

Urinary Excretion of an Oxidative Stress Marker, 8-hydroxyguanine (8-OH-Gua), among Nickel-cadmium Battery Workers

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Abstract: Urinary Excretion of an Oxidative Stress Marker, 8-hydroxyguanine (8-OH-Gua), among Nickel-cadmium Battery Workers: Noriyuki YOSHIOKA, et al. Department of Preventive Medicine and Public Health, School of Medicine, Keio University—The relationship between oxidative stress and carcinogenic metals including nickel and cadmium is a matter of interest. To assess the oxidative stress status of workers exposed to nickel and cadmium simultaneously, we determined urinary excretion of 8-hydroxyguanine (8-OH-Gua), a urinary oxidative stress marker. Our subjects were 66 (64 males and 2 females) nickel-cadmium battery workers. Spot urine and blood samples were collected. The levels of cadmium in blood (Cd-B) and nickel in urine (Ni-U) were determined by graphite furnace atomic absorption spectrophotometry. 8-OH-Gua in urine was analyzed using a high performance liquid chromatography-electrochemical detector (HPLC-ECD) system. Data on age, sex, duration of present work and smoking status were also obtained from each subject. Creatinine-adjusted 8-OH-Gua was significantly correlated with age, Ni-U and Cd-B in univariate analysis, while multivariate analysis revealed that Ni-U and Cd-B were significant independent variables and that these two biological exposure indices were positively correlated with 8-OH-Gua. The data were also analyzed in the context of mixture toxicity. The subjects were divided into groups based on median level of Ni-U and Cd-B (2.86 µg/g creatinine and 0.23 µg/dl, respectively). Workers with high Ni-U/high Cd-

B (Group IV) had the highest levels of 8-OH-Gua levels (GM (GSD), 21.7(2.0)), followed by those with high Ni-U/low Cd-B (11.5(1.6) Group III), those with low Ni-U/high Cd-B (8.9(1.9) Group II), and those with low Ni-U/low Cd-B (8.5(1.5) Group I). The *p* values of Students' *t*-tests between Group I and Group II, III and IV were 0.847, 0.050 and <0.001, respectively. The combined effect of Cd and Ni on the urinary excretion of 8-OH-Gua departed from additivity. (*J Occup Health 2008; 50: 229–235*)

Key words: 8-hydroxyguanine, Oxidative stress, Nickel, Cadmium, Mixture toxicity

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The relationship between oxidative stress and carcinogenic elements, such as Cr, As, Ni and Cd is a matter of interest both in occupational settings and with respect to the environment. Among carcinogenic metals, reactive oxygen species (ROS) are assumed to be involved in Ni carcinogenicity¹. On the other hand, Cd is thought to impair DNA damage repair, especially base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR)². BER includes multiple-step enzymatic reactions, in which incision of the modified base is followed by cleavage of the apurinic/apyrimidinic site and nucleotide insertion and ligation. Although the effect of Cd on the initial steps of BER has been extensively studied, inhibition in the later steps has not yet been fully investigated². Cd, however, is also believed to induce ROS by mechanisms other than a Fenton-type reaction³.

Both 8-hydroxyguanine (8-OH-Gua) and 8-hydroxy-2'-deoxyguanosine (8-OH-dG) are urinary markers of oxidative stress, however, they have somewhat different biological meanings. Oxidized guanine residue in DNA is eliminated through BER initiated by glycosylase, from which urinary 8-OH-Gua is derived⁴. NER also plays a

