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Toxicological assessment of dust from sanding micronized copper-treated lumber *in vivo*☆

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ABSTRACT

Micronized copper azole (MCA) is a lumber treatment improve longevity. In this study, the *in vivo* response to $PM_{2.5}$ sanding dust generated from MCA-treated lumber was compared to that of untreated yellow pine (UYP) or soluble copper azole-treated (CA-C) lumber to determine if the MCA was more bioactive than CA-C. Mice were exposed to doses (28, 140, or 280 μg/mouse) of UYP, MCA, or CA-C sanding dust using oropharyngeal aspiration. Bronchoalveolar lavage fluid (BALF) lactate dehydrogenase activity was increased at 1 day post-exposure to 280 μg/mouse of MCA and CA-C compared to UYP. BALF polymorphonuclear cells were increased by MCA and CA-C. There were increases in BALF cytokines in MCA and CA-C-exposed groups at 1 day post-exposure. Lung histopathology indicated inflammation with infiltration of neutrophils and macrophages. Pulmonary responses were more severe in MCA and CA-C-exposed groups at 1 day post-exposure. MCA caused more severe inflammatory responses than CA-C at 1 day post-exposure. These findings suggest that the MCA and CA-C sanding dusts are more bioactive than the UYP sanding dust, and, moreover, the MCA sanding dust is more bioactive in comparison to the CA-C sanding dust. No chronic toxic effects were observed among all observed sanding dusts.

1. Introduction

Pressure treating lumber is a low-cost process that increases the durability of outdoor wood structures for several years [1]. Previously, the pressurized wood treatment was arsenic-based; however, starting in the 1990s, it was phased out. The arsenic treatment was later replaced with copper-treated lumber with an additional organic co-biocide treatment for fungi that were copper resistant [2,3]. Originally, the copper used in wood treatment was in water soluble forms: alkaline copper quaternary (ACQ) and copper azole type-C (CA-C) [2,4]. However, in order to increase the final copper concentration reached in the treated wood, particle copper carbonate was introduced into the industry in 2006. In this new technique, the particle copper carbonate is ground into fine particles and suspended for pressure treatment along with a dispersant and an organic co-biocide. The average diameter of this micronized copper azole (MCA) is approximately 300–500 nm [2]. Compared to the CA-C treatment, the MCA treatment exhibits a much lower copper solubility. The use of micronized copper increases copper concentration in the treated wood, prolonging the effective half-life [2,5,6]. Particle copper carbonate used in the MCA wood treatment has become a concern for the environment and workers handling it. Particularly, sanding the MCA-treated wood may release MCA along with the wood dust, which could potentially pose a hazard to human health.

Our previous *in vitro* studies showed that sanding the MCA-treated wood, as well as the CA-C-treated wood and untreated yellow pine (UYP), released dust with a number-based mean diameter (d_{pg}) of approximately1 μm and the mass-based d_{pg} mean of about 4–5 μm [4], which is within the human respirable range. Sanding of the MCA-treated wood may release MCA from the dust as soluble copper (Cu^{2+}) or as copper carbonate nanoparticles [7,8]. Copper carbonate

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention or the Consumer Product Safety Commission.

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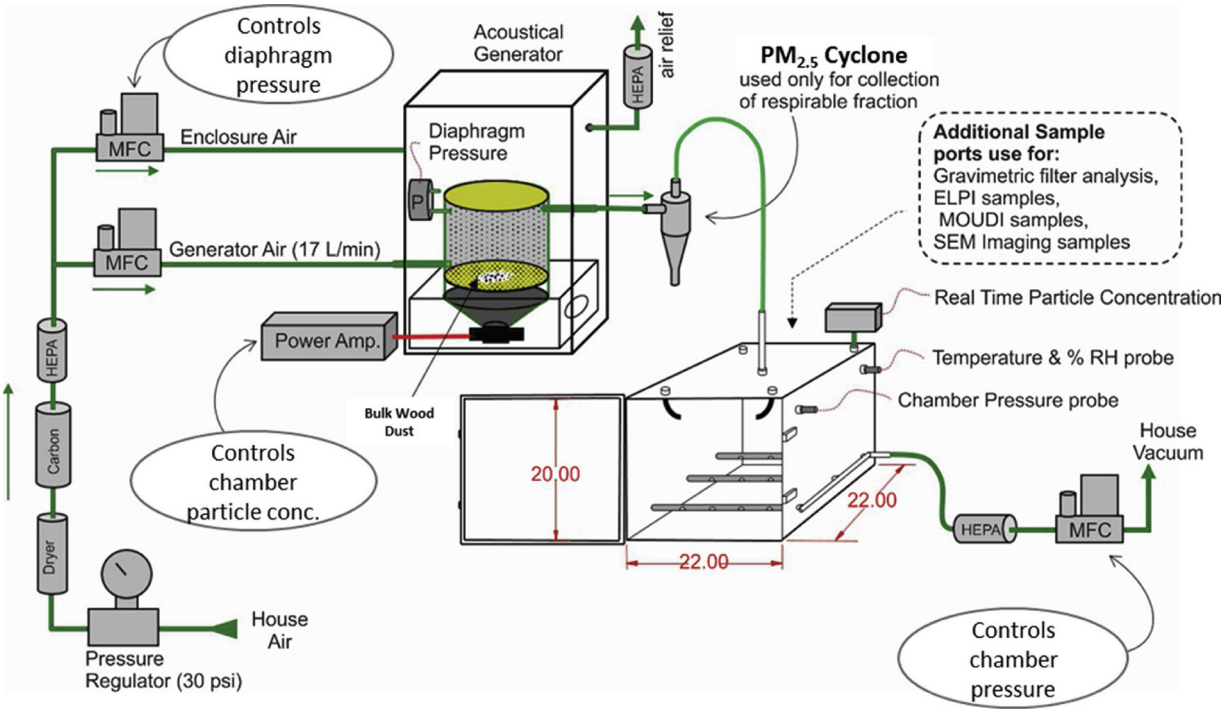

Fig. 1. Acoustic generator inhalation chamber. The acoustic generator inhalation chamber was used to re-aerosolize the sanding dust with the addition of the $PM_{2.5}$ cyclone used to collect the respirable fraction.

nanoparticles have been shown to induce cytotoxicity and DNA damage [9]. It has also been demonstrated that ingestion of copper nanoparticles induced adverse effects in mouse organs, including kidney, liver, and spleen [10,11]. Our previous results indicated that sanding MCA-treated wood released a greater number of nanoparticles than sanding CA-C and UYP-treated wood, with the particle size range between 0.4 µm to 2 µm containing the highest copper concentration [4]. Further analysis found that nanoparticles were present in the fraction of particles less than or equal to 2.5 µm ($PM_{2.5}$) of the MCA sanding dust whereas no nanoparticles were seen in the $PM_{2.5}$ of the CA-C and UPY sanding dust. Furthermore, the $PM_{2.5}$ from the MCA sanding contained more copper than the $PM_{2.5}$ from the CA-C and UYP sanding dust even though the bulk lumbers of MCA and CA-C contained similar copper contents. Inductively coupled plasma mass spectrometry (ICP-MS) analysis revealed that the $PM_{2.5}$ of the MCA sanding dust released copper nanoparticles into the cell culture medium. The cell-based *in vitro* toxicity analysis found that in high concentrations of copper ($\geq$ 5 µg/ml), the supernatants of both MCA and CA-C suspensions were capable of inducing toxicity *in vitro*.

