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## Alterations in Leukocyte Telomere Length in Workers Occupationally Exposed to Benzene

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### Abstract

Exposure to benzene, a known leukemogen and probable lymphomagen, has been demonstrated to result in oxidative stress, which has previously been associated with altered telomere length (TL). TL specifically has been associated with several health outcomes in epidemiologic studies, including cancer risk, and has been demonstrated to be altered following exposure to a variety of chemical agents. To evaluate the association between benzene exposure and TL, we measured TL by monochrome multiplex quantitative PCR in 43 workers exposed to high levels of benzene and 43 age and sex-matched unexposed workers in Shanghai, China. Benzene exposure levels were monitored using organic vapor passive dosimetry badges before phlebotomy. The median benzene exposure level in exposed workers was 31 ppm. The mean TL in controls, workers exposed to levels of benzene below the median (< 31 ppm), and above the median (>31 ppm) was  $1.26 \pm 0.17$ ,  $1.25 \pm 0.16$ , and  $1.37 \pm 0.23$ , respectively. Mean TL was significantly elevated in workers exposed to >31 ppm of benzene compared with controls ( $P = 0.03$ ). Our findings provide evidence that high levels of occupational benzene exposure are associated with TL. *Environ. Mol. Mutagen.* 55:673–678, 2014.

### Keywords

benzene; telomere length; telomeres; occupational exposure

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#### AUTHOR CONTRIBUTIONS

Q.L., N.R., L.Z., M.T.S., S.Y., G.L., S.R., and R.V. conceived of and designed the study. R.M.C. conducted the laboratory analyses. B.A.B., Q.L., N.R., W.H., M.S., and F.B.-A. were involved in data preparation and/or analysis. B.A.B. prepared the manuscript draft with input from Q.L., N.R., L.Z., F.B.-A., and R.V. All authors reviewed the manuscript.

## INTRODUCTION

Benzene is a commonly used industrial solvent and ubiquitous environmental contaminant found in cigarette smoke and automobile emissions. Benzene is a Group 1 carcinogen and a known human leukemogen, with the strongest associations observed for acute myeloid leukemia (AML), and has also been associated with non-Hodgkin lymphoma (NHL) [IARC, 2012]. Benzene exposure is associated with hematotoxic and genotoxic effects at relatively low levels, including declines in peripheral blood cell counts and myeloid progenitor cells, and with an increase in chromosomal aberrations in occupationally exposed workers that are specifically associated with the development of AML [Smith et al., 1998; Lan et al., 2004].

Telomeres maintain chromosomal integrity by capping the ends of the chromosome and providing protection from degradation and fusion during the cell cycle. Epidemiologic studies have suggested that telomere length (TL) is associated with cancer risk, but the evidence has been inconsistent [Savage et al., 2013]. In particular, studies that have used a case-control design have generally suggested an increased risk of cancer associated with shorter TL [Prescott et al., 2012], whereas some recent prospective studies have provided evidence that longer TL may also be associated with cancer susceptibility, including for NHL and cancers of the lung and pancreas [Lan et al., 2009, 2013; Lynch et al., 2013].

Several epidemiologic studies have evaluated the effect of environmental and occupational exposures on TL [Zhang et al., 2013]. A positive relationship with TL has been demonstrated in studies of organic pollutants [Shin et al., 2010], arsenic [Li et al., 2012], and short-term exposure to ambient particulate matter [Dioni et al., 2011; Hou et al., 2012], whereas other studies have demonstrated a decline in TL in workers exposed to lead [Wu et al., 2012], polycyclic aromatic hydrocarbons [Pavanello et al., 2010], black carbon [McCracken et al., 2010], and traffic pollution [Hoxha et al., 2009]. These studies have further indicated that the relationship with TL for some exposures may be dependent on the exposure level or the length of the exposure period [Zhang et al., 2003; Dioni et al., 2011].

Given that exposure to benzene results in oxidative stress and can alter various components of the immune system, two factors demonstrated to influence telomere dynamics, we hypothesized that TL would be altered in factory workers occupationally exposed to a wide-range of benzene exposure levels. Here, we evaluated this association and used a monochrome multiplex quantitative PCR procedure to measure TL, which has both high assay precision and correlation with the Southern blot method of measuring TL [Cawthon, 2009].

