

Effects of elevated CO₂ levels on lung immune response to organic dust and lipopolysaccharide

David Schneberger

University of Saskatchewan College of Medicine

Upkardeep Singh Pandher

University of Saskatchewan

Brooke Thompson

University of Saskatchewan College of Medicine

Shelley Kirychuk (✉ shelley.kirychuk@usask.ca)

University of Saskatchewan College of Medicine <https://orcid.org/0000-0002-3645-793X>

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Abstract

Workplaces with elevated organic dust levels such as animal feed barns also commonly have elevated levels of gasses, such as CO₂. Workers exposed to such complex environments often experience respiratory effects that may be due to a combination of respirable factors. We examined the effects of CO₂ at the ASHRAE recommended limit (1000 ppm) as well as the EPA 8hr time weighted average limit (5000 ppm) on lung innate immune responses in mice with exposure to inflammatory lipopolysaccharide and organic dust. Mice were nasally instilled with dust extracts or LPS and immediately put into chambers with a constant flow of room air (approx. 430 ppm CO₂), 1000 ppm, or 5000 ppm CO₂ enriched air. Organic dust exposures tended to show decreased inflammatory responses with 1000 ppm CO₂ and increased responses at 5000 ppm CO₂. Conversely, LPS with addition of CO₂ as low as 1000 ppm tended to inhibit several inflammatory markers. In most cases saline treated animals showed few changes with CO₂ exposure, though some changes in mRNA levels were present. This shows that CO₂ as low as 1000 ppm CO₂ was capable of altering innate immune responses to both LPS and organic dust extracts, but each response was altered in a different fashion.

Background

Organic dust exposures in animal feed operations has long been shown to be detrimental to the health of workers in these facilities^{1,2}. Experimental approaches to determining the factors present in these dusts that may be responsible for respiratory inflammation have yielded considerable data, particularly on the role of microbial products such as endotoxins (LPS) and proteoglycans, and others as potential causes of these lung problems^{3,4}. Such dust exposures however are often in the context of facilities with a variety of elevated gases such as ammonia and hydrogen sulfide, both of which have been implicated in causing lung problems or being immuno-modulatory^{5,6}.

Carbon dioxide (CO₂) has often been overlooked despite significant data showing routine elevations of CO₂ in animal feed operations⁷ often as high as the OSHA 8hr time weighted average (TWA) limit of 5000 ppm,. CO₂ at levels as low as 1000 ppm have been shown to induce cognitive changes in humans^{8,9}. Indeed, elevated CO₂ is a broader problem, and common in many facilities such as schools⁹, daycares^{10,11}, prisons¹², cars¹³, airplanes¹⁴, and many other locations¹⁵.

Previously we have shown that innate immune responses to organic dust extracts were changed when co-exposed to CO₂ at 5000 ppm¹⁶. These changes were at the protein and mRNA levels and in most cases were indicative of enhanced inflammation. Subsequent to this study others showed that neutrophils were altered by similar levels of CO₂¹⁷. At present however these are the only studies we are aware of to address this issue in lung immunology. There have been several studies related to behavior¹⁸ and at higher levels of CO₂ where there is induction of hypercapnic acidosis in animals and cell cultures¹⁹⁻²¹. Results for these latter studies have been somewhat mixed, particularly in situations of LPS

exposure versus more complex challenges such as tobacco extracts^{21,22}. These studies also do not address CO₂ at levels applicable to most work environments and crowded spaces^{23,24}.

The aims of the current study were to extend the previous co-exposure model to assess lower levels of CO₂ exposure while at the same time testing both a defined single inflammatory stimuli (LPS) and a complex one (organic dust extracts). We hypothesize that the lung alters innate immune responses when presented with a combined exposure of an inflammatory agent and CO₂ as low as 1000 ppm.

Methods

Organic Dust Extracts and Treatments

Organic dust extracts (HDE) were produced from settled dust samples collected from two separate swine confinement facilities in Nebraska. These dust extracts have been characterized previously for muramic acid, endotoxin, and protein²⁵, and the bacterial composition has also been described²⁶. Extracts were prepared by mixing 1 g dust with 10 ml HBSS (without calcium, Sigma, St. Louis, MS) and incubating for 1 hr at room temperature before centrifugation twice, and subsequent filter sterilization through a Nalgene 0.2µM SFCA membrane syringe filter (ThermoFisher, Rochester, NY) as described previously²⁷. This results in a solution of approximately 0.105 g/ml dust that is stable at -20C or lower for at least a year or more. Dust extracts were given to mice at a final concentration of 12.5% vol/vol or 0.005 g/ml dust delivered in 40 µl dropwise to the nares, and mice allowed to inhale under light (1%) isoflurane anesthesia.

LPS from *Escherichia coli* 0111:B4 (lot 095M4163V, Sigma, St. Louis) was diluted in HyClone HBSS(1X) (HyClone Laboratories; Logan, UT) to 0.1 µg, and delivered in 40 µl to the nares, and mice allowed to inhale under light (1%) isoflurane anesthesia. Control groups were given 40 µl HBSS in the same manner under light anesthesia.

Animals

Six to seven week old female C57BL/6 mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Mice were housed in standard cages in a controlled room with ambient temperature of 23°C +/- 2°C and a 12-hour light/dark cycle. Mice were fed standard laboratory chow and water ad libitum. Mice were acclimatized in their first week in facility for 2 hours per day for 5 days to plethysmography chambers (part 601-1425-001; DSI, Minneapolis, MN) ventilated by a Buxco Finepointe whole body plethysmography 4-site system (DSI, Minneapolis Minnesota). All intranasal treatments were given between 8:00–8:30AM to minimize any potential circadian rhythm differences between treatments. At end of the experiment mice were euthanized.

CO₂ Delivery and Whole Body Plethysmography

All mice upon intranasal treatment were placed immediately into whole body plethysmography (WBP) chambers (DSI, Minneapolis, MN) for the duration of exposure under positive airflow of 5psi. The biasflow air consisted of either of room air (~ 500 ppm CO₂), or air from tanks consisting of room air enriched for CO₂ to 1000, or 5000 ppm CO₂ (Praxair; Mississauga, Canada). Airflow was constant through the exposure period to prevent any accumulation of CO₂ due to respiration from animals. Mice were provided with food and water in exposure chambers for the duration of exposures. CO₂ readings of the treatment room provided an average ambient CO₂ level of 430 ppm averaged over 8 using a Q-Trak Indoor Air Quality Monitor 7575 (TSI; Shoreview, MN).