The present study aimed to determine the *in* vivo pulmonary effects of exposure to the sanding dusts from MCA, CA-C and UYP-treated lumber, delivered *via* oropharyngeal aspiration. To accomplish this, bronchoalveolar lavage fluid (BALF) from exposed mice was analyzed for lactate dehydrogenase (LDH) activity, polymorphonuclear cell (PMN) infiltration, and inflammatory cytokine/chemokine production. Lung histopathology was examined for inflammatory and fibrotic responses in mouse lungs after up to 84 days post-exposure.

2. Materials and methods

2.1. Materials

Three types of lumber were used to assess the bioactivity of the micronized copper: UYP (EACOM Timber Corporation, Montreal, Quebec, Canada) and two types of treated lumber: MCA “ground contact” and CA-C “ground contact”. The MCA-treated lumber tested was manufactured by Madison Wood Preservers, Inc. (Madison, VA), and had a preservative content retention of 0.14 pound per cubic foot (PCF, equivalent to 2.2 kg/m^3). The CA-C-treated lumber tested was manufactured by CN Tucker Lumber Company (Pageland, SC), and had a retention of 0.15 PCF (2.4 kg/m^3). The UYP was used as a negative control, while CA-C was used for comparison with the bioactivity of the micronized copper used in MCA. All lumber tested originated from yellow pine. MCA-treated lumber is normally labeled either “µCA-C” or “MCA”; throughout this paper, MCA will be used as the abbreviation.

2.2. Methods

2.2.1. Production, aerosolization, collection, and analysis of wood dust particles

The details of production, collection and analysis of wood dust have been published [4].

Sanding wood generally produces a substantial amount of particles that are too large (greater than 10 µm) to be inhaled and deposited into the respiratory zone of the lungs. We used a specially designed stainless steel cyclone with a 2.5 µm cut point (URG-2000-30EHS, URG corp., Chapel Hill, NC) to separate and collect the $PM_{2.5}$ fraction of the re-aerosolized particles so that it could be used for both *in vitro* assays and oropharyngeal aspiration exposure in mice. Wood dust particle size distributions (PSD) were measured with and without the cyclone using the MOUDI to ensure Supplemental Fig. 1), its effectivness (A bank of 5 sample filters (Whatman 25 mm, 0.2 µm pore size polycarbonate filters), each with sample flow rates of 1 l/min, was used to collect $PM_{2.5}$ from the sample chamber. The cyclone was removed and cleaned after 20 min of use. After 5 rounds of 20 min sampling, the filters were removed. This process was repeated several times for each type of wood dust until a sufficient amount of material for animal exposures was collected (Fig. 1).

2.2.2. Suspension of wood dust

Wood dust, in varying amounts, was re-suspended in U.S.P. saline. Samples were then sonicated for 1–2 min or until the wood dust suspension was uniform. Samples were vortexed immediately before use.

2.2.3. Copper in aspiration media

Measurement of copper leaching from the wood dust into the saline suspension media has been previously described [4]. Enhanced-darkfield microscopy was used to detect the presence of free copper nanoparticles in the intermediate, aqueous layer.

2.2.4. Animals

Eight week old (weighing 22.65 ± 0.36 g) male C57BL/6 J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animal welfare and housing information may be found in supplemental section S1.1.

Mice were treated with UYP, MCA, or CA-C at the doses of 28, 140 or 280 μg dust/mouse using oropharyngeal aspiration following the previously published protocol [12]. The mice were given wellness checks and weights were measured monthly to monitor health. At 1 and 7 days post-exposure, whole lungs were analyzed for inflammatory and damage markers in bronchoalveolar lavage fluid (BALF) (n = 10–12 animals per treatment group). At 1 and 84 day post-exposure, whole lungs from a separate group of animals were analyzed for histopathological changes at the dose of 280 μg/mouse (n = 10–12 animals per treatment group).

2.2.5. Bronchoalveolar lavage

At 1 and 7 days post-exposure, mice were administered an overdose of sodium pentobarbital (> 100 mg/kg body weight) (Fatal Plus, Vortech Pharmaceuticals Ltd., Dearborn, MI) *via* intraperitoneal injection (i.p.) injection, followed by exsanguination. The protocol used for this study has been previously described [13], with detail in supplemental section S1.2.

2.2.6. BALF cytokine and chemokine measurement

BALF collected from mice sacrificed at 1 and 7 days post-exposure was used to analyze cytokine and chemokine protein expression according to manufacturer guidelines. Details may be found in supplemental section S1.3.

2.2.7. Histopathology

At 1 and 84 days post exposure, mice from the saline and 280 μg/mouse sanding dust groups were euthanized. At necropsy, the lung was inflated with 1 ml 10% neutral buffered formalin. Lungs were processed, embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin (H&E) for microscopic analysis. Sections of the lung were analyzed by a board-certified veterinary pathologist at NIOSH (Morgantown, WV) for histopathological analysis. H&E stained slides were examined using bright-field microscopy to evaluate morphologic changes and by polarizing light microscopy for identifying foreign material. Morphologic changes were characterized and scored according to the criteria provided in Table 1.

2.2.8. Enhanced-darkfield microscopy (EDM) of wood dust in tissue sections

Untreated wood dusts, as well as the micronized copper particles embedded in the MCA wood particles were examined using EDM [14]. The EDM images light scattered due to the crystalline nature of particles and differences in refractive index from the background medium. Further details for EDM analysis can be found in supplemental section S1.4.

Table 1
Histopathology Scoring System.

Score	Meaning
WNL	Within Normal Limits
1	Minimal, change barely exceeds that which is considered normal
2	Mild/slight, the lesion is easily identified but is of limited severity
3	Moderate, the lesion is prominent but there is significant potential for increased severity
4	Severe, the change is as complete as possible, occupies the majority of the organ

2.2.9. BALF LDH measurement

Acellular BALF LDH activity was measured using a COBAS MIRA Plus (Roche Diagnostic Systems; Montclair, NJ).