## MATERIALS AND METHODS

### Study Population

The study population and design has been described previously [Rothman et al., 1996b] along with our observations in this study population that benzene is associated with significant declines in peripheral blood cell counts and increased rates of several types of chromosomal aberrations [Rothman et al., 1996a; Smith et al., 1998; Zhang et al., 2007].

Briefly, three factories that used products containing benzene in Shanghai, China and had a wide range of benzene air levels (1 to 25 ppm as an 8-hr time-weighted average) were selected for the study, which was conducted in 1992. Two workplaces in the same geographic area that did not use benzene or other chemicals associated with bone marrow toxicity were selected as control factories. Workers with a prior history of cancer, history of chemotherapy or radiotherapy, and/or a current pregnancy were not eligible for the study. Eligibility criteria for exposed workers included current employment for at least 6 months in a factory that used benzene, minimal exposure to other aromatic solvents, and no exposure to other known marrow-toxic chemicals or ionizing radiation. Control workers with previous occupational exposure to benzene were ineligible for the study. Controls were frequency-matched by age ( $\pm 5$  years) and gender to workers currently exposed to benzene in one of the three exposed factories. Each worker enrolled in the study was administered a questionnaire inquiring about demographic and lifestyle characteristics, as well as a medical and occupational history. Informed consent using the IRB approved procedures was obtained for all eligible subjects in the study. In total, 88 of 94 eligible subjects (94%) agreed to participate in the study.

### Exposure Assessment

Current benzene exposure levels were monitored using organic vapor passive dosimetry badges (3M, Saint Paul, MN) in exposed workers as well as in a subset of controls as described elsewhere [Rothman et al., 1996a]. Briefly, the badges were worn by each worker for a full work shift on five separate days during the 1–2 weeks before biologic samples were collected. The badges were subsequently analyzed by gas chromatography with flame ionization detection for benzene, toluene, and xylene. Trace amounts of other aromatic solvents were detected (i.e., toluene, xylene), and a detailed assessment of factory records showed that no other chemicals or physical agents that are known or suspected to be toxic to the bone marrow, including hematotoxic pesticides and exposure to ionizing radiation, were present in these factories. A high and significant correlation was observed between urinary benzene metabolite levels and benzene air levels measured on the same day in 38 exposed subjects ( $r = 0.66$ – $0.71$  for specific metabolites;  $P < 0.0001$ ) [Rothman et al., 1996a].

### Sample Collection and Laboratory Analysis

The protocol for biologic sample collection has been previously described [Rothman et al., 1996b]. Briefly, each enrolled subject provided a 27 mL sample of blood obtained by venous phlebotomy following completion of the questionnaire, and peripheral blood samples were delivered to the processing lab within 4–6 hr of collection. DNA was extracted from buffy coat samples, which were stored at  $-80^{\circ}\text{C}$ , by phenol–chloroform extraction.

TL was measured in 43 exposed workers and 43 unexposed controls in the laboratory of Dr. Richard Cawthon at the University of Utah and was assessed using a monochrome multiplex quantitative PCR assay as described elsewhere [Cawthon, 2009]. MyiQ software (Bio-Rad iQ5 2.0 Standard Edition Optical System Software) was used after each run to determine the T (telomere) and S (single gene copy) values for each sample by the Standard Curve method, and the average TL per cell was determined by taking the ratio of the telomere PCR signal to the single copy gene ( $\beta$ -globin) PCR signal (T/S ratio). This ratio is proportional to

the average TL per cell, with T/S values > 1.0 for a given sample corresponding to an average TL greater than that of the standard DNA. Quality control duplicate samples were interspersed in each batch to evaluate assay reproducibility. The overall coefficient of variation for the TL assay was 8.7%.

