was identical for the PM samples and pure LPS (Lonza, Switzerland). As shown in Figure 1, heating of the layer hen barn PM samples (25 or 50 μg) removed most of the pro-inflammatory activity from the PM.

However, not all of the pro-inflammatory activity in the PM is due to endotoxin. When the differential cell counts after instillation of various samples of heat-treated PM are examined for their activity in mouse lung, we see residual neutrophils in all of the samples, especially the winter particles. None of the differences between seasonal samples are significantly different from each other when statistical comparisons (*t*-tests) are corrected for multiple comparisons, unequal variances, and non-Gaussian distribution of the data, as shown in Figure 4.

To further confirm the role of endotoxin in the pro-inflammatory response of the mouse lung to the layer hen PM, we incubated macrophages containing a reporter gene for NF-κB activation in tissue culture

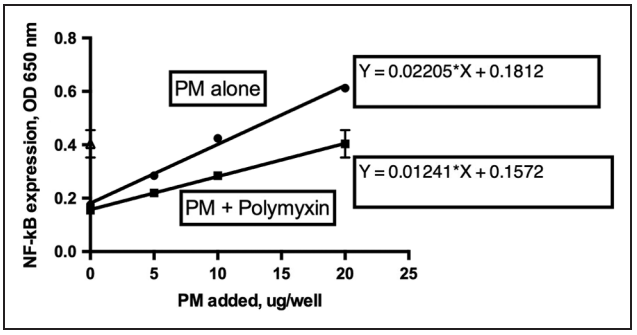


Figure 5. Dose–response for expression of a reporter gene for NF-κB activation in a macrophage cell line exposed to PM isolated during the winter season from a layer hen barn with aviary housing. The response to PM with or without preincubation with polymyxin B to prevent LPS binding to its cognate cell membrane receptor (TLR-4) is shown. PM: particulate matter; LPS: lipopolysaccharide; NF-κB: nuclear factor κB.

plates with 0–25 μg/well of layer hen PM (winter aviary housing). A clear dose–response curve for activation of NF-κB is apparent with increasing amounts of the PM (Figure 5). To further confirm LPS (endotoxin) as an active component of the PM, we added the antibiotic polymyxin B to the wells prior to incubation with the PM preparations. Inhibition by the antibiotic, which forms a complex with LPS that is unable to bind to the TLR-4 receptor to initiate the signaling pathway to NF-κB, is apparent at all of the doses of PM tested. However, the slope of the line

Table 1. Recovery of PM from filters for this study, total milligram recovered.[a]

Layer hen facility sampled, type			
Conventional	Enriched	Aviary (free range)	Blank
7.5	5.1	18.7	0.0

PM: particulate matter.
[a]PM was scraped from the sampling filters recovered from each type of barn into a plastic tube and weighed.

generated with added polymyxin B is still significantly greater than 0, again suggesting a role for components of the particulate fraction other than endotoxin in the response of macrophages to the layer hen barn PM samples.

In additional studies, we exposed macrophages containing a reporter gene for activation of NF-κB in cell culture to PM isolated from layer hen barns with different styles of caging. This assay measures PM-induced activation of this ubiquitous signaling pathway for selective activation of transcription of diverse genes, including several genes associated with responses of the cell to oxidative stress. In winter and spring, the PM from the barns with conventional caging produced significantly less response from the identical dose of PM than did the particles from Proposition-2 compliant caging. In summer, the response of the reporter gene to PM was low and was identical within experimental error for all three types of caging. Based on inhibition experiments with polymyxin B added to the particles, most or all of the reporter gene response observed with summer PM were caused by endotoxin, whereas only about half of the total oxidative stress was endotoxin driven in the spring and winter PM (data not shown).

The total yield of PM we were able to recover from the filters (calculated from the pooled amount in all of the seasonal samples) for the studies described herein, separated on the basis of type of barn, is indicated in Table 1.

Discussion

The issue of layer hen facility workers' exposure to indoor air contaminants has been a subject of concern and study in the previous literature. Most of the previously published papers we found in the literature have measured either respiratory effects in workers, concentrations of PM and/or endotoxin in the indoor air, or both in poultry raising facilities with conventional caging. For example, a major study in Canada (Senthilselvan et al., 2011) reported a significant correlation between respiratory symptoms in workers and total dust-associated endotoxin exposure.

