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Elevated urinary mutagenicity among those exposed to bituminous coal combustion emissions or diesel engine exhaust

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AUTHOR CONTRIBUTIONS

Jason Y.Y. Wong conducted epidemiologic analyses. Judy Mumford, Debra T. Silverman, Roel Vermeulen, Nathaniel Rothman, and Qing Lan designed the study. W. Kyle Martin prepared the organic extracts of the urines, Hannah K. Liberatore performed the solvent-exchange of the extracts, Sarah H. Warren performed the mutagenicity experiments, and David M. DeMarini designed the mutagenicity experiments and analyzed the data to generate the mutagenic potencies. Jason Y.Y. Wong, Roel Vermeulen, Yufei Dai, Wei Hu, W. Kyle Martin, Sarah H. Warren, Hannah K. Liberatore, Dianzhi Ren, Huawei Duan, Yong Niu, Jun Xu, Wei Fu, Kees Meliefste, Jufang Yang, Meng Ye, Xiaowei Jia, Tao Meng, Bryan A. Bassig, H. Dean Hosgood, Douglas I. Walker, Jiyeon Choi, Mohammad L. Rahman, Yuxin Zheng, Judy Mumford, Debra T. Silverman, Nathaniel Rothman, David M. DeMarini, and Qing Lan contributed to preparation of the manuscript.

CONFLICT OF INTEREST

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

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Abstract

Urinary mutagenicity reflects systemic exposure to complex mixtures of genotoxic/carcinogenic agents and is linked to tumor development. Coal combustion emissions (CCE) and diesel engine exhaust (DEE) are associated with cancers of the lung and other sites, but their influence on urinary mutagenicity is unclear. We investigated associations between exposure to CCE or DEE and urinary mutagenicity. In two separate cross-sectional studies of non-smokers, organic extracts of urine were evaluated for mutagenicity levels using strain YG1041 in the *Salmonella* (Ames) mutagenicity assay. First, we compared levels among 10 female bituminous (smoky) coal users from Laibin, Xuanwei, China, and 10 female anthracite (smokeless) coal users. We estimated exposure-response relationships using indoor air concentrations of two carcinogens in CCE relevant to lung cancer, 5-methylchrysene (5MC) and benzo[*a*]pyrene (B[*a*]P). Second, we compared levels among 20 highly exposed male diesel factory workers and 15 unexposed male controls; we evaluated exposure-response relationships using elemental carbon (EC) as a DEE-surrogate. Age-adjusted linear regression was used to estimate associations. Laibin smoky coal users had significantly higher average urinary mutagenicity levels compared to smokeless coal users (28.4±14.0 SD vs. 0.9±2.8 SD rev/ml-eq, $p=2 \times 10^{-5}$) and a significant exposure-response relationship with 5MC ($p=7 \times 10^{-4}$). DEE-exposed workers had significantly higher urinary mutagenicity levels compared to unexposed controls (13.0±10.1 SD vs. 5.6±4.4 SD rev/ml-eq, $p=0.02$) and a significant exposure-response relationship with EC (p-trend$=2 \times 10^{-3}$). Exposure to CCE and DEE is associated with urinary mutagenicity, suggesting systemic exposure to mutagens, potentially contributing to cancer risk and development at various sites.

Keywords

Complex mixtures; coal combustion; smoky coal; diesel exhaust; *Salmonella* mutagenicity; urinary genotoxicity biomarkers

INTRODUCTION

Indoor coal combustion emissions (CCE) and diesel engine exhaust (DEE) are known human carcinogens based largely on findings from epidemiologic and mechanistic studies of lung cancer (IARC 1989, 2012, 2014; Silverman 2018). These environmental exposures are among the most prominent non-smoking related risk factors for lung cancer (Samet et al., 2009; IARC 2014) and contribute to air pollution-associated lung carcinogenesis (IARC 2016). Furthermore, there is evidence that exposure to CCE and DEE may be related to risk of tumor development at other sites besides the lung (Sharpe et al., 1989; Kim et al., 2016; Koutros et al., 2020; Sapkota et al., 2008).

With respect to indoor CCE, we have shown that female lifetime users of bituminous (smoky) coal had substantially elevated lung cancer mortality compared to anthracite (smokeless) coal users in a cohort study conducted in the Xuanwei region of China (Barone-Adesi et al., 2012), which was further supported by evidence from parallel case-control studies (Lan et al., 2008; Kim et al., 2014; Seow et al., 2014; Vermeulen et al., 2019;

Wong et al., 2019). The smokeless coal users were largely from the southwestern region of Xuanwei near Reshui. Additionally, we found that exposure to a specific type of smoky coal sourced from geologic deposits near Laibin township of Xuanwei had a particularly strong relationship with lung cancer risk (Lan et al., 2008; Wong et al., 2019). Much of the evidence for the carcinogenicity of DEE was derived from the Diesel Exhaust in Miners Study and a study of trucking industry workers, which found significant increasing trends in lung cancer risk with increasing cumulative DEE exposure (Garshick et al., 2012; Silverman et al., 2012; Silverman 2017, 2018) and average intensity (Silverman et al., 2012; Silverman 2017, 2018).

Polycyclic aromatic hydrocarbons (PAHs) and their methyl-derivatives play an important role in CCE-associated lung cancer (IARC 2012; Vermeulen et al., 2019). Nitroarenes (nitro-PAHs) and aromatic amines appear to contribute substantially to the carcinogenicity and mutagenicity of DEE (IARC 1989, 2014). Studies conducted in Xuanwei found that residents were highly exposed to PAHs at near occupational levels in their homes from domestic coal combustion (Mumford et al., 1995; Downward et al., 2014). In a subsequent epidemiologic study, we found strong associations between a cluster of 25 PAHs and lung cancer risk in this region (Vermeulen et al., 2019). Among these PAHs, a particularly carcinogenic methyl-derivative, 5-methylchrysene (5MC) (Hecht et al., 1974), was associated with the highest risk (Vermeulen et al., 2019).