2.2.10. Light microscopy examination of wood dust in tissue sections stained with grocott methenamine silver stain (GMS)

While EDM was ideal for examination of micronized copper particles in isolated wood dust samples, visualization of small, respirable particles in tissue sections proved more difficult. To facilitate visualization, tissue sections were stained with GMS which imparts a black stain to lignin, a complex organic polymer found in the cell walls of plants. Light microscopy of GMS-stained tissue sections was then used [15] to measure particle diameter of respirable wood fibers in alveoli from mice exposed UYP, MCA, CA-C at 280 μg or saline. Further details can be found in supplemental section S1.5.

2.2.11. Measurement of area-equivalent diameter of cyclone separated wood dusts

Given that wood is a porous material and has the potential to take up water and swell in aqueous solution and *in vivo*, measurement of wood particle area-equivalent diameter was used to compare the size distribution of wood particles in the untreated cyclone separated samples with those of wood particles in the alveolar region of treated lungs. EDM micrographs were taken for dry, untreated samples of UYP, MCA and CA-C (30 micrographs from 3 slides). Further details can be found is supplemental section S1.5.

2.2.12. Statistical analysis

Statistical comparisons for UYP, MCA and CA-C-exposed groups, across three exposure levels, were performed for each of three post-exposure times using analysis of variance (ANOVA). Further details can be found in supplemental section S1.6.

3. Results

3.1. Characterization and collection of PM2.5 from sanding wood dusts

The particle size distributions based on both mass and number concentration for all three types of wood dusts are plotted in Fig. 2. There were no statistically significant differences between mass- or number-based mean particle sizes. The mass and number-based size distributions data suggests that the copper within the treated wood does not separate into smaller particles from sanding dust, and that it stayed embeded within larger wood particles. There was a lack of such particles detected with a field emission scanning electron microscope (FESEM), and all 3 types of wood dusts had very similar sizes and shapes. Supplemental Fig. 2 shows images of wood dust particle taken with the FESEM.

3.2. Copper content in lumber and the $PM_{2.5}$ fraction of sanding wood dust

ICPMS analysis was applied to measure the copper content within the lumber, $PM_{2.5}$ fraction of wood sanding dust, and saline dispersion medium. Metal content for bulk UYP, MCA and CA-C samples has been previously reported [4]. The copper content (μg/g) within the bulk lumber dust is shown in Fig. 3A. The copper content in MCA and CA-C bulk lumber dust is similar, but not detectable in UYP. Isolated $PM_{2.5}$ fractions were also analyzed for the total metal contents (Supplemental Table 1) including copper (Fig. 3B). The $PM_{2.5}$ fraction of MCA dust contained higher amounts of copper compared to CA-C, while the $PM_{2.5}$ fraction of UYP did not contain copper in detectable amounts. These data suggest that both MCA-treated lumber and CA-C-treated lumber contained equal amounts of copper. However, in the $PM_{2.5}$ fraction,

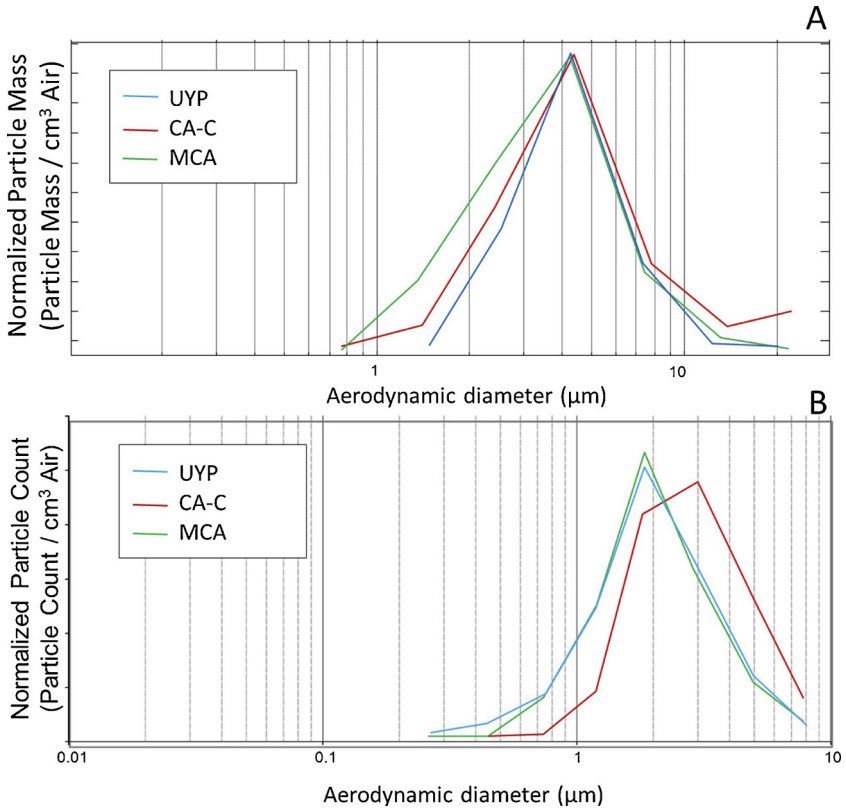

Fig. 2. Particle size distribution (PSD) of re-aerosolized wood dust. (A) Mass–based PSD measured by a micro-orifice uniform deposit impactor (MOUDI), and (B) Number-based PSD measured by an Electrical Low Pressure Impactor (ELPI).

MCA contained more copper. The saline extract samples were analyzed for metal using ICPMS (Supplemental Table 1) and it revealed that CA-C samples had a slightly higher level of copper leached into the saline in comparison to MCA (Fig. 3C). Copper was not detected in the saline control and UYP samples. Therefore, the copper measured within the saline samples was due to the copper from the pressurized treated lumber and not from the saline or wood. No free nanoparticles were revealed when the saline samples were imaged with EDM microscopy. These data suggest that copper is released from the pressurized treated lumber sanding dust of both MCA and CA-C.

Representative EDM images of untreated, cyclone-separated wood particles for UYP, MCA, and CA-C particles are given in Fig. 4. No qualitative differences were noted in size or shape between the different particles. Micronized copper particles were found within the MCA wood particles. Micronized copper particles efficiently scattered light in the MCA wood particles and were demonstrated in both cyclone-separated MCA wood dust (Fig. 4A) and in the MCA-treated lungs.

3.3. Analysis of pulmonary toxicity of wood dust in vivo

3.3.1. Particle deposition and clearance from the lungs

GMS staining of wood particles was used to assess the deposition and clearance of wood particles in lung sections and representative images are shown in Fig. 5. Wood particles in lungs of mice exposed to UYP, MCA and CA-C wood dusts were found widely distributed in the alveolar region at 1 day after exposure (left column of Fig. 5). By 84 days after exposure to all three wood particle types, the majority of particles were cleared from the lungs.

