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Oxidative DNA Damage Estimated by Plasma 8-hydroxydeoxyguanosine (8-OHdG): Influence of 4, 4'-methylenebis (2-chloroaniline) Exposure and Smoking

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Abstract: Oxidative DNA Damage Estimated by Plasma 8-hydroxydeoxyguanosine (8-OHdG): Influence of 4, 4'-methylenebis (2-chloroaniline) Exposure and Smoking: Hong-I CHEN, *et al.* Division of Urology, Department of Surgery, Tri-Service General Hospital, Taiwan, R.O.C—Oxidative DNA damage may play an important role in the human carcinogenic process. Recently, we reported a case of bladder cancer among 4, 4'-methylenebis (2-chloroaniline) (MBOCA)-exposed workers. By measuring the plasma level of 8-hydroxydeoxyguanosine (8-OHdG), we investigated the association between oxidative DNA damage and MBOCA exposure. In addition, we examined the effects of different confounders on the plasma level of 8-OHdG. We undertook a cross-sectional survey at four MBOCA-producing factories in Taiwan (158 subjects). Plasma 8-OHdG levels and urinary MBOCA concentrations were measured by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Personal characteristics were collected by questionnaire. The workers were classified according to their job titles as exposed (n=57) or unexposed (n=101) groups as well as classified according to urinary MBOCA levels as high urinary MBOCA (>20 μg/g creatinine) (n=45) or low urinary MBOCA (n=108) groups. Neither the MBOCA-exposed workers nor the high urinary MBOCA workers had a significant increase in the mean plasma 8-OHdG level, even after adjustment for potential confounders. Age and gender were significantly positively correlated with plasma 8-OHdG levels. Smokers among high urinary MBOCA workers also had significantly higher 8-OHdG levels than non-smokers among high urinary MBOCA workers. Our study provides evidence that smoking rather than MBOCA exposure induces elevation of plasma 8-OHdG levels among workers exposed to MBOCA, indicating that oxidative DNA damage does not play an important role in the carcinogenic processes of MBOCA.
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Key words: 4, 4'-methylenebis (2-chloroaniline) (MBOCA), Oxidative DNA damage, 8-hydroxydeoxyguanosine (8-OHdG), Smoking, Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS)

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4, 4'- methylenebis (2-chloroaniline) (MBOCA) is a synthetic aromatic amine used in industry primarily to make castable polyurethane products. Additionally, MBOCA also has a coating application in chemical reactions by helping glues, plastics, and adhesives set[1)]. Because plastics have many applications, MBOCA is widely used[1)]. Most exposure to MBOCA occurs in the workplace. Workers may inhale small particles of MBOCA in the air or absorb it through the skin after contact with MBOCA dust or vapor[1)]. The International Agency for Research on Cancer has determined that MBOCA is probably carcinogenic to humans (2A). The US Environmental Protection Agency (EPA) has determined that MBOCA is a probable human carcinogen (2A). The National Toxicology Program 10th Report on Carcinogens (NTP, 2002) also declares that MBOCA may

reasonably be anticipated to be a human carcinogen[2, 3]. Our recent paper reported a sentinel case of transitional cell carcinoma of the urinary bladder in one worker with 14 yr of occupational exposure to MBOCA in Taiwan[3].

Although the carcinogenicity of MBOCA has been recognized, the mechanism leading to the development of bladder cancer is unclear. DNA adduct formation is thought to be a major cause of DNA damage by carcinogenic aromatic amines[1, 4]. Several pathways are involved in MBOCA metabolism, including *N*-acetylation and *N*-hydroxylation, which may be followed by N-oxidation, and ring hydroxylation[1]. Studies have shown that most of MBOCA's mutagenic activity appears to be due to the N-hydroxy metabolite[1, 5]. Activating N-hydroxy-MBOCA to reactive electrophilic species that bound to DNA, strongly suggest that MBOCA could be a human bladder carcinogen[1, 5]. Another possible mechanism of MBOCA carcinogenecity is induction of oxidative DNA damage by the hydroxyl radical, one kind of reactive oxygen species (ROS)[6, 7]. Studies have found that, in addition to DNA adduct formation, oxidative DNA damage may play an important role in the carcinogenic process of aromatic amines[8, 9]. We investigated whether the N-hydroxy metabolite of MBOCA can cause oxidative DNA damage or not. ROS can induce a variety of DNA damage, including single and double strand breaks, abasic sites and base modification, and they are thought to play an important role in the mechanism of aging and carcinogenesis[10, 11]. Among the diverse oxidative lesions, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one the most abundant base modifiers and has gained much attention because of its mutagenic potential for G to T transversions[10, 11]; thus, the presence of 8-OHdG may result in mutagenesis. In addition, the repair process for 8-OHdG-inflicted damage results in excised 8-OHdG adduct being excreted in urine[10, 11]. Due to easy collection, urinary 8-OHdG has been widely studied as a biomarker of oxidative stress[10, 11]. In contrast, the data on plasma 8-OHdG as a marker of oxidative damage is still lacking.

