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Pro-Inflammatory Effects of Cook Stove Emissions on Human Bronchial Epithelial Cells

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Abstract

Approximately half the world's population uses biomass fuel for indoor cooking and heating. This form of combustion typically occurs in open fires or primitive stoves. Human exposure to emissions from indoor biomass combustion is a global health concern, causing an estimated 1.5 million premature deaths each year. Many 'improved' stoves have been developed to address this concern; however, studies that examine exposure-response with cleaner-burning, more efficient stoves are few. The objective of this research was to evaluate the effects of traditional and cleaner burning stove emissions on an established model of the bronchial epithelium. We exposed well-differentiated, normal human bronchial epithelial (NHBE) cells to emissions from a single biomass combustion event using either a traditional three-stone fire or one of two energy-efficient stoves. Air-liquid interface cultures were exposed using a novel, aerosol-to-cell deposition system. Cellular expression of a panel of three pro-inflammatory markers was evaluated at 1 and 24 hours following exposure. Cells exposed to emissions from the cleaner burning stoves generated significantly fewer amounts of pro-inflammatory markers than cells exposed to emissions from a traditional, three stone fire. Particulate matter emissions from each cookstove were substantially different, with the three-stone fire producing the largest concentrations of particles (by both number and mass). This study supports emerging evidence that more efficient cookstoves have the potential to reduce respiratory inflammation in settings where solid fuel combustion is used to meet basic domestic needs.

Keywords

biomass fuels; *in vitro*; wood smoke; pro-inflammatory; air-liquid interface; NHBE

Introduction

Indoor biomass combustion is relied upon as a cooking and heating practice to nourish and sustain approximately half of the world's population (Rehfuess, 2006). The burning of biomass fuel (e.g., wood, dung, agricultural waste) indoors is typically carried out in an open fire pit (i.e., a three-stone fire), or in a simple cook stove. Air pollution from this form of combustion poses a significant global health problem (Bruce et al., 2000). The World Health Organization (WHO) reported in 2006 that women, men, and children living in homes relying upon biomass burning receive an exposure equivalent to smoking two packs of

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Competing Interests

The authors declare they have no competing interests.

cigarettes each day. The same WHO report estimated that smoke from open fires and simple cookstoves causes 1.5 million premature deaths each year (Rehfuess, 2006).

Wood accounts for 50–60% of the biomass fuel used for domestic combustion (Smith, 2008). Exposure to wood smoke has been associated with increased incidence of chronic obstructive pulmonary disease, cancers of the trachea, bronchus, and lung, chronic infectious diseases of the lung like Tuberculosis, and acute lower respiratory tract infections (ALRIs) (Bruce et al., 2000). ALRIs cause more lost life-years than any other disease, since they affect children most severely (WHO, 2004). Furthermore, respiratory inflammation has been shown to represent the first step in a known biologic pathway that eventually results in systemic inflammation, autonomic and vascular dysfunction, and subsequent clinical manifestation of pulmonary and cardiovascular disease states (Brook et al., 2010). Repeated and sustained periods of acute inflammation can lead to a chronic inflammatory state and can eventually contribute to chronic bronchitis, emphysema, pulmonary fibrosis, or cancers of the respiratory tract (Coussens and Werb, 2002, Gross, 1990, Malkinson, 2005, Schottenfeld and Beebe-Dimmer, 2006), and each of the aforementioned respiratory pathologies has been associated with indoor cook stove exposure (Bruce et al., 2000, Naeher et al., 2007).

Epidemiological associations between particulate matter (PM) levels and the morbidity and mortality among populations exposed to wood smoke (Bruce et al., 2000, Naeher et al., 2007, Smith, 2008) are often hampered by relatively small sample sizes and the logistical difficulties associated with field work in developing countries (Smith and Peel, 2010). An alternative approach to understand and characterize the human health burden related to indoor cook stove exposure relies upon investigating molecular pathways by which wood smoke PM exerts damaging effects on the cardiopulmonary system. Increased susceptibility to respiratory infections has been attributed to reduced mucociliary and macrophage clearance of pathogens following PM exposure (Zelikoff et al., 2002). Further, acute exposure to PM from biomass combustion increases irritation, inflammation, and reactivity of the airways (Bruce et al., 2000, Naeher et al., 2007). Persistent inflammation can cause necrosis and fibrosis, two cellular pathologies that are associated with respiratory disease (Ballaz and Mulshine, 2003). Furthermore, growing evidence suggests that chronic inflammation from repetitive PM exposure promotes a genotoxic, carcinogenic environment within the lung (Azad et al., 2008, Mantovani et al., 2008, Gungor et al., 2010, Lee et al., 2007).

