Published in final edited form as:

Environ Mol Mutagen. 2008 July ; 49(6): 453–457. doi:10.1002/em.20402.

Association Between Mitochondrial DNA Copy Number, Blood Cell Counts, and Occupational Benzene Exposure

Min Shen^{1,*}, Luoping Zhang², Matthew R. Bonner³, Chin-San Liu⁴, Guilan Li⁵, Roel Vermeulen⁶, Mustafa Dosemeci¹, Songnian Yin⁵, and Qing Lan¹

¹Division of Cancer Epidemiology and Genetics, NCI, NIH, DHHS, Bethesda, Maryland ²School of Public Health, University of California, Berkeley, California ³Department of Social and Preventive Medicine, University at Buffalo the State University of New York, Buffalo, New York ⁴Department of Neurology and Vascular and Genomic Center, Changhua Christian Hospital, Changhua, Taiwan ⁵Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention, Beijing, China ⁶Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

Abstract

Benzene is a recognized hematotoxicant and carcinogen that produces genotoxic damage. Benzene metabolites can produce reactive oxidative species. Mitochondrial DNA (mtDNA) copy number may be increased in response to oxidative stress to compensate for damaged mitochondria. We carried out a cross-sectional study of 40 benzene-exposed workers and 40 controls to evaluate the association between benzene exposure and mtDNA copy number. Copy number of mtDNA in leukocyte DNA was determined by real-time PCR. Compared with controls, the copy number of mtDNA increased by 4% and by 15% in workers exposed to ≤ 10 ppm ($n = 20$) and > 10 ppm ($n = 20$) benzene, respectively. After adjusting for recent infection, the factor that was significantly correlated with mtDNA, the increase of mtDNA was statistically significant in the high exposed group ($P = 0.016$) with a significant linear trend ($P = 0.024$). To our best knowledge, this is the first report that benzene exposure was associated with increased mitochondria DNA copy number. Benzene exposure may induce mtDNA amplification, possibly in response to oxidative stress caused by benzene. The finding needs to be replicated by other studies.

Keywords

benzene; mitochondrial DNA; oxidative stress

INTRODUCTION

Benzene is an important industrial chemical and also a significant environmental pollutant [Wallace, 1996]. Epidemiological studies have shown that exposure to benzene results in an increased risk of aplastic anemia, myelodys-plastic syndromes, leukemia, and other blood disorders [Goldstein, 1988]. Benzene has to be metabolized to elicit toxicity to the blood and bone marrow [Yoon et al., 2001]. These benzene metabolites, i.e., reactive quinones, are able

to bind to and damage macromolecules, including DNA. Additionally, benzene metabolites may give rise to reactive oxygen species (ROS) [Kolachana et al., 1993].

Benzene metabolites and ROS may affect mitochondrial function. Mitochondria generate energy for the cell by synthesizing adenosine 5'-triphosphate (ATP). Mitochondria also play a role in cell apoptosis. ATP is produced when the major products of glycolysis are oxidized through the electron transport chain in mitochondria. ROS from exogenous sources like benzene metabolites may impair electron chain transport and damage mitochondrial DNA (mtDNA). mtDNA is more susceptible to damage than the nuclear genome because mtDNA does not have intron and mitochondria lack protective histones [Penta et al., 2001]. Mitochondria have developed defenses against ROS including superoxide dismutases, glutathione peroxidase, and glutathione. However, the repair is limited.

Mitochondria are not only the target of ROS but also one of the major intracellular sources of ROS. Some of the electrons passing through the mitochondrial electron chain transport can leak out to molecular oxygen (O_2) to form superoxide which is quickly dismutated to H_2O_2 by superoxide dismutase.

Human mtDNA-encoded genes are involved in the production of proteins essential for cellular respiration and normal mitochondrial function. Human normal cell mitochondria contain 10^3 – 10^4 copies per cell of their mtDNA genome. The mtDNA copy number strongly depends on the energy demand of the tissue. The mitochondrial mass and mtDNA copy number of individual cells vary with the type of cell and tissue, and are altered during cell differentiation, hormone treatment and exercise [Lee and Wei, 2005].