### Statistical Analysis

Arithmetic means and standard deviations were calculated for TL in workers with benzene exposure levels the median (< 31 ppm) and greater than the median (>31 ppm) based on the air benzene exposure level in the exposed workers, as well as for unexposed control workers. Spearman correlation coefficients were calculated for TL and continuous baseline characteristics, including age and BMI, and differences between exposed and control workers with respect to baseline characteristics were evaluated using a chi-square test for categorical variables or a *t*-test for continuous variables.

Multiple linear regression using the natural logarithm of TL to approximate a normal distribution was conducted to test for differences in TL between workers exposed to benzene according to their exposure level and unexposed controls as well as to evaluate for an exposure-response trend across air exposure levels based on the median level (control, < 31 ppm, and >31 ppm). All models were adjusted for the matching factors age (continuous) and sex, and variables relating to recent smoking (yes/no), alcohol use (yes/no), recent infection (yes/no), and BMI (continuous) were further considered as potential confounders in the models. Variables that altered the regression coefficient of the benzene exposure variable by  $\pm 15\%$  when included in the age and sex adjusted model were included in the final model. All statistical tests were two-sided, with  $P < 0.05$  considered statistically significant. Statistical analyses were conducted using SAS v.9.1.3 (Cary, NC).

## RESULTS

Exposed and control workers were similar with respect to sex, age, BMI, and other characteristics including recent smoking, alcohol use, and recent infection, and there were no significant differences between exposed and control workers with respect to any baseline characteristic ( $P > 0.05$ ) (Table I). Study participants had a mean age of 35 years, and the majority did not drink alcohol or have evidence of a recent infection (Table I). The mean benzene air exposure level in exposed workers was 62.7 ppm (SD  $\pm$  70.8). The median air benzene level in exposed workers was 31.5 ppm (range 1.6–328.5 ppm) and was 13.5 ppm (range 1.6–30.6 ppm) and 92.0 ppm (range 31.5–328.5 ppm) in the low (< 31 ppm) and high (>31 ppm) exposure groups, respectively (Table I).

As expected, TL was inversely correlated with age ( $r$  in overall study population =  $-0.19$ ,  $P = 0.09$ ;  $r$  in controls =  $-0.12$ ,  $P = 0.45$ ) and was also inversely correlated with BMI ( $r$  in overall study population =  $-0.18$ ,  $P = 0.09$ ;  $r$  in controls =  $-0.28$ ,  $P = 0.07$ ). The relationship between TL and air benzene levels is shown in Figure 1. The mean TL was marginally higher in workers currently exposed to benzene ( $1.32 \pm 0.20$ ) compared with unexposed control workers ( $1.26 \pm 0.17$ ), adjusted for age and sex ( $P = 0.21$ ). Analyses according to benzene exposure levels showed that while mean TL was similar in workers exposed to < 31 ppm of benzene ( $1.25 \pm 0.16$ ;  $P = 0.93$ ) compared with unexposed workers, workers

exposed to >31 ppm of benzene had a statistically significant increase in TL ( $1.37 \pm 0.23$ ) compared with unexposed workers, adjusted for age and sex ( $P = 0.03$ ;  $P_{\text{trend}} = 0.05$ ; Fig. 1). Further adjustment of the age and sex adjusted model for BMI and recent smoking did not alter the significance of the finding in workers exposed to >31 ppm of benzene ( $P = 0.04$ ).

## DISCUSSION

We conducted a cross-sectional study of factory workers exposed to a wide-range of benzene exposure levels and unexposed controls, and found evidence for a statistically significant increased mean TL in workers exposed to >31 ppm of benzene compared to controls. Our findings provide some evidence that high levels of occupational benzene exposure are associated with TL, and add to the increasing body of literature that suggests that chemical exposures may alter telomere dynamics.