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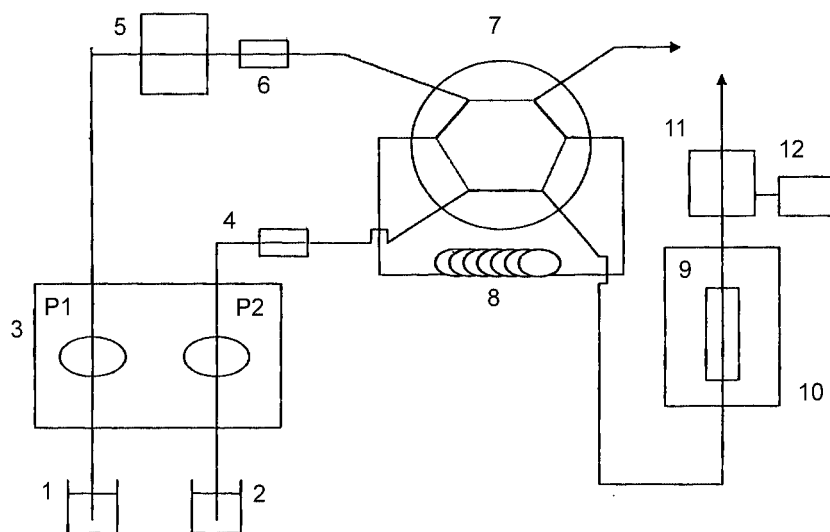


Fig. 1. Schematic diagram of the HPLC-ECD system. 1: pretreatment eluent (2.5% acetonitrile and 1.5% methanol in 10 mM borate buffer at pH 7.5); 2: analytical eluent (0.5% methanol and 10 mg/l EDTA- Na_2 in 50 mM KH_2PO_4 at pH 5.8); 3: pump 1 (0.6 ml/min) and pump 2 (0.7 ml/min); 4: guard cell; 5: auto sample injector; 6: pretreatment column; 7: six-port switching valve; 8: sample loop (100 μl); 9: analytical column; 10: analytical column oven; 11: electrochemical detector (150 mV (electrode 1) and 300 mV (electrode 2) and 100 nA as full-range deflection); 12: data processor.

role in the elimination of oxidized bases in that it contributes to the excretion of 8-OH-dG in urine, and another source of 8-OH-dG is the nucleotide pool⁴). While many studies have been performed on 8-OH-dG, the number of researchers who have examined 8-OH-Gua is limited^{5,6}). Recently, however, Svoboda *et al.* examined urinary excretion of 8-OH-Gua and 8-OH-dG using a high performance liquid chromatography-electrochemical detector (HPLC-ECD) system⁴). They collected urine samples from various animals including humans, and their results suggest that 8-OH-Gua is a better marker than 8-OH-dG for predicting the life span of species. They also showed that 8-OH-Gua is more abundant than 8-OH-dG in urine.

Following Svoboda *et al.*⁴) we determined 8-OH-Gua levels in Ni-Cd battery workers using an HPLC-ECD system featuring a reverse phase column, in order to elucidate the status of oxidative stress levels in workers who are exposed to cadmium and nickel simultaneously. The Ni-Cd battery plant offers a unique opportunity in that workers are simultaneously exposed to both Ni and Cd. Thus, the present results are also discussed in terms of mixture toxicity.

Materials and Methods

Study population

The present study population consisted of 64 males and 2 females working in a nickel-cadmium battery plant.

Information on age and duration of present work was obtained from each participant. The subjects were also asked about their daily use of tobacco and current smoking status (yes or no) was evaluated for each participant. Urine and blood were collected during working hours (Monday through Friday, 8:30 a.m. to 5:30 p.m.). Urine samples were maintained at a temperature of -80°C until analysis. The recruitment of the participants and the collection of blood and urine samples were carried out in 2000. All measurements were completed by April of 2001.

Determination of 8-OH-Gua

1) Reagents

Potassium dihydrogen phosphate was purchased from Merck Ltd., Japan (Tokyo, Japan). For the standard solution, 8-OH-Gua (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in water and prepared at a concentration of 10 $\mu\text{g}/\text{ml}$, which was then diluted by 1 M Tris-HCl buffer (pH 7.5) to obtain the standard solution. All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan): ethanol (for HPLC), methanol (for HPLC), acetonitrile (for HPLC), Tris (hydroxymethyl) amino methane, ethylene diamine tetraacetic acid (EDTA)- Na_2 and sodium tetraborate.

