Acidic Urine pH Is Associated with Elevated Levels of Free Urinary Benzidine and *N*-Acetylbenzidine and Urothelial Cell DNA Adducts in Exposed Workers[1]

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Abstract

We evaluated the influence of urine pH on the proportion of urinary benzidine (BZ) and *N*-acetylbenzidine present in the free, unconjugated state and on exfoliated urothelial cell DNA adduct levels in 32 workers exposed to BZ in India. Postworkshift urine pH was inversely correlated with the proportions of BZ ($r = -0.78$; $P < 0.0001$) and *N*-acetylbenzidine ($r = -0.67$; $P < 0.0001$) present as free compounds. Furthermore, the average of each subject's pre- and postworkshift urine pH was negatively associated with the predominant urothelial DNA adduct ($P = 0.0037$, adjusted for urinary BZ and metabolites), which has been shown to cochromatograph with a *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine adduct standard. Controlling for internal dose, individuals with urine pH <6 had 10-fold higher DNA adduct levels compared to subjects with urine pH ≥7. As reported previously, polymorphisms in *NAT1*, *NAT2*, and *GSTM1* had no impact on DNA adduct levels. This is the first study to demonstrate that urine pH has a strong influence on the presence of free urinary aromatic amine compounds and on urothelial cell DNA adduct levels in exposed humans. Because there is evidence that acidic urine has a similar influence on aromatic amines derived from cigarette smoke, urine pH, which is influenced by diet, may be an important susceptibility factor for bladder cancer caused by tobacco in the general population.

Introduction

N-Glucuronides of *N*-hydroxy derivatives of 2-naphthylamine, 1-naphthylamine, and ABP[3] are hydrolyzed under acidic conditions and can bind to DNA (1). Acidic urine has a similar influence on the hydrolysis of *N*-glucuronides of BZ and several of its metabolites (2, 3), which can bind to DNA after subsequent metabolic activation in the bladder (4, 5). For example, at 37°C, the half-lives of BZ and ABZ glucuronides at pH 7.4 were 104 and 150 min, respectively, which decreased to 4 and 5 min at pH 5.3 (2, 3).

Although there is substantial *in vitro* and animal evidence that urine pH influences aromatic amine carcinogenesis (1, 6–9), we know of no studies that have directly tested this hypothesis in humans. The advent of noninvasive testing to measure DNA adduct levels in the urinary bladder (10) has made investigation of this concept possible. We have previously carried out a cross-sectional study of 33 workers exposed to BZ and BZ-based dyes in India to evaluate genetic susceptibility for exfoliated urothelial cell DNA adduct formation (11, 12). Here, we report the influence of urine pH on the proportion of BZ, ABZ, and DBZ present in free, unconjugated form and on levels of the predominant urothelial DNA adduct, which has been shown to cochromatograph with a *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine adduct standard (11).

Materials and Methods

Subject Enrollment. The field phase of the study was conducted in 1993 under the auspices of the National Institute of Occupational Health in Ahmedabad, India, and has been described in detail (11). The study was explained to the subjects, and informed consent was received. BZ-exposed subjects worked in factories that manufactured BZ dihydrochloride or BZ-based dyes. Workers were relatively young (mean, 25 years; SD, 4.5), and all were male. BZ and BZ-dye manufacturing and use were banned in India in 1994, and these factories were subsequently closed. Control subjects (15 of 20 eligible individuals were enrolled into the study) worked at a construction company and were excluded from this report, because they had no detectable urinary BZ, ABZ, or DBZ (11).

Subjects provided a first morning void sample for 2 successive days and a 14-ml peripheral blood sample. On 1 day, they provided a preworkshift spot urine sample at approximately 8:30 a.m, voided again about two-thirds through the

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[3] The abbreviations used are: ABP, 4-aminobiphenyl; BZ, benzidine; ABZ, *N*-acetylbenzidine; DBZ, *N,N*′-diacetylbenzidine; RAL, relative adduct labeling; cr, creatinine.

workshift, and then provided a postworkshift spot urine sample at approximately 7:30 p.m. After collection, all urine and blood samples were immediately placed on ice and kept cold until processed as described (11).