Readings from the WBP chambers were captured every 2 seconds for the entire duration of exposure. Results were averaged for 20 minute intervals using the Finepointe system software (DSI, Minneapolis, MN).

Bronchoalveolar Lavage

Lungs were lavaged as detailed earlier (Romberger et al. 2002). Briefly, lungs were washed three times with 0.5 ml HBSS each time. Bronchoalveolar lavage (BAL) fluid was centrifuged at 1000 X g for 10 minutes, and supernatant transferred to new tubes and stored at -80oC. Cell pellets were resuspended in HBSS and stained with trypan blue (Life Technologies, Grand Island, NY) and counted using a hemocytometer. Cells were resuspended to 100 ul in HBSS and adhered to glass slides (Fisher Scientific; Pittsburgh, PA) via cytocentrifugation for 10 min at 10000 X g (Cytopin, Shandon Elliott, Great Britain). Cells were dried overnight and fixed and stained using Diff Quik kit (Siemens Healthcare Diagnostics, Newark, DE) and mounted using MM24 mounting media (Leica Biosystems; Buffalo Grove, IL). A differential count of 100 cells was made based on morphological assessment of cells and expressed as absolute cell numbers.

Lung collection

After BAL collection lungs were excised. The right lung was tied off at the primary bronchus, removed, flash frozen in liquid nitrogen, and stored at -80oC. The left lung was slowly inflated with 200 ul of 4% PFA (Sigma; St. Louis, MO) and stored in 4% PFA overnight, followed by 100% ethanol. The fixed lung was embedded the next day in paraffin in a Intelsint RVG/1 tissue processor (Intelsint; Turin, Italy) followed by mounting in paraffin blocks in Tissue Tek II tissue embedder (Sakura Finetek; Nagano, Japan).

Lung Histology

Lung sections from paraffin embedded lungs were sectioned in 5 uM slices on a Microm 350S microtome (Microm, Germany). Tissue sections were then stained with Hemotoxylin and Eosin as previously described (Rolls and Sampias 2019) and mounted using Surgipath MM24 (Leica Biosystems, Richmond, IL). Sections were imaged using an Aperio CS2 virtual microscopy system (Leica Biosystems, Concord ON Canada) and examined for any notable changes to lung structure.

ELISA

Quantification of MCP-1, MIP-2, IL-6, KC, IL-1b, TNF-a, IL-4, IL-5, IL-13, IL-33, and IL-10 in BAL fluid was determined by Luminex xMAP multiplex ELISA (Procartaplex, ThermoFisher) according to manufacturer's specifications. Samples were read using a Bioplex 200 system (Bio-Rad, Hercules, California) and Bioplex Manager Software (Bio-Rad)

RNA purification and RT-PCR analysis

RNA was purified from lung tissue samples using a Qiagen RNeasy Plus Mini kit (Qiagen, Chatsworth CA) according to the manufacturer's instructions for tissue extraction. Briefly, 20–25 mg of lung tissue was put into 350 ul of RLT lysis buffer and 2.0 mm zircon beads (BioSpec, Bartlesville, OK). Samples were placed in a Biospec Mini-BeadBeater-24 (BioSpec) and run twice for 2 minutes, with a pause between runs of 5 minutes where samples were held on ice to prevent heat buildup. Supernatant was removed to DNA eliminator column, and the remainder of purification done according to the kit protocol. RNA was quantified by Take3 plate (Biotek, Winooski, VT) in a Synergy HT plate reader (BioTek). cDNA synthesis was done using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) with 300 ng of template mRNA. Samples were incubated at 25oC for 5 min, 46oC for 20 minutes, and finally at 95oC for 1 minute in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (BioRad). RT-PCR was done using probes for TLR2 (Mm00442346_m1), TLR4 (Mm445273_m1), Hsp72 (Mm01159846_S1), A20/TNFAIP3 (Mm00437121_m1) (Life Technologies, Grand Island, NY). Ribosomal RNA (Life Technologies) was used as an endogenous control in all reactions. PCR was conducted using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Reactions were all carried out in duplicate and performed 2 minutes at 50oC, 10 minutes at 95oC, then 40 cycles of 15 seconds at 95oC and 1 at 60oC minute each using ddPCR Supermix for Probes kit (Bio-Rad). Relative comparison of selected targets to the ribosomal endogenous control cycle threshold (CT) value was analyzed with the DDcT method.

Statistical Analysis

Data was analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Error bars represent mean +/- SEM. For values outside the assay limit of detection either the LOD/2 or a minimum value below the lowest attained value was designated. Statistical significance was determined using one-way ANOVA with follow-up Tukey test for multiple comparisons. If the assumption of equal variance was not met, the data was either log transformed followed by one-way ANOVA and multiple comparison tests, or nonparametric tests (Kruskal-Wallis) were conducted. For all tests, a p-value <= 0.05 was considered significant for differences between groups.

Results

Plethysmography Data

Monitoring of breathing parameters of mice in exposure chambers showed changes in several parameters over the six hours of exposure, across all treatments (Fig. 1).

Animals given saline showed elevations in peak inspiratory and expiratory flow (Fig. 1c, 1d), tidal volume, total (Fig. 1e), and minute volume (Fig. 1g) at 1000 ppm CO₂, and tidal volume and total inspiratory time (Figs. 1e and 1f) at 5000 ppm. Animals treated with HDE showed significant increased peak inspiratory flow ($p < 0.05$) (Fig. 1c) and tidal volume ($p < 0.01$) (Fig. 1e) over ambient air at 5000 ppm CO₂, whereas at 1000 ppm only tidal volume was elevated ($p < 0.05$). LPS treatment to animals showed increases in tidal volume over ambient air co-exposure ($p < 0.05$) at 1000 and 5000 ppm CO₂ co-exposure. CO₂ meanwhile resulted in a decrease in LPS-induced increase in PenH ($p < 0.01$) (Fig. 1a).

Comparing within and between treatments there was increased tidal volume with all co-treatments with CO₂ compared to treatments with no CO₂.

None of the treatments appeared to have any significant effect on breathing frequency (Fig. 1b), total inspiratory time (Fig. 1f), or minute volume (Fig. 1g).

Bronchoalveolar Lavage Cells

Cell counts from BAL fluid showed several patterns (Fig. 2). First, mice given saline intranasally showed no significant changes in BAL cell composition or number with increased CO₂ (Fig. 2a).