There is evidence supporting the role of PAHs in CCE-induced lung cancer. For example, we have found that this malignancy is linked to the nucleotide excision repair (NER) pathway (Shen et al., 2005), which repairs PAH-induced DNA damage; the aldo-keto reductase (AKR) pathway, which activates PAH dihydrodiols to form redox-active *o*-quinones (Lan et al., 2004); and the base-excision repair pathway, which plays a role in the generation or repair of oxidative damage (Lan et al., 2004). Additional supporting evidence comes from mutation spectra studies of lung tumors from women exposed to smoky coal emissions, notably from the patterns of *TP53* mutations (DeMarini et al., 2001; Granville *et al.*, 2003; Keohavong et al., 2005).

Urinary mutagenicity is an integrated, overall measure that reflects systemic exposure to complex mixtures of genotoxic/carcinogenic agents. Urinary mutagenicity has been linked to colorectal adenoma risk (Peters et al., 2003) and correlates with biomarkers among those exposed to a variety of pollutants, including cigarette smoke, benzidine dyes, heterocyclic amines, nitrotoluenes, and woodsmoke (Yamasaki and Ames 1977; De Meo et al., 1987; Clonfero et al., 1995; Cerná et al., 1997; DeMarini et al., 1997; Kato et al., 2004; Sabbioni et al., 2006; Shaughnessy et al., 2011; Long et al., 2014; Keir et al., 2017; Adetona et al., 2019; Wu et al., 2021).

The reported associations between exposure to coal products, DEE, and urinary mutagenicity have been inconsistent, which could be due to low exposure levels and/or limited exposure variation. Although Mielzynska and Snit (1992) found an association with coal, other studies did not (Moller and Dybing 1980; Recio et al., 1984). We have found high concentrations of the PAH metabolite 9-hydroxybenzo[*a*]pyrene, a known mutagen (Lubet et al., 1979), in urine samples collected from smoky coal-exposed subjects (Mumford

et al., 1995); however, these samples were not assessed for mutagenicity. Furthermore, although DEE is highly mutagenic (IARC 2014), two studies of individuals occupationally exposed to DEE did not detect relationships with urinary mutagenicity (Williems et al., 1989; Schenker et al., 1992).

To explore further, we performed two separate air monitoring studies for relevant pollutants and determined urinary mutagenicity levels among non-smokers with high indoor exposure to either CCE or DEE. One study was conducted among women from the general population who domestically burned either smokeless coal or a particularly carcinogenic form of smoky coal sourced from geologic deposits near Laibin township of Xuanwei, China (Laibin smoky coal). We examined exposure-response relationships with two prominent carcinogenic PAH-constituents of CCE, namely 5MC and benzo[*a*]pyrene (B[*a*]P). We measured 5MC because of *a priori* evidence for its association with lung cancer from our previous study conducted in Xuanwei (Vermeulen et al., 2019), and B[*a*]P was analyzed because it is a known human carcinogen that is used as a surrogate for other PAHs (IARC 2010a). The second study was among men who worked in a diesel truck engine-testing facility in China, where we examined an exposure-response relationship with elemental carbon (EC), a well-recognized surrogate for exposure to DEE (IARC 2014).

MATERIALS AND METHODS

Indoor coal burning study

We analyzed a subset of non-smoking women who domestically burned particularly carcinogenic Laibin smoky coal or smokeless coal from a community-based cross-sectional study conducted in Xuanwei, China, in August-September 1993. We investigated these women because we found that smoky coal sourced from geologic deposits near Laibin township were associated with a high risk of lung cancer, even greater than smoky coal from other regions of Xuanwei (Lan et al., 2008; Wong et al., 2019).

Briefly, the subjects of the parent community-based cross-sectional study were administered questionnaires and 120 participants aged 45-60 years were classified into four groups: (1) 30 women from Chengguan or Laibin who burned smoky coal in their homes without chimneys, (2) 30 women from Chengguan or Laibin who burned smoky coal with chimneys, (3) 30 women from Reshui who burned smokeless coal without chimneys, and (4) 30 women from Yilihe who used electricity. We excluded those with (a) unknown demographic, occupational, smoking, reproductive, and dietary information; (b) known occupational exposure to toxicants; (c) serious infectious diseases such as AIDS, hepatitis, or active tuberculosis; and (d) treatment with chemotherapy, radiation, or other medications that could affect PAH levels in blood or urine. In the current study, we analyzed 10 Laibin smoky coal users without chimneys who had the highest possible indoor air pollution exposure levels and 10 smokeless coal users, all of whom had urine samples with sufficient volume for the urinary mutagenicity assay. This study was conducted in accordance with the World Medical Association Declaration of Helsinki's recommendations for human subject protection. The research protocol was approved by a United States Environmental Protection Agency Human Subjects Research Review Official.

Detailed exposure assessments have been performed in previous studies of subjects exposed to indoor CCE (Mumford et al., 1993; Mumford et al., 1995; Downward et al., 2014; Hu et al., 2014; Wong et al., 2017). Here, we collected indoor area air samples from the participants' homes for 3 days, 24 h/day, at a flow rate of 4 L/min. There were four indoor air samplers per study site and one for quality assurance. Air filter samples were collected and placed in desiccators for 24 h, weighed, and stored in polyethylene bags. PAHs (ng/m^3) from air samples were identified using gas chromatograph connected to a mass spectrometer (GC/MS) as described by Wong et al. (2017). Urine samples (~75 ml) were collected twice a day (in the morning after the subject cooked breakfast and in the evening before dinner) from the participants on the same days as the air sampling and stored at below −10°C until processing. We pooled the repeated urine samples for each subject to obtain sufficient volume for the urinary mutagenicity assay.