To determine whether there were changes in the diameter of wood dust particles in lung sections due to swelling in aqueous solution, particle diameters were measure in lung sections from treated mice and compared to untreated particles. Fig. 6 depicts the cross-sectional diameter of wood particles in untreated cyclone-separated wood particles and in the alveolar region of the lungs at 1 day post-exposure. Arithmetic means ± standards deviation of particle diameter for untreated cyclone-separated wood particles were: 2.76 ± 3.36, N = 247; 2.55 ± 3.69, N = 110; and 2.20 ± 3.25 µm, N = 259 for UYP, MCA

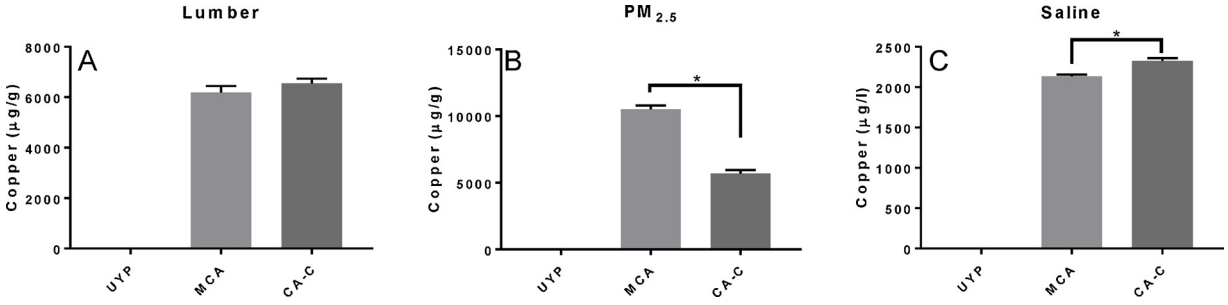

Fig. 3. Copper content measured by ICPMS. ICPMS analysis of UYP, MCA and CA-C in lumber (A), $PM_{2.5}$ (B), and saline extract (C) for elemental copper. n = 3 experiments, values are means ± standard error. * represents $p < 0.05$ significance between MCA and CA-C.

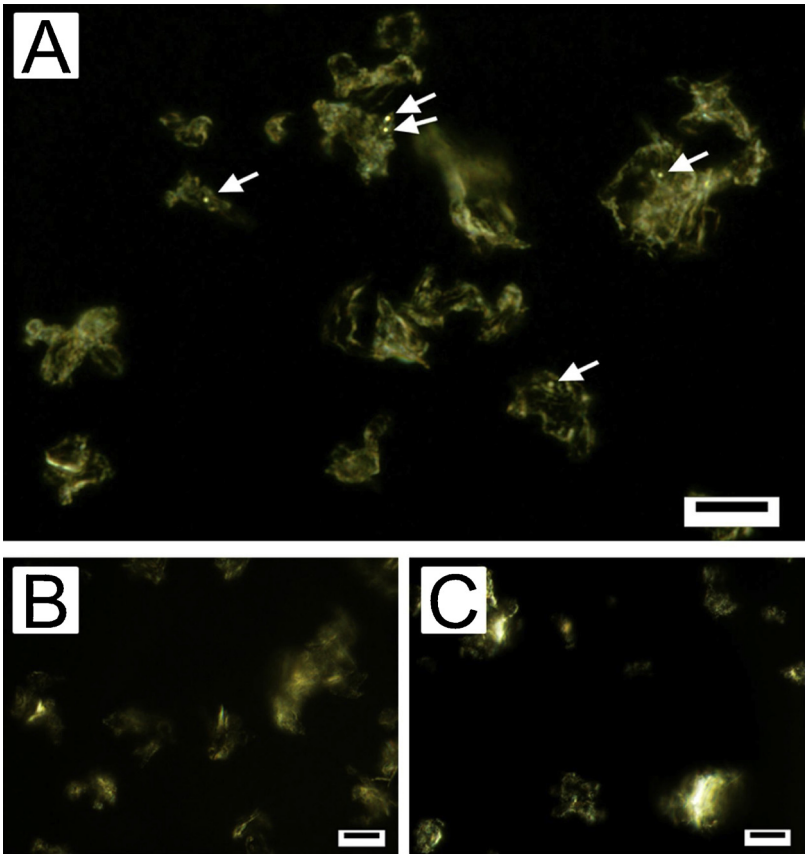

Fig. 4. EDM images of cyclone-separated wood particles. White arrows in the MCA image (A). indicate micronized copper particles, which are absent in UYP (B) or CA-C (C) Calibration marker is 10 µm.

and CA-C particles, respectively (N represents number of particles measured). Means ± standard deviations for particle diameters of alveolar region particles at day 1 after exposure were: 1.39 ± 1.66, N = 555; 2.78 ± 5.73, N = 510; and 1.43 ± 1.46 µm, N = 520 for UYP, MCA and CA-C particles, respectively. The mean diameter of the wood dust particles deposited in the lung alveoli was smaller for CAC and UYP wood particles than the comparable untreated cyclone-separated wood particles. Median particle diameters were 0.87, 0.92, and 0.96 µm for the alveolar-deposited wood particles (UYP, MCA and CA-C, respectively), and 0.96, 1.10, and 0.91 µm for the untreated cyclone-separated wood particles (UYP, MCA and CA-C, respectively). As determined in wood-free control sections, the GMS staining was found to produce minimal artifactual stain in the alveolar region. However, there was positive staining of secretory granules within airway