LPS treatment showed an increase in macrophages/monocytes compared to saline control. This increase was significant or near significant compared to treatment at a similar CO₂ level (Saline vs. LPS ($p = 0.0637$), Saline vs. LPS + 1000 ppm CO₂ each ($p = 0.0852$), Saline vs. LPS + 5000 ppm CO₂ each ($p < 0.05$). No significant increases in neutrophils were noted between saline and LPS treatments, except between Saline + 5000 ppm CO₂ and LPS + 5000 ppm CO₂ ($p < 0.01$).

HDE exposure (Fig. 2c) on its own or with room air was not able to induce a significant rise in monocytes/macrophages. However co-exposure of HDE with 1000 ppm CO₂ was able to induce an increased numbers of cells over saline given at a similar level of CO₂ ($p = 0.0732$), which was significantly increased with 5000 ppm co-exposure ($p < 0.05$). Levels of neutrophils were elevated with HDE, but not significantly different as compared to saline. However, co-exposure of HDE with 5000 ppm CO₂ caused a substantial increase of the neutrophil population as compared to HDE and room air ($p < 0.001$), which was also significantly different from the HDE and 1000 ppm CO₂ co-exposure ($p < 0.01$), and from the saline and 5000 ppm CO₂ co-exposure ($p < 0.0001$).

ELISAs

As shown in Figs. 3 – 1 and 3 – 2, the expression of cytokines appeared to follow three general patterns. First, no difference in cytokines was noted when CO₂ was co-exposed with saline.

For LPS, increased CO₂ provoked a decrease in cytokines/chemokines as compared to co-exposure with room air. Co-delivery of 1000 ppm CO₂ with LPS resulted in significant decreases in IL-6 ($p < 0.01$), KC ($p < 0.001$), TNF- α ($p < 0.01$), and IL-1 β ($p < 0.01$) as compared to co-exposure with room air. Similar trends were noted at 5000 ppm CO₂ co-exposure with LPS with reductions in IL-6 ($p < 0.05$), KC ($p < 0.001$), and TNF- α ($p < 0.01$) as compared to room air co-exposure to LPS. Cytokine/chemokines were not significantly different between co-exposure of LPS to CO₂ at 1000 ppm and 5000 ppm as compared to saline co-exposures at these same CO₂ levels.

The situation was more complex with HDE. Compared to co-exposure with room air, at 1000 ppm CO₂ co-exposure HDE showed no significant differences between cytokines/chemokines though there was some apparent drop in production. However at 5000 ppm CO₂ co-exposure there was a significant rise in MCP-1 and MIP-2 ($p < 0.05$) compared to HDE on room air. There was also a raise in MCP-1 ($p < 0.001$), MIP-2 ($p < 0.01$), and IL-1 β ($p < 0.01$) at 5000 ppm co-delivery as compared to co-delivery of 1000 ppm CO₂. This suggested an increase in inflammatory response at higher CO₂ delivery as opposed to the reductions seen with all CO₂ co-delivery levels when CO₂ was co-exposed with LPS. Further, with HDE co-exposure with 1000 ppm CO₂ there was a tendency to reduced cytokines whereas 5000 ppm CO₂ co-delivery with HDE showed increases in cytokines, suggesting differential responses with increased CO₂ concentrations when co-exposed with HDE.

RT-PCR

We looked for evidence of changes to signaling in lung cells first at the receptor level with two of the receptors most commonly associated with response to HDE, TLR2 and TLR4, the latter of which is critical to responses to LPS.

TLR2 (Fig. 4A), which is implicated in HDE induced inflammation^{28,29} showed no significant change with HDE co-exposure with any level of CO₂. However, LPS with room air induced a significantly large increase in TLR2 mRNA compared to saline and room air ($p < 0.05$), and this increase was inhibited by addition of 1000 ppm CO₂ ($p < 0.01$) and 5000 ppm CO₂ (NS) co-exposures.

TLR4 (Fig. 4B) showed no significant change in mRNA expression across all LPS and HDE treatments and CO₂ co-exposures.

With little work having been done on CO₂ signaling pathways we next looked at a negative regulator of the TLR/NF- κ B pathway, TNFAIP3 (A20) (Fig. 4c), which has been implicated by others in the prevention of asthma in rural populations³⁰. While there was no increase in mRNA production with LPS exposure, there was an increase in A20 mRNA expression with HDE compared with control ($p < 0.01$). Interestingly, for HDE, this increase was inhibited by CO₂ co-delivery at 1000 ppm ($p < 0.05$), and less inhibitory at 5000 ppm.

mRNA expression of another protein shown to have effects on MAPK and NF- κ B signaling, Hsp72 (Hsp1A) was also tested (Fig. 4d). CO₂ at 5000 ppm co-exposure with saline was able to induce some

Hsp72 increase in mRNA, but not a significant increase as compared to saline and room air. Co-exposed with room air, HDE induced a similar non-significant Hsp72 increase and LPS a significant increase ($p < 0.01$). However, Hsp72 mRNA was decreased when HDE or LPS stimuli were co-exposed with either CO₂ concentration ($p < 0.05$ for LPS + CO₂ treated groups).

Histology

Tissue sections (Fig. 5) overall showed little in the way of overt changes due to inflammation. This may be in part due to the limited time frame of exposure, and longer exposures are likely to produce more significant changes. No consistent overt differences were noted in mice for saline, LPS, or HDE treatments compared to their similarly treated but CO₂ co-exposed duplicates.

Discussion

In high intensity animal production operations, there are buildups of both particulates in the air as well as a variety of gasses, mostly as a result of the animals housed there. Given the problems of respiratory effects in exposed workers^{1,31} numerous studies have tried to recreate these conditions in animal models² to determine responsible agents for health effects. Given that CO₂ elevation is common in many environments, not just in barns, we previously looked at the effects of workplace relevant (5000 ppm) CO₂ on mice treated with barn dust extract to try to devise a more holistic model of barn exposure inflammation. The results from this study suggested that we look at both a more simple innate inflammatory molecule (LPS), as well as CO₂ concentrations that are more reflective of a number of environments, making the results more meaningful for a variety of facilities, such as homes and offices.