We conducted this cross-sectional study in MBOCA-producing factories in Taiwan to investigate whether oxidative DNA damage plays an important role in the carcinogenic process of MBOCA. We used plasma activity of 8-OHdG as a marker to investigate possible associations between MBOCA exposure, different confounders and oxidative DNA damage in workers exposed to MBOCA.

Material and Methods

Study population and data collection

The study population was recruited from four MBOCA-producing factories in Taiwan[12]. A total 158 workers participated in this study. All participants in this study were recruited on a voluntary basis, and they gave their informed consent. Trained interviewers administered questionnaires that contained information pertaining to this study. Subjects reported age, gender, height, weight, educational level, nutritional history, past and personal medical history, alcohol drinking and smoking, drug history, detailed occupational history (such as job history, job classification, etc.), and regular use of personal protectors.

The workers were divided into the following two groups by job title: the exposed group and the unexposed group. Exposed group workers were directly involved in the MBOCA manufacturing process, including reaction, neutralization, washing, purification, and packing processes, or indirectly involved in the research & development (R&D) laboratory. The exposed group consisted of 57 workers. The unexposed group were another 101 workers who were not involved in MBOCA manufacturing or R & D laboratory work and did not work in the same building. Of the 158 workers, 153 were available for urine MBOCA concentrations measurement. A cut-off point set at 20 μg/g creatinine of total MBOCA concentration, based on the study of Robert *et al*.[13], was used to divide the study population into high urinary MBOCA (>20 μg/g creatinine) and low urinary MBOCA groups.

Measurements of urinary MBOCA and acetyl MBOCA using LC/MS/MS

A solid phase extraction (C_{18} cartridge) (100 mg/1 m*l*; Waters) was used for sample clean -up, and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was used for quantitation of urinary MBOCA and acetyl-MBOCA. For analysis, a C_{18} cartridge was conditioned using 5 m*l* of pure methanol, followed by 5 m*l* of 5% methanol solution. Then 2 m*l* of urine was loaded, washed with 5 m*l* of 5% methanol, and eluted with 5 m*l* of pure methanol. After being dried under Speed Vac for 2 h, samples were rinsed in 0.1 M formic acid in 50% methanol solution and in preparation for LC/MS/MS instrument analysis. The recovery rate was 56%.

N-acetyl MBOCA, the N-acetyl metabolite of MBOCA and deuterium-labeled N-acetyl MBOCA were synthesized, purified, and characterized using nuclear magnetic resonance (NMR). The urinary samples were spiked with d3-labeled N-acetyl MBOCA to serve as an internal standard and cleaned using the C_{18} cartridge. The eluents were dried in a Speed Vac, rinsed in 0.1 M formic acid in 50% methanol solution, and also analyzed by LC/MS/MS. The detection limit of this method was 0.02 ppb and the recovery rate was 60%.

MBOCA and N-acetyl MBOCA were analyzed by injection of 20 μ*l* of the sample solution into the specific LC/MS/MS instrument. The HPLC system consisted of a PE 200 autosampler and two PE 200 micropumps (Perkin Elmer, Norwalk, CT, USA), and a Polyamine-II

endcapped HPLC column (150 × 2.0 mm, 5 mm, YMC) with an identical guard column (10 × 2 mm, YMC). The mobile phase was 95% acetonitrile with 0.1% formic acid, delivered at a flow rate of 300 μl/min. The eluent of the HPLC system was connected to a triple-quadrupole mass spectrometer (API 3000, Applied Biosystems, Foster City, CA, USA) equipped with a TurboionSpray™ source.

The concentrations of MBOCA and N-acetyl MBOCA were adjusted the urinary creatinine level. The concentrations of MBOCA and N-acetyl MBOCA were added for the total MBOCA concentration.