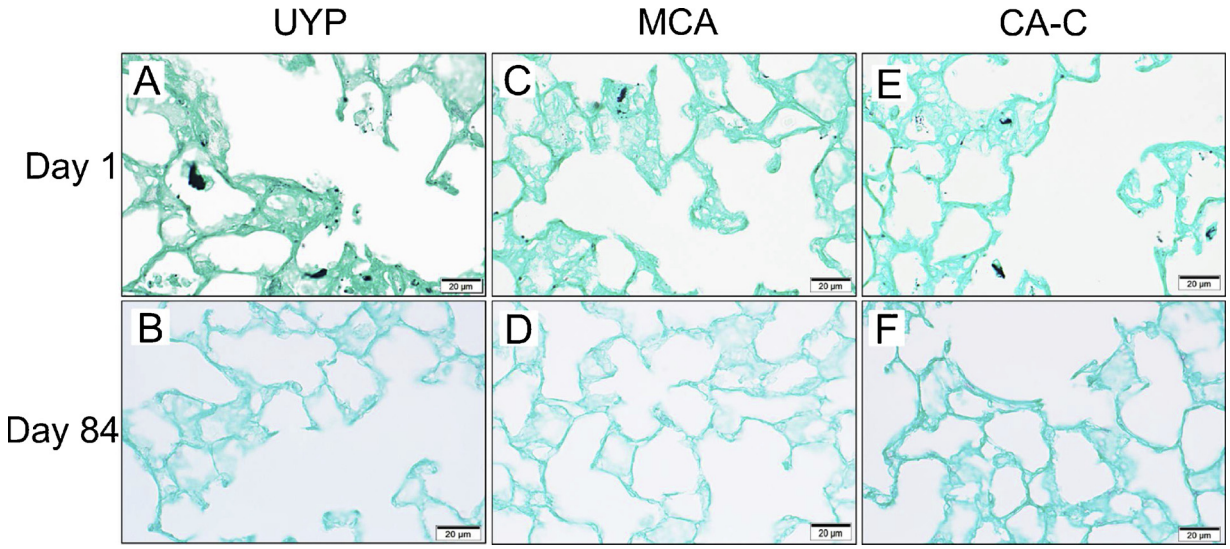

Fig. 5. Light microscope images of GMS-treated sections of the alveolar region from mice lung at 1 and 84 days after instillation. As shown the top row of Fig. 5, GMS treatment of sections at 1 day post-exposure demonstrated and widespread pattern of wood deposition (black stain). Except for rare cases, to be described in the histopathology section, by 84 days post-exposure (bottom row), the alveolar region was essentially cleared of wood particles for all three groups.

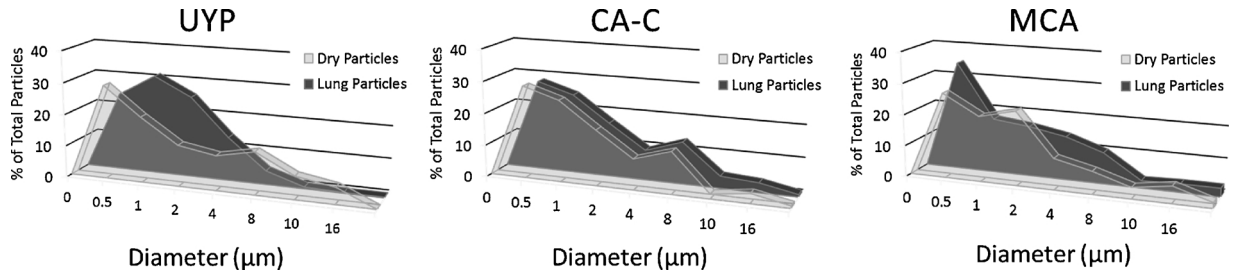

Fig. 6. Cross-sectional diameter in lungs and raw wood particles. Particle cross-sectional diameter for untreated, cyclone-separated (Dry) and day 1 alveolar region (Lung) wood particles.

epithelial cells. There were no statistically significant differences in the mean particle size for any group. Thus, it does not appear that dispersing wood dust particles in an aqueous solution significantly changed the mean particle diameters compared to the comparable untreated dry particles.

3.3.2. Pulmonary damage and inflammatory cell influx

LDH in BALF was used as a marker of pulmonary damage in murine lung at 1 and 7 days post-exposure to the three types of wood dust. The 280 μg dose of MCA elicited a significant increase in BALF LDH activity and day 1 post-exposure (Fig. 7A). This particle-induced LDH activity returned to control levels at 7 days post-exposure (Fig. 7B). Neither UYP or CA-C elicited any LDH changes at any dose or time point. This finding suggests that MCA causes more acute pulmonary damage than UYP or CA-C at 1 day post-exposure, but this enhanced cell damage resolved as sanding dust is rapidly cleared from the conducting airways

The number of PMNs was calculated from total cell counts and cell differentials in BALF. As seen in Fig. 8A, PMNs were significantly increased at 1 day post-exposure for both 140 and 280 μg MCA and 280 μg CA-C doses; however, this was resolved by 7 days post-exposure (data not shown). PMNs cell count was higher at 280 μg MCA than either UYP or CA-C at 1 day post-exposure. These data are consistent with the LDH data and suggest that MCA caused a greater inflammatory response than either UYP or CA-C. Alveolar macrophages were significantly decreased in the BAL samples at 1 day after the 280 μg MCA exposure, but this was not seen at 7 days post-exposure, which suggests transient macrophage activation (Fig. 8B). BAL lymphocytes increased 1 day after the CA-C 280 μg exposure (Fig. 8C), but were not different from saline control. This effect did not persist at 7 day post-exposure (data not shown). There was a significant dose-dependent increase in eosinophils (Fig. 8D) 1 day after MCA and CA-C exposure that was not seen at 7 day post-exposure (data not shown). The induction of eosinophils was significantly greater at the 280 μg MCA exposure in comparison to CA-C, again suggesting that MCA is more bioactive and causes greater acute pulmonary inflammatory cell influx and damage. There were no significant effects in the UYP treated groups, which suggests that the inflammatory cell influx and damage observed in the MCA and CA-C groups were due to the pressurized treatment and not the wood itself. There were also no time-dependent changes seen in the saline control groups. At 7 days post-exposure, there were no significant differences in BAL leukocytes in any exposure group (data not shown).

To characterize the pulmonary immune response to wood dust exposure, the induction of 10 pro-inflammatory cytokines and chemokines was measured in the BALF (Fig. 9). The analytes measured were: IL-2, IL-4, IL-5, IL-6, IL-1β, KC/GRO, and TNFα, IFN-γ, IL-10, and IL-12. IFN-γ, IL-10, and IL-12 levels were not affected at any time point among the treatment groups, suggesting their respective downstream pathways are most likely not involved in the responses to treated wood dusts. In general, the dust treatments all evoked dose-dependent increases in IL-2, IL-4, IL-5, IL-6, IL-1β, and KC/GRO levels. However, when comparing treatment groups at the highest dose of 280 μg/mouse, IL-1β, IL-4, IL-6, KC/GRO, and TNFα levels were elevated in the MCA group compared to UYP or CA-C groups. Also, UYP did not induce significant changes in cytokines and chemokines, again suggesting that the induction observed is due to the treatment of the lumber and not the wood dust itself. No differences in the measured cytokines were observed between groups or exposure levels at day 84. Taken together, these data confirm the LDH and cell count data that indicates that MCA causes a more robust acute immune response than UYP or CA-C.