The first questions was if breathing parameters showed any changes, as might be expected from an elevation in ambient CO₂ levels. Others have reported potential stress after long repeated exposures to elevated CO₂ in rats (700 ppm, 6hr/day over 15 days)¹⁸, whereas others have shown changes in minute volume associated with stressful procedures³². There were no significant changes to minute volume for any of the treated animals over the course of our studies, suggesting the treatments were reasonably tolerated. Similarly, breath frequency was unaffected across all treatments and CO₂ levels. Tidal volume appeared to be the most sensitive of the tested parameters to treatments and CO₂ exposure. Changes however appeared to be due to presence of CO₂ alone as the secondary treatment had no effect. This is unsurprising as volume of air moved in and out of the lung should logically increase to counteract elevated CO₂. Interestingly, within the HDE exposed animals the peak expiratory flow, peak inspiratory flow, and tidal volume measures increased with increasing ppm co-exposure to CO₂, suggesting that when co-exposed, the increasing doses of carbon dioxide may be resulting in increases in airways responsiveness. While this would fit with the barn dust extract and CO₂ treatment being one of the most inflammatory treatments tested here, the changes were nearly immediate within the monitoring time (within 40 minutes, data not shown) which may suggest very early inflammatory mechanisms could be playing a role.

Looking at cellular levels in the lung extracellular space we see that similar to our previous study, increasing CO₂ appeared to have no effect on saline treated animals. HDE by comparison showed a trend to increases in total cells and a trend to increased monocytes with CO₂ co-treatment, even at levels as low as 1000 ppm CO₂, and by 5000 ppm a significant increase in total BAL cells and neutrophils in the lung. This general increased inflammatory profile is similar to our previous report¹⁶. A further similarity to that report was that in lung tissue histology (Fig. 5) there was no discernable changes seen with any of the treatments with or without added CO₂. Given that this was a 6 hour exposure, we did not expect to see significant histological changes. We suspect a more chronic exposure may yield different results though, given other changes in cytokines and chemokine expression. The lack of significant physical changes to the lung also suggests that any changes to breathing parameters were likely not due to gross mechanical changes in the lungs over the course of the experiment.

Past work by our lab has shown that CO₂ co-exposure did cause significant changes at the molecular level, increasing inflammatory responses to organic dusts. As LPS is a common constituent of agricultural dusts it was thought that LPS exposures and co-exposures may respond similarly to HDE. Our results showed a relatively common pattern across several cytokines with regards to LPS as compared to saline, showing significant increase in inflammatory cytokine production with LPS exposure. However, for the proinflammatory cytokines, the LPS with room air response tended to be greater than the HDE with room air response. These responses changed with co-exposure to CO₂. For LPS, there were significant decreases in cytokines with both CO₂ co-exposures. By contrast, when HDE was co-exposed with CO₂, at 1000 ppm there was a decreased cytokine response compared to the room air HDE + CO₂ co-exposure, however, at 5000 ppm HDE + CO₂ co-treatment the cytokines were greater as compared to the co-exposures to room air and 1000 ppm CO₂. This results are similar to the BAL cellular responses, particularly with well-known monocyte and macrophage chemoattractors such as MCP-1, MIP-2, KC, TNF- α , and IL-1B^{33,34}. IL-1B should warrant particular attention given past work in ex-vivo systems with neutrophil microparticles that shows increased levels of IL-1b per microparticle with increasing CO₂ up to 4000 ppm (Thom et al 2017). While we did not see this with unstimulated animals, we do see this increased IL-1B with HDE co-exposure. The similar results they show with human and mouse neutrophils in response to CO₂ also suggest the mouse model does reflect what occurs in human cells. Their further proof of iNOS activation provides yet another avenue of exploration in our next studies.

Of the cytokines tested TNF- α , MCP-1, MIP-2 and KC are all transcriptionally controlled by NF- κ B and AP-1 (Nicholson 95, Redhu 11)³⁵⁻³⁸, suggesting a possible target for further testing as we try to deduce mechanisms of CO₂ inflammation. Very little is currently known about a CO₂ "sensor" or signaling pathway in cells. Several studies have reported effects on certain proteins such as ERK and JNK activation³⁹. The most promising results for immunological effects have looked at changes to the IKK α and NF- κ B alternate pathway signaling (P100 and RelB) in response to CO₂ exposure^{40,41}. These results implicate the NF- κ B pathway as being involved. For this reason, we decided to examine expression of receptors and regulators of this pathway associated with organic dust exposure and LPS. TLR2 and TLR4 are two of the most heavily implicated receptors associated with HDE exposure^{3,28,29,42}. TLR4 of

course is also the primary receptor associated with LPS detection ⁴³. Interestingly we can see that TLR4 mRNA expression tended to be similar for all treatments. TLR2 on the other hand appears to exhibit a pattern of expression that follows a pattern similar to that seen with many of the proinflammatory cytokines we tested for, though other than for LPS + 1000 ppm CO₂, the changes were not significant. As TLR2 can bind to LPS ⁴⁴, the reduction in TLR2 mRNA may suggest a mechanism for reduced LPS responses with higher CO₂ but would not explain the increased indicators of inflammation seen with HDE. An examination at the mRNA and protein level of TLR2, TLR4, as well as MD-2 and CD14 ^{43,45,46}, all of which are involved in the binding of LPS to TLR2 and TLR4 ⁴⁷ could assist in further delineating pathway involvements.

More recently an important upstream regulator of NF-κB signaling, TNFAIP3 (A20), was found to be vital in development of protection to asthma in rural children exposed to LPS ³⁰. While this may suggest that farm work may be protective against some respiratory problems, those working in concentrated animal feed operations receive high endotoxin exposure but show increased susceptibility to a variety of respiratory problems, including asthma ³⁰. Thus, we examined A20 mRNA expression in response to CO₂. While there was little expression of A20 when exposed to LPS alone, there was a significant increase in expression with exposure to organic dust, but this was inhibited by CO₂ co-exposure, particularly at higher levels. While this result needs to be explored further it does suggest one way in which elevated CO₂ may enhance HDE innate immune responses, by reducing expression of the inhibitor A20. Extrapolating to the previously mentioned study ³⁰ such an A20 reduction may result in a non-protective exposure, if A20 is as critical to inhibiting future atopic responses as has been reported. This association of CO₂ with worse asthma ⁴⁸ and wheeze is shown in several daycare and school studies ^{10,11}.