Measurements of plasma 8-OHdG levels

Blood samples from both the exposed and unexposed groups were obtained from the cubital vein in the morning. Subjects were instructed not to eat for at least 6 hours prior to blood sampling. The sample of plasma 8-hydroxydeoxyguanosine (8-OHdG) was measured by isotope-dilution LC/MS/MS following the method developed by one of the authors (KY Wu)[14]. Synthesis of $^{15}N_5$-8-OHdG was performed in Dr. Wu's laboratory. Using LC/MS/MS, 500 μl of plasma was first diluted with 500 μl of water, followed by the addition of 20 μl of a $^{15}N_5$-8-OHdG solution (42.6 pg /ml) as the internal standard. After the addition of 150 μl of 1 M ammonium acetate buffer (pH 5.25) and vigorous vortexing, the sample was loaded into a Sep-Pak C_{18} cartridge (100 mg/ 1 ml; Waters) preconditioned with 1 ml methanol and 1 ml distilled water. The column was then washed with 1 ml of water. The fraction containing 8-OHdG was eluted with 1 ml of 40% (v/v) methanol, collected, dried under a vacuum for 2 h, and dissolved in 500 μl of 80% acetonitrile containing 0.1% formic acid.

Twenty microliters of the sample solution was injected into the HPLC/MS/MS instrument. The HPLC system consisted of a PE 200 autosampler, two PE 200 micropumps (Perkin Elmer, Norwalk, CT, USA), and a polyamine-II endcapped HPLC column (150 × 2.0 mm, 5 μm, YMC) with an identical guard column (10 × 2 mm, YMC). The mobile phase was 80% acetonitrile with 0.1% formic acid, delivered at a flow rate of 300 μl/min. The eluent of the HPLC system was connected to a triple-quadrupole mass spectrometer (API 3000, Applied Biosystems, Foster City, CA, USA) equipped with a TurboionSpray™ source. Electrospray ionization was performed in the positive mode. For all samples, the $[M+H]^+$ ion was selected by the first mass filter. After collisional activation, the $[M+H-116]^+$ ions, corresponding to BH_2^+, were selected by the last mass filter. Nitrogen was used as the nebulizing, curtain, heater (6 l/min), and collision gases. The Turboion-Spray™ probe temperature was set to 300°C.

Statistical analyses

Statistical evaluation was carried out with the SPSS statistical software (version 13.0). Mean, range, and the distribution of personal characteristics (age, gender, BMI, education level, marital status, duration of employment, smoking status, alcohol consumption, tea drinking, coffee drinking) were described. Arithmetic means, medians, and standard deviations were calculated for plasma 8-OHdG activities and urinary total MBOCA concentration in both the exposed and unexposed groups. Student's *t*-test and the non-parametric test were used to compare the means of plasma 8-OHdG and urinary total MBOCA concentration between subgroups. A cut-off point of 20 μg/g creatinine of total MBOCA was set to divide the study population into high urinary MBOCA and low urinary MBOCA groups. Student's *t*-test and the non-parametric test were used to compare the means of plasma 8-OHdG between high and low urinary MBOCA subgroups. Linear regression was used to examine the association between urinary MBOCA levels and mean plasma 8-OHdG levels after adjusting for gender, age and smoking.

Plasma 8-OHdG was classified into high and low levels based on the median value in the low MBOCA level group (6.528 pg /ml). A high 8-OHdG level was defined as a plasma 8-OHdG level exceeding 6.528 pg /ml. The crude odds ratio (ORs) and 95% confidence intervals (95% CIs) of having a high plasma 8-OHdG level was calculated for the exposed group. In addition, we used unconditional logistic regression methods to examine the association between MBOCA exposure and high plasma 8-OHdG levels after adjusting for gender, age, and smoking.

Results

Comparison of plasma 8-OHdG between the MBOCA-exposed and unexposed groups classified by job titles

First, we compared the plasma 8-OHdG between the MBOCA-exposed and unexposed groups as defined by job title. No significant difference in mean plasma 8-OHdG levels was found between the MBOCA-exposed (6.29 ± 1.98, n=57) and the unexposed groups (6.72 ± 3.03, n=101) (p=0.28). When stratified by risk factors (table not shown), MBOCA-exposed males had significantly lower plasma 8-OHdG concentrations than unexposed males. Among individuals with a body mass index (BMI) of >24, MBOCA-exposed workers had significantly lower plasma 8-OHdG concentrations than unexposed male. Among alcohol consumers, MBOCA-exposed workers had significantly lower plasma 8-OHdG concentrations than unexposed workers. There was no significant difference in mean 8-OHdG levels between the MBOCA-exposed and the unexposed groups for other variables. After adjusting for gender, smoking, and age using a linear regression model, the 8-OHdG level in the exposed group was lower than that in the unexposed group, but the difference was not statistically significant. Age and gender were significantly positively correlated