There have been previous industrial hygiene studies of potential worker exposure to endotoxin in layer hen facilities with alternative housing systems. Huneau-Salaün et al. (2011) reported higher endotoxin levels in aviary barns than in conventional caging facilities. The ratio of endotoxin in aviary to conventional caging was 565:98 endotoxin units per cubic meter (5.8:1) in experimental facilities and 684:204 (3.4:1) in a field study. These values are lower than, but consistent with, our findings of a 7.6:1 ratio for the TSP concentrations in aviary versus conventional caging facilities. We should note the layer hen facilities in the French study were considerably smaller than in our study (about 1/10th as many total hens per barn) and that different size fractions of PM were compared in the different studies. Shepherd et al. (2015) found relative emission rates (mg/hen/day) for coarse PM in the barns of 100:16:16, a ratio of about 6.3:1:1 for aviary: enriched: conventional in the same facilities we studied in this article. Ahammed et al. (2014) compared laying performance and egg quality in aviary, the so-called "barn," and conventional cage raised layer hens in Korea. They found little difference between the three systems studied. However, caging type also has been reported to affect egg production in perhaps a counterintuitive way. Fewer hens are needed to produce 1000 kg of eggs in a conventional caging barn than in organic or free-range conditions (Leinonen et al., 2012).

Our study site contains separate barns stocked with either conventional battery cages or Proposition 2-compliant–enriched cages or aviary (free range) enclosures. Toxicological assays of bronchoalveolar lavage fluid from mice intratracheally instilled with PM collected from the layer hen facilities suggest that the inflammatory activity of the PM samples was similar per unit weight of particles regardless of the type of caging used. For the facilities tested during the winter sampling campaign, from which we had the most PM available for these experiments, the relative exposures to workers in the barns were approximately 35:6.4:4.6 for the aviary: enriched: conventional caging based upon the relative recovery of total PM from the test filters. Thus, based upon exposure to the airborne PM available, the aviary facility exposed layer hen facility workers to about 8 times more risk of inhaling PM capable of causing lung inflammation than they would have been exposed to in a

comparable conventional cage facility and about 6 times more risk than the exposure in a comparable enriched cage facility. Based upon the combination of toxicity per unit dose of PM and total dose of PM the workers would receive in the barns, we would rank the relative risk to workers in these barns containing the three different types of caging as follows: conventional < enriched < aviary.

Based upon our inhibition studies with polymyxin B and/or heat treatment of the PM, from half to all of the pro-inflammatory activity and NF-κB activation caused by the PM from the layer hen barns could be attributed to endotoxin in the particles. Previous studies by others have demonstrated the occurrence of endotoxin in PM from these facilities (Arteaga et al., 2015). However, our studies are, to the best of our knowledge, the first to do whole animal toxicological studies on these particles. The unique strength of animal toxicology experiments such as those we report here is they allow quantitative analysis of PM toxicity and the means to allocate the toxicity to specific components of the particles themselves. To our knowledge, this is the first report demonstrating the presence of inflammatory components other than endotoxin in the PM collected from the various types of layer hen barns.

Since the total PM emissions varied by caging source, these results lead to the conclusion that workers' lung responses to inhaled toxic particles in a layer hen facility will be directly proportional to the measured concentrations of the PM in the indoor air. Based on these experiments, we can conclude that strategies aimed at reduction of ambient PM levels in the indoor air of the layer hen facilities will be protective of worker health. This conclusion suggests that there will be a positive worker health benefit to the use of enriched cages as a strategy for compliance with Proposition 2 in preference to the promotion of free-range (aviary) enclosures, in agreement with previous recommendations of Mitchell et al. (2015) based upon measurements of respiratory symptoms in workers exposed to PM during shifts in the same three types of layer hen facilities in our study.