Biological samples provided by study subjects in the morning were frozen approximately 5 h after collection, and postworkshift samples provided in the evening were frozen about 2 h after collection.

Storage of urine samples with pH ≥7.0 on ice prevented hydrolysis of conjugated BZ and ABZ, as noted below. In urine samples with acidic pH, *N*-glucuronides of BZ and ABZ would have gone through multiple hydrolysis half-lives at 37°C (2, 3) during storage in the bladder prior to voiding. As such, any additional hydrolysis that occurred while samples were stored on ice prior to freezing could have made only a negligible contribution to the total amount of free BZ and ABZ present.

Urine BZ Metabolite Analysis. After assessing the initial (baseline) pH of urine samples, a 1-ml aliquot of each postworkshift sample was incubated for 60 min either on ice with pH adjusted to 7–7.4 (which prevented hydrolysis of conjugated BZ and ABZ, based on preliminary studies carried out for the current investigation) or at 37°C with pH adjusted to 5.5. After incubation, BZ, ABZ, and DBZ were measured by a highly sensitive and specific capillary gas chromatography/negative chemical ion mass spectrometry method (13). Because previous studies have shown that *N*-glucuronides of BZ and ABZ are the only known metabolites of BZ that are hydrolyzed rapidly by acid pH to their respective parent amines (2, 3), the values obtained in samples incubated at neutral pH on ice were considered free amine, and those obtained at acid pH were considered total (free and glucuronidated) amine. This distinction allowed calculation of free urinary amine as a percentage of total urinary amine (*i.e.*, free amine/total amine × 100). DBZ is not glucuronidated (3), and its analysis was used to assess the influence of the experimental conditions. We have reported previously on genetic susceptibility for urine mutagenicity in this population (12); a study similar to that reported here could not be conducted, because an adequate quantity of urine was not available.

Exfoliated Urothelial Cell DNA Adduct Analysis. Processing, cell isolation, DNA extraction, and ^{32}P postlabeling of first morning void urine samples were carried out as described (11). Chromatographic conditions could resolve *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine, *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-BZ, and the major smoking-associated DNA adducts (*e.g.*, N-(3′-phosphodeoxyguanosin-8-yl)-*N*′-ABP). Adduct levels are expressed as RAL (calculated as $cpm_{adducts}$/$cpm_{unadducted\ nucleotides}$) × 10^9.

NAT1, NAT2, and GSTM1 Genotype Analysis. *NAT1* (*NAT1*3, NAT1*4, NAT1*10,* and *NAT1*11*), *NAT2* (*NAT2*4, NAT2*5B, NAT2*6A, NAT2*7A,* and *NAT2*14A*), and *GSTM1* (*GSTM1*-null) genotypes were determined by PCR-RFLP as described (11, 12).

Statistical Analysis. Pearson's correlation was used to test the relationship between baseline urine pH, categorized into moderately acidic (<6), mildly acidic (≥6 and <7), and neutral/basic (≥7) and the ratio of free:total BZ, ABZ, and DBZ. ANOVA was used to compare ratios at pH <6 to ratios at higher pH. Urine pH cutpoints were chosen prior to analysis and are used to clarify graphical presentation and interpretation of results. Analyses were repeated using urine pH as a continuous measure and resulted in similar results that are provided in figure and table legends. Linear regression was used to test the association between urine pH and exfoliated urothelial cell DNA adduct levels, adjusting for internal dose (*i.e.*, sum of total urinary BZ, ABZ, and DBZ). For this analysis, the pH of each subject's pre- and postworkshift urine sample was averaged under the assumption that it would be a better estimate of usual urine pH than a single measure taken at the beginning or end of the day. The optimal regression model was chosen using stepwise and R^2 linear regression procedures in SAS for PC (14). Transformation of independent or dependent variables by the natural logarithm was used where appropriate to normalize distributions. Two-sided *P*s were calculated throughout, with *P*s < 0.05 considered statistically significant.