3.3.3. Histopathology

At day 1 post exposure, microscopic changes in lung of exposed mice were characterized by multifocal mild to moderate acute bronchiolitis and alveolitis, characterized by infiltrates of neutrophils and macrophages at terminal bronchioles with extension into alveolar ducts (Fig. 10). The observed mild-moderate inflammation was similar among the different dust particles tested, but tended to be more severe in mice exposed to dust from MCA-treated lumber when compared to dust from wood treated with CA-C or UYP (Summarized in Table 2). At this time point, polarizing light microscopy showed numerous birefringent wood dust particles within foci of inflammation and (Fig. 11), similar to what was seen with GMS staining. Resolution of acute inflammation was seen in all treatment groups at 84 days post exposure (Fig. 10 and Table 3). At this time point, wood dust particles were occasionally seen in alveolar macrophages without associated inflammation (Fig. 11). No evidence of pulmonary fibrosis was seen in

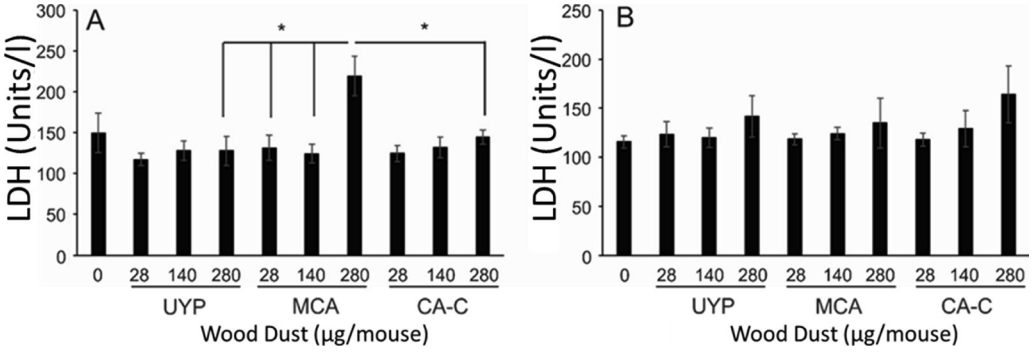

Fig. 7. Pulmonary damage in mice exposed to UYP, MCA and CA-C wood dust. BALF was analyzed for LDH activity from mice exposed to saline, UYP, MCA or CA-C. At (A) 1 day and (B) 7 days post-exposure. Values are means ± standard errors. n = 10–12 mice per group. Asterisks represents significant difference between indicated treatments $p < 0.05$.

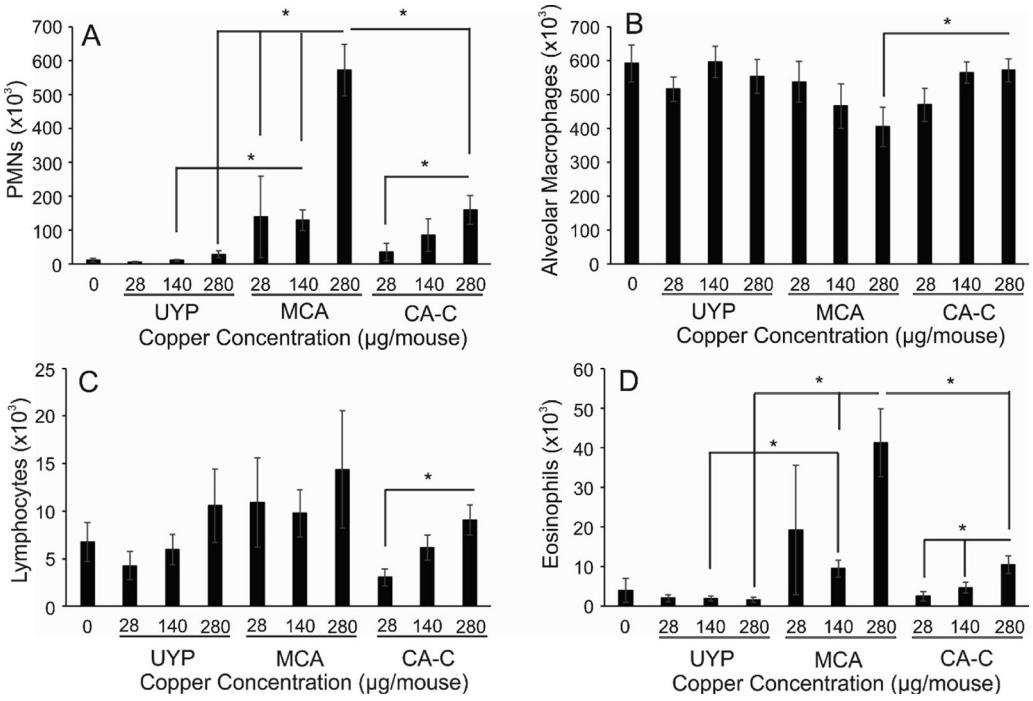

Fig. 8. Induction of pulmonary inflammation by UYP, MCA and CA-C dust. BALF was analyzed for (A) PMNs, (B) alveolar macrophages, (C) lymphocytes, and (D) eosinophils from mice exposed to saline, UYP, MCA or CA-C at 1 day post-exposure. Values are means ± standard errors. n = 10–12 mice per group. Asterisks represents significant difference between indicated treatments $p < 0.05$.

any lung sections examined. There were no treatment-related abnormalities in the lungs of saline exposed mice, nor were any wood dust particles detected in these animals at either time point examined.

4. Discussion

This study sought to compare pulmonary responses to $PM_{2.5}$ sanding dust from MCA-treated lumber to those of the CA-C-treated wood and the UYP in mice. The dose range (28, 140, 280 µg) was selected in order to provide an estimated range of exposures that would elicit low/no effect, and maximal effect. A single aspiration exposure to UYP did not cause substantial alterations in the lungs at any dose. However, at 280 µg/mouse, MCA caused more pulmonary inflammation at 1 day post-exposure than either CA-C or UYP as evidenced by increased BALF cellularity and proinflammatory cytokine levels. Similarly, MCA treatment (280 µg/mouse) induced a significant increase in BALF LDH activity, greater numbers of BALF PMNs and lymphocytes, and a decrease in macrophages recovered by lavage than mice exposed to the same dose of dust from CA-C treated lumber. This suggested that MCA lumber treatment dust induced greater acute pulmonary inflammation. Pulmonary inflammation was confirmed through the analysis of pro-inflammatory cytokines and chemokines, where the greatest induction was caused by MCA compared to CA-C or UYP treatments. Lung histopathology analysis revealed that all three exposures induced a similar acute inflammatory response in the mouse lung. Our findings suggest that exposure to the MCA sanding dust induces a slightly more severe acute pulmonary response in comparison to the CA-C and UYP dust at 1 day post-exposure. These results are consistent with earlier *in vitro* studies [4].

The decrease in BALF macrophages at the highest MCA exposure at day 1 post-exposure seems counter to our other observations of increased acute inflammation associated with this dose. However, it may in fact reinforce these findings. Others have observed that particle exposure may activate alveolar macrophages such that they adhere strongly enough to the epithelium and are less likely to be dislodged and collected in the BALF fluid, resulting in an unexpectedly low cell count [16]. In these cases, the increase in alveolar macrophages can be detected by histopathologic means.

