Exposure to heavy metals and polycyclic aromatic hydrocarbons and DNA damage in Taiwanese traffic conductors

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Running title: Metals, Polycyclic aromatic hydrocarbons, and DNA damage

Keywords: Heavy metals, Polycyclic aromatic hydrocarbons, Oxidative stress

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1 figure and 3 tables

39 references
Abstract

Background: Exposure to traffic-related air pollutants, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals, has been associated with the etiology and prognosis of many illnesses. However, the specific causal agents and underlying mechanisms for different health outcomes remain unclear. The aims of this study were to assess the relations between urinary biomarkers of exposure to PAHs (1-hydroxypyrene-glucuronide, 1-OHPG) and heavy metals (cadmium, Cd; nickel, Ni; arsenic, As; lead, Pb; copper, Cu) and their interaction effect on DNA damage (8-oxo-7,8-dihydro-guanine, 8-oxodG).

Methods: We recruited 91 traffic conductors and 53 indoor office workers between May 2009 and June 2011 in Taipei, Taiwan. Post-shift urine samples from 2 consecutive days were analyzed for 1-OHPG, Cd, Ni, As, Pb, Cu and 8-oxodG. To estimate the effects from PAHs and metals on DNA damage, we constructed a linear mixed model adjusted for confounding variables.

Results: We found that urinary 1-OHPG and Cd levels were independent predictors of urinary 8-oxodG levels ($\beta=0.112$, $p=0.015$ for 1-OHPG; $\beta=0.138$, $p=0.031$ for urinary Cd). The joint effect of urinary 1-OHPG and Cd levels was associated with urinary 8-oxodG levels ($p=0.001$).

Conclusions: Co-exposure to environmental PAHs and Cd could cause oxidative DNA damage.
damage.

Impact: These findings suggest that the additive interaction between exposure to environmental PAHs and Cd could enhance the burden of oxidative stress.
Introduction

In urban environments across the globe, air pollution from traffic exhaust has been considered an important public health issue. Although there is accumulating epidemiologic evidence that exposure to traffic-related air pollutants plays a role in the etiology and prognosis of many illnesses, including cardiovascular disease and cancer, the role of specific causal agents and the underlying mechanisms for different health outcomes remain unknown (1). Among these pollutants, polycyclic aromatic hydrocarbons (PAHs) and heavy metals adsorbed in the pores and surfaces of the particles could easily penetrate into the respiratory tract and reach the circulatory system (2).

Previous studies have reported that exposure to PAHs or metals could generate reactive oxidative species (ROS) to induce oxidative stress in vitro or vivo (3, 4). Oxidative stress could play an important role in the initiation and promotion of carcinogenesis. However, few studies have explored the effect of co-exposure to PAHs and metals on oxidative stress in humans (5, 6).

Applying biomarkers of exposure and outcome could be useful both in the quantification of the association and in providing insight into the specific causal agents and underlying biological mechanism. A major product with a clear mutagenic potential, deoxyguanosine (dG) in urine has been commonly used as a biomarker of
oxidative stress in studies on ambient air pollution (7-10). Conversely, urinary
1-hydroxypyrene-glucuronide (1-OHPG), a PAH metabolite, has been used as a
biomarker of exposure to PAHs (9, 11, 12). Urinary metals, including cadmium (Cd),
arsonic (As), nickel (Ni), lead (Pb) and copper (Cu), indicate short-term or long-term
levels of internal dose of exposure to metals (13). Most studies showed that exposure
to either PAHs or metals could increase levels of urinary 8-oxodG in occupational or
environmental fields (7-9, 14). Only a few studies have investigated their interaction
effect on biomarkers of oxidative stress, but a joint effect between PAHs and metals
was not found (5, 6).

Therefore, we carried out a study of traffic conductors to examine the relation
between urinary biomarkers of PAHs and heavy metals and their interaction effect of
DNA damage.
Materials and methods

Study population. We recruited 91 traffic conductors as the exposed group and 53 indoor office workers as the reference group, between April 2009 and June 2011, in Taipei, Taiwan. The group members were 20 to 63 years of age. All participants were free of cancer and pulmonary disease, and they had all been working in their current position for at least 3 mo.

Data collection. The participants underwent health examinations and completed a self-administered questionnaire on demographic information, lifestyle habits, and history of previous and current diseases. Post-shift urine was collected from participants on 2 consecutive days. This study was approved by the Institutional Review Board of the National Health Research Institutes in Taiwan, and written informed consent from participants was obtained prior to study enrollment.