Table 1. Distribution and comparison of characteristic between high urinary MBOCA (>20 μg/g creatinine) and low urinary MBOCA groups

Variables	Low urinary MBOCA group (n=108)	High urinary MBOCA group (n=45)	Total (n=153)	*p*-value*
Urine MBOCA (μg/g creatinine) range				
	1.86 ± 4.10	278.46 ± 315.53		<0.001
	N.D. – 19.65	21.48 – 1264.19		
	37.73 ± 9.18	38.01 ± 8.84	37.81 ± 9.05	0.28
Age (yr)				
<40 yr	70 (64.8%)	25 (55.6%)	95 (62.1%)	
≥40 yr	38 (35.2%)	20 (44.4%)	58 (37.9%)	
Gender				0.01
Male	79 (73.1%)	41 (91.1%)	120 (78.4%)	
Female	29 (26.9%)	4 (8.9%)	33 (21.6%)	
Height (cm)	166.47 ± 7.84	167.88 ± 7.28	166.86 ± 7.69	0.34
Weight (Kg)	65.41 ± 12.52	69.97 ± 13.20	66.68 ± 12.83	0.06
BMI (Kg/m^2)	23.52 ± 3.70	24.89 ± 4.91	23.90 ± 4.10	0.30
<22	38 (38.4%)	11 (28.9%)	49 (35.8%)	
≥22	61 (61.6%)	27 (71.1%)	88 (64.2%)	
Duration of employment	9.31 ± 6.58	6.08 ± 5.72	8.36 ± 6.49	<0.01
<4 yr	28 (25.9%)	24 (53.3%)	52 (34.0%)	
4–10 yr	40 (37.0%)	13 (28.9%)	53 (34.6%)	
>10 yr	40 (37.0%)	8 (17.8%)	48 (31.4%)	
Education				<0.01
Less than high school	38 (36.5%)	28 (42.4%)	66 (44.9%)	
University	66 (63.5%)	15 (34.9%)	81 (55.1%)	
Marital status				0.91
Single	33 (32.4%)	14 (33.3%)	66 (44.9%)	
Married	69 (67.6%)	28 (66.7%)	81 (55.1%)	
Smoking				<0.01
No	71 (67.6%)	15 (34.9%)	86 (58.1%)	
Yes	34 (32.4%)	28 (65.1%)	62 (41.9%)	
Alcohol drinking				<0.01
No	87 (83.7%)	25 (58.1%)	112 (76.2%)	
Yes	17 (16.3%)	18 (41.9%)	35 (23.8%)	
Tea drinking				0.40
No	57 (55.3%)	19 (47.5%)	76 (53.1%)	
Yes	46 (44.7%)	21 (52.5%)	67 (46.9%)	
Coffee drinking				0.39
No	84 (82.4%)	37 (88.1%)	121 (84.0%)	
Yes	18 (17.6%)	5 (11.9%)	23 (16.0%)	

*: Chi-square test or Student's *t* test.

with plasma 8-OHdG levels. Smokers also had higher 8-OHdG levels than non-smokers, but the difference was not significant (data not shown).

Distribution of characteristics among the study population classified by urinary MBOCA level (Table 1)

Since no significant difference in mean plasma 8-OHdG levels was found between the MBOCA-exposed and unexposed groups as defined by job titles, we further divided the study population into a high urinary MBOCA group (>20 μg/g creatinine) and a low urinary MBOCA group (<20 μg/g creatinine) in order to study the dose-response relationship between MBOCA concentrations and plasma 8-OHdG levels. The mean urinary MBOCA concentration was 1.86 ± 4.10 μg/g creatinine in the low urinary MBOCA group and 278.46 ± 315.53 μg/g creatinine in the high urinary MBOCA group.