Epithelial cells within the respiratory tract serve as the first line of defense against inhaled PM and can initiate localized inflammation through the release of cytokines (IL-1, IL-6, GM-CSF), chemokines (IL-8, MIP-1), prostaglandins (COX-2), adhesion molecules (ICAM-1) and through activation of stress response genes (HSP-27, 70, 90, HOX-1) (Danielsen, 2009, Rahman, 2003). *In vitro* models of the respiratory epithelium have advanced significantly in recent years, exhibiting morphologic, histologic, and functional features of the bronchial lung *in vivo* (Le Visage et al., 2004, Ross et al., 2007, Rothen-Rutishauser et al., 2005). Such models, when used with direct, particle-to-cell deposition systems, provide a more physiologically-relevant means for evaluating cellular injury, inflammation, and repair in response to environmental air pollutants, especially when compared to traditional *in vitro* models which employ submerged cultures, immortalized cell lines, and exposures to air pollution extracts (Devlin et al., 2005, Teeguarden et al., 2007). Therefore, we chose to examine cellular inflammation as a benchmark for comparing the effects of improved and traditional cook stove emissions upon a well-differentiated model of the bronchial epithelium.

Improved cookstoves are designed to operate with increased thermal efficiency, meaning more efficient heat transfer and less fuel consumption, and increased combustion efficiency, meaning reduced emissions of PM and gaseous species (per gram of fuel burned).

Hazardous PM emissions consist of species like elemental carbon (also referred to as black carbon or soot), redox-active metals (e.g., zinc, iron, chromium, etc.) and organic carbon species such as polycyclic aromatic hydrocarbons. Examples of toxic gasses emitted by cookstoves include oxides of carbon and nitrogen, and volatile and semi-volatile organic compounds such as aldehydes, quinones, alkanes, and aromatics (Naeher et al., 2007, Reid et al., 1986, Schmidl et al., 2008).

The objective of this research was to evaluate acute inflammation in well-differentiated cultures of normal human bronchial epithelial (NHBE) cells after exposure to wood combustion particles and gases emitted by traditional and improved cookstoves. The NHBE cell culture is considered an established, organo-typic model of the human bronchial epithelium (BéruBé et al., 2010) that has been used for numerous medical and toxicological investigations (Lin et al., 2007, Kim et al., 2005, Rothen-Rutishauser et al., 2005, Ehrhardt et al., 2008). When carried over a period of several weeks at air interface, human bronchial epithelial cells differentiate into a pseudo-stratified columnar structure, with signs of ciliation and mucus production *in vivo* (Ross et al., 2007). To our knowledge, this is the first laboratory-based evaluation of the pro-inflammatory effects of cook stove emissions on human lung cells. We characterized cellular expression of three pro-inflammatory genes in response to a single combustion event, in addition to characterizing PM emissions by mass, size, and count. We hypothesized that cells exposed to PM emitted by improved cookstoves would accumulate fewer pro-inflammatory markers compared to cells exposed to PM emissions from a traditional cook stove (i.e., a three-stone fire).

Materials and methods

Cell Culture

Normal human bronchial epithelial (NHBE) cells were obtained by brush biopsy from three healthy, non-smoking human volunteers (EPA, Research Triangle Park, NC) in accordance with a human studies protocol approved by the Institutional Review Board at the University of North Carolina. Cell populations were expanded through two passages in Petri dishes with Bronchial Epithelial Growth Media (BEGM kit, Lonza, Walkersville, MD) before being plated onto collagen-coated, porous, polycarbonate membranes (0.4 µm Snapwell membrane, Corning, Inc.) at a seeding density of approximately 70,000 cells per cm². Air-liquid interface cultures were carried for a minimum of 21 days (prior to exposure) to promote cellular differentiation into ciliated, mucin-producing, and basal-like cell types within a pseudo-stratified columnar epithelium (Ross et al., 2007). Culture progression was evaluated by formalin fixing and paraffin embedding a subset of NHBE cultures at days 1, 10, 24, and 28 of air-liquid interface and also by quantifying the relative expression of mucin-producing (MUC5AC, MUC5B) and ciliogenesis (TUBA3) genes at each time period (see Supplementary Material for further detail).