The only additional study to our knowledge that assessed TL in the context of benzene exposure was a study of traffic and office workers in Italy that measured airborne benzene and toluene levels as tracers for traffic exposure [Hoxha et al., 2009]. A significant decline in leukocyte TL was observed with increasing exposure to traffic pollution and among workers exposed to high traffic intensity compared with low intensity, as well as for benzene and toluene specifically [Hoxha et al., 2009]. Several distinct differences in terms of the benzene exposure levels and potential for co-exposures between this previous study and ours might help explain the conflicting findings. Specifically, our study included very highly exposed factory workers with a mean air benzene level of ~62.7 ppm, compared with a mean personal benzene exposure level of <1 ppm reported in the traffic workers. Second, traffic officers are likely exposed to multiple agents, including long-term exposure to particulate matter and black carbon that has been demonstrated to shorten TL in occupationally exposed workers [McCracken et al., 2010; Hou et al., 2012]. We conducted exposure monitoring for other chemicals and detected only trace amounts in exposed workers, which suggests that our findings are unlikely to be due to exposure to other solvents in the workplace.

The increase in TL associated with occupational benzene exposure in our study follows similar epidemiologic observations previously observed for some other chemicals, namely arsenic, a Group 1 carcinogen, specific classes of organic pollutants, and short-term exposure to particulate matter [Zhang et al., 2003; Shin et al., 2010; Dioni et al., 2011]. A recent evaluation of the effect of arsenic exposure on TL in 202 Argentinian women exposed to arsenic in drinking water with exposure ranges of 3.5–200  $\mu\text{g/L}$  suggested a significant positive relationship between urinary arsenic levels and both TL and expression of *TERT* [Li et al., 2012]. Similarly, a study of healthy Koreans showed an increase in leukocyte TL following low exposures to organic pollutants, with the strongest effects observed for *p,p'*-DDE and several classes of PCBs [Shin et al., 2010]. However, in contrast to our findings for benzene, in vitro studies have indicated that higher exposure levels of arsenic exposure increased telomere attrition in both cell lines, while the effects of organic pollutants and particulate matter on TL in the previous epidemiologic studies were primarily apparent at lower exposure levels or in subjects with shorter exposure durations [Zhang et al., 2003; Ferrario et al., 2009; Shin et al., 2010].

Mechanistic information concerning the effect of benzene on TL is currently limited, although some evidence indicates that benzene can alter telomerase activity. Telomerase activity was previously demonstrated to be enhanced in human lung (LL24) cells treated in vivo with low concentrations of benzene as well as in lymphocytes of mice with increasing concentrations of benzene [He et al., 2008; Giuliano et al., 2009]. Moreover, whereas telomerase activity is absent or present at low levels in most somatic cells, experimental evidence has indicated that telomerase activity is upregulated in certain inflammatory cells, including mature lymphocytic cells following antigenic stimulation [Weng et al., 1998]. Given our previous observations demonstrating immune suppression in subjects exposed to benzene, additional experimental studies are needed to elucidate the mechanism underlying our findings, including further evaluation of whether benzene induces changes in telomerase expression in humans. Whereas upregulation of telomerase was previously associated with increasing arsenic levels, no association was apparent between *TERT* expression and TL in that study [Li et al., 2012]. Thus, this may suggest that environmental contaminants including benzene influence TL via other mechanisms independent of *TERT* expression. For instance, telomere elongation was recently observed in naïve T-cells and granulocytes, but not in memory B or T cells, in individuals following initial exposure to intense oxidative stress [Shlush et al., 2011]. These observations in granulocytes in particular were suggested to be the result of repopulation of the peripheral hematopoietic compartment with undifferentiated hematopoietic cells carrying longer telomeres [Shlush et al., 2011]. Other factors, such as the cellular composition of peripheral blood at the time of collection and degree of immune suppression in study subjects, also may affect the observed TL [Svenson et al., 2011].

In contrast to other hematotoxic and genotoxic effects of benzene, which have been demonstrated, including in our study, to occur at relatively low levels of exposure [Lan et al., 2004], our data suggest that the effect on TL occurred only in the highest exposed workers although the small size of our study could potentially explain the absence of an association in workers exposed to levels less than the median ( 31 ppm). Both shorter and longer TL may have implications for cancer risk depending on the microenvironment of the cell and the cell's history of somatic mutations. Whereas shorter telomeres may lead to carcinogenesis through the development of cytogenetic abnormalities or mechanisms involving DNA damage signaling or repair [Wu et al., 2003], longer telomeres in some cases may contribute to delayed senescence and/or apoptosis enabling the cell more time to develop the somatic mutations that may precede carcinogenesis [Mooi and Peepers, 2006]. Therefore, it is plausible that an increase in TL associated with benzene exposure could have implications for carcinogenicity particularly given that benzene is a probable lymphomagen and NHL has previously been associated with longer leukocyte TL in a prospective study [Lan et al., 2009].