Based upon the nature of the PM samples available to us, the design of the layer hen facilities tested and the experimental design several significant assumptions had to be made for these studies. These assumptions include the following: (1) Layer hen number and density differ between the various types of barns. If differences in layer hen density in the barn introduce an uncontrolled variable in these data, we need to account for the effect of twice as many hens in the conventional caging as in the enriched or aviary barns. In all likelihood, if such an error exists, we would be systematically underestimating aviary PM toxicity. (2) The results presented in this study were obtained from PM samples collected over a single year in a single facility. Our conclusions arise from extrapolating the results from one location of barns and one sampling campaign (three seasons) over a single year. (3) TSP was used for these studies, not size fractionated into the OSHA-regulated coarse and fine PM fractions. We are tacitly assuming comparable size distribution in all three types of barn tested. The agreement of our findings with those of Shepherd et al. (2015) who measured size fractionated (coarse and fine PM) PM in the same barns from which we obtained out TSP samples suggests this assumption is reasonable. The relative proportions of TSP emitted in the three types of barn (approximately threefold higher from aviary facilities compared to conventional or enriched caging, which were similar to each other; Table 1 of this article) were comparable to the findings of Shepherd et al. with size fractionated PM from the same facilities (aviary barns approximately 5- to 6-fold higher for coarse PM and sixfold higher for fine PM; table 3 of Shepherd et al.) (4) The data we could obtain were limited by the low total amount of PM we had available for testing. Only representative data are shown from pooled PM obtained from the seasons and types of layer hen barns indicated. (5) Mice instilled intratracheally with PM are not the same as people inhaling the equivalent kind and amount of PM from the ambient air inside the barns. Differences in breathing patterns and immune systems between humans and mice could influence our conclusions.

AQ5

Finally, the question could also arise as to the relevance of our studies in mice to actual worker exposures and current OSHA standards. The 8-h daily TWA exposure limits for workers are 15 mg/m^3 for total dust and 5 mg/m^3 for respirable dust (OSHA, 2006). PM was collected on our filters for a 48-h shift. For the aviary barns, we found about 5 mg of total dust per filter. Thus, the workers in the spring and winter aviary barns were exposed to a total dose of approximately 1400–1500 mg/kg per 8-h shift. This dose may be compared with a 25-g mouse receiving an intratracheal dose of 50 μg of PM or 2 mg/kg in our studies. On the other hand, 2 mg/kg of PM is administered to the mouse as a bolus dose over about 30 s, while the human dose is spread over an 8-h shift, so

the dose rate is much higher in the mice. Conventional industrial hygiene studies performed by Zhao et al. (2015) found PM10 concentrations (mg/m^3) in the same barns we studied of 0.59 ± 0.16, 0.44 ± 0.18, and 3.95 ± 2.83 (conventional caging, enriched caging, and aviary), all under the relevant OSHA standards.

We performed animal toxicological studies on PM isolated from ambient air in three styles of layer hen facility: conventional caging (now banned by Proposition 2 in California and comparable legislation elsewhere), enriched caging, and aviary. Our results are completely consistent, both qualitatively and quantitatively, with published studies of the acute respiratory health impact of similar PM on workers in these barns. Because we had access to lung lavage fluid from the mice obtained by invasive procedures, we were able to determine the quantitative importance of lung inflammation and of endotoxin in the response elicited in these animals. Most importantly, because we knew the precise dose of PM administered to the lungs of the animals by intratracheal instillation, we were able to determine that the PM from all three types of facility had the same intrinsic toxicity. Therefore, the measured concentration of PM in the ambient air inside the barn is an appropriate surrogate measurement upon which to base occupational health standards for regulating permissible PM levels in the various types of layer hen barns.

In conclusion, our study demonstrates that there is significantly greater exposure to total PM in the aviary facilities than in conventional or enriched facilities, that the relative toxicity of the PM from all three facilities is directly comparable, and that a major contributor to the total toxicity of the PM is endotoxin. Thus, we suggest preferential use of enriched caging rather than aviary housing would be more protective of worker health.

Acknowledgments

We thank Frank Mitloehner for the PM samples we analyzed.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: National Institute of Occupational Safety and Health (NIOSH) U54 OH007550.

References

Ahammed M, Chae BJ, Lohakare J, et al. (2014) Comparison of aviary, barn and conventional cage raising of chickens on laying performance and egg quality. *Asian and Australasian Journal of Animal Science* 27: 1196–1203.

Arteaga V, Mitchell D, Armitage T, et al. (2015) Cage versus noncage laying-hen housings: respiratory exposures. *Journal of Agromedicine* 20: 245–255.

Bruschi M, Pirri G, Giuliani A, et al. (2010) Synthesis, characterization, antimicrobial activity and LPS interaction properties of SB041, a novel dendrimeric peptide with antimicrobial properties. *Peptides* 31: 1459–1467.

CDC (2005) NIOSH pocket guide to chemical hazards: Ammonia. Available at: www.cdc.gov/niosh.

Franzi LM, Bratt JM, Williams KM, et al. (2011) Why is particulate matter produced by wildfires toxic to lung macrophages? *Toxicology and Applied Pharmacology* 257: 182–188.