2) Urine sample preparation

The urine samples were separated in a centrifugal

Table 1. Characteristics of the study population

Age (yr)	40.4 ± 9.3
Years working	8.8 ± 7.6
Smoking status	
Yes	35
No	29
Number of cigarettes/day	8.7 ± 9.2
Ni-U (µg/g creatinine)	4.05 ± 4.77
Cd-B (µg/dl)	0.35 ± 0.26

Representative values of numerical variables are shown as mean ± SD.

separator operated for 5 minutes at 5,000 rpm, and the supernatant was diluted 4-fold using 1 M Tris-HCl buffer at pH 7.5 of which 20 µl was applied to the HPLC-ECD system.

3) Apparatus

The application of the HPLC-ECD system was based on Loft *et al.*^{7,8)}. The analytical system was composed of the following: two metering pumps, pump 1 (L-6200) and pump 2 (L-6000), a guard cell (Model 5020; ESA Biosciences, Inc., Chelmsford, MA, USA), an autosampler (AS-4000), a six-port switching valve (655-0730), an air compressor (SPS-8), a column oven (L-5025), an electrochemical detector (Coulochrome II; ESA Biosciences) and a data processor (D-2500) (Fig. 1). For the analytical column, a TSKgel ODS-80Ts (4.6 mm × 250 mm, Tosoh Co., Tokyo, Japan) was used with TSKprecolumn PW (4.6 mm × 35 mm, Tosoh) as the pretreatment column. The pretreatment column allows the passage of hydrophilic components such as proteins first and hydrophobically retains substances of lower molecular weight in the small pores. All devices for which a company name is not listed above were obtained from Hitachi High-Technologies Co. (Tokyo, Japan).

4) Analytical conditions

Automatic time control of the six-port valve switching was carried out following the method devised by Hashimoto *et al.*⁹⁾ The samples were carried to the pretreatment column by the solvent arranged for pretreatment. The valve was switched 1.9 min after injection of the sample and the target component was conveyed from the sample loop to the analytical column using the solvent for analysis and separation.

Determination of Ni-U, Cd-B and urinary creatinine excretion

Cadmium in blood (Cd-B) and nickel in urine (Ni-U) were determined by graphite furnace atomic absorption spectrophotometry¹⁰⁾. The concentration of creatinine in

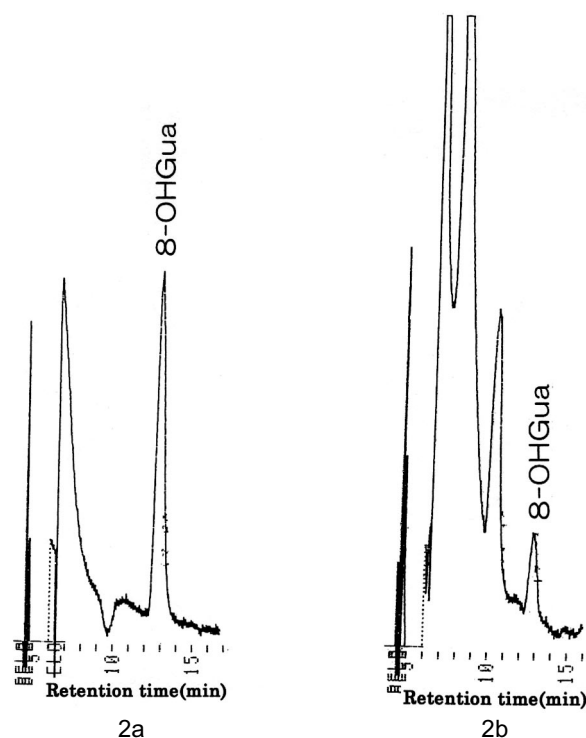


Fig. 2. Examples of chromatograms. Chromatogram of 25 ng/ml standard solution (Fig. 2a) and that of a human sample (Fig. 2b).

urine was determined by a commercial clinical chemistry laboratory test (SRL, Inc., Tachikawa, Tokyo, Japan). The values of 8-OH-Gua and Ni-U were divided by the value of creatinine to obtain creatinine-adjusted 8-OH-Gua and Ni-U.