Diesel biomarker study

We assessed the influence of DEE exposure among non-smoking participants sampled from a previously described occupational study (Lan et al., 2015). Briefly, we recruited 54 male workers highly exposed to DEE in a truck engine-testing facility in China. These workers spent most of their shift in direct proximity to the engines being tested. The exposure assessment survey was conducted from October 2012 to March 2013 and included all workers in the testing facility. Repeated full-shift personal air samples for EC exposure were collected using a cyclone attached to the lapel near the breathing zone, with an aerodynamic cut-off of 2.5 μm and a flow rate of 3.5 L/min using quartz filters. EC was measured on the quartz filters using NIOSH Method 5040 (Birch 2002). Additionally, 55 unexposed male workers, frequency-matched to the exposed workers by age ±5 years and smoking status (never, former, current) were recruited from control workplaces (brewery, water treatment plant, meat packing facility, and an administrative facility) in the same local region of China, with work processes that did not involve exposure to DEE, other types of particulates, or any known or suspected genotoxic, hematotoxic, or immunotoxic chemicals. Overnight urine samples (~450 ml) were collected from the subjects following their work shift. For the current study, we analyzed a study sample of 20 non-smoking diesel engine testing workers and 15 non-smoking unexposed controls who provided urine samples with sufficient volume to conduct the mutagenicity assay.

The study was approved by Institutional Review Boards at the U.S. National Cancer Institute and the National Institute of Occupational Health and Poison Control, China Center for Disease Control. Participation was voluntary and all subjects provided written informed consent.

Urinary mutagenicity assay

Deconjugated urine contains both un-conjugated and formerly conjugated mutagens, resulting in mutagenic potencies ~2- to 15-fold higher than those of urines not deconjugated (Shaughnessy et al. 2011), increasing the ability to detect urinary mutagenicity. Thus, we performed the urinary mutagenicity assay as described by Kato et al. (2004) using enzymatic deconjugation. Briefly, 50 ml of urine were thawed, filtered through 0.25-μm filters, and de-conjugated enzymatically in 0.2-M (10% v/v) sodium acetate buffer (pH 5.0) (Sigma, St.

Louis, MO) containing β-glucuronidase (6 U/ml urine; Cat. No. G-7017, Sigma, St. Louis, MO) and sulphatase (2 U/ml urine, Cat. No. S-9751, Sigma, St. Louis, MO) for 16 h at 37°C. The urine was then poured through a C-18 silica-gel column (Waters Corp, Milford, MA), and the organics were eluted from the column with 10 ml of methanol, which was then solvent-exchanged into dimethyl sulfoxide (DMSO) to produce an organic concentrate at 150X; concentrates were stored at 4°C until used for bioassays. Methanol/C-18 blanks were also prepared and tested for mutagenicity.

We evaluated the extracts in the *Salmonella* (Ames) plate-incorporation mutagenicity assay (Maron and Ames 1983) with strain YG1041, which is a frameshift strain derived from strain TA98 that over-expresses both *O*-acetyltransferase and nitro-reductase, enhancing the detection of mutagenic aromatic amines, nitroarenes, and PAHs (Hagiwara et al., 1993), all of which are present in CCE and DEE. The extracts were tested at 0.3, 0.6, 1.2, 3, 6, and 12 ml-equivalents (ml-eq) of urine per plate at one plate per dose. Duplicate experiments were performed for all 35 DEE samples, but for only 19 of the 20 CCE samples due to limited amounts of urine. All experiments were performed with metabolic activation (S9) mix made from Aroclor-induced, Sprague-Dawley rat-liver S9 (Moltox, Boone, NC). Plates were incubated at 37°C for 3 days, after which the colonies were counted on an automatic colony counter (ProtoCOL 3, Synbiosis, Frederick, MD). We used DMSO at 100 μl/plate as the negative control, along with 2-nitrofluorene in DMSO at 3 μg/plate in the absence of S9 and 2-aminioanthracene in DMSO at 0.5 μg/plate in the presence of S9 as positive controls.

We used Prism (GraphPad, San Diego, CA) to construct mutagenicity dose-response curves using data from either single experiments or else replicate experiments. Linear regressions over the linear portion of the dose-response curves were used to determine the mutagenic potency, which was the slope of the curve expressed as rev/ml-eq. The linear portion of the curve was defined by the initial doses that gave the highest r^2-value. A dose-related response of an experimental sample that approached or exceeded a 2-fold increase in rev/plate relative to the DMSO control and that had a p-value ≤ 0.05 based on a trend test (Prism, GraphPad, San Diego, CA) was considered a mutagenic response. Samples that did not achieve these requirements were assigned mutagenic potencies of zero. The archived urines from the CCE study were collected 27 years ago and were not assessed for creatinine concentrations. In the DEE study, urinary creatinine concentrations were only available for post-shift samples, but not the overnight samples used for the mutagenicity assay. As such, direct comparisons between the CCE and DEE studies could be made based only on rev/ml-eq.

To assess reproducibility between replicate experiments, intraclass correlations (ICC) were calculated separately for the CCE and DEE studies using linear mixed models with revertant counts as the dependent variable, replicate experiment (i.e., time 1 and 2) as the independent variable, along with random intercepts for study subject and dose (ml-eq/plate). The estimated ICC for the CCE and DEE studies were 84% and 92%, respectively.

Statistical analyses

In the CCE study, unadjusted and age-adjusted linear regression models were used to assess associations between type of coal used (Laibin Smoky vs. Smokeless) and continuous urinary mutagenicity levels. We considered education as a covariate; however, its inclusion

did not change the findings >10%, and a parsimonious model was chosen. Additionally, we considered exposure to secondhand smoke as a covariate, but it lacked statistical variation and was not included in the models. We also evaluated exposure-response relationships between 5MC and B[*a*]P from indoor area air samples and urinary mutagenicity using single pollutant models. Additional sensitivity analysis was conducted using a bi-pollutant model. Spearman correlations were estimated among the PAHs. B[*a*]P and 5MC were specified *a priori* for analysis based on previous findings (IARC 2010a; Vermeulen et al., 2019).