The distribution of characteristics among the high urinary MBOCA and low urinary MBOCA groups is shown in Table 1. The distribution of age, marital status,

Table 2. The influence of risk factors on plasma 8-OHdG (pg/m*l*) in high urinary MBOCA (>20 μg/g creatinine) and low urinary MBOCA groups

Variables	Low urinary MBOCA group N	mean ± SD	High urinary MBOCA group N	mean ± SD	*p*-value*
Total mean (pg/m*l*)	108	6.53 ± 2.98	45	6.70 ± 2.05	0.68
Age (yr)					
<40 yr	70	5.99 ± 2.59	25	6.49 ± 2.12	0.39
≥40 yr	38	7.52 ± 3.40	20	6.97 ± 1.99	0.44
p-value**		0.01		0.45	
Gender					
Males	79	7.37 ± 2.81	41	6.73 ± 2.14	0.19
Females	29	4.22 ± 2.07	4	6.47 ± 0.87	0.01#
p-value		<0.01		0.72#	
Duration of employment					
<4 yr	28	6.74 ± 2.37	24	6.35 ± 2.08	0.54
4–10 yr	40	6.11 ± 3.34	13	5.56 ± 2.14	0.65
>10 yr	40	6.80 ± 2.99	8	8.00 ± 1.45	0.13#
p-value		0.53		0.06#	
BMI					
≤22	38	5.80 ± 2.46	11	6.78 ± 1.07	0.23
>22	61	7.14 ± 3.08	27	6.72 ± 2.09	0.52
p-value		0.03		0.93	
Education					
Lower than high school	38	6.76 ± 2.95	28	6.66 ± 2.07	0.88
Higher than college	66	6.29 ± 2.80	15	6.83 ± 2.17	0.48
p-value		0.42		0.79	
Marital status					
Single	33	5.98 ± 2.27	14	6.63 ± 2.50	0.39
Married	69	6.76 ± 3.17	28	6.77 ± 1.93	0.97
p-value		0.21		0.85	
Smoking					
No	71	6.10 ± 2.79	15	5.72 ± 1.79	0.62
Yes	34	7.39 ± 2.97	28	7.25 ± 2.06	0.84
p-value		0.03		0.02	
Alcohol drinking					
No	87	6.09 ± 2.68	25	6.95 ± 2.11	0.14
Yes	17	8.41 ± 3.10	18	6.40 ± 2.05	0.03
p-value		<0.01		0.40	
Tea drinking					
No	57	6.65 ± 2.74	19	6.56 ± 2.26	0.89
Yes	46	6.46 ± 3.11	21	6.73 ± 2.05	0.71
p-value		0.73		0.80	
Coffee drinking					
No	84	6.47 ± 2.66	37	6.59 ± 2.01	0.81
Yes	18	7.09 ± 3.95	5	6.74 ± 1.82	0.82#
p-value		0.54		0.86#	

*: Comparison between high urinary MBOCA and low urinary MBOCA groups by Student's *t* test or Mann-Whitney U test (#). **: Comparison between categories of each variable for high urinary MBOCA and low urinary MBOCA groups by Student's *t* test or Mann-Whitney U test (#).

and tea and coffee consumption habits did not significantly differ between the high urinary MBOCA and low urinary MBOCA groups, but the distribution of gender, educational level, durations of employment, smoking habit, and the alcohol drinking habit significantly differed between the high urinary MBOCA and low urinary MBOCA groups. The high urinary MBOCA group had more males than the low urinary MBOCA group (p=0.01), had lower education level (lower than college degree) than the low urinary MBOCA group (p<0.01), and had a short working duration than the low urinary MBOCA group (p<0.01). The high urinary

Table 3. Linear regression model of risk factors of plasma 8-OHdG (pg/m*l*)

Items	Coefficient (β)	Standard error (S.E.)	*p*-value
Model 1 (total population)			
Exposure (high/low urinary MBOCA group)	–0.65	0.44	0.15
Gender (male/female)	2.71	0.50	<0.01
Smoking (smoker/nonsmoker)	0.78	0.47	0.10
Age (>40 yr vs. ≤40 yr)	1.51	0.40	<0.01
Alcohol drinking (yes/no)	0.15	0.50	0.76
Model 2 (high urinary MBOCA group only)			
Working years	0.04	0.06	0.46
Gender (male/female)	0.13	1.08	0.90
Smoking (smoker/nonsmoker)	2.27	0.68	<0.01
Age (>40 yr vs. ≤40 yr)	1.00	0.76	0.19
Alcohol drinking(yes/no)	–1.13	0.62	0.07
Model 3 (high urinary MBOCA group only)			
MBOCA concentration (μg/g creatinine)	0.00	0.001	0.80
Gender (male/female)	0.33	1.11	0.77
Smoking (smoker/nonsmoker)	2.33	0.68	<0.01
Age (>40 yr vs. ≤40 yr)	1.32	0.64	0.05
Alcohol drinking (yes/no)	–1.19	0.62	0.06

MBOCA group had a higher percentage of smokers (65.1%) than the low urinary MBOCA group (32.4%) (p<0.01), and a higher percentage of persons drinking alcohol more than one time per week (41.9%) than the low urinary MBOCA group (16.3%) (p<0.01).