Analysis of urine samples for 1-OHPG. The urine samples were collected within half an hour of the end of a work shift. We divided the urine samples into several small volume aliquot and stored them at -80 °C in a freezer until analysis. Urinary 1-OHPG was measured using the assay developed by Strickland et al. (1994) (15). Urine samples (2 ml) were treated with 0.1 N HCl (90°C) to hydrolyse acid-labile metabolites. The hydrolysed samples were loaded onto Sep-Pak C18
cartridges (Waters) and washed with methanol (30% in water). The relatively non-polar metabolites were eluted with methanol (80% in water; 4ml) and the volume was reduced to 0.5 ml by a centrifugal and vacuum evaporator (Eyela CVE-100, Tokyo, Japan). The concentrated samples were diluted to 4ml with 15mM phosphate buffered saline (PBS). Immunoaffinity columns were prepared using poly-prep columns (0.8×4 cm) filled with CNBr-activated Sepharose 4B (0.8 ml) coupled with monoclonal antibody 8E11, which recognizes several PAH-DNA adducts and metabolites. Monoclonal antibody 8E11 was obtained from Trevigen, Inc; Gaithersburg, MD, USA. It was originally produced against benzo[a]pyrene-diolepoxy-modifed DNA, and has been shown to recognize 1-OHPG. After washing the columns two times with 4ml of 15mM PBS, samples in phosphate-buffered saline were loaded on columns and bound material was eluted with 2ml of 40% methanol. Eluted fractions were quantified by synchronous fluorescence spectroscopy (SFS) using a Perkin-Elmer LS 50B luminescence spectrometer. The excitation-emission monochromators were driven synchronously with a wavelength difference of 34nm. 1-OHPG, purchased from National Cancer Institute (NCI) Chemical Carcinogen Repository (MRI, Kansas City, MO, USA), produces a characteristic fluorescence emission maximum at 381nm (347nm excitation). Fluorescence intensity was used to quantify 1-OHPG. The recovery of the
assay was 91%. The coefficient of variation of the assay was less than 5% during sample analysis. The limit of detection was 0.05 ng/ml.

**Analysis of urine samples for 8-oxodG** Urinary 8-oxodG concentrations were measured using liquid chromatography/MS/MS, as described elsewhere (16). Briefly, 20 µL of urine was diluted 10-fold with 5% methanol containing 0.1% formic acid. After the addition of 40 µL of $^{15}$N$_5$-8-oxodG solution (20 µg/L in 5% methanol/ 0.1% formic acid) as internal standard, a 100 µL of prepared urine sample was directly injected into the on-line solid-phase extraction LC-MS/MS. After automatic sample cleanup, LC-MS/MS analysis was done using a PE Series 200 HPLC system interfaced with a PE Sciex API 3000 triple quadrupole mass spectrometer with electrospray ion source. The samples were analyzed in the positive ion multiple reaction monitoring mode, and the transitions of the precursors to the product ions were as follows: m/z 284→168 (quantifier ion) and 284→140 (qualifier ion) for 8-oxodG, and m/z 289→173 (quantifier ion) and 289→145 (qualifier ion) for $^{15}$N$_5$-8-oxodG. A detection limit of 5.7 ng/L was obtained using 7 repeated analyses of deionized water. The coefficients of variation in interday and intraday tests were <5%. Mean recovery of 8-oxodG in urine was 99%–102%. Analyses of field blanks found no significant contamination. Each participant’s urinary 1-OHPG and 8-oxodG concentrations were normalized to urine creatinine.
**Analysis of urinary metals.** Urinary metal concentration was analyzed by ICP-MS (Agilent 7700X series ICP-MS, Agilent Technologies, Inc., Palo Alto, CA, USA), as previously described (17). ICP-MS was used to determine the Cd, Ni, As, Pb, and Cu concentrations in the urine. Briefly, the frozen urine samples were first moved to a refrigerator and stored at 4 °C for several hours, and then thawed at room temperature. Two milliliters of thawed urine were diluted 15 times with 1% HNO₃ diluent containing 1 μg/L yttrium (Y) internal standard in the final sample dilution, then mixed well, and finally aspirated from plastic containers into the ICP-MS. Standard solutions of 0.1, 0.5, 1, 2, 5, 10, 20, and 50 μg/L were used for sample quantification. The spike recoveries using 5 μg/L urinary matrix were: Cd, 94.1±2.1; Ni, 100.5±1.4; As, 99.7±4.6; Pb, 99.3±4.8; Cu, 98.5±3.0. The method detection limits were: Cd, 0.075; Ni, 0.022; As, 0.184; Pb, 0.095; Cu, 0.023 μg/L. The outcome figures below the limit of detection (LOD) were set to LOD/(square root of 2).