In the DEE study, unadjusted and age-adjusted linear regression models were used to assess associations between diesel exposure (DEE-exposed workers vs. unexposed controls) and continuous urinary mutagenicity levels (rev/ml-eq). We also analyzed exposure-response relationships using the unexposed controls and personal EC measurements from the DEE-exposed workers. EC was analyzed as: 1) geometric means ($\mu g/m^3$) corrected for background levels among the unexposed measured during initial walkthrough surveys of control workplaces, 2) tertiles as specified previously (Lan et al., 2015): low (6.1–39.0 $\mu g/m^3$), medium (39.1–54.5 $\mu g/m^3$), and high (54.6–107.7 $\mu g/m^3$), and 3) categories based on the new European Union (EU) occupational exposure limit (OEL) of 50 $\mu g/m^3$: (<25 $\mu g/m^3$), (25 - <50 $\mu g/m^3$), and (≥50 $\mu g/m^3$). We considered body mass index, work years, and packyears of past smoking as potential covariates; however, these factors were not associated with urine mutagenicity in our analyses and were not included in the final models. Additionally, we conducted sensitivity analyses restricted to subjects exposed to EC levels below the new OEL of 50 $\mu g/m^3$ to determine if exposure-response trends could be detected among those with lower DEE exposure. All analyses were conducted using SAS v9.4, and a two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Indoor coal combustion emissions

The demographic characteristics are shown in Table 1. Laibin smoky coal users were slightly more advanced in age (47.9±5.4 SD vs. 45.4±4.1 SD years) and had a higher proportion of illiteracy (80% vs. 50%) than smokeless coal users. However, there was no considerable difference in exposure to secondhand smoke.

Urinary mutagenicity levels by coal use, along with categories of 5MC and B[*a*]P, are shown in Table 2. We found that Laibin smoky coal users were exposed to high concentrations of B[*a*]P (Median (IQR)=134.3 (99.7-152.4) ng/m^3) and 5MC (Median (IQR)=71.3 (54.8-96.3) ng/m^3) in household air, whereas smokeless coal users had relatively lower exposure to B[*a*]P (Median (IQR)=37.0 (28.4-46.4) ng/m^3) and 5MC (Median (IQR)=4.5 (3.2-6.2) SD ng/m^3). Those who domestically burned carcinogenic Laibin smoky coal had strikingly higher urinary mutagenicity compared with smokeless coal users: 28.4±14.0 SD vs. 0.9±2.8 SD rev/ml-eq; $\beta_{unadjusted} = 26.6$ (95%CI: 18.1-37.0, $p=9 \times 10^{-6}$); $\beta_{age\text{-}adjusted} = 28.3$ (95%CI: 18.3-38.4, $p=2 \times 10^{-5}$) (Figure 1A).

There was a significant exposure-response relationship between 5MC and urinary mutagenicity in the single pollutant model: $\beta_{age\text{-}adjusted} = 0.2$ (95%CI: 0.1-0.3, $p=7 \times 10^{-4}$), along with a suggestive association with B[*a*]P (Figure 1A and 1B). In the bi-pollutant

model with 5MC and B[*a*]P, the effect of 5MC remained significant: $\beta_{age\text{-}adjusted} = 0.2$ (95%CI: 0.1-0.3, $p=5 \times 10^{-3}$). Furthermore, 5MC and B[*a*]P concentrations were modestly correlated (Spearman *rho*=0.36).

Diesel engine exhaust

The demographic and anthropometric characteristics are shown in Table 3. DEE-exposed subjects were more advanced in age (43.2±6.5 SD vs. 39.4±8.8 SD years), worked more years (18.7±7.9 vs. 12.8±9.1 years), and had a higher proportion of never-smokers (45% vs. 33%) than unexposed controls.

Urinary mutagenicity levels by categories of DEE are shown in Table 4. We found that DEE-exposed factory workers had significantly higher urinary mutagenicity levels compared to unexposed controls: 13.0±10.1 SD vs. 5.6±4.4 SD rev/ml-eq; $\beta_{unadjusted} = 7.4$ (95%CI:1.7-13.1, $p=0.01$); $\beta_{age\text{-}adjusted} = 7.2$ (95%CI:1.2-13.2, $p=0.02$) (Figure 2A). Moreover, we found a significant DEE exposure-response relationship with urinary mutagenicity, which further supported the association: mean urinary mutagenicity levels for unexposed subjects were 5.6±4.4 SD, $EC_{tertile\text{-}1}$=6.7±4.8 SD, $EC_{tertile\text{-}2}$=15.5±9.4 SD, and $EC_{tertile\text{-}3}$=19.6±12.9 SD rev/ml-eq (Figure 2B); $p\text{-trend}_{age\text{-}adjusted}=2 \times 10^{-3}$ (Figure 2C). Subjects who were exposed to 25 - <50 μg/m^3 of EC, which was below the new EU OEL of 50 μg/m^3, had significantly higher urinary mutagenicity compared to the unexposed (p=0.03, Figure 3A). Further, positive exposure-response trends between EC and urinary mutagenicity ($p_{slope\ age\text{-}adjusted} = 0.01$) were detected among subjects exposed to EC concentrations below the new EU OEL (Figure 3B).

DISCUSSION

Urinary mutagenicity reflects systemic exposure to complex mixtures of genotoxic agents or probable carcinogens (DeMarini et al., 1997; Kato et al., 2004; Sabbioni et al., 2006; Wu et al., 2021; Peters et al., 2004). Further, urinary mutagenicity has been linked to tumor development (Peters et al., 2003) and urinary metabolites of carcinogenic PAHs were found to be associated with lung and gastric cancer risk (Yuan et al., 2014; Liao et al., 2014) in epidemiologic studies. Our findings show, for the first time, strong evidence for systemic exposure to mutagens among people exposed to CCE or DEE, as well as significant associations between the levels of urinary mutagenicity and the concentrations of relevant marker pollutants in the air for CCE or DEE. Exposure to CCE and DEE has been found to be associated with lung cancer risk as well as development at malignancies of other sites (Kim et al., 2016; Koutros et al., 2020; IARC 2014; IARC 2010c). Indoor CCE has been associated with renal cell carcinoma (kidney cancer) (Sharpe et al., 1989) and esophageal cancer (Pan et al., 1999), and associated non-significantly with cervical (Wu et al., 2004) and salivary gland cancer (Zheng et al., 1996). DEE has been associated with cancers of the bladder (Koutros et al., 2020), colon/colorectum (Siemiatycki et al., 1988), prostate (Seidler et al., 1998), larynx (Elci et al., 2003), and non-Hodgkin lymphoma (Karunanyake et al., 2008), and suggestively associated with kidney cancer (Peters et al., 2018) and leukemia (IARC 2014). Our findings provide further evidence that systemic exposure to mutagens

from CCE or DEE could be involved in the etiologic mechanisms of lung cancer as well as tumors at other sites.