Finally, we looked at a very general heat shock protein, Hsp72 (HspA1). This particular protein has been shown to be produced in response to a broad array of stress stimuli ⁴⁹. Links between HspA1 and control of a more recently examined player in dust inflammation, HMGB1, made us interested in its induction. Interestingly, there were significant reductions in HspA1 for when LPS was co-exposed with CO₂, even at levels as low as 1000 ppm CO₂, and a trend in HDE treated animals in the same direction. As localization of HspA1 is a strong indicator of whether it is a pro or anti-inflammatory agent, longer exposures will need to be done in the future, looking at protein expression and localization. Our initial mRNA results suggest that addition of a CO₂ co-exposure appears to reduce HspA1 mRNA when given in conjunction with an inflammatory stimulus.

While this work shows interesting results related to co-exposures to inflammatory agents, additional work is necessary. There is a need for a proteomic assessment of these inflammatory measures, to determine the breadth and scope of changes CO₂ induces. Assessment of NF-κB signaling, including use of its alternate signaling pathway should be conducted. There is of course the need to assess chronic exposures, as well as situations of removing CO₂ co-exposure at the start or end of an exposure period, to see if there is a time in which this increased CO₂ exposure is critical to alteration of innate immunity. Several of these parameters are part of ongoing work in our lab.

While this study was to look at exposures common in animal feed facilities, there is ample evidence that people are exposed in a number of environments to elevated CO₂ that exceed 1000 ppm¹⁵. This work highlights that immunological exposures may not just be a factor of the introduced inflammatory agent, but also the environmental context in which it is given, and in which the subject lives and works. This may be particularly important where indications of respiratory symptoms in children such as wheeze, asthma^{10,11}, and respiratory infection⁵⁰ could be exacerbated by increased CO₂ exposure in their environment in addition to more commonly tested irritants. We show here that expression of mRNA of some innate immune receptors may be altered with just CO₂ alone, but that response to inflammation can be significantly altered by these indoor CO₂ levels at 1000 ppm or higher. Interestingly, our current study suggests that these responses will not necessarily be modified in the same manner across different inflammatory agents. This highlights the further need to deduce signaling pathways or commonalities in responses to certain immunological insults to better predict the impact of CO₂. If response to an infection, or progression of certain chronic diseases (ex. COPD, asthma) can be impacted by elevated CO₂, the possibility exists of ameliorating some effects of these same illnesses by improving ventilation.

Declarations

Ethics

All experiments and procedures were approved by and conducted according to the protocols of the University of Saskatchewan Committee on Animals Care Assurance (AUP 20160104).

Consent for Publication

Not applicable

Availability of Data

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare they have no actual or potential competing financial interests.

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Author Contributions

DS conceived the study and study design, performed treatments and most assays, collected and analyzed data. UP helped with data collection, analysis, and animal treatment. BT helped with data collection, sample processing and analysis of data and helped editing the manuscript. SK supervised the project and helped with data analysis, statistical measures, and in writing and editing the manuscript.

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Figures

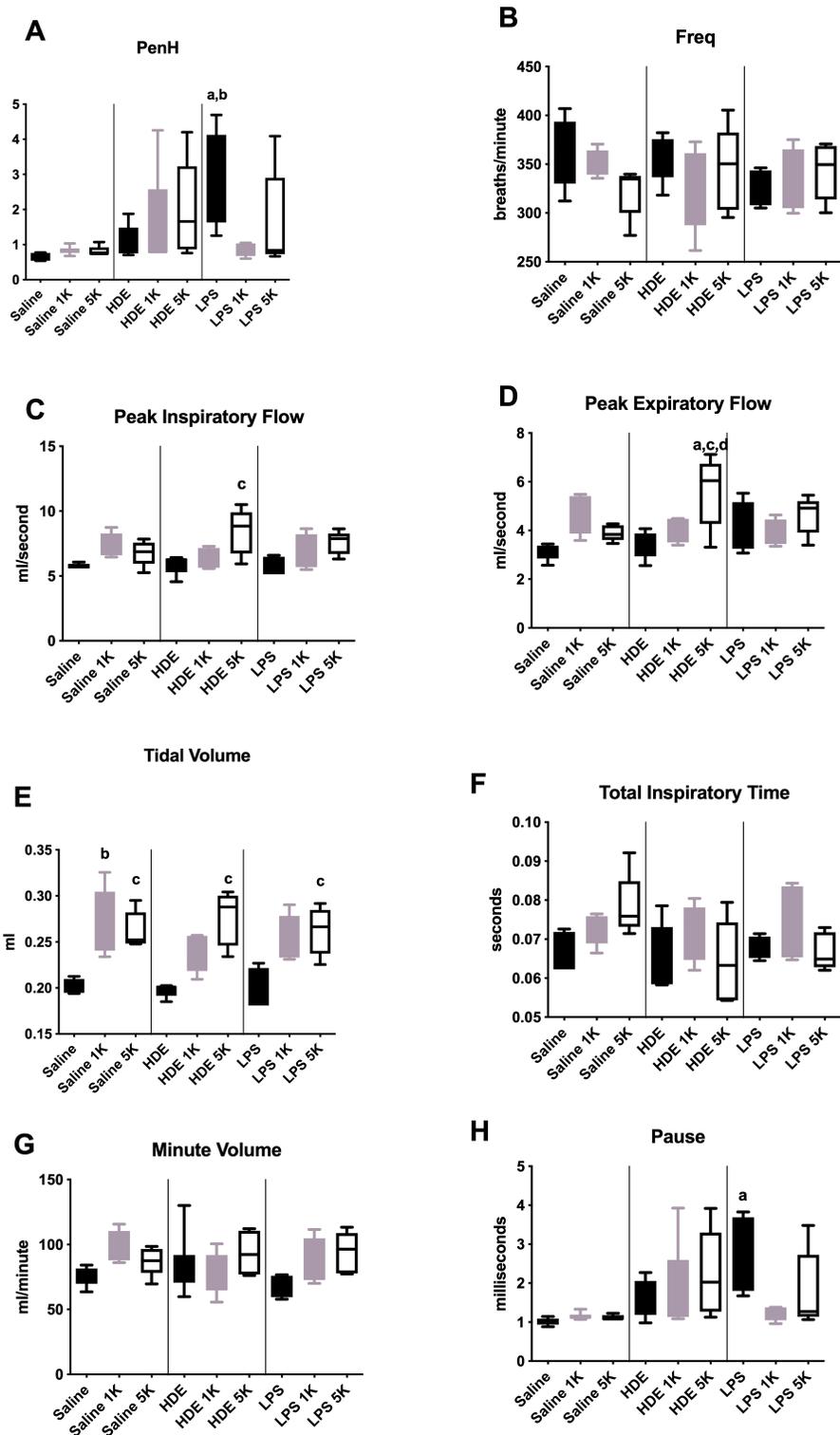


Figure 1

Plethysmography data: Mice were monitored during exposure period and PenH, peak inspiratory volume, peak expiratory volume, volume per minute, and breath frequency. Results are given as average values over 6 hours exposure with averaged readings taken at 20 minute intervals. Statistical significance denoted by “a” (treatment vs respective saline control), “b” (1000 ppm vs room air treatment), “c” (5000

ppm vs room air treatment) and “d” (5000 ppm vs 1000 ppm treatment) and error bars denoting SEM. N=5 mice/group.