**Statistical methods.** The levels of urinary metals, urinary 1-OHPG, and urinary 8-oxodG were compared between the exposed and reference groups by mixed-model repeat measure analysis (Proc mixed). This model was used to investigate the relations between urinary metals, urinary 1-OHPG, and 8-oxodG after adjusting for fixed covariates (such as age, sex, education level, smoking habit, season of data collection, and exposure status). These models treated the participants as random
effects, and model selections were based on Akaike’s Information Criterion. The compound symmetry and variance components were constructed as the covariance structures. The dependent variables were transformed by the natural logarithm (Ln). Residual and influence analyses were conducted.

We also assessed the interaction effect from exposure to PAHs and metals on urinary 8-oxodG in nonsmoking workers. We categorized the exposures as greater or less than the 50th percentile among nonsmoking workers. We compared the geometric mean (GM) levels of urinary 8-oxodG according to the combination of these exposures in the model. A two-sided $p$-value $< 0.05$ was considered statistically significant. All statistical analyses were performed using SAS (version 9.1.3; SAS Institute Inc., Carry, NC, USA.).

**Results**

**Study population.** Certain characteristics of the exposed and reference groups of the study population were compared (Table 1). The mean age was 49.2 y (SD 9.17) in the exposed group and 42.9 y (SD 8.86) in the reference group. The exposed group had a lower percentage of women and a lower education level than the reference group. The most common season for data collection was spring for the exposed group, but spring and winter for the reference group. The distributions of lifestyle factors, such as
smoking habits, alcohol consumption habits, and cooking habits, were similar
between the exposed and reference groups.

Comparison of urinary heavy metals, 1-OHPG and 8-oxodG levels between
the exposed and reference groups. Regardless of whether the workers were
nonsmoking or smoking, the geometric mean levels of urinary Ni, Cu, 1-OHPG and
8-oxodG among the exposed group were significantly higher than those among the
reference group (Table 2). No differences of geometric mean levels of urinary Cd, As
and Pb were observed between the exposed and reference groups.

Relations between biomarkers. Table 3 presents the relations of urinary heavy metals
and 1-OHPG levels with urinary 8-oxodG levels. After controlling for the fixed
covariates, we found that the urinary Cd and 1-OHPG levels were positively
associated with urinary 8-oxodG levels in all workers and nonsmoking workers. Due
to small sample size in smokers, further analysis was limited. We also examined this
association using data separately for each day (Supplemental Table A). The
conclusion is the same with similar beta and p values.

For further analysis of the interaction of exposure to both PAHs and Cd, we
grouped participants according to the median levels of urinary 1-OHPG and Cd
among nonsmoking workers. Workers with high urinary 1-OHPG and high urinary Cd
levels had on average 60.3% higher levels of urinary 8-oxodG than did those with low
urinary 1-OHPG and low urinary Cd levels in the adjusted model. Workers with high urinary 1-OHPG and low urinary Cd levels and workers with low urinary 1-OHPG and high urinary Cd levels had on average 25.4% and 36.3% higher levels of urinary 8-oxodG, respectively, than workers with low urinary 1-OHPG and low urinary Cd levels. The *p*-value for the interaction term of both exposures (*p*=0.001) is consistent with an additive interaction between PAHs and Cd on urinary 8-oxodG levels. Figure 1 shows GM and 95% CI of adjusted urinary 8-oxodG levels by urinary 1-OHPG and urinary Cd concentrations.

**Discussion**

To our understanding, this is the first study to indicate that levels of urinary 1-OHPG and Cd had significant associations with levels of urinary 8-oxodG. With increasing levels of urinary 1-OHPG and Cd, elevated levels of 8-oxodG in urine were observed. These results provide evidence that co-exposure to PAHs and Cd could increase levels of oxidative stress.