Indoor coal combustion emissions

This is the first report showing that non-smoking women who domestically burned a particularly carcinogenic form of smoky coal sourced from geologic deposits near Laibin township of Xuanwei, China, had significantly elevated urinary mutagenicity levels compared to those who used less toxic smokeless coal. We found positive exposure-response relationships for the carcinogenic 5MC constituent of Laibin smoky coal. 5MC had higher and more consistent mutagenic potency than B[*a*]P in our analyses and has been found to be highly carcinogenic (IARC 2010a), even compared to other PAH species (Hecht et al., 1974).

Several human studies have examined the relationship between coal products and urinary mutagenicity, most of which were conducted among coke oven workers. De Méo et al. (1987) found that among non-smokers, steel workers exposed to coke oven emissions had higher urinary mutagenicity compared to unexposed controls. This finding was further supported by another study of non-smokers (Simioli et al., 2004), along with a repeated-measures study that found that highly exposed coke oven workers had higher overall urinary mutagenicity levels compared to those with lower exposure over the work week (Chao et al., 2008). Although Recio et al. (1984) found no clear evidence among non-smokers that urinary mutagenicity was higher among U.S. coal liquefaction pilot plant workers compared with controls, they did find differences by smoking status. Our investigation expands upon the findings from these occupational studies by examining subjects from a general population who had indoor air pollution exposure from domestic coal combustion at levels comparable to those of occupational studies.

Our findings of associations between urinary mutagenicity and the concentrations of carcinogenic PAHs in the air among CCE-exposed women are concordant with studies of other exposures in which a variety of genotoxicity biomarkers were associated with concentrations of PAHs in the air (DeMarini 2013). 5MC has been evaluated by IARC (2010a) as a Group 2B (possible) human carcinogen, and B[*a*]P is a well-studied Group 1 (known) human carcinogen (IARC 2010a). Although some studies for urinary mutagenicity reported null or equivocal findings (Venier et al., 1985; Clonfero et al., 1995; Mielyńska et al., 1997), others found positive associations between urinary mutagenicity and PAH exposure, such as in bus drivers (Hansen et al., 2004), coke oven workers (Simioli et al., 2004; Siwińska et al., 2004), rubber manufacturers (Peters et al., 2008), policeman (Ledda et al., 2018), and municipal firefighters (Keir et al., 2017).

Women in our study with CCE exposure had the highest increase in urinary mutagenicity relative to controls (31.5-fold) compared with subjects exposed to other types of agents (Table 5). Although their level of urinary mutagenicity was approximately an order-of-magnitude lower than that of subjects exposed to benzidine, charcoal production emissions, or nitrotoluenes (Table 5), women using smoky coal in Xuanwei, China, have an extremely elevated risk for lung cancer due to the emissions from smoky coal (IARC 2010a). Because urinary mutagenicity has been linked to increased colorectal cancer risk (Peter et al., 2003),

the systemic exposure to mutagens, including mutagenic/carcinogenic PAHs, by women in this study suggests that exposure to CCE might pose risks for cancers to additional organs (Kim et al., 2016; IARC 2010c; Sharpe et al., 1989; Pan et al., 1999) along with the lung.

Diesel engine exhaust

Our results contribute evidence to an inconclusive body of literature on urinary mutagenicity from DEE-exposed subjects, showing that male DEE-exposed workers had significantly higher (3.5-fold) levels of urinary mutagenicity compared to unexposed controls (Table 5). Further, we observed a positive exposure-response relationship between EC and urinary mutagenicity. Previous human studies have shown that exposure to DEE or atmospheres containing DEE induces a range of genotoxicity biomarkers, including DNA adducts, DNA damage, *HPRT* mutations, and micronuclei (DeMarini 2013; IARC 2014). However, relative to controls, increased levels of urinary mutagenicity were not found in occupational studies of diesel-exposed car mechanics (Willems et al., 1989), railroad workers (Schenker et al., 1992), or road tanker drivers (Nylander and Berg 1991). These null findings may have been due to limited sample size, low diesel exposure levels, and/or limited exposure variation. In contrast, our study of diesel engine testing workers was conducted in a high-exposure workplace, and the controls were from workplaces determined empirically to have negligible DEE exposure based on walkthrough surveys (Lan et al., 2015), which likely improved the likelihood of detecting effects. DEE levels in our study were considerably higher than those found in a study of U.S. trucking industry workers (Davis et al., 2006, 2007; Smith et al., 2006; Garshick et al. 2008).