Cell Exposures

The experimental matrix for the cell exposures is shown in Table 1. Wood smoke was generated from three stove types: the Envirofit G3300 stove, an energy efficient model with a rocket-elbow combustion chamber designed and distributed by Envirofit International; the Philips Gasifier stove, an energy efficient model with a two-stage combustion chamber designed and distributed by Philips Inc.; and a three-stone fire designed to model a traditional, yet inefficient cook stove (images of these stoves are provided in Supplementary Material). All experiments were conducted at the Colorado State University Engines and

Energy Conversion Laboratory using a constant-displacement fume hood designed specifically for cook stove research.

Cells were exposed to emissions during a simulated cooking operation. On each test day, a stove model was randomly selected, and a standardized water boil test was begun by following a protocol similar to the water boil test used by Household Energy and Health Programme, Shell Foundation (Bailis et al., 2007). Both the Envirofit G3300 stove and the three-stone fire burned 7% moisture content Douglas fir, whereas the Philips Gasifier burned West Slope Wood Pellets with moisture content of 6.4%, as per manufacturer instructions. The water boil test uses a stove to bring a 6 L pot containing 5 kg of water to boil. Once boiling, the pot is maintained at a simmer (95.5 °C in Fort Collins, CO) for 45 minutes. Fuel is fed to the stove on an as-needed basis during the test, and the total fuel consumption, by mass, is recorded. Wood smoke emissions during the 45-minute simmer phase were used for the cell exposures, described below. Immediately following an exposure, a matched set of control cells (i.e., from the same donor) were placed in the chamber and exposed to HEPA-filtered room air for the same duration (45 min). Cells from each donor were exposed to smoke from each of the three stove types.

***In Vitro* Exposure System**

Cultures were exposed to cook stove emissions using an electrostatic aerosol *in vitro* exposure system (EAVES) (Volckens et al., 2009). The EAVES (and similar direct, air-to-cell exposure systems) have substantial advantages over traditional particle exposure models, as these newer systems are designed to deposit aerosol directly onto air-liquid interface cultures (Lenz et al., 2009, Aufderheide et al., 2002, Grass et al., 2010, Tippe et al., 2002, de Bruijne et al., 2009). Traditional particle exposure systems, which use particulate matter extracts and submerged cell culture, lack physiologic relevance; problems with these traditional models have been discussed in detail previously (Devlin et al., 2005, Teeguarden et al., 2007).

Aerosol entering the EAVES was heated to 37°C and humidified to 80–90% relative humidity to maintain cell viability during the exposures. Cook stove exhaust was pulled from the fume hood at 3.8 L/min and directed into the EAVES. This exhaust sample was then supplemented with CO₂ (5% by volume, 0.2 L/min) to maintain cellular pH during exposures. Total airflow through the chamber was maintained at 4 L/min by a mass flow controller (FMA 5400/5500, Omega Engineering, Inc.) that was previously calibrated with a primary flow standard (DryCal DC-2, Bios International Corporation). A corona source, located upstream of the cell cultures applied a positive charge to the incoming aerosol. Charged particles were then deposited directly on the cells through an applied electric field using a repeller plate charged to + 1.5 kV. de Bruijne et al. characterized a similar electrostatic aerosol exposure system (operated at 2 L/min) and reported efficient aerosol charging and collect efficiency: 90% of all particles between 19 and 882 nm are effectively charged and collected (2009). For our experiments conducted at 4 L/min of flow, collection efficiency of the EAVES was reduced to approximately 70% of the total aerosol concentration between 10 and 1000 nm. Furthermore, because multiple cell culture inserts are used in the EAVES during a given experiment, each insert receives only 2% of the incoming aerosol mass. These measurements were verified using a fluorescent aerosol tracer assay (of similar size distribution), as reported previously (Volckens et al., 2009).

Emissions Characterization

Separate samples of gas and particle emissions were characterized by mass and number concentration. Particulate matter emissions by mass, for particles of aerodynamic diameter less than 10 µm (PM₁₀), were collected on 47 mm Teflon filters (PALL, LifeSciences, Ann

Arbor, Michigan) following a sampling protocol similar to that of Subramanian et al. (Subramanian et al., 2004). The total flow rate through the cassette containing the Teflon filter used for gravimetric analysis was 8.4 L/min and was within 10% of the flow rate needed for isokinetic sampling. The total mass collected on the filter was used to estimate the total PM_{10} emitted (PM_{10}) from each stove by following equation 1,

$$PM_{10} = \frac{PM_{10,filter} \cdot V_{total}}{V_{sampled}} \quad (1)$$

where $PM_{10,filter}$ represents the PM_{10} mass collected on the 47mm Teflon filter, V_{total} represents the total volume of air exhausted from the fume hood, and $V_{sampled}$ represents the volume of air passed through the 47mm Teflon filter during the forty-five minute simmer phase of each test.