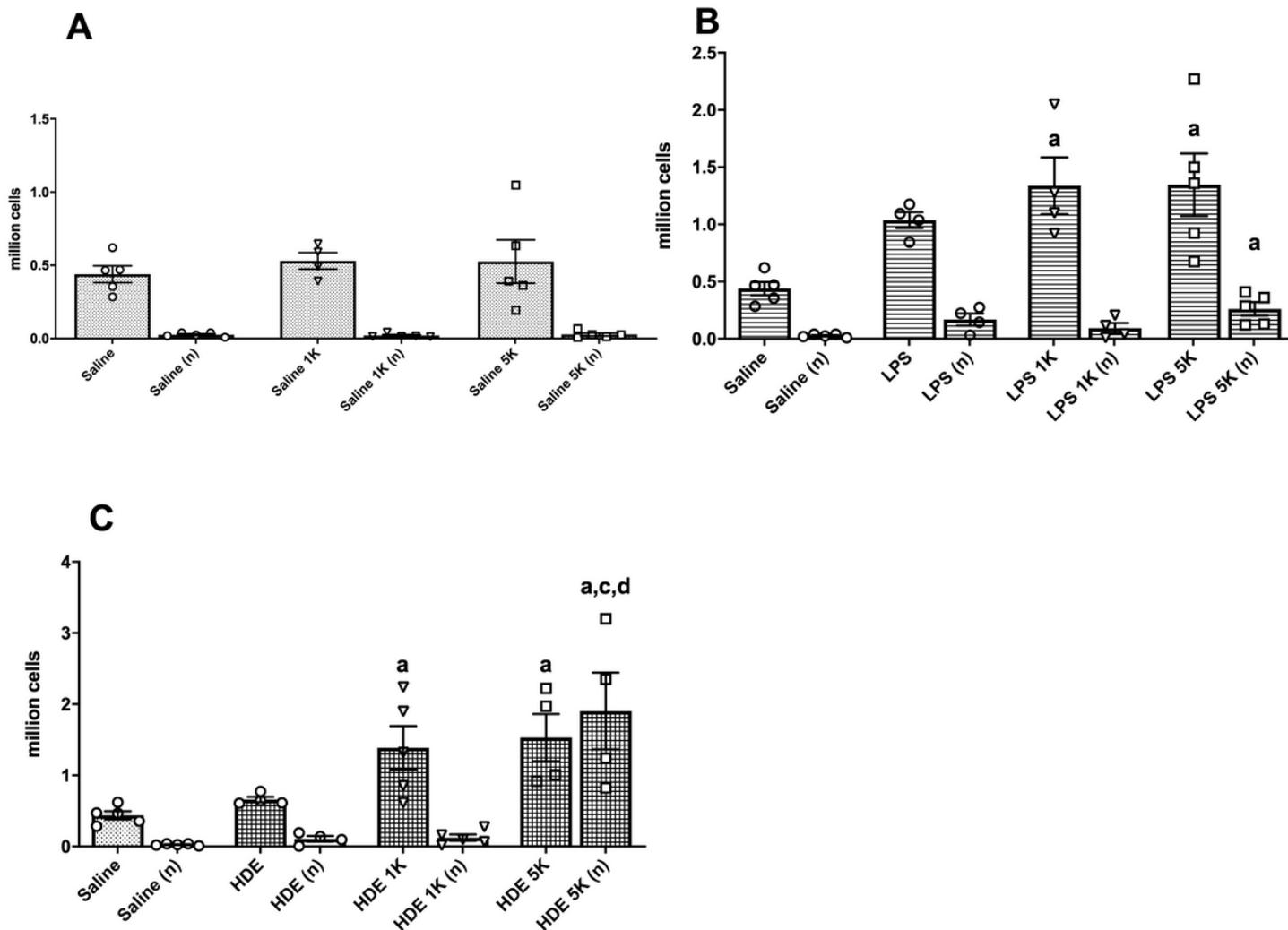


Figure 2

Effects of CO₂ on BAL fluid cells. Mice were treated intranasally with saline, organic dust (HDE), or lipopolysaccharide (LPS) and then exposed to either room air (~500ppm), 1000ppm (1K), or 5000ppm (5K) CO₂. Values given are based on number of cells in BAL fluid multiplied by the fraction of monocytes/macrophages (black bars) or neutrophils (open bars) in [A] saline, [B] HDE or [C] LPS treated animals. Bar graphs represent averages and error bars the standard deviation. N= 4-5 mice/group. Statistical significance denoted by “a” (treatment vs respective saline control), “b” (1000 ppm vs room air treatment), “c” (5000 ppm vs room air treatment) and “d” (5000 ppm vs 1000 ppm treatment) and error bars denoting SEM.