In summary, we observed a significant increase in TL associated with very high levels of benzene exposure in factory workers compared to unexposed control workers. Our results provide additional insight into the early biologic effects of benzene and further evidence that exposure to chemical agents may alter telomere dynamics. However, given the relatively



small size of our study, these findings and the underlying biologic mechanism will require further investigation.

## Acknowledgments

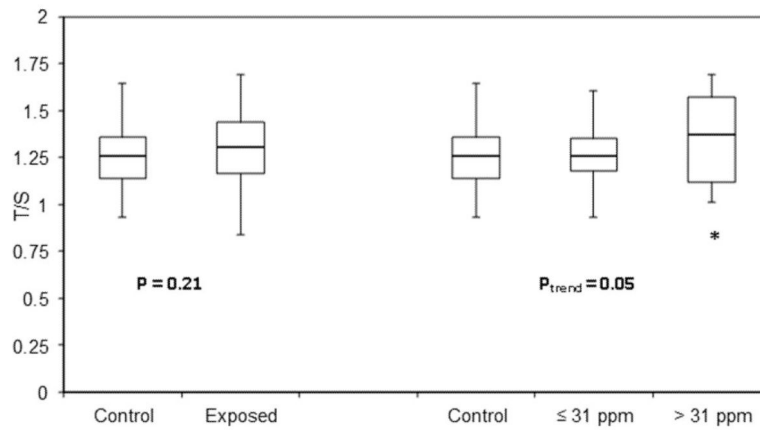
Nathaniel Rothman, Roel Vermeulen, and Qing Lan co-supervised this work.

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**Fig. 1.** Box plots of TL in relation to air benzene exposure levels in occupationally exposed workers and controls. Median (line), interquartile range (box), and whiskers to the lowest and highest TL values in relation to benzene air exposure levels are shown for workers exposed  $\leq 31$  ppm and  $>31$  ppm of benzene versus controls. 31 ppm was the median air benzene level in exposed workers. *P*-values are indicated as  $*P = 0.03$ . All final models were adjusted for age and sex.

TABLE 1

Selected Characteristics of Workers Exposed to Benzene and Unexposed Controls

Characteristic <sup>a</sup>	Control (n = 43)	Exposed (n = 43)	Lower exposed (n = 21)	High exposed (n = 22)
	(n, %)	(n, %)	31 ppm <sup>b</sup> (n, %)	>31 ppm <sup>b</sup> (n, %)
Sex (n, %)				
Male	22 (51)	22 (51)	13 (62)	9 (41)
Female	21 (49)	21 (49)	8 (38)	13 (59)
Mean age (SD)	36 (±7)	35 (±8)	34 (±8)	36 (±8)
BMI (SD)	22 (±2)	22 (±3)	21 (±2)	22 (±4)
Smoking (n, %)				
Yes	20 (47)	20 (47)	11 (52)	9 (41)
No	23 (53)	23 (53)	10 (48)	13 (59)
Alcohol use (n, %)				
Yes	16 (37)	14 (33)	8 (38)	6 (27)
No	27 (63)	29 (67)	13 (62)	16 (73)
Recent infection (n, %)				
Yes	13 (30)	17 (40)	5 (24)	12 (55)
No	30 (70)	26 (60)	16 (76)	10 (45)
Mean benzene air exposure, ppm (SD)	n/a	62.7 (±70.8)	14.0 (±8.9)	109.2 (±73.0)
Median benzene air exposure, ppm (Range)	n/a	31.5 (1.6–328.5)	13.5 (1.6–30.6)	92.0(31.5–328.5)

<sup>a</sup>  $P > 0.05$  for exposed versus control for all baseline characteristics.

<sup>b</sup> Based on median benzene exposure of 31 ppm in exposed workers.