Particle size distributions were measured using a Sequential Mobility Particle Sizer (SMPS +C, Grimm Technologies, Douglasville, GA); gaseous emissions (CO , CO_2) were quantified with a Fourier transform infra-red spectrometer (Magna 560, Thermo-Scientific Inc., Waltham, MA).

Estimation of PM Mass Delivered to Cells

The mass of particles deposited per unit cellular growth area (PM_{dep}) was calculated from Equation 2,

$$PM_{dep} = \frac{[PM_{10}] \cdot Q_E \cdot t \cdot E \cdot D_i}{A_{growth}} \quad (2)$$

where $[PM_{10}]$ represents the measured PM_{10} concentration in the emissions from each cook stove, Q_E represents the flow through the EAVES chamber, t represents the duration of exposure, E represents the overall collection efficiency of the EAVES, D_i represents the fractional deposition efficiency of PM to each cell culture insert, and A_{growth} represents the area of cellular growth in each insert.

Pro-inflammatory Gene Expression Analysis

Combustion-derived particles can sequester cytokines produced by epithelial cells making them unavailable for analysis by ELISA (Seagrave et al., 2004, Kocbach et al., 2008). Therefore, we chose to quantify mRNA transcripts coding for a panel of three proteins associated with cellular inflammation or injury: interleukin-8 (IL-8), a chemokine associated with attracting inflammatory cells into the lung (Baggiolini et al., 1995); cyclooxygenase-2 (COX-2), an enzyme in the prostaglandin pathway for signaling inflammation and pain (Tilley et al., 2001); and heme oxygenase-1 (HOX-1), an oxidative stress response enzyme (Choi and Alam, 1996). Total mRNA transcripts from exposed and clean-air control cells were isolated at 1 and 24 hours following exposure using a standardized kit and protocol (RNeasy mini kit, Qiagen, Valencia, CA). A DNase digestion step was added to minimize DNA contamination (RNase Free DNase Set, Qiagen, Valencia, CA). The purity and quantity of the mRNA was assessed by spectrophotometry at wavelengths of 260 and 280 nm (Nanodrop ND-1000, ThermoScientific, Wilmington, DE). A standard real-time RT PCR protocol (Biorad One-Step RT-PCR Kit with SYBR Green, Bio-Rad Laboratories, Hercules, CA) was followed for each gene of interest (see Supplementary Material for further detail). Expression profiles for each transcript are normalized to the expression of GAPDH (Barber et al., 2005) and to average expression profiles of the clean air controls.

Cytotoxicity

Lactate dehydrogenase (LDH) is expressed universally in these cells. The loss of membrane integrity during cell injury/death causes extracellular release of LDH, which can be used as an indicator of cytotoxicity (Allan and Rushton, 1994). Cellular release of LDH was assayed at 1 and 24 hours post exposure by using a standardized LDH protocol (Promega Cytotox96 Non-radioactive Cytotoxicity Assay).

Statistical Analyses

All statistical analyses were conducted with SAS software (SAS Institute Inc., Cary, NC) with a type I error rate of $\alpha=0.05$. Transcript data were log transformed to satisfy model assumptions of normality and homoscedasticity. Pollutant emissions between different stove pairs (e.g., traditional vs. Gasifier, Gen3300 vs. Gasifier, etc.) were compared by using a one-way ANOVA MEANS procedure in SAS. Comparisons were made for total particle number concentration, particle count median diameter, PM₁₀ mass, and fuel consumption rate. A Tukey's adjustment was made to account for the error due to multiple comparisons. The effects of stove model, time post-exposure, donor phenotype, burn day, and their interactions, on the expression of HOX-1, IL-8, COX-2 genes and the release of LDH were evaluated using the MIXED procedure. Cell donor and burn day were treated as random effects.

Results

Pro-Inflammatory Effects of Cook Stove Emissions

The relative expression of HOX-1, IL-8, and COX-2 transcripts by NHBE cells exposed to emissions from each cook stove is shown in Figure 1 as box and whisker plots. The center line within the box indicates the median, the top and bottom of the box indicate the lower and upper quartiles, and the whiskers extending above and below the box indicate the data range (excluding outliers). Levels greater than one indicate an increase in expression relative to the clean air controls (below, we report the median relative expression for each mRNA transcript with one standard deviation reported in parentheses). No significant differences were observed in mRNA expression due to effects of either the donor phenotype or the burn day. The LDH measured in cell exudate did not differ significantly between cells exposed to control (HEPA-filtered) air, emissions from the three-stone fire, the G3300 stove, and the Gasifier stove ($p = 0.49$ – 1.00). This finding was expected, as these experiments were designed to produce exposure levels below what is expected to induce cell death.