In addition to lung cancer, bladder cancer has also been positively associated with DEE exposure (IARC 2014; Koutros et al., 2020). The most plausible mechanism is that nitroarenes (nitro-PAHs), which account for much of the mutagenic activity of diesel exhaust (DeMarini et al., 2004), are metabolized to aromatic amines (IARC 2014), which are well-known bladder carcinogens (IARC 2010b). DEE induced a significant 3.5-fold increase in urinary mutagenicity relative to controls (Table 5), although the level (19.6 rev/ml-eq) was more than an order-of-magnitude lower than that of subjects exposed to benzidine (496 rev/ml-eq), a known bladder carcinogen (IARC 2010b). However, we used *Salmonella* strain YG1041, which we have shown to be the most sensitive of all the *Salmonella* strains for detecting the mutagenicity of organic extracts of diesel exhaust particles (Mutlu et al., 2015a,b). This increased sensitivity is because YG1041 over-expresses both nitroreductase, which metabolizes nitroarenes to aromatic amines, as well as *O*-acetyltransferase, which metabolizes activated aromatic amines (S9 was used in the mutagenicity assay) to acetylamines, which can bind covalently to DNA to form mutagenic DNA adducts (Hagiwara et al., 1993). Our finding of elevated levels of urinary mutagenicity among subjects exposed to DEE supports the association between exposure to DEE and bladder cancer (IARC 2014; Koutros et al., 2020).

The United States (US) has not set a permissible exposure limit for diesel exhaust for general industry; however, the US Mine Safety and Health Administration (MSHA) currently enforces diesel particulate matter standards at underground metal/nonmetal mines and at underground coal mines. In the US, a miner's personal exposure must not exceed

an 8-h time-weighted average of 160 μg/m^3 of total carbon (TC; EC conversion factors range from 1.19-1.44 (MSHA 2001; Noll et al., 2015). The EU adopted an OEL of 50 μg/m^3 for EC in December 2018, which will take effect in 2026 for underground mines and tunnel construction and in 2023 for other industries (Directive 2019). There has been concern that this new EU OEL for EC leaves a high residual risk of DEE-related cancers. Even at EC concentrations below 50 μg/m^3, we detected a marked biological effect on urinary mutagenicity in our study. As such, the need for lower OELs for EC may be justified to protect workers' health. In 2001, the American Conference of Governmental Industrial Hygienists (ACGIH) proposed a recommended threshold limit value of 20 μg/m^3 of EC. Although ACGIH recommendations are not legal standards, a number of regulatory authorities consider these recommendations when drafting legislation. In July 2020, the Netherlands adopted an even lower OEL for EC of 10 μg/m^3, which is the most stringent in Europe (Diesel Engine Exhaust 2019).

Strengths and limitations of studies

Our study had notable strengths. First, our study subjects were exposed to high levels of CCE or DEE, which increased the likelihood of detecting effects. Further, we were able to estimate a DEE exposure-response using EC measurements from personal air samples, which are correlated with diesel particulate matter (Noll et al., 2007). Second, the study subjects were current non-smokers, which reduced potential confounding by smoking. Current active smoking has been found to influence short-term urinary mutagenicity levels (Yamasaki and Ames 1977; Recio et al., 1984; De Meo et al., 1987; Kawano et al., 1987; Clonfero et al., 1995). Third, we used a well-established method to detect urinary mutagenicity, which improved the comparability of our results to other similar studies.

Our study had some limitations. First, our sample size was small; however, the statistical power to detect effects was improved by analyzing subjects with very high versus low exposure levels. Second, we had limited information on potential confounders and did not have the sample size to accommodate more covariates. However, we assessed the influence of some potentially influential variables, *e.g.*, body mass index, work years, and packyears of past smoking in the diesel study, and these factors had little effect on the findings. Third, our lack of creatinine measurements precluded the expression of urinary mutagenicity data in terms of rev/creatinine concentration, which prevented comparisons of our data to a wider literature. However, expression of urinary mutagenicity as rev/ml-eq is sufficient to conclude if subjects have mutagenic urine or not, which was the key issue addressed in our study.

Diesel exhaust and coal emissions are composed of both organic and inorganic compounds. Although inorganics such as inorganic carbon nanoparticles might contribute to the carcinogenicity of both diesel exhaust (IARC 1989, 2014) and coal emissions (IARC 2010C, 2012), inorganic carbon would not have contributed to the urinary mutagenicity because we evaluated organic extracts of the urine, which did not contain inorganic carbon.

Conclusions

In summary, we found that exposure to indoor smoky coal combustion emissions or diesel engine exhaust can result in systemic exposure to mutagens. Further confirmation of these

results with larger sample sizes, measurement of additional exposure biomarkers, and comprehensive identification of chemical constituents of air samples would improve our understanding of the potential risk that these exposures present to organs other than the lung. The established carcinogenicity to the lung of coal combustion emissions (IARC 2012) and diesel engine exhaust (IARC 2014) may extend to other organs given the observed elevated urinary mutagenicity caused by these exposures. Indeed, coal combustion emissions and diesel engine exhaust have been linked to risk of cancer of several sites in addition to lung (Sharpe et al., 1989; Pan et al., 1999; Kim et al., 2016; Koutros et al., 2020; IARC 2014; IARC 2010c), and our findings suggest that systemic exposure to mutagens could be potentially involved in carcinogenesis at these sites.

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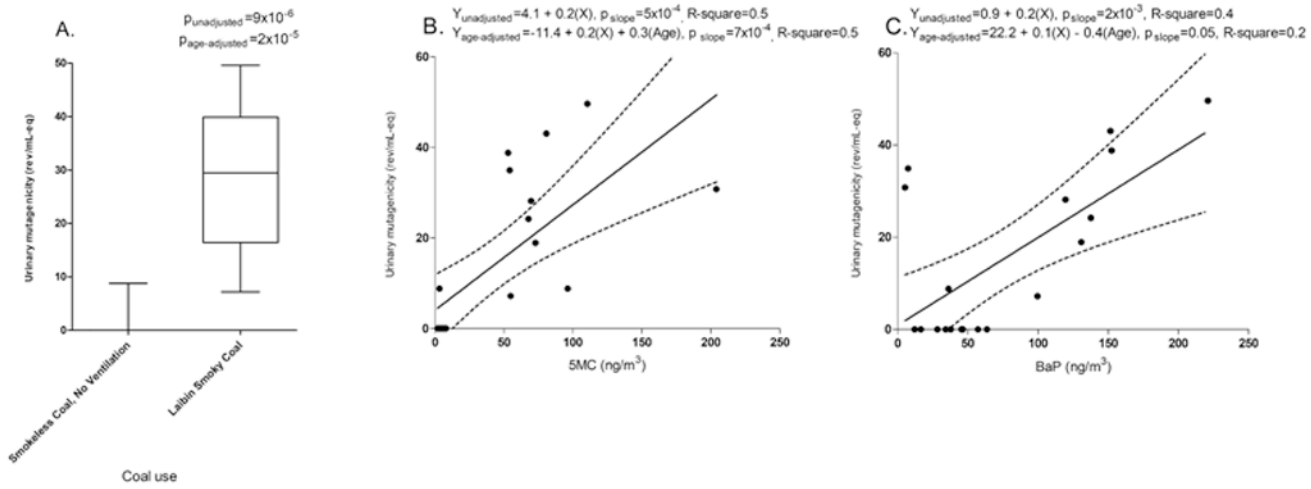

Figure 1.