Statistical analysis

The value of 8-OH-Gua was log-transformed to obtain normality. SPSS 14.0J (SPSS Japan Inc., Tokyo, Japan) was used for statistical analysis. All statistical analyses were performed using a two-tailed test. A value of $p < 0.05$ was considered to be statistically significant.

Results

The study population included only two females. Since differences by sex have been reported for 8-OH-dG, another urinary oxidative stress marker¹¹⁾, these 2 subjects were excluded from the present analysis. The characteristics of the present study population are shown in Table 1. The level of 8-OH-Gua ranged from 6.0 to 48.0 ng/ml, and 8-OH-Gua values adjusted for creatinine ranged from 3.3 to 53.3 µg/g creatinine. Examples of the chromatograms are shown in Fig. 2. Univariate analysis revealed that age, Ni-U and Cd-B were correlated with 8-OH-Gua level (Table 2). Step-wise multiple regression analysis was performed with age, current

Table 2. Results of univariate and multivariate analyses

	Univariate analysis	Multivariate analysis
Age	Standardized coefficient =0.260 ($p=0.038$) Simple regression analysis	Not adapted
Years working	Not significant Simple regression analysis	
Smoking (yes or no)	Not significant Student's <i>t</i> -test	Not adapted
Daily tobacco consumption (/day)	Not significant Simple regression analysis	
Ni-U ($\mu\text{g/g}$ creatinine)	Standardized coefficient=0.440 ($p<0.001$) Simple regression analysis	Standardized coefficient=0.421 ($p<0.001$)
Cd-B ($\mu\text{g/dl}$)	Standardized coefficient=0.557 ($p<0.001$) Simple regression analysis	Standardized coefficient=0.543 ($p<0.001$)

Multiple regression analysis with years working and daily consumption of tobacco instead of age and current smoking status generated similar results.

smoking status (yes or no), Ni-U and Cd-B as possible independent variables, and Ni-U and Cd-B were adopted (Table 2). Multiple regression analysis with years working and daily consumption of tobacco instead of age and current smoking status generated similar results (data not shown).

In order to analyze the combined effect of Ni and Cd, the subjects were divided into four groups based on their median values of Ni-U and Cd-B (2.86 $\mu\text{g/g}$ creatinine and 0.23 $\mu\text{g/dl}$, respectively): Group I (low Ni-U/low Cd-B), Group II (low Ni-U/high Cd-B), Group III (high Ni-U/low Cd-B) and Group IV (high Ni-U/high Cd-B)¹²(Fig. 3a). Group IV had the highest 8-OH-Gua levels at 21.7(2.0) $\mu\text{g/g}$ creatinine (GM (GSD)), followed by group III (11.5(1.6)), Group II (8.9(1.9)), and Group I (8.5(1.5)) (Fig. 3b). The *p* values of Students' *t*-tests between Group I and Group II, III and IV were 0.847, 0.050 and <0.001 , respectively. The difference in 8-OH-Gua levels between Groups IV and I (13.2 $\mu\text{g/g}$ creatinine) was greater than the summation of the differences of Groups III and I (3.0 $\mu\text{g/g}$ creatinine), and those of Groups II and I (0.4 $\mu\text{g/g}$ creatinine). The differences between the Ni-U level of Groups I and II, and between those of Groups III and IV were not statistically significant ($p=0.314$, *t*-test and $p=0.239$, U-test, respectively), nor were the differences between Groups I and III, and between Groups II and IV for Cd-B ($p=0.842$, *t*-test and $p=0.244$, U-test, respectively). To further examine the interaction of Ni-U and Cd-B, multiple linear regression analysis was carried out as modified by Cronbach¹³ in order to reduce multicollinearity. The following formula was applied: $y = b_0 + b_1x_1 + b_2x_2 + b_3x_1x_2$, where x_1 is a newly designated independent variable calculated by subtracting the mean Ni-U concentration from the individual Ni-U value, and x_2 is similarly calculated for Cd-B. While both b_1 and b_2