At one hour post-exposure, IL-8 transcription for cells exposed to emissions from the three-stone fire increased by a factor of $1.8 (\pm 0.47)$ ($p = 0.02$). IL-8 transcription in cells exposed to either the G3300 or Gasifier stove did not increase relative to cells exposed to room air ($p=0.14$, $p=0.58$, respectively). At twenty-four hours post exposure, none of the exposed cells showed a significant change in IL-8 transcription relative to room-air controls ($p=0.12$ for three-stone fire, $p=0.5$ for the G3300 stove, and $p=0.39$ for the Gasifier stove).

HOX-1 transcription in cells exposed to emissions from the three-stone fire was significantly elevated relative to the improved stoves, at one hour post-exposure. Fold changes in HOX-1 transcription were: $3.2 (\pm 1.5)$ in cells exposed to emissions from the three-stone fire ($p < 0.0001$), $1.2 (\pm 0.4)$ in cells exposed to emissions from the G3300 stove ($p = 0.99$), and $0.6 (\pm 0.3)$ in cells exposed to the Gasifier emissions ($p = 0.40$). At twenty-four hours, relative levels of HOX-1 transcription were markedly reduced and not statistically different among different stoves or between exposures and clean-air controls.

Changes in COX-2 transcription in cells exposed to emissions from the three-stone fire were also significantly elevated when compared to either of the improved stoves. At one hour post-exposure, COX-2 transcription increased by a factor of $2.6 (\pm 1.3)$ for cells exposed to emissions from the three-stone fire ($p = 0.0015$), $0.9 (\pm 0.5)$ for cells exposed to emissions from the G3300 stove ($p = 0.86$), and $1.2 (\pm 0.3)$ for cells exposed to emissions from the Gasifier stove ($p = 0.28$) relative to the clean air controls. At twenty-four hours post exposure, these differences were markedly reduced and non-significant.

Cellular exposure-response curves for mRNA expression are shown as a function of PM concentration deposited to cell surfaces ($\mu\text{g PM}/\text{cm}^2$ cellular growth area) in Figure 2. A similar, linear pattern is evident for HOX-1 and COX-2 transcripts, with the highest mass loading eliciting the greatest cellular response. Cellular expression of IL-8 also follows a general linear trend with PM exposure, albeit at a more attenuated rate. Only the highest exposure levels elicited a significant increase in mRNA expression (above controls) for each transcript ($p < 0.05$ for each).

Characterization of Particulate and Gaseous Emissions

Stove emissions data, chamber concentrations, fuel consumption rates, and deposited PM concentrations are summarized in Table 2. The Philips Gasifier stove emitted an average of 134 mg of PM_{10} during the simmer phase (average chamber concentration of $496 \mu\text{g}/\text{m}^3$), the lowest concentration emitted by any stove. The gasifier was also the most fuel-efficient stove, requiring 195 ± 15 grams of wood pellets to maintain a pot at a simmer temperature (95.5°C in Fort Collins, CO) for 45 minutes. Average particle size distributions (normalized to the width of each measurement bin) emitted by each stove are depicted in Figure 3. The gasifier produced fewer, smaller sized particles, with an average, count median diameter (CMD) of 19.4 ± 1.8 nm as compared to the other models. Emissions from the gasifier stove resulted in an average of $0.24 \mu\text{g}/\text{cm}^2$ of PM delivered to cells during the simmer phase of the water boil test.

The Envirofit G3300 stove required more wood (314 ± 12 grams) to maintain a pot at a simmer for 45 minutes, which resulted in greater overall PM emissions (327 mg total; $1210 \mu\text{g}/\text{m}^3$) than the gasifier stove ($p < 0.01$). Particles produced by the G3300 stove were also slightly larger, with a CMD of 26.0 ± 3.3 nm, as seen in Figure 1. On average, NHBE cells exposed to G3300 emissions received approximately $0.58 \mu\text{g}/\text{cm}^2$ of PM mass.