Garcia J, Bennett D, Tancredi D, et al. (2012) Characterization of endotoxin collected on california dairies using personal and area-based sampling methods. *Journal of Occupational and Environmental Hygiene* 9: 580–591.

Huneau-Salaun A, Le Bouquin S, Bex-Capelle V, et al. (2011) Endotoxin concentration in poultry houses for laying hens kept in cages or in alternative housing systems. *British Poultry Science* 52: 523–530.

Kenyon NJ, Last MS, Eiserich JP, et al. (2006) Differentiation of the roles of NO from airway epithelium and inflammatory cells in ozone-induced lung inflammation. *Toxicology and Applied Pharmacology* 215: 250–259.

Leinonen I, Williams AG, Wiseman J, et al. (2012) Predicting the environmental impacts of chicken systems in the United Kingdom through a life cycle assessment: egg production systems. *Poultry Science* 91: 26–40.

Li H, Xin H, Burns RT, et al. (2008). Effects of bird activity, ventilation rate and humidity on pm10 concentration and emission rate of a turkey barn. In: Stowell RR, Wheeler EF and Xin H (eds) *Proceedings of the 8th International Livestock Environment Symposium, Sept 1-3, 2008*, Iguassu Falls, Brazil. St Joseph, Michigan.

Martensson L and Pehrson C (1997) Air quality in a multiple tier rearing system for layer type pullets. *Journal of Agricultural Safety and Health* 3: 217–228.

Mench JA, Sumner DA and Rosen-Molina JT (2011) Sustainability of egg production in the United States—The policy and market context. *Poultry Science* 90: 229–240.

Mitchell D, Arteaga V, Armitage T, et al. (2015) Cage Versus Noncage Laying-Hen Housings: Worker Respiratory Health, *Journal of Agromedicine* 20: 256–264.

Mitloehner FM and Calvo MS (2008) Worker health and safety in concentrated animal feeding operations. *Journal of Agricultural Safety and Health* 14: 163–187.

Mitloehner FM and Schenker MB (2007) Environmental exposure and health effects from concentrated animal feeding operations. *Epidemiology* 18: 309–311

Moshell AN, Tarone RE, Newfield SA, et al. (1981) A simple and rapid method for evaluating the survival of xeroderma pigmentosum lymphoid lines after irradiation with ultraviolet light. *In Vitro* 17: 299–307.

Nimmermark S, Lund V, Gustafsson G, et al. (2009) Ammonia, dust and bacteria in welfare-oriented systems for laying hens. *Annals of Agricultural and Environmental Medicine* 16: 103–113.

OSHA (2006) Occupational safety and health administration. http://www.osha.gov/dts/sltc/methods/inorganic/id188/id188.html

Shoenfelt J, Mitkus RJ, Zeisler R, et al. (2009) Involvement of TLR2 and TLR4 in inflammatory immune responses induced by fine and coarse ambient air particulate matter. *Journal of Leukocyte Biology* 86: 303–312.

Shepherd T, Zhao Y and Xin H (2015) Environmental assessment of three laying-hen housing systems–Part II: air emissions. *Poultry Science* 94: 534–543.

Takai H, Pedersen S, Johnsen JO, et al. (1998) Concentrations and emissions of airborne dust in livestock buildings in Northern Europe. *Journal of Agricultural Engineering Research* 70: 59–77.

Wathes CM, Holden MR, Sneath RW, et al. (1997) Concentrations and emission rates of aerial ammonia, nitrous oxide, methane, carbon dioxide, dust and endotoxin in UK broiler and layer houses. *British Poultry Science* 38: 14–28.

Wegesser T and Last JA (2008) Lung response to coarse PM: bioassay in mice. *Toxicology and Applied Pharmacology* 230: 159–166.

Wegesser T and Last JA (2009) Mouse lung inflammation after instillation of particulate matter collected from a working dairy barn. *Toxicology and Applied Pharmacology* 236: 348–357.

Wenger I, Senthilselvan A, Beach J, et al. (2011) A prospective evaluation of air quality and workers' health in broiler and layer operations. *Occupational and Environmental Medicine* 68: 102–107.

Zhao Y, Shepherd TA, Swanson J, et al. (2015a) Comparative evaluation of three egg production systems: Housing characteristics and management practices. *Poultry Science* 94: 475–484.

Zhao Y, Shepherd TA, Li H, et al. (2015b) Environmental assessment of three laying-hen housing systems–Part I: monitoring system and indoor air quality. *Poultry Science* 94: 518–533.

AQ 8

AQ 9