Experimentally, cells challenged with ROS may synthesize more copies of their mtDNA. In a study by Liu et al. [2003], the mtDNA copy number increased with increasing biomarkers of oxidative stress. The increase in mitochondrial mass and mtDNA content may be an early molecular event of human cells in response to endogenous or exogenous oxidative stress [Lee et al., 2000] and mitochondrial mass was increased to about 1.4-fold, in skin fibroblasts of myoclonic epilepsy patients compared with those of normal skin fibroblasts [Lee et al., 2006].

Little evidence is available about the effect that benzene may have on mitochondria and mtDNA. Exposure to benzene may shift the balance between oxidants and anti-oxidants leading to oxidative stress and cellular damage. It may increase the generation of ROS in the mitochondria and increase the risk of leukemia [Klaunig and Kamendulis, 2004] and other benzene related-malignancies. We examined the hypothesis that mitochondria and mtDNA are involved in benzene-related toxicity in a cross-sectional occupational study.

MATERIALS AND METHODS

Study Population and Exposure Assessment

The details of this cross-sectional study have been described elsewhere [Lan et al., 2004]. Briefly, the study subjects were enrolled in 2000 (pilot study, 57 benzene-exposed workers and 31 controls) and in 2001 (main study, 221 exposed workers and 109 controls). Twenty-eight exposed workers from the pilot study were also studied in the main study. They were treated as independent observations. In total, there were 278 observations in 250 unique workers who were exposed to benzene in two shoe manufacturing factories, and 140 unexposed controls from comparable populations who worked in three clothing-manufacturing factories in the same region of China. Controls were frequency-matched on sex and age to exposed workers. Blood samples were collected from all workers. Individual exposure to benzene and toluene, as well as other organic solvents, was monitored repeatedly up to 16 months before

phlebotomy by wearing an organic vapor passive monitor badge, and postshift urine samples were collected from each subject [Lan et al., 2004]. Subjects were administered a questionnaire for information on lifetime occupational history, hobbies, environmental exposures, medical history and current medications, and past and current tobacco and alcohol use.

For this mtDNA study, 39 benzene-exposed workers were randomly picked including 38 workers from either the pilot or the main study and 1 worker who was studied in both pilot and main studies. Therefore, there are 40 observations (20 > 10 ppm and 20 1–10 ppm, based on the exposure in the previous year before phlebotomy) in total. Forty controls were frequency-matched to the 40 exposed workers on sex and age (± 2 years).

Hematology

Blood samples were delivered to the lab within 6 hr of being collected, the complete blood cells and differentials were analyzed using a T540 blood counter, and the major lymphocyte subsets were analyzed by a FACS Calibur flow cytometer (Software: SimulSET v3.1).

mtDNA Assay

Leukocyte DNA extracted with phenol/chloroform was used to determine the mtDNA copy number by fluorescence-based quantitative PCR. Primers for the PCR determined both the nuclear gene β -globin and the mitochondrial gene ND1 threshold cycle number (Ct). These Ct values were then used in a linear regression model to measure the number of mitochondrial gene copies [Liu et al., 2003]. The copy number of mtDNA was determined in two DNA duplicate samples for every study subject, whose identity and exposure level were blinded to lab investigators.

Statistical Analysis

The reliability between the two measurements was examined by correlation coefficient and intraclass correlation coefficient (ICC) using a two-way random effects model. Arithmetic mean of the two measurements for each subject was used in the analysis. Correlation between demographic factors (sex, continuous variable of age), tobacco smoking (current smoking status, pack-year, year of smoking, and pack per day), alcohol drinking (current alcohol drinking status, drinks in the past day and drinks in the past week), infection (current infection status), benzene exposure level (continuous exposure in the past month and in the past year), and mtDNA copy number was determined with the Spearman Correlation. A potential confounder was defined to be correlated significantly with mtDNA copy number and to alter regression coefficient of benzene exposure by 15% after adjustment. The copy number of mtDNA was correlated with recent infection ($r = -0.22$, $P = 0.045$) significantly, but not with age, sex, body mass index, tobacco smoking, and alcohol drinking. generalized estimating equations were used to model the relationship between blood cell counts in natural log, mtDNA copy number and benzene exposure categories (none, low, and high) to take into account the autocorrelation between the repeats [Zeger and Liang, 1986]. The model of mtDNA copy number and benzene exposure was adjusted for recent infection, the only potential confounder. Data were analyzed with the Statistical Analysis Software 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Demographic characteristics were essentially the same between benzene-exposed workers and controls. The majority of the study subjects was females (70%) and was relatively young (33 ± 9). The subjects in the benzene-exposed and nonexposed groups were comparable for alcohol use, recent infection, smoking status, and body mass index (Table I). The average benzene air exposure for all exposed workers in the month and in the year prior to phlebotomy were 14.3 (SD: 20.4) ppm and 15.7 (SD: 17.3) ppm, respectively. White blood cells (WBCs) and most