The three-stone fire produced the largest size (CMD of 92.3 ± 13.0 nm) and concentration (1190 mg emitted; $4410 \mu\text{g}/\text{m}^3$) of particles and required the most fuel (523 ± 14 grams of wood) to maintain a pot at a simmer for 45 minutes ($p < 0.01$ for all comparisons). The three-stone fire produced approximately three to four times more PM_{10} mass than the Envirofit G3300 stove ($p < 0.001$) and approximately eight to nine times more PM_{10} mass than the Philips Gasifier stove ($p < 0.001$), resulting in a proportionate increase in particulate matter delivered to cell cultures (Table 2). The variability in the estimated PM_{10} mass delivered to the cells is a result of both test-to-test variability as well as variations in particle deposition rates within the EAVES chamber (Volckens et al., 2009). Accounting for fuel consumption, the three-stone fire produced twice as much PM_{10} per gram of wood burned as the G3300 stove ($p < 0.01$), and three times as much PM_{10} per gram of wood as the Philips Gasifier stove ($p < 0.001$) during the simmer phase.

Discussion

The expression profiles shown in Figures 1 and 2 include data pooled from multiple donors and test repeats. The large variability in observed exposure-response for a given marker was not unexpected, given the following considerations. First and foremost, we used primary

NHBE cells taken from different donors and the relative response varied between subjects (though not significantly). Second, day to day variability in cook stove emissions ($\pm 25\%$ from average) resulted in differential exposure concentrations experienced on separate test days. Yet, despite these sources of variability, statistically significant differences were observed in the responses among NHBE cells exposed to emissions from different stove types (HOX-1 and COX-2 at 1 hour following exposure).

The electrostatic deposition of particles carrying a net positive charge may alter how the NHBE cells detect and respond to wood smoke PM. However, a similar electrostatic exposure system run with similar charging conditions and flow rates was used by de Bruijne *et al.* to study the biological response of air-liquid interfaced cells after exposure to charged polystyrene latex (PSL) particles. They found that charged PSL particles did not increase the production of pro-inflammatory markers or cytotoxicity when compared to controls.

The aim of this study was not to elucidate which factors most strongly predict cellular response, but instead to compare the inflammatory effects of different stove technologies on an *in vitro* model of the bronchial lung. For that reason, we did not control the amount of fuel burned (or the levels of PM deposited to cells) during a given test, nor the type of fuel used. Instead, we chose to mimic a common activity for domestic wood burning, specifically: maintaining a pot of water at boil for a pre-determined time (as would be carried out during normal cooking practices). This choice reflects a compromise between experimental rigor and real-world applicability. The Philips Gasifier stove requires the use of pelletized wood, per manufacturer's instructions, as compared to the three-stone fire and G3300 stoves, which burn more traditional wood kindling. The use of a different fuel type may have influenced the size and composition of the PM emitted from the Gasifier stove. Therefore, we cannot rule out fuel type as a factor affecting the cellular responses for this stove. We chose to include a gasifier stove, despite the differences in fuel type, because this technology is considered 'cleanest' among wood burning stoves. We also note, however, the marked differences in response between the G3300 stove and the three-stone fire, both of which burned the same fuel but produced significantly different levels of emissions.

In normal human bronchial epithelial cells, increased cytokine release in response to PM exposure is correlated with increased production of mRNA transcripts that code for these proteins (Wyatt *et al.*, 2007). However, combustion-derived PM has been shown to sequester cytokines released from epithelial cells, which can result in a negative bias when these cytokines are quantified using ELISA (Seagrave *et al.*, 2004, Seagrave, 2008, Kocbach *et al.*, 2008). Therefore, we chose not to quantify protein levels directly but instead to assess levels of mRNA transcripts as a proxy for cytokine production.

Three cellular transcripts were evaluated for this study among many possible candidate markers. These transcripts represent a panel of genes involved in lung injury and inflammation and each covers a different mode of cellular response. Interleukin-8 (IL-8) is a protein that recruits neutrophils to sites of inflammation (Schraufstatter *et al.*, 2001). Neutrophil infiltration and activation within the lung is associated with asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, acute respiratory distress syndrome, and lung tumorigenesis (Kunkel *et al.*, 1991, Gungor *et al.*, 2010, Rahman, 2003). Heme oxygenase-1 is an intracellular enzyme involved in the cellular response to oxidative stress and in mediating oxidative damage in the lung (Choi and Alam, 1996). Cyclooxygenase-2 is a chemokine involved in the prostanoïd (pain signaling) pathway and has been indicated to play a role in asthmatic responses of the bronchial epithelium and also in the development of lung cancer (Lipsky *et al.*, 2000, Carey *et al.*, 2003, Campa *et al.*, 2004, Castelao *et al.*, 2003).