Several factors may contribute to the greater pro-inflammatory response of the MCA sanding dust at 1 day post-exposure. First, as previously established using a Fast Mobility Particle Sizer Spectrometer, the MCA dust tended to have a higher proportion of nanoparticles than the CA-C [4], Greater bioactivity by mass for particulates containing larger proportion of smaller particles is well established [17], including for copper particles [18]. Second, while the copper contents between the MCA- and CA-C-treated woods in lumber form are relatively similar, the analysis of the copper contents of the different fractions of the sanding dust demonstrated that the $PM_{2.5}$ from the MCA-treated lumber had a higher percentage of copper than that of the CA-C-treated lumber. Our previous *in vitro* studies showed that higher concentrations (≥ 5 µg/ml) of copper in the supernatants of the MCA and CA-C sanding dust tended to induce a more severe cellular toxicity [4]. Third, copper in the CA-C-treatment is soluble, whereas copper in the MCA-treated lumber is remains in a less-soluble copper carbonate particle form. While both the CA-C and MCA leached copper into the saline, slightly more copper was detected in the saline CA-C-treated lumber dust. In contrast, the copper content in the dry $PM_{2.5}$ particles was substantially higher in the dust from MCA-treated lumber, by weight (Fig. 3), suggesting that the solubility of the copper in the CA-C treatment is greater than that of the MCA. These observations are similar to those reported in another recent study of MCA-treated wood [8]. Lower solubility of MCA is in line with the industrial design of the product, as the CA-C lumber treatment involves dissolved copper in solution, while the MCA particles are merely suspended before impregnation *via* high pressure. Because the copper contained in the MCA is less soluble and leaches into solution at a lower rate, it may be held in close proximity to the lung epithelium longer than the copper from the CA-C, allowing for greater for oxidative damage and macrophage activation to occur. Fourth, although the status of the copper particles in the MCA treatment formula as engineered nanoparticles (ENP) or a fine particles is a matter of debate, our previous studies showed that sanding MCA-treated wood released a greater number of nanoparticles than sanding CA-C and UYP (Fig. 3 in Sisler et al. 2018) [4]. Some embedded nanoparticles have been found in the $PM_{2.5}$ MCA sanding dust, and, moreover, free nanoparticles have been found in the supernatants of the cell culture media with the $PM_{2.5}$ MCA sanding dust [4]. However, analysis of the lungs by field emission scanning electron microscope (FESEM) for free nanoparticles at 1 and 84 days after exposure revealed no definitive free

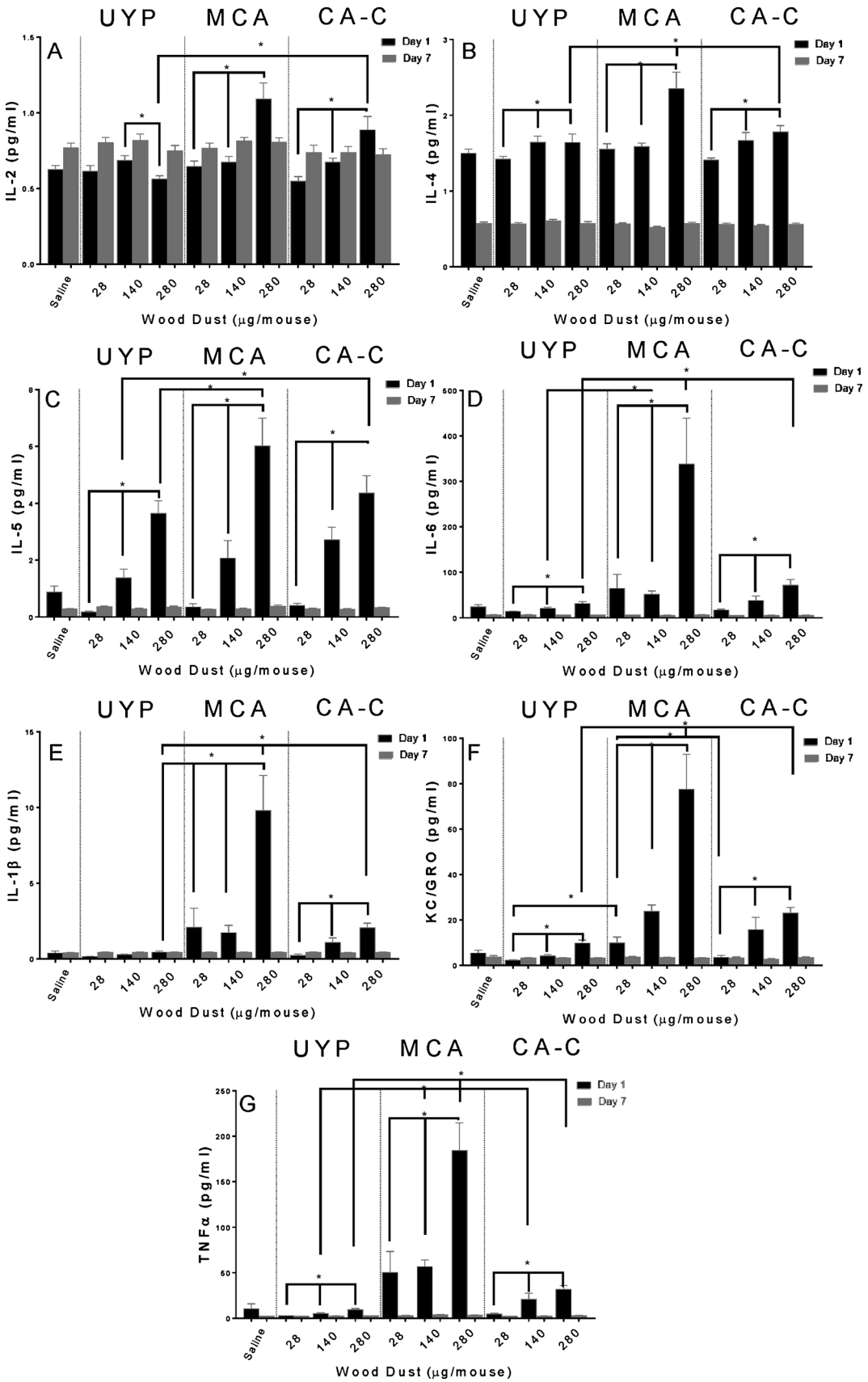

Fig. 9. Induction of BALF cytokines and chemokines by UYP, MCA and CA-C dust. (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-1β, (F) KC/GRO, and (G) TNFα, were measured in BALF of mice exposed to saline, UYP, MCA or CA-C at 1 day (black bars) or 84 days (gray bars) post-exposure. Asterisks represent significant difference between indicated treatments $p < 0.05$. Values are means ± standard errors. n = 10–12 mice per group.