were statistically significant ($p<0.001$), the coefficient of the interaction term was found to be insignificant ($p=0.182$).

Discussion

Group I, the low Ni-U/low Cd-B group, showed a level of metals similar to that of the general population^{14, 15}. In a study by Svoboda *et al.*⁴), human urine samples were taken from people not occupationally exposed to Ni and Cd, and their 8-OH-Gua level of 11 ± 2.4 $\mu\text{g/g}$ creatinine is comparable to that of our Group I, which validates the present methodology.

There are several limitations to the present study. First, we asked about the participants' smoking habits, but did not ask about their habitual ethanol intake. Intake levels of antioxidants such as vitamin A, C or E can also be confounding factors. Another stress marker, 8-OH-dG, has been studied well with respect to the effect of those possible confounders. Although habitual alcohol consumption is reported to induce the elevation of urinary 8-OH-dG levels¹⁶), Yoshida *et al.*¹⁷) saw a reduction in excretion level among drinkers who consumed alcohol the day before sample collection. Additionally, studies on anti-oxidant vitamins or carotenoids have sometimes failed to observe the relationship¹⁸⁻²⁰). Future studies on the effect of Ni and Cd on 8-OH-Gua should take into consideration the possible confounding factors listed above.

Second, we did not take urine and blood samples at the end of the workshift. Usually, sampling for biological monitoring is performed at the end of workshift for possible cumulative or "carry-over" effects. The biological half-time of urinary Ni excretion was estimated in a study of four welders and four electroplaters²¹) to range from 17 to 39 h, while a very long whole-body

Group III (high Ni-U/low Cd-B) n=14 Ni-U: 5.56 ± 5.84 Cd-B: 0.17 ± 0.05	Group IV (high Ni-U/high Cd-B) n=18 Ni-U: 6.86 ± 6.17 Cd-B: 0.59 ± 0.27
Group I (low Ni-U/low Cd-B) n=20 Ni-U: 1.73 ± 0.53 Cd-B: 0.17 ± 0.05	Group II (low Ni-U/high Cd-B) n=12 Ni-U: 1.94 ± 0.59 Cd-B: 0.49 ± 0.22

Fig. 3a. Characteristics of subgroups. The subjects were divided into four groups based on the median values of Ni-U and Cd-B ($2.86 \mu\text{g/g}$ creatinine and $0.23 \mu\text{g/dl}$, respectively). Ni-U: mean \pm SD ($\mu\text{g/g}$ creatinine), Cd-B: mean \pm SD ($\mu\text{g/dl}$).

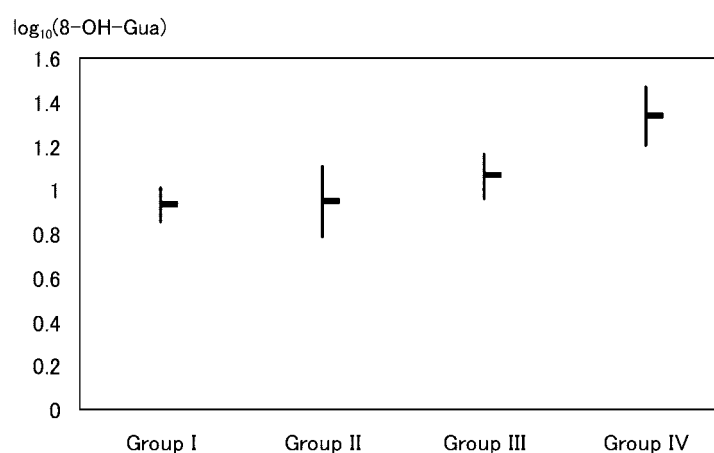


Fig. 3b. Effect of Ni-U and Cd-B on urinary excretion levels of 8-OH-Gua. The mean \log_{10} (8-OH-Gua) of each subgroup is shown with its 95% confidence interval.