Results

Thirty-three of 48 eligible subjects were enrolled into the study from factories that manufactured BZ dihydrochloride or BZ-based dyes. One subject had nondetectable levels of BZ, ABZ and DBZ in his postworkshift urine sample after incubation at both neutral and acidic pH and was excluded from these analyses. Among the BZ-exposed workers, 42% of total BZ (mean, 1.6 ng/μmol cr), 66% of total ABZ (mean, 19.6 ng/μmol cr), and 97% of total DBZ (mean, 1.0 ng/μmol cr) were present in the free, unconjugated state in postworkshift urine samples. There was a strong and highly significant inverse relationship between the baseline urine pH of these samples and the proportion of BZ and ABZ present as free compounds (Fig. 1, *a* and *b*). For example, 27% of BZ was present in the free state in samples with pH ≥7, which increased to 61% in samples with pH <6 ($P < 0.0001$); similarly, the proportion of ABZ present in free form increased from 51% in samples at pH ≥7 to 79% in samples at pH <6 ($P < 0.0001$). In contrast, there was no significant relationship between urine pH and the proportion of DBZ present in the free state (Fig. 1*c*).

We evaluated the influence of urine pH (average of pre- and postworkshift samples) on levels of the predominant exfoliated urothelial cell DNA adduct detected in this population (Table 1), which has been shown to cochromatograph with a *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine standard (11). There was a significant inverse relationship between urine pH and DNA adduct levels ($P = 0.035$; Table 1, model 2) that became more significant after adjustment for internal dose ($P = 0.0037$; Table 1, model 3). As reported previously (11, 12), *NAT1, NAT2,* and *GSTM1* genotypes were not significantly associated with DNA adduct levels and did little to improve the model R^2 (Table 1, model 4). Further adjustment for age, bidi (a form of tobacco), and alcohol use also had a negligible influence on the results. The optimal regression model included only urine pH and internal dose (Table 1, model 3) and predicted adduct levels of 32.0 RAL × 10^9 for a worker with urine pH <6.0 and 3.2 RAL × 10^9 for a worker with urine pH ≥7 (at an internal BZ dose of 22.1 ng/μmol cr, the mean for all workers).

Discussion

We report that urine pH had a strong and highly significant inverse relationship with the proportion of urinary BZ and ABZ present in free form and with exfoliated urothelial cell *N*′-acetylbenzidine DNA adduct levels among BZ-exposed workers. This is the first study to demonstrate an effect of urine pH on levels of free, unconjugated aromatic amines and on the level of a specific DNA adduct in humans and suggests that urine pH may have a significant impact on risk of BZ-associated bladder cancer. These results are consistent with the observation that BZ and ABZ *N*-glucuronides, which are formed