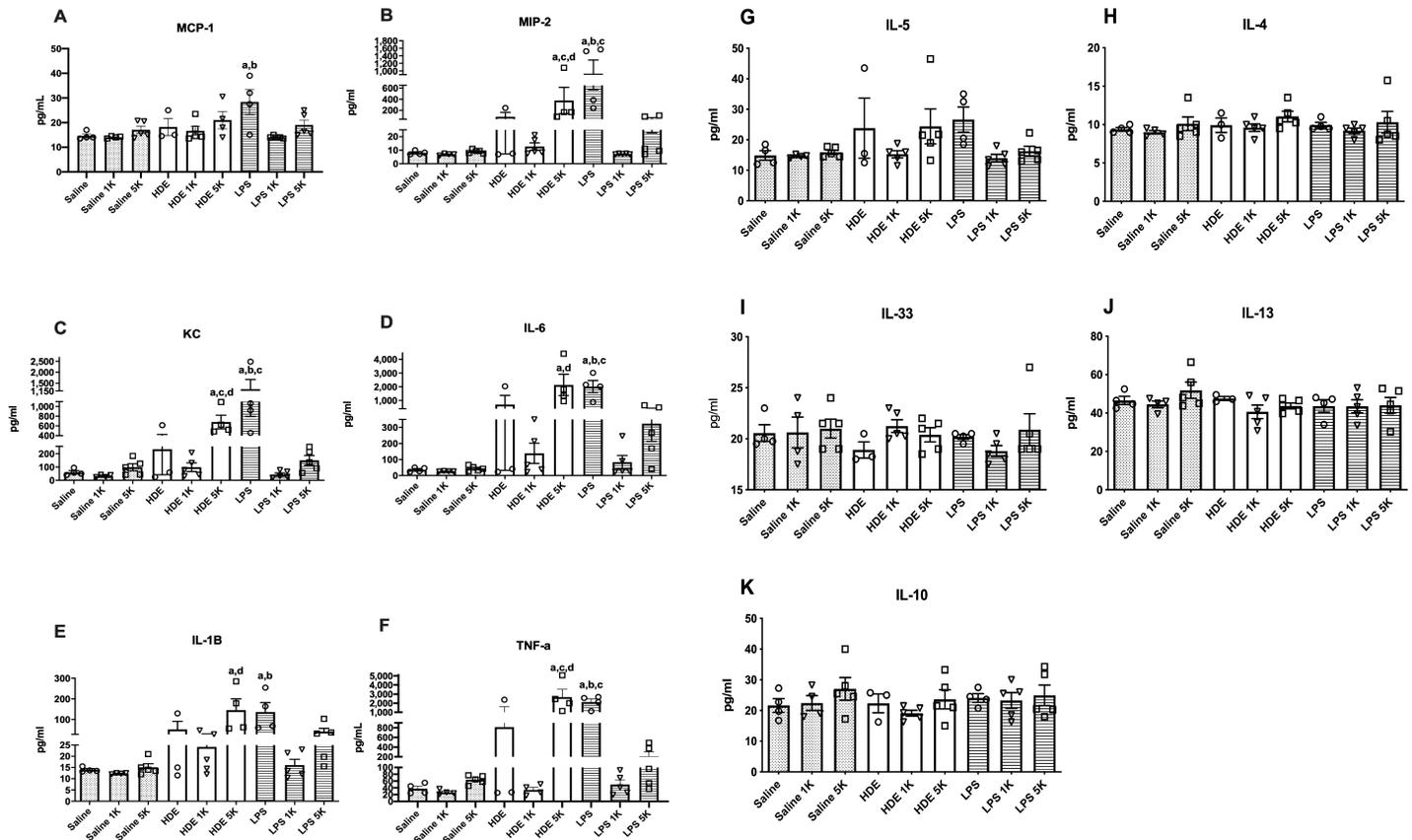


Figure 3

Protein expression in BAL fluid. Values given are in OD reading [A] MCP-1, [B] MIP-2, [C] IL-6, [D] KC, [E] IL-1B, [F] TNF-a, [G] IL-5, [H] IL-4, [I] IL-13, [J] IL-13, [K] IL-33, and [L] IL-10. Mice were treated intranasally with saline, organic dust (OD), or lipopolysaccharide (LPS) intranasally and then exposed to either room air (~500ppm), 1000ppm (1K), or 5000ppm (5K) CO₂. BAL fluid was centrifuged, cells removed and tested by ELISA. Results show reductions in KC and MIP-2 with LPS treated animals plus CO₂, and an increase in all three for OD plus 5000ppm CO₂. Bar graphs represent averages and error bars the SEM. N= 3-5 mice/group. by "a" (treatment vs respective saline control), "b" (1000 ppm vs room air treatment), "c" (5000 ppm vs room air treatment) and "d" (5000 ppm vs 1000 ppm treatment).