Risk factors associated with plasma 8-OHdG in the high urinary MBOCA and low urinary MBOCA groups

The association between confounders and plasma 8-OHdG levels in the high urinary MBOCA and low urinary MBOCA groups is shown in Table 2. Age, gender, BMI, smoking and alcohol drinking had significant impacts on plasma 8-OHdG concentrations in the low urinary MBOCA group. The greater the age, the higher the plasma 8-OHdG concentration was. Males had significantly higher plasma 8-OHdG concentration than females. The greater the BMI, the higher the plasma 8-OHdG concentration was. Smokers had higher plasma 8-OHdG concentrations than non-smokers. Alcohol consumers also had higher plasma 8-OHdG concentrations than teetotallers. Smoking status had a significant impact on plasma 8-OHdG concentrations in the high urinary MBOCA group. Smokers had higher plasma 8-OHdG concentrations than non-smokers.

Comparison of mean plasma 8-OHdG levels between the high urinary MBOCA and low urinary MBOCA groups

We compared the plasma 8-OHdG between the high urinary MBOCA and low urinary MBOCA groups. No significant difference in mean plasma 8-OHdG levels was found between the high urinary MBOCA group (6.70 ± 2.05 pg/m*l*, n=45) and the low urinary MBOCA group (6.53 ± 2.98 pg/m*l*, n=108) (p=0.68) (Table2). The impact of MBOCA exposure on the plasma 8-OHdG levels was studied using a linear regression model adjusted for potential risk factors (Table 3). After adjusting for gender, smoking status, age and alcohol drinking, the 8-OHdG level in the high urinary MBOCA group was lower than that in the low urinary MBOCA group, but the difference was not statistically significant (Table 3, Model 1). Age and gender were significantly positively correlated with plasma 8-OHdG levels. Smokers also had higher 8-OHdG levels than non-smokers, but the increase was not statistically significant.

In order to study the dose-response relationship between years of MBOCA exposure and plasma 8-OHdG levels, another linear regression model was applied to the high urinary MBOCA group only (Table 3, Model 2). The working duration (in years) was not significantly correlated with 8-OHdG levels. Age and gender were also not significantly correlated with plasma 8-OHdG levels. Smoking was significantly positively correlated with 8-OHdG levels in the high urinary MBOCA group, an association which was not seen in the total population. Smokers among high urinary MBOCA workers had significantly higher 8-OHdG levels than non-smokers among high urinary MBOCA workers.

Another model (Table 3, Model 3) was used to study the dose-response relationship between urinary MBOCA concentrations and plasma 8-OHdG levels in the high urinary MBOCA group only. The MBOCA concentration (in μg/g creatinine) was not significantly correlated with

Table 4. Logistic regression model of risk factors of having high plasma 8-OHdG*

Items	Odds ratio	Coefficient (β)	Standard error (S.E.)	p-value
Model 1 (total population)				
Exposure (high/low urinary MBOCA)	0.85	–0.16	0.42	0.70
Gender (male/female)	8.91	2.19	0.62	<0.01
Smoking (smoker/nonsmoker)	1.81	0.59	0.43	0.17
Age (>40 yr vs. ≤40 yr)	3.26	1.18	0.41	<0.01
Alcohol drinking (yes/no)	1.04	0.03	0.47	0.94
Model 2 (high urinary MBOCA group only)				
Working years	1.08	0.07	0.09	0.40
Gender (male/female)	1.42	0.35	1.24	0.78
Smoking (smoker/nonsmoker)	16.87	2.83	1.08	<0.01
Age (>40 yr vs. ≤40 yr)	4.79	1.57	0.99	0.12
Alcohol drinking (yes/no)	0.19	–1.65	0.85	0.05
Model 3 (high urinary MBOCA group only)				
MBOCA concentration (μg/g creatinine)	1.00	–0.00	0.001	0.50
Gender (male/female)	1.89	0.63	1.27	0.62
Smoking (smoker/nonsmoker)	16.02	2.77	1.05	<0.01
Age (>40 yr vs. ≤40 yr)	6.69	1.90	0.95	0.05
Alcohol drinking (yes/no)	0.19	–1.65	0.85	0.05

*Plasma 8-OHdG was classified into high 8-OHdG (>6.528 pg/m*l*) and low 8-OHdG (<6.528 pg/m*l*) groups based on the median value 6.528 pg/m*l* in the low urinary MBOCA group.