A forty-five minute exposure was sufficient to produce statistically-significant increases without affecting cell viability. The expression profiles for the mRNA transcripts for IL-8, HOX-1, and COX-2 observed in the cells exposed to either of the two improved stoves were not significantly elevated when compared to filtered-air controls. These results do not necessarily indicate that emissions from either of the improved stoves are benign. Rather, the lack of any observed difference between exposures to improved stoves and filtered room air may be an artifact of the duration of exposure. Also, the selection of 1 and 24 hour time points may not have sufficiently captured peak IL-8 expression. Complicating this issue is the fact that each gene likely underwent peak production at different times following exposure. Our experimental resources precluded the evaluation of mRNA expression at additional time points, but such investigations represent a promising avenue for further research.

Winkler-Heil and Hoffman (2002) estimated deposition fractions in the bronchial region of the human lung to range from 0.000025–0.000225 per cm² of bronchial airway for particles from 1000 to 10 nm in size (Winkler-Heil and Hofmann, 2002). Assuming an average tidal volume of 0.5 L, a concentration of 4410 µg/m³ (reported here as the average concentration from the three stone fire), a human being would inhale and deposit between 5.5·10⁻⁵ and 4.0·10⁻⁴ µg per cm² of bronchial area with each breath. Assuming a respiratory rate of 12–20 breaths per minute, and a 45 minute exposure duration, the estimated deposition *in vivo* would be approximately 0.03–0.36 µg per cm² of bronchial airway. The estimated dose delivered to cells exposed in the EAVES was roughly 10 to 100 times higher than what would be estimated to deposit in the lungs of a human during the same duration of exposure. Thus, we expect the cell exposures used here are on the order of one to two weeks exposure *in vivo*. However, for individuals who operate such stoves, these exposures typically continue throughout the course of their lifetime.

The three-stone fire tested here simulated a “traditional” stove or open fire that is typical in the developing world. However, considerable variation exists among the type, fuel, and usage of traditional stoves worldwide. Although the three-stone fire tested here does not replicate every traditional stove or open fire in use, emissions from this stove fell into the range of emissions reported for typical, inefficient cookstoves. Typical time-averaged PM levels in households with open burning range from 100 to 2000 µg/m³, with peaks during cooking five to ten times higher (Smith, 2008). The PM₁₀ levels measured from the three-stone fire tested here corroborate with PM₁₀ concentrations observed during peak cooking use in homes with open fires.

The three-stone fire consumed the most wood fuel per task ($p < 0.001$) and emitted significantly more PM₁₀ mass per gram of wood burned than either of the other two stove models tested ($p < 0.001$). The Envirofit G3300 stove and the Philips Gasifier stove demonstrated increased fuel efficiency and reduced particulate matter emissions when compared to the three-stone fire (Table 2). The number concentration of particles did not differ significantly between either of the two improved stoves ($p = 0.21$). However, because the gasifier tended to emit smaller particle sizes (Figure 2), PM₁₀ emissions by mass were lower for this stove compared to the G3300 ($p < 0.01$).

PM₁₀ was collected rather than PM_{2.5} since particulate matter smaller than 10 µm is capable of depositing in the bronchial region of the lungs. However, previous studies have shown that most combustion PM is smaller than 1 µm, with the majority of particle mass residing between 0.15 and 0.4 µm (Hays et al., 2002, Kleeman et al., 1999). Particle size distributions shown in Figure 2 indicate that the majority of the PM emitted by these stoves was smaller than 1 µm.

Conclusions

Results from this study indicate that NHBE cells exposed to emissions from improved cookstoves produce fewer pro-inflammatory markers as compared to emissions from a traditional three-stone fire. Although not surprising, given the increased emissions (and decreased efficiency) of the traditional three-stone fire, this finding is significant because repeated and sustained periods of acute inflammation have been found to lead to a chronic inflammatory state and can eventually contribute to chronic bronchitis, emphysema, pulmonary fibrosis, and/or cancers of the respiratory tract (Ballaz and Mulshine, 2003, Azad et al., 2008).

To the extent of our knowledge, this study is the first *in vitro* study to provide evidence that improved cookstoves have the potential to reduce respiratory inflammation in settings where open wood burning is used to meet basic domestic needs. Such laboratory-based studies help confirm those studies conducted in the field, which have, to date, been limited by relatively small sample sizes and difficulties associated with field work in developing countries (Energy, 2011).