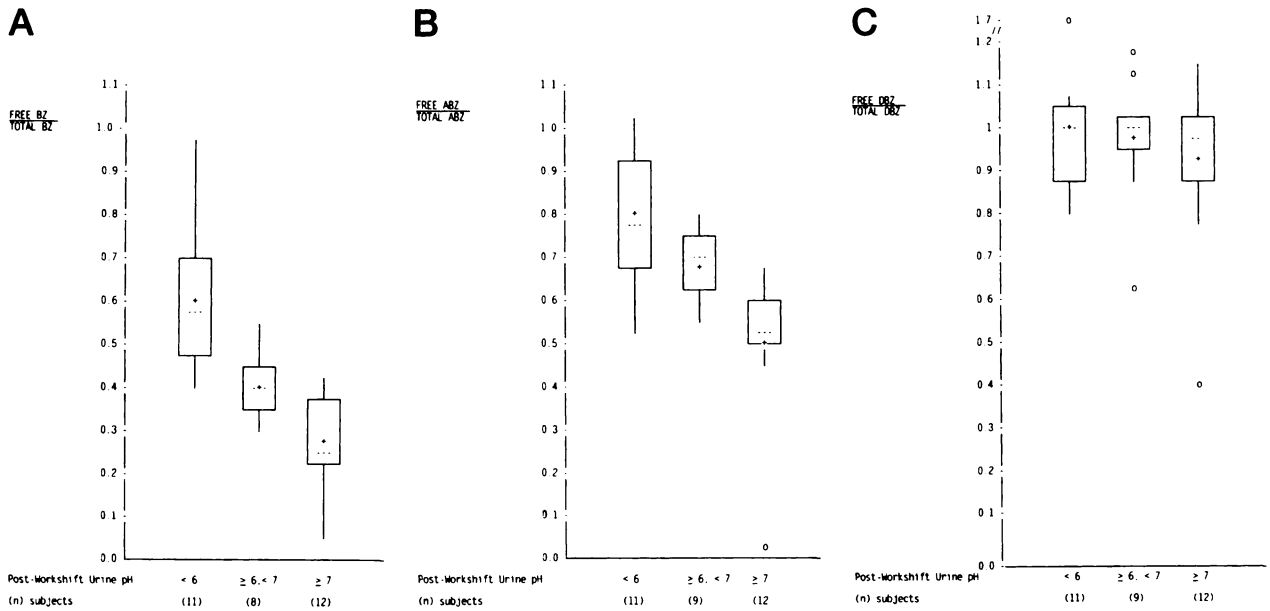

Fig. 1. Box and whisker plots for proportions of free:total BZ (*a*), ABZ (*b*), and DBZ (*c*) in postworkshift urine samples, by baseline urine pH. Whiskers include highest and lowest 25th percentiles of data. +, mean; - - -, median; *o*, outlier, defined as >1.5 × height of box, which represents the middle 50% of the data. *a*, (n = 31; one subject with nondetectable total BZ levels was excluded). Correlation with urine pH in categories, $r = -0.78$ ($P < 0.0001$); as continuous measure, $r = -0.68$ ($P < 0.0001$). *b*, (n = 32). Correlation with urine pH in categories, $r = -0.67$ ($P < 0.0001$); as continuous measure, $r = -0.56$ ($P < 0.0001$). *c*, (n = 32). Correlation with urine pH in categories, $r = -0.20$ ($P = 0.20$); as continuous measure, $r = -0.12$ ($P = 0.52$).

Table 1 Influence of internal BZ dose, urinary pH, and *NAT1*, *NAT2*, and *GSTM1* genotypes on *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine adduct levels in exfoliated urothelial cells of 31[a] Indian workers studied in Ahmedabad, India, 1993

Model[b]	Independent variable	b[c]	Standard error[d]	P[e]	Model R^2[f]	Model P
1	Internal dose[g]	0.73	0.16	<0.0001	0.42	<0.0001
	Intercept	0.43	0.40			
2	Urinary pH[h]	−1.13	0.51	0.035	0.14	0.035
	Intercept	3.70	0.89			
3	Internal dose	0.70	0.13	<0.0001	0.58	<0.0001
	Urinary pH[i]	−1.15	0.36	0.0037		
	Intercept	2.45	0.67			
4	Internal dose	0.70	0.15	<0.0001	0.59	0.0003
	Urinary pH	−1.15	0.38	0.0062		
	NAT1 genotype[j]	0.04	0.56	0.94		
	NAT2 genotype[k]	0.30	0.58	0.60		
	GSTM1 genotype[l]	−0.009	0.57	0.99		
	Intercept	2.0	1.32			