8-OHdG levels. The correlation coefficient between plasma 8-OHdG and urinary MBOCA levels was 0.02 for the total population and was 0.16 for the high urinary MBOCA group. Smoking was also significantly positively correlated with 8-OHdG levels in the high urinary MBOCA group, but age and gender were not significantly correlated with plasma 8-OHdG levels. This result implies that smoking had a significant impact on oxidative DNA damage seen in the high urinary MBOCA workers.

Comparison of high plasma 8-OHdG levels between the high urinary MBOCA and low urinary MBOCA groups

Plasma 8-OHdG was classified into high and low levels based on the median value in the control group (6.528 pg/m*l*). A high 8-OHdG level was defined as one exceeding 6.528 pg/m*l*. The impact of MBOCA exposure on the odds of having high plasma 8-OHdG levels was studied using a logistic regression model adjusted for potential risk factors (Table 4). After adjusting for gender, smoking, age and alcohol drinking, the odds ratio of having a high 8-OHdG level was 0.85 in the high urinary MBOCA group compared to the low urinary MBOCA group, but it was not statistically significant (Table 4, Model 1). Age and gender were significantly associated with having a high plasma 8-OHdG level. Smokers had a higher odds ratio of having a high 8-OHdG level than non-smokers, but the increase was not statistically significant.

In order to study the dose-response relationship between having a high plasma 8-OHdG level and years of MBOCA exposure, another logistic regression model was applied to the high urinary MBOCA group (Table 4, Model 2). The working duration (in years) was not significantly associated with having a high 8-OHdG level, while smoking was significantly associated with having a high plasma 8-OHdG level in the high urinary MBOCA group.

Another model (Table 4, Model 3) was used to study the dose-response relationship between urinary MBOCA concentration and having a high plasma 8-OHdG level in the high urinary MBOCA group. The MBOCA concentration (in μg/g creatinine) was not significantly correlated with having a high 8-OHdG level. Smoking was also significantly positively correlated with having a high 8-OHdG level in the high urinary MBOCA group, but age and gender were not.

Discussion

We previously reported one incidence of MBOCA-induced bladder cancer for a 52-yr-old male who had worked in one of the four MBOCA-producing companies of the present study for 14 yr[3]. In addition, we conducted a bladder cancer screening program in the four MBOCA-producing factories[12]. Although no further bladder cancer cases were found and the prevalence of atypical urinary cells and the NMP22 biomarker did not differ between MBOCA-exposed workers and unexposed workers for

both male and female workers, the prevalence of positive occult blood was higher in male exposed workers (18%) than in male unexposed workers (7%)[12]. To the best of our knowledge, this is the first study which has attempted to investigate the relationship between plasma 8-OHdG levels and occupational exposure[10]. However, neither MBOCA-exposed workers nor high urinary MBOCA workers had an elevated level of plasma 8-OHdG, even after adjusting for potential confounders.