(Panel A) Urinary mutagenicity levels among female non-smokers who used particularly carcinogenic Laibin smoky coal or smokeless coal in the 1993 community-based cross-sectional study in Xuanwei, China. Differences between exposure groups were tested with unadjusted and age-adjusted linear regression models. Exposure-response relationships between urinary mutagenicity and air 5-methylchrysene (5MC) (Panel B) and benzo[*a*]pyrene (B[*a*]P) (Panel C). Associations were tested with unadjusted and age-adjusted linear regression models.

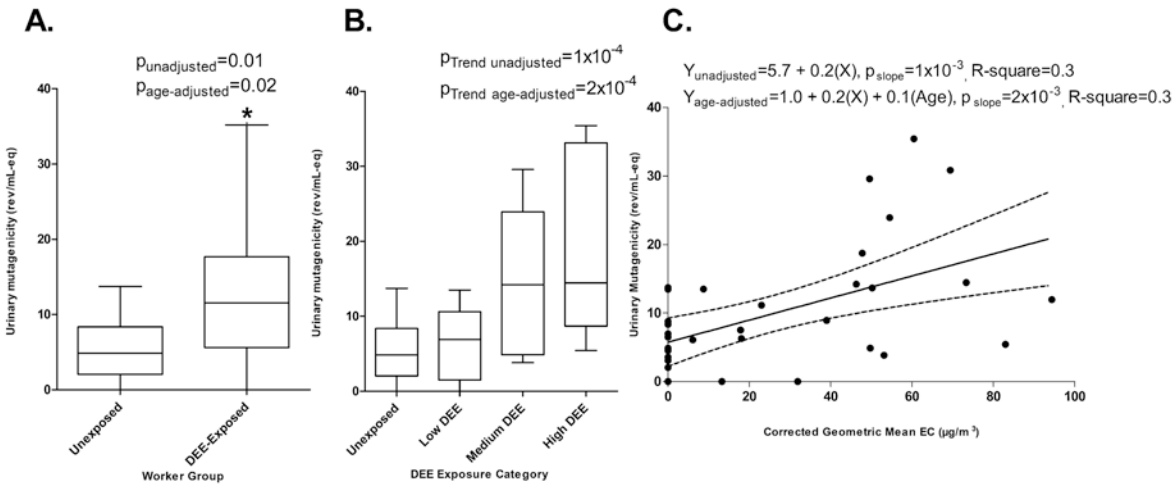

Figure 2.

(Panel A) Urinary mutagenicity levels among male engine testing factory workers exposed to diesel engine exhaust and unexposed controls. Differences between exposure groups were tested with unadjusted and age-adjusted linear regression models. (Panel B) DEE exposure was categorized as tertiles by EC concentrations: low (6.1–39.0 µg/m^3), medium (39.1–54.5 µg/m^3), and high (54.6–107.7 µg/m^3). (Panel C) DEE exposure as reflected by EC concentrations was analyzed continuously as geometric mean (µg/m^3) corrected for background levels among the controls.

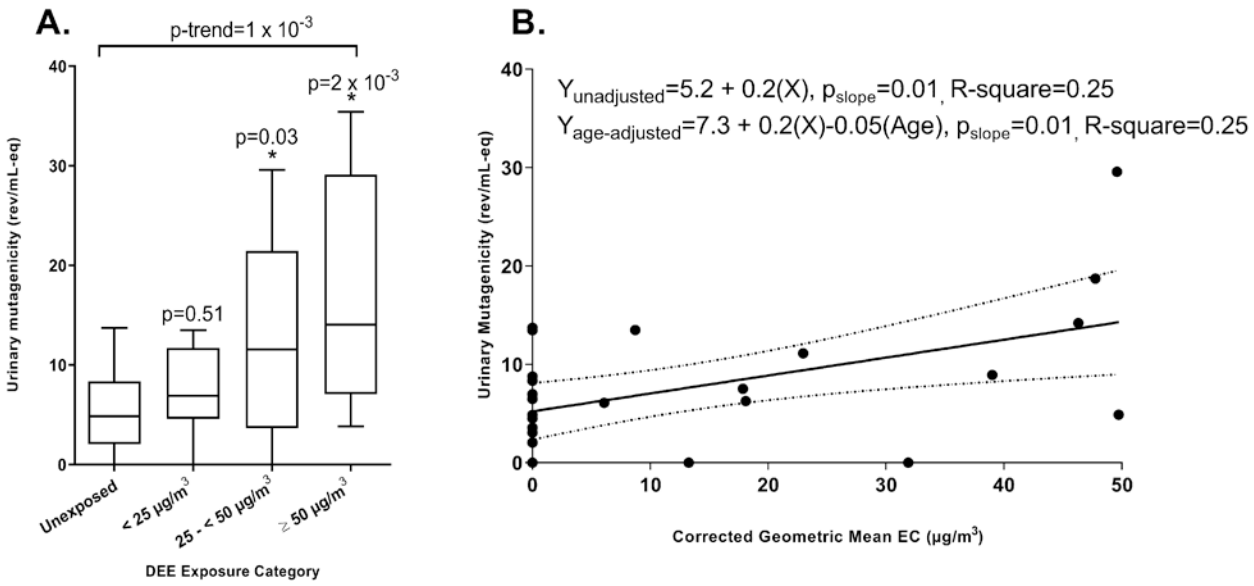

Figure 3.