WBC subtypes, as well as the platelet counts, were reduced in benzene-exposed workers compared with controls, especially in high exposed workers (Table II). However, the linear trend was not statistically significant for some of them as shown in the entire study population because of the small sample size [Lan et al., 2004]. The counts of CD8⁺-T cells and hemoglobin did not vary with benzene exposure levels.

The two measurements of mtDNA copy number were in good correlation ($r = 0.93$, $P < 0.0001$) with an ICC of 0.93 (95% CI: 0.90–0.95). The copy number of mtDNA was inversely correlated with WBC counts ($r = -0.22$, $P = 0.05$) in the study population.

The copy number of mtDNA was 117 (SD: 32) in controls and there were a 4% and a 15% increase in workers exposed to ≤ 10 ppm and > 10 ppm benzene, respectively. The increase of mtDNA was statistically significant in the high exposed group with a significant linear trend ($P = 0.024$). When the benzene exposure was classified based on the previous month before phlebotomy, the relationship between mtDNA and benzene exposure level remained unchanged.

DISCUSSION

In this study, we found that the mtDNA copy number was increased proportionally with benzene exposure level, which may be a biological adaptation in response to the oxidative stress caused by benzene. It may also indicate benzene-induced damage to mitochondria that is relevant to benzene-induced hematotoxicity. It may be a biological marker of response to benzene exposure.

When physiological conditions are changed, the mtDNA copy number can be modulated. ROS and free radicals generated by environmental exposures (e.g., UV radiation, cigarette smoke, and air pollutants) and xenobiotics (e.g., drugs and betel quid) may induce the accumulation of mtDNA mutations in human tissue. Human mtDNA is more susceptible to oxidative damage and consequently acquires mutations at a higher rate than does nuclear DNA [Richter et al., 1988] because of exposure to high levels of ROS generated during respiration, a lack of protective histones, and a limited capacity for the repair of DNA damage. As a result, mitochondrial function is compromised. Oxidative stress-induced increase in mitochondria and mtDNA molecules may compensate for the decline of mitochondrial respiratory function. At the same time, ROS would be generated from the increased mitochondria in these cells. Consequently, it can cause more oxidative damage to mitochondria and other intracellular constituents [Chen et al., 1998].

In addition, benzene metabolites can increase mitochondria membrane permeability potential [Inayat-Hussain and Ross, 2005]. With the increase in membrane permeability, cytochrome c is released into the cytosol and reacts with Apaf-1 to initiate apoptosis [Green and Reed, 1998]. Apoptosis has been proposed as a potential hematotoxic mechanism through which benzene causes this constellation of hematological conditions whereby progenitor cells are depleted in the bone marrow [Moran et al., 1996]. Experimentally, mitochondria and mtDNA copy number has been shown to increase in HL-60 cells treated with etoposide to induce apoptosis, suggesting that proliferation of mitochondria may be a fundamental characteristic of apoptosis [Eliseev et al., 2003]. Our study supported the hypothesis that mtDNA may be a target of benzene metabolites and mitochondria may contribute to hematological toxicity through the induction of apoptosis.

Besides apoptosis, mitochondria and/or mtDNA can play a role at multiple stages in the carcinogenesis. The mitochondrial production of ROS may be involved in both the initiation and promotion of carcinogenesis [Klaunig and