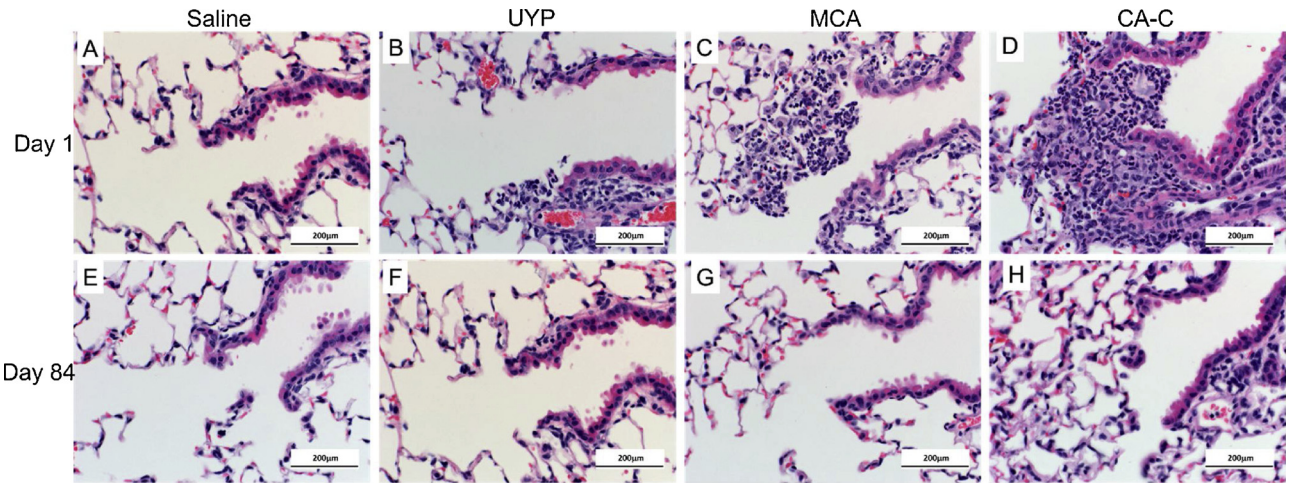

Fig. 10. Histopathology in murine lung exposed to wood dust particles. Infiltrates of inflammatory cells are seen at terminal bronchioles and alveolar ducts at day 1 following exposure to all particles with resolution of inflammation at day 84.

Table 2
Summary of Incidence and Mean Severity from Mice Exposed to Saline, UYP, MCA and CA-C at 1 Day Post Exposure.

Exposure	Number Examined	Particle Incidence	Inflammation Incidence	Mean inflammation severity
Saline	10	0/10	0/10	0
UYP	11	11/11	9/11	1.6
MCA	11	10/11	10/11	2.1
CA-C	11	10/11	10/11	1.9

Table 3
Summary of Incidence and Mean Severity from Mice Exposed to Saline, UYP, MCA and CA-C at 84 Days Post Exposure.

Exposure	Number Examined	Particle Incidence	Inflammation Incidence	Mean inflammation severity
Saline	11	0/11	0/12	0
UYP	12	12/12	1/12	1
MCA	12	11/12	2/12	1
CA-C	12	11/12	4/12	1.5

copper nanoparticles.

Exposure to the different sanding wood dusts induced acute pulmonary inflammation, markers of which (in the BALF) resolved ay 7 days post-exposure. No chronic pulmonary inflammation was observed, likely due to the rapid clearance of the sanding wood dust particles from the airways of the mouse lungs. Indeed, the results of the EDM analysis and polarized light microscopy demonstrated that most of the sanding wood dust particles were located near terminal bronchioles and there were only minimal amounts particles located in the alveolar region at 1 day post-exposure (Fig. 4). By 84 days post-exposure, the majority of dust particles were cleared from the mouse lungs, correlating with resolution of pulmonary inflammation. The potential mechanisms involved in the rapid clearance of the sanding wood dusts from the mouse lungs are: 1) mucociliary escalator of bronchi and bronchioles and; 2) phagocytosis by airway macrophages. Any sanding wood dusts engulfed by the alveolar macrophages could be removed by the mucociliary escalator by 84 days post-exposure, or be cleared from the lung by transport through the lymphatics.

All of the changes observed *in vivo* at 1 day post-exposure were resolved by the seventh day post-exposure. All histopathologic and inflammatory biomarker changes were resolved by day 84. Several studies demonstrated that accumulations of lymphocytes, eosinophils, macrophages, and neutrophils have been observed in the BAL from the lungs of mice [19] and humans [20] following pulmonary wood dust exposure. Consequently, the lack of alteration in these parameters in our UYP group compared to these studies is likely due to differences in wood source, species, mouse strain, and exposure method. We understand that this work is limited by several factors. No consideration could be made for the variability of wood dusts generated across different trees growing in different rainfall regions and lumber storage conditions. Variability in preservative retention in different types of lumbers, and variability within and along the length of the board [21] could also not be controlled. However, the objective of this study was not to look the lumber variability, but to address two questions: 1) would the sanding of lumber generate inhalable wood particles, and 2) does copper treatment enable the pulmonary response of the $PM_{2.5}$ fraction of sanding particles?

Taken together, our *in vivo* studies suggest that, at the highest dose,

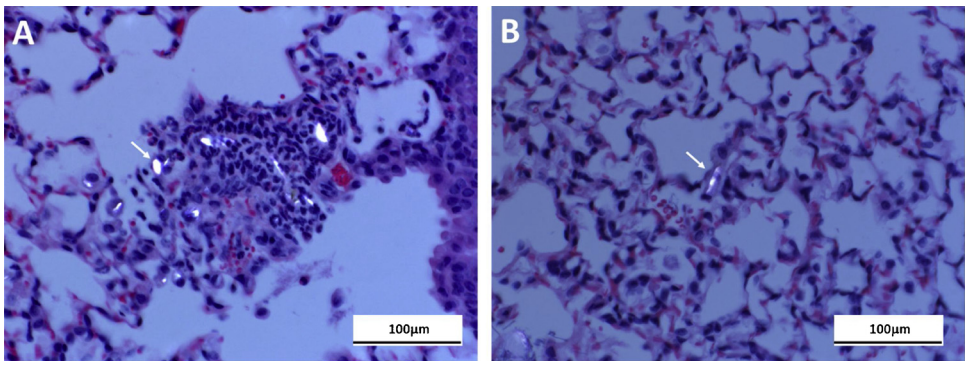

Fig. 11. Polarizing light highlights wood dust particles. Many birefringent wood dust particles can be seen within foci of acute inflammation in murine lung at day 1 post exposure, irrespective of treatment group (Panel A). Resolution of acute inflammation is associated with clearance of particles, with only a few remaining particles present within alveolar macrophages (Panel B). Wood dust particles appear white when viewed with polarized light (arrows).

the respirable dust from sanding MCA-treated lumber was the most pro-inflammatory when compared to CA-C or UYP, and caused the greatest pulmonary inflammatory response. Our findings also suggest that, while the MCA induced greater acute pulmonary response, the pulmonary inflammation was transitory.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.02.068.

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