Concentrations of urinary Cd could be regarded as a well-recognized biomarker of chronic Cd exposure (18). Cd elicits oxidative stress by inducing the generation of ROS, reducing the antioxidant defense systems of cells by depleting glutathione and decreasing the activities of cellular antioxidant enzymes, leading to mitochondria...
injuries and increasing the susceptibility of cells to oxidative attack by altering the
membrane integrity and fatty acid composition (18, 19). Consequently, it is plausible
that the toxic effect from Cd can be partially responsible for the impaired balance
between oxidants and antioxidants. The strong association observed between urinary
Cd levels and oxidative stress markers in the third U.S. National Health and Nutrition
Survey suggests that Cd could increase the burden of oxidative stress (20). Bae et al.
(2010) also reported that exposure to Cd from air pollution was associated with
oxidative stress in schoolchildren (5). However, the lack of correlation between
exposure to these metals, including nickel, arsenic, lead and copper, and oxidative
stress could be attributed to a low biologically relevant dose in the study population.
Although there were the consistent negative associations between other metals (Ni, As
and Pb) and urinary 8oxodG, these negative associations might be generated by
statistical random variations.

We found no differences in urinary Cd levels between the traffic conductors
and indoor offices workers. Although air pollution from traffic exhausts was
identified as a possible source of Cd (21), contribution from diets cannot be ruled out.
Dietary intake is one of important source of exposure to Cd in the general
nonsmoking population, especially in consumption of some foods such as rice, other
crops, and shellfish (22-25). The urinary Cd concentrations in our study population
were higher than those reported in the third U.S. National Health and Nutrition Survey (geometric mean: 0.37 μg/g creatinine) (20) and similar to those reported for the Japanese population (geometric mean 0.8 μg/g creatinine) (26). Overall, most participants (99%) had urinary Cd levels of <5 μg/g creatinine, the World Health Organization (WHO) standard for urinary Cd (27).

The urinary 1-OHPG levels in traffic conductors were significantly higher than those in indoor office workers, which were similar to the findings in toll station workers (9), bus drivers (7), and police officers (28). These results suggest that traffic sources have been recognized as major contributors to PAHs and have an effect on urinary 1-OHPG levels. Conversely, the urinary 1-OHPG concentrations in traffic conductors (range: 0.08-0.11 μmol/mol) were lower than those reported in toll station workers (range: 0.12-0.16 μmol/mol) (9), painters in shipyards (range: 0.99-1.4 μmol/mol) (12) and incinerator workers (mean: 0.24 μmol/mol) (11), indicating a moderate exposure to PAHs in the present study. The main pathways of metabolic activation of PAHs are the formation of anti- and syn-diol epoxides, the formation of radical cations, and the formation of o-quinones (3). The radical cations can generate ROS and cause oxidative stress by redox cycling (29, 30). Epidemiological studies found that exposure to PAHs from air pollution could be positively associated with urinary 8-OHdG levels (9, 31, 32).
Urinary Cd levels and urinary 1-OHPG levels as independent predictors of urinary 8-OHdG levels indicate the hypothesis that Cd and PAHs could induce oxidative stress through different mechanisms. Environmentally relevant pollutants seldom occur alone. Little is known on the exact mechanism of carcinogenesis of 2 or more pollutants when they are present together. Our results support the assumption that the concept of additivity is operative on moderate or low-level exposures to chemical mixtures. Valavanidis et al. (2005) demonstrated that transition metals, redox cycling quinoids and PAHs act synergically to produce ROS, to increase the burden of oxidative stress (33). Cd inhibits several enzymes involved in DNA repair, and this has been identified as a major mechanism underlying the carcinogenic potential of Cd (19, 34). Thus, the DNA damage might be induced by oxidative stress from exposure to Cd and/or PAHs, and further enhanced by impaired repair from one or both. In this way, the interaction effect was observed though the concentrations were moderate. Future studies are required to clarify these findings.

The urinary excretion of products of damaged nucleotides in cellular nucleotide pools or in DNA may be important biomarkers of exposure to relevant carcinogens and may predict cancer risk. Urinary 8-oxodG in steady state reflects products of DNA damage and repair (35), and could also be regarded as biomarkers of oxidative stress in occupational or environmental fields (6-10). Our results also showed that
traffic conductors could have higher levels of urinary 8-oxodG than indoor office workers, which were in agreement with previous studies (7, 9). Air pollution from traffic exhaust could increase levels of oxidative DNA damage. Other factors, such as cell death and diets, might contribute to increase urinary 8-oxodG levels. However, the evidence was limited (36, 37). Although genetic background (such as DNA repair capability) from each participant could confound the estimated effect, the present study of two measurements on the consecutive days could minimize these effects.