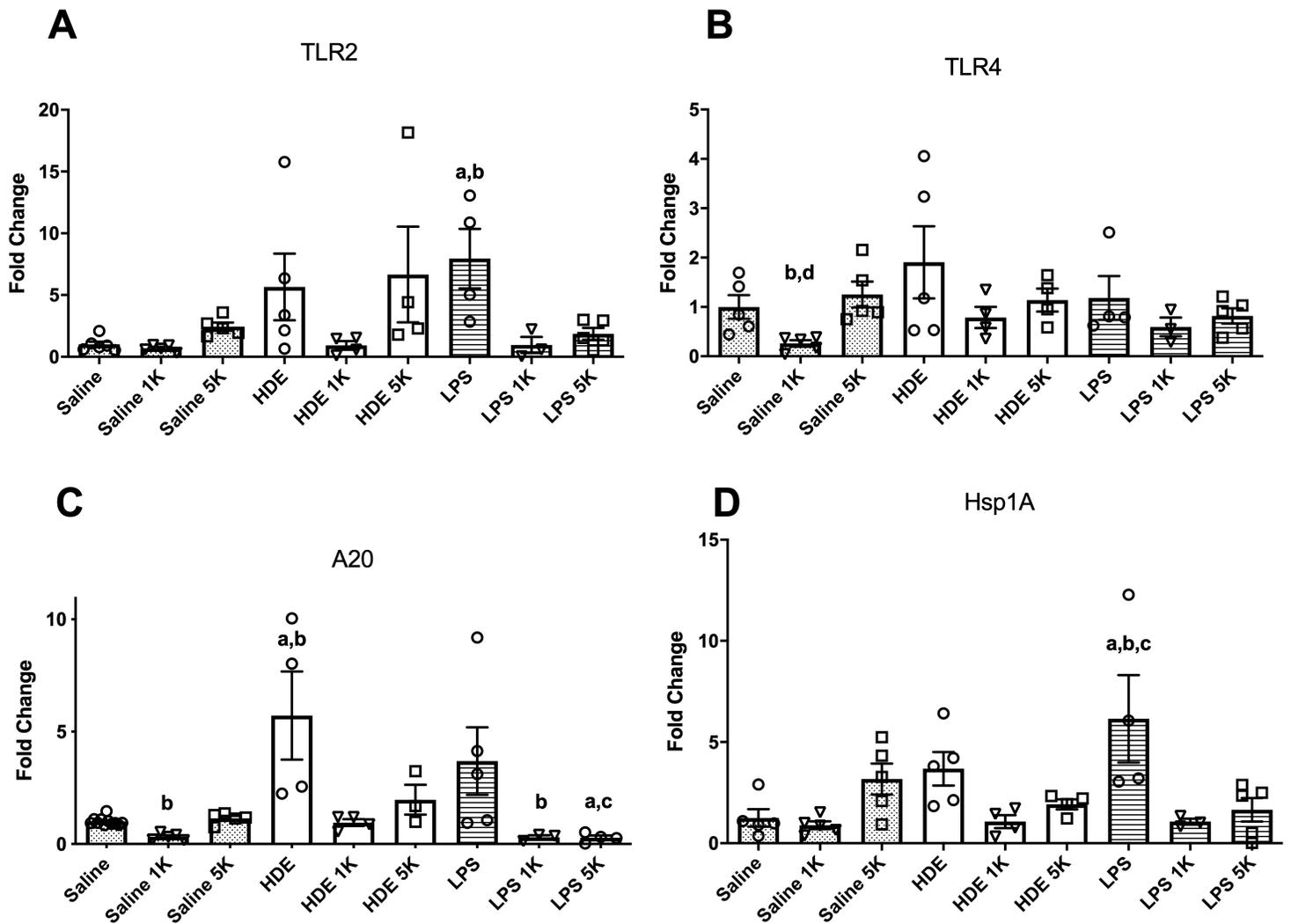


Figure 4

mRNA expression in mouse lung tissue. Values given are fold change in mRNA compared to a control value. mRNA harvested from lungs of mice given saline, organic dust (OD), or lipopolysaccharide (LPS) intranasally and then exposed to either room air (approximately 500ppm), 1000ppm (1K), or 5000ppm (5K) CO₂. Tissue was tested for the receptor TLR2 [A], TLR4 [B], cytoplasmic protein A20 [C] and Hsp1A [D]. Results show reductions in TLR2, and Hsp1A with LPS treated animals plus CO₂, and general decreases in A20 and Hsp1A for OD plus CO₂. Hsp1A was elevated with CO₂ delivery to control, to date the only protein mRNA found to do this. Bar graphs represent averages and error bars the SEM. N= 3-5 mice/group. by “a” (treatment vs respective saline control), “b” (1000 ppm vs room air treatment), “c” (5000 ppm vs room air treatment) and “d” (5000 ppm vs 1000 ppm treatment).

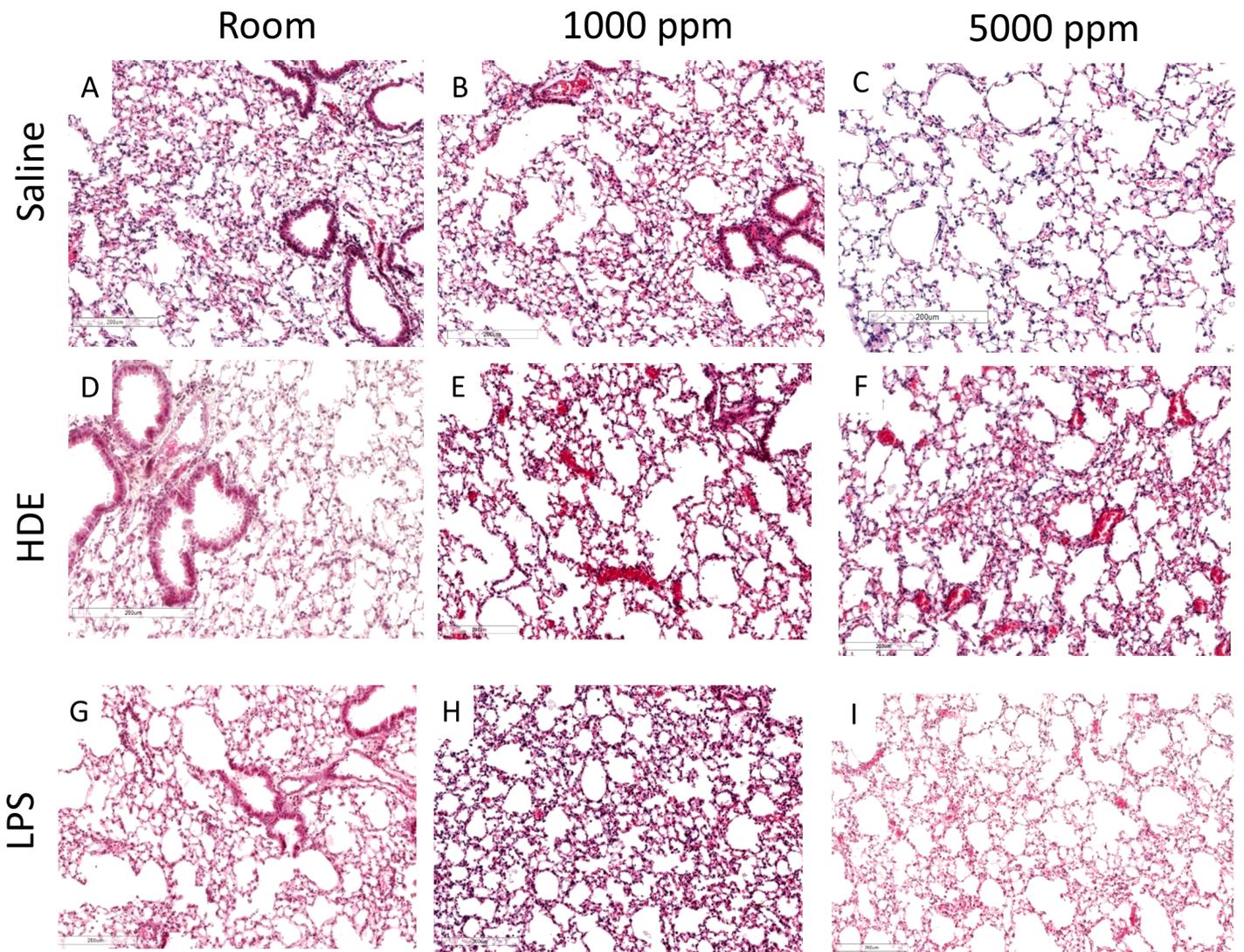


Figure 5

Histological examination of mouse lungs. Left lungs of mice were fixed in 4% PFA and processed for paraffin blocks. 5µm tissue slices were cut and stained with hematoxylin and eosin and imaged. Images at 100X are included for saline (A-C), HDE (D-F), and LPS (G-I). Changes in inflammation were apparent between saline, HDE, and LPS, treatments, but CO₂ co-treatment appeared to have little effect.