MBOCA is considered to probably be carcinogenic to humans by the International Agency of Research on Cancer and other Agents[2, 3]. The precise mechanism of action of MBOCA is not completely understood. DNA adducts have been found following oral and dermal administration of radiolabeled MBOCA to rats, following the incubation of radiolabeled MBOCA with explants of dog and human bladder urothelium, and following incubation of rat DNA and radiolabeled *N*-hydroxy-MBOCA[4]. At least six adducts have been found in canine bladder epithelium, and four adducts have been found in human bladder epithelium[15–17] The capacity of MBOCA to form adducts with hemoglobin, tissue DNA, and globin and serum albumin in rats may play a role in its carcinogenicity[1]. In addition to adduct formation, another possible mechanism of MBOCA carcinogenicity may be the induction of oxidative DNA damage by the hydroxyl radical, one powerful reactive oxygen species (ROS)[6, 7]. *N*-acetylation and *N*-hydroxylation are mainly involved in MBOCA metabolism[1]. One study analyzed 23 urine samples from workers occupationally exposed to MBOCA and showed that all contained MBOCA, but only 10 of 23 contained *N*-acetyl MBOCA. The ratio of *N*-acetyl MBOCA to the parent MBOCA ranged from 0.005 to 0.09 indicating that *N*-acetyl MBOCA is a relatively minor urine metabolite in humans[18]. Studies have found that oxidative DNA damage resulting from *N*-hydroxy metabolites may also play another important role in the carcinogenic process of aromatic amines[8, 9]. Another study has noted that most of MBOCA's mutagenic activity appears to be due to the *N*-hydroxy metabolite, which causes dose-dependent increases in mutations of the *S. typhimurium* strains, TA100 and TA98[5]. The mononitroso derivative of the *N*-hydroxy metabolite appears to be a direct-acting mutagen but is much less potent. Neither the *o*-hydroxy nor the dinitroso derivatives of the *N*-hydroxy metabolite is a direct-acting mutagen[5]. Our present study was designed to test the hypothesis that the *N*-hydroxy metabolite of MBOCA can cause oxidative DNA damage. 8-OHdG has been widely studied as a biomarker for detecting oxidative DNA damage, and such damage has been suggested as being important in aging processes and several human cancers, including bladder cancer[10, 11].

The concentration of 8-OHdG was 6.29 ± 1.98 pg/m*l* in the exposed group and 6.72 ± 3.03 pg/m*l* in the unexposed group; the mean plasma 8-OHdG levels was 6.70 ± 2.05 pg/m*l* in the high urinary MBOCA group and 6.53 ± 2.98 pg/m*l* in the low urinary MBOCA group. Neither the MBOCA-exposed workers nor the high urinary MBOCA workers had a significant increase in the mean plasma 8-OHdG level, even after adjustment for potential confounders. These results suggest that oxidative DNA damage does not play an important role in the carcinogenic process of MBOCA. In addition, levels of oxidized bases can change not only because of changes in the rate of oxidative DNA damage, but also because of alterations in the rate of repair[10, 11]. The amount of plasma 8-OHdG is suggested to represent the deleted oxidative DNA damage through repair processes occurring throughout the entire body[10, 11]. The lack of an increase in plasma 8-OHdG levels in MBOCA-exposed workers may possibly be due to a reduction in the rate of DNA repair in workers exposed to MBOCA.

Analysis of 8-OHdG levels has been performed by methods such as high performance liquid chromatography with electrochemical detection (HPLC-ECD), gas chromatography with mass spectrometry (GC/MS), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography with tandem mass spectrometry (LC/MS/MS)[10]. GC/MS and HPLC-ECD have the disadvantage of artifacts caused by oxidation of normal DNA constituents. Although ELISA has the advantage of being easy and rapid, and requiring little equipment, analysis time, sample preparation, and sample volume, its sensitivity is lower than LC/MS/MS[10]. A previous study showed that a statistically significant difference in urinary levels of 8-OHdG between exposed and control workers was detected by the LC/MS/MS method, but not by the ELISA method[14]. The LC/MS/MS method provides high selectivity, sensitivity, and no derivatization step is required in the analysis. In addition, the targeted analyte can be quantitated reliably and accurately with the application of the isotope-dilution method[14]. With respect to concentration, the plasma 8-OHdG level is about only 0.1% of urinary 8-OHdG level. This extremely low level of 8-OHdG can suitably be determined by the isotope-dilution LC/MS/MS method[14].

Although there was no significant difference in adjusted 8-OHdG levels between the MBOCA-exposed and unexposed groups, this study found that a male gender, a history of smoking, and being older were associated with increased plasma 8-OHdG. The risk factors which have been shown to be associated with elevation of 8-OHdG includes age, gender, smoking, and other lifestyle factors[19–25]. Our findings were consistent with the previous studies. Smoking was significantly positively correlated with 8-OHdG levels in the high urinary MBOCA group, an association which was not seen in the total population. Smoking high urinary MBOCA workers had significantly higher 8-OHdG levels than non-

smoking high urinary MBOCA workers. This result implies that smoking rather than MBOCA exposure is the factor inducing oxidative damage in MBOCA-exposed workers.

In conclusion, this study did not find evidence of oxidative DNA damage among MBOCA-exposed workers. It did however show that smoking induced oxidative damage in high urinary MBOCA workers. Although the precise mechanism of MBOCA in inducing bladder cancer is not completely understood, the results of this study do not support suggestions that oxidative DNA damage plays an important role in the carcinogenic process of MBOCA.

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