An early prospective study had reported that elevated levels of urinary 8-oxodG were significantly associated with increasing risk of lung cancer among never-smokers (38). Recently, a nested case-control study of lung cancer and diesel exhaust indicated that workers in highly polluted cities over a lifetime had at least a 50% increased lung cancer risk (39). Exposures to PAHs and Cd are known as risk factors of many illnesses. Therefore, traffic conductors in urban environments could be more susceptible to injury and disease caused by exposure to PAHs and metals.

In summary, our results indicate that co-exposure to urinary 1-OHPG and Cd could increase the levels of urinary 8-oxodG, suggesting that the additive interaction between exposure to environmental PAHs and Cd could enhance the burden of oxidative stress.

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References


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<th>Variables</th>
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<th>Reference group (N=53)</th>
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<sup>a</sup> χ² test

<sup>b</sup> Student’s t test

<sup>c</sup> Fisher’s exact test
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<th>Biomarkers (µg/g creatinine)</th>
<th>All participants</th>
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<th>Reference group</th>
<th>p-value</th>
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### Table 2. (cont.)

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<td>3.84 (3.35-4.40)</td>
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aAbbreviations: number of observations (No)
bMixed model was used to test for differences between the exposed and reference groups.
Table 3. Relations of urinary metal and 1-OHPG levels with urinary 8-oxodG levels

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ln urinary 8-oxodG (µg/g creatinine)</th>
<th>β</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1 (all workers) a</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln Cd</td>
<td>0.129</td>
<td>0.026</td>
<td>to 0.231</td>
<td>0.014</td>
</tr>
<tr>
<td>Ln Ni</td>
<td>-0.063</td>
<td>-0.126</td>
<td>to 0.006</td>
<td>0.060</td>
</tr>
<tr>
<td>Ln As</td>
<td>-0.018</td>
<td>-0.105</td>
<td>to 0.069</td>
<td>0.676</td>
</tr>
<tr>
<td>Ln Pb</td>
<td>-0.022</td>
<td>-0.070</td>
<td>to 0.026</td>
<td>0.362</td>
</tr>
<tr>
<td>Ln Cu</td>
<td>0.003</td>
<td>-0.098</td>
<td>to 0.103</td>
<td>0.956</td>
</tr>
<tr>
<td>Ln urinary 1-OHPG (µg/g creatinine)</td>
<td>0.095</td>
<td>0.018</td>
<td>to 0.173</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Model 2 (nonsmoking workers) b</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ln Cd</td>
<td>0.138</td>
<td>0.013</td>
<td>to 0.264</td>
<td>0.031</td>
</tr>
<tr>
<td>Ln Ni</td>
<td>-0.068</td>
<td>-0.142</td>
<td>to 0.006</td>
<td>0.072</td>
</tr>
<tr>
<td>Ln As</td>
<td>-0.019</td>
<td>-0.119</td>
<td>to 0.081</td>
<td>0.707</td>
</tr>
<tr>
<td>Ln Pb</td>
<td>-0.024</td>
<td>-0.083</td>
<td>to 0.035</td>
<td>0.424</td>
</tr>
<tr>
<td>Ln Cu</td>
<td>-0.005</td>
<td>-0.124</td>
<td>to 0.115</td>
<td>0.939</td>
</tr>
<tr>
<td>Ln urinary 1-OHPG (µg/g creatinine)</td>
<td>0.112</td>
<td>0.023</td>
<td>to 0.202</td>
<td>0.015</td>
</tr>
</tbody>
</table>

a Adjusted for age, sex, smoking habit, season, educational level and group.
b Adjusted for age, sex, season, educational level and group.
Figure legends

**Figure 1.** Interaction effects of urinary 1-OHPG and Cd levels with urinary 8-oxodG levels in nonsmoking workers. Value showed was geometric mean. Cut points were determined according to medians (1-OHPG, 0.23 μg/g creatinine; Cd, 0.88 μg/g creatinine) of urinary creatinine-adjusted levels among nonsmoking workers. (*P <0.05, **P <0.01, ***P <0.001)
Figure 1.

Geometric mean and 95% CI of adjusted urinary 8-oxodG (ug/g creatinine)

- low 1-OHPG / low Cd
- low 1-OHPG / high Cd
- high 1-OHPG / low Cd
- high 1-OHPG / high Cd

Values:
- 2.01
- 2.52
- 2.74
- 3.22
Cancer Epidemiology, Biomarkers & Prevention

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Han-Bin Huang, Guan-Wen Chen, Chien-Jen Wang, et al.

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