biological half-time for Cd has been reported²²). In occupational settings, Ni levels are thought to become higher toward the end of the week, while those of Cd are thought to remain stable. We do not know the exact retention time between metal exposure and the emergence of 8-OH-Gua in urine. Unless Ni and Cd work antagonistically, however, 8-OH-Gua levels are theoretically expected to rise gradually toward the weekend. With respect to consecutive urinary 8-OH-dG monitoring, data on boilermaker construction workers who are exposed to residual oil fly ash and fumes from welding are available²³); their urine was monitored for Ni, Cr, Pb, Mn, Cu, vanadium and 1-hydroxypyrene, a marker of PAH (polycyclic aromatic hydrocarbon) exposure²³). Urinary Ni excretion in these workers increased toward the weekend. Although their urinary Ni excretion was comparable to that observed in the present study, that of 8-OH-dG was relatively stable from Monday through Friday especially among smokers²³). The effect of the timing of sampling might be small. However,

structured urine collection should be performed in future studies.

The urinary Ni level is reported to be a predictor of urinary 8-OH-dG excretion²³); this was also the case for urinary 8-OH-Gua excretion. The involvement of ROS is assumed for Ni carcinogenicity¹). ROS are known to injure DNA, causing single-strand breaks, DNA-protein cross-links and the modification of base residues such as the introduction of a hydroxy group into the C-8 position of a guanine residue. An increase in the urinary excretion of 8-OH-Gua can be thought to reflect an increased number of modified DNA bases for Ni exposure.

Both Cd-B and Ni-U were adopted for multiple linear regression analysis. Multiple steps of BER are known to be inhibited by Cd²). Since 8-OH-Gua in urine is believed to be derived from guanine residues into which a hydroxy group has been introduced, inhibition of DNA glycosylase can be thought to reduce urinary 8-OH-Gua levels. On the other hand, it seems implausible that inhibition in later steps affects urinary excretion. In addition to DNA

repair impairment, Cd is believed to induce ROS by mechanisms other than a Fenton-type reaction³, which can cause direct DNA damage. Thus, it is possible to conceive that an increase in the urinary excretion of 8-OH-Gua is a reflection of an increased number of modified DNA bases, although the exact mechanism involved remains unclear.

The combined effect of Cd and Ni on the urinary 8-OH-Gua excretion departed from additivity, although the subsequent multiple linear regression analysis failed to show statistical significance for the coefficient of the interaction term. Wong *et al.*¹² observed a synergistic effect of combined arsenic and chromium exposure on DNA oxidative stress among Taiwanese school children living in the vicinity of a coal power plant. The authors sought the reason for the synergism in the combination of ROS and impairment of DNA repair. It is noteworthy that diverse mechanisms of DNA damage are conceivable both in Taiwanese school children and in the workers in the present study. Additionally, Hengstler *et al.* examined DNA single-strand breaks in the cryopreserved lymphocytes of workers co-exposed to cadmium, cobalt and lead²⁴. Using the logistic regression model, they identified more than multiplicative effects of co-exposure. The concept of additivity is generally accepted in the regulation of exposure to chemical mixtures at low concentrations. Therefore, the synergism found in these studies is notable in the context of regulation of occupational and environmental settings. Further studies are required to clarify these findings.

Both cadmium and nickel were shown to be determinants of a urinary oxidative stress marker, 8-OH-Gua. In the context of mixture toxicity, the present data suggest departure from additivity, which requires further clarification in future studies.

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