(Panel A) Associations between exposure to elemental carbon (EC) and urinary mutagenicity using EC categories defined by the new European Union occupational exposure limit of <50 µg/m^3: unexposed controls (n = 15), <25 µg/m^3 (n = 6), 25 - <50 µg/m^3 (n = 6), and ≥50 µg/m^3 (n = 8). (Panel B) Associations between exposure to elemental carbon (EC) and urinary mutagenicity among subjects below the new European Union occupational exposure limit of <50 µg/m^3 of EC. EC concentrations were analyzed continuously as geometric mean (µg/m^3) corrected for background levels among the controls.

TABLE 1

Characteristics of subjects from the cross-sectional indoor coal burning study

Characteristic	Laibin smoky coal users (*n* = 10)	Smokeless coal users (*n* = 10)
Age, years, mean (SD)	47.9 (5.4)	45.4 (4.1)
Education[a], *n*, %		
Illiterate	8 (80%)	5 (50%)
Elementary	2 (20%)	4 (40%)
Exposed to ≥1 person who smoked at home, *n, %*	9 (90%)	10 (100%)
Smoking, *n*, %		
Current	0	0
Former	0	0
Never	10 (100%)	10 (100%)
Pack-years, mean (SD)	No data	No data

[a]One smokeless coal user had missing education data.

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TABLE 2

Urinary biomarkers in subjects from the cross-sectional indoor coal burning study

Biomarker	Comparison Group	Laibin smoky coal user exposure category		
	Smokeless coal users[¥] n=10	Overall Laibin smoky coal users n=10	Low PAH subgroup 5MC <71.3 ng/m^3 B[*a*]P <134.3 ng/m^3 n=5	High PAH subgroup 5MC ≥71.3 ng/m^3 B[*a*]P ≥134.3 ng/m^3 n=5
Mutagenicity, mean rev/ml-eq (SD)	0.9 (2.8) [¥]	28.4 (14.0)		
Difference %, compared to smokeless coal	Reference	3055.6		
Wilcoxon *p*-value	Reference	1.7 x 10^{-4}		
5MC				
Urinary mutagenicity, mean rev/ml-eq (SD)	0.9 (2.8) [¥]		26.7 (12.3)	30.2 (16.8)
Difference %, compared to smokeless coal	Reference		2866.7	3255.6
Wilcoxon *p*-value	Reference		1.2 x 10^{-3}	1.2 x 10^{-3}
B[*a*]P				
Urinary mutagenicity, mean rev/ml-eq (SD)	0.9 (2.8) [¥]		24.0 (11.1)	32.9 (16.4)
Difference %, compared to smokeless coal	Reference		2566.7	3555.6
Wilcoxon *p*-value	Reference		1.2 x 10^{-3}	1.2 x 10^{-3}

$p < 0.05$ was considered statistically significant.

[¥]A common reference/comparison group of smokeless coal users was used for the three separate analyses of: 1) Laibin smoky coal users, 2) 5MC concentrations, and 3) B[a]P concentrations.

TABLE 3

Characteristics of subjects from the cross-sectional diesel biomarker study

Characteristic	DEE-Exposed (*n* = 20)	Unexposed Controls (*n* = 15)
Age, years, mean (SD)	43.2 (6.5)	39.4 (8.8)
Body mass index, kg/m^2 (SD)	25.4 (3.8)	25.4 (4.0)
Work years, mean (SD)	18.7 (7.9)	12.8 (9.1)
Geometric mean of elemental carbon (EC) exposure among individuals, corrected for background levels, μg/m^3, mean (SD)	44.5 (24.9)	Not applicable
Smoking, *n*, %		
Current	0	0
Former	11 (55%)	10 (67%)
Never	9 (45%)	5 (33%)
Pack-years, mean (SD)	5.2 (8.3)	3.3 (10.6)

Abbreviations: diesel engine exhaust (DEE).

TABLE 4

Urinary mutagenicity levels among subjects from the cross-sectional diesel biomarker study

Measurements	Comparison Group	DEE Exposure Category			
	Unexposed Controls (n=15)	Overall DEE-Exposed (n=20)	Low DEE subgroup 6.1-39.0 µg/m^3 (n=8)	Medium DEE subgroup 39.1-54.5 µg/m^3 (n=7)	High DEE subgroup 54.6-107.7 µg/m^3 (n=5)
Mutagenicity, mean rev/ml-eq (SD)	5.6 (4.4)	13.0 (10.1)	6.7 (4.8)	15.5 (9.4)	19.6 (12.9)
Difference %, compared to controls	Reference	132.1	19.6	176.8	250
Wilcoxon *p*-value	Reference	0.02	0.58	0.01	0.01

Exposure tertile categories are based on elemental carbon (EC) concentrations from Lan et al. (2015). Abbreviations: diesel engine exhaust (DEE).

TABLE 5

Comparisons of urinary mutagenicity from various exposures[a]

Exposure	Mean rev/ml-eq	Fold increase	Reference
Smoky coal combustion emissions	24.8	31.5	Present study
Diesel engine exhaust	19.6	3.5	Present study
Benzidine	496.0	20.7	DeMarini et al. (1997)
Charcoal production emissions	276.0	2.3	Kato et al. (2004)
Nitrotoluenes	127.0	Not reported	Sabbioni et al. (2006)
Wildfire	11.0	2.6	Wu et al. (2021)

[a]Other than for benzidine, all other data were generated in *Salmonella* frameshift strain YG1041, which over-expresses both *O*-acetyltransferase and nitroreductase. The benzidine data were generated in the related strain YG1024, which over-expresses only *O*-acetyltransferase. Data for benzidine were extrapolated from published dose-response curves, and data for charcoal emissions were estimated from published data.