Results from this research, however, should be tempered with the following limitations. First, the complete chemical composition of the combustion pollutants used for exposure was not analyzed. Some of the inflammatory effects observed may have been due to differences in the chemical composition of the fuel types used in this study and their aerosolized combustion byproducts. Second, only a single cell population (NHBE) was studied. Within the lungs, increased transcription of IL-8, HOX-1, and COX-2 in NHBE cells might recruit other immune cell populations like macrophages and neutrophils in response to inhaled toxicants (Gungor et al., 2010). The *in vitro* model tested here (21-day NHBE cells cultured at air-liquid interface) represents a state-of-the art model of the bronchial epithelium. However, this model lacks other characteristics of the human lung *in vivo*, such as: macrophages, neutrophils, endothelial cells, smooth muscle structures, and innervation – all of which can act in concert to produce inflammation (Karp et al., 2002).

The mechanisms associated with the inflammatory response observed in NHBE cells exposed to three-stone fire emissions are not fully understood. Our data suggest that the three-stone fire produced particles that were larger in size, mass, and greater in number than the particles emitted from either of the improved stove models. Whether it is wood smoke particle size, mass, or particle number that was most significant in stimulating inflammation in NHBE cells after exposure to emissions from the three-stone fire is unknown. Further investigations into the role that aerosol size, mass, surface area, particle shape, redox potential, and gaseous species play in predicting the inflammatory response of human bronchial epithelial cells may help cook stove designers to develop cookstoves that reduce the human health burden from exposure to indoor biomass combustion emissions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ALRI	Acute lower respiratory tract infections
CMD	count median diameter
COX-2	Cyclooxygenase-2
EPA	Environmental Protection Agency
HOX-1	Heme oxygenase-1
IL-8	Interleukin-8
LDH	Lactate dehydrogenase
NHBE	Normal human bronchial epithelial
PM	Particulate matter

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Practical Implications

Emissions from more efficient, cleaner burning cookstoves produced less inflammation in well-differentiated bronchial lung cells. The results support evidence that more efficient cookstoves can reduce the health burden associated with exposure to indoor pollution from the combustion of biomass.

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Figure 1. Box-whisker plots of relative mRNA expression (ratio of exposure to control) at one-hour (left panel) and twenty-four hours (right panel) following exposure to cook stove emissions. The center line within the box indicates the median and the top and bottom of the box indicates the upper and lower quartiles, respectively. Each box-whisker plot represents data from 18 samples. The (*) symbols indicate expression profiles that were significantly elevated above control cells exposed to filtered room air.

Figure 2.

Exposure-response curves for HOX-1, COX-2, and IL-8 mRNA at one hour post exposure. The estimated PM₁₀ exposure is shown on the horizontal axis in terms of mass delivered per cm² of cellular growth area. The vertical axis indicates the average increase in expression, relative to control cells exposed to filtered room air. Error bars indicate one standard deviation. Data points are staggered for clarity.

Figure 3.
Distribution of particle number emissions by size and stove type for a typical simmer test.
Error bars represent one standard deviation.

Table 1

Experimental matrix for NHBE cell exposure-response tests.

Experimental Variable	Levels	Details
Normal Human Bronchial Epithelial Cells (NHBE)	3	Donors 1, 2, 3
Stove Type	4	Improved Stoves (2), Traditional, Three-Stone Fire (1), Filtered Air Control (1)
Experimental Replicates	2	NHBE cells exposed to each stove type on two separate days
Cellular Marker Timepoints	2	1, 24 hrs Post-Exposure
Cellular Markers	4	IL-8, HOX-1, COX-2, LDH
Biological Replicates	3	Three wells per condition

Table 2

PM₁₀ emissions and cellular exposure data (± 1 S.D.) by stove type for the simmer phase of a water boil test.

	Three Stone Fire	Envirofit G3300	Philips Gasifier
Total PM₁₀ Emissions (mg)	1190 (± 289)	327 (± 20)	134 (± 37)
Fuel Consumed (g)	523 (± 14)	314 (± 12)	195 (± 15)
PM₁₀ Air Conc. ($\mu\text{g}/\text{m}^3$)	4410 (± 1070)	1210 (± 73)	496 (± 138)
Emissions Factor (mg PM₁₀/ g Fuel)	2.28 (± 0.55)	1.04 (± 0.63)	0.69 (± 0.19)
Cellular Exposure ($\mu\text{g PM}_{10}/\text{cm}^2$)	2.1 (± 0.5)	0.58 (± 0.17)	0.24 (± 0.07)
Total CO Emissions (g)	22.6 (± 8.9)	12.7 (± 3.2)	6.0 (± 0.6)
CO Air Conc. (ppm)	68.0 (± 26.7)	38.0 (± 9.7)	18.1 (± 1.9)
CO Emissions Factor (mg CO/ g Fuel)	43.6 (± 17.8)	40.6 (± 12.1)	30.9 (± 0.7)