[a] Preshift urine pH was not available for one subject.
[b] Dependent variable in all models, ln(DNA adduct levels).
[c] Linear regression coefficient.
[d] "Standard error" of the regression coefficient.
[e] P for the independent variable.
[f] The amount of variance in DNA adduct levels explained by the model.
[g] ln(total urinary BZ, ABZ, and DBZ in ng/μmol cr).
[h] Average of pre- and postworkshift urine pH: 1, pH < 6; 2, pH ≥ 6 and < 7; 3, pH ≥ 7; for urine pH as continuous measure, $P = 0.022$.
[i] For urine pH as continuous variable, $P = 0.011$.
[j] No copies of *NAT*10* = 1 (n = 11); one or two copies of *NAT*10* allele = 2 [associated with elevated enzyme activity (15); n = 20].
[k] Two copies of any combination of *NAT2*5B*, *NAT2*6A*, *NAT2*7A*, and *NAT2*14A* = 1 (associated with slow activity; n = 21); no copies or one copy of any of these alleles = 2 (associated with rapid activity; n = 10).
[l] Wild-type/heterozygous = 1 (n = 15); homozygous for *GSTM1*-null genotype = 2 (n = 16).

in the liver to enhance their excretion and minimize hepatic toxicity (3, 9, 16), are unstable under acidic conditions in the bladder (2, 3) and after hydrolysis to the parent amines can be metabolically activated and bind DNA (4, 5).

An alternative pathway for formation of the *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine adduct observed in this study is via hepatic formation of *N*-hydroxy-*N*′-acetylbenzidine-*N*-glucuronide, transportation to the bladder, pH-

dependent hydrolysis, and DNA binding. Wang *et al.* have shown that *N*-hydroxy-*N'*-acetylbenzidine and its *N*-glucuronide are carcinogenic in the heterotopic bladder model (17). However, the *N*-glucuronide of *N*-hydroxy-*N'*-acetylbenzidine is substantially less acid labile than is the *N*-glucuronide of ABZ (*i.e.*, at 37°C and pH 5.5, half-lives are 210 min and 7.5 min, respectively) and may play a relatively smaller role in the formation of pH-dependent urothelial cell adducts (18).

Another important factor that could influence DNA adduct levels is voiding frequency (8). As voiding frequency decreases and urinary stasis increases, more time is available for hydrolysis of *N*-glucuronides. Data were not available in our study on voiding frequency. Although a substantial portion of the variation in DNA adduct levels was explained by internal dose and urine pH (Table 1, model 3; $R^2 = 0.59$), voiding frequency also may have influenced adduct levels.

As reported previously, polymorphisms in *NAT1, NAT2,* and *GSTM1* had no influence on BZ DNA adduct levels (11, 12). This is consistent with the observation that acetylation of BZ was not rate limiting over a wide range of exposure levels in this population (11), that the predominant DNA adduct in urothelial cells was *N*-acetylated (11), and that neither *NAT2* nor *GSTM1* genotype influenced bladder cancer risk among subjects identified from a cohort of Chinese workers exposed only to BZ and related compounds (12, 19).

Acidic urine hydrolyzes glucuronidated aromatic amines derived from tobacco smoke (1, 6–9) and thus may also be an important susceptibility factor for cigarette-associated bladder cancer. Using a physiological pharmacokinetic model, Bois *et al.* (20) predicted that interindividual variation in urine pH, ABP *N*-oxidation, ABP *N*-acetylation, and voiding frequency could result in large differences in ABP adduct formation, and that urine pH had the strongest influence on DNA adduct levels.

Diet is an important determinant of urine pH in the healthy, general population (21). Cheese, meat, fish and grain products contribute to urine acidification, whereas most fruits and vegetables contribute to urine alkalinization (21). Interestingly, intake of fruits and vegetables consistently has been associated with decreased bladder cancer risk in epidemiological studies (22). Although a role for fruit and vegetable-associated micronutrients in bladder cancer prevention has been proposed, the influence of fruits and vegetables on urinary pH as a modifier of bladder cancer risk needs further evaluation.

In conclusion, acidic urine was associated with a higher proportion of urinary BZ and ABZ present in the free state and with higher levels of urothelial cell *N'*-acetylated BZ DNA adducts among workers occupationally exposed to BZ. Urine pH may be an important susceptibility factor for bladder cancer in certain occupational settings and may also play a role as a risk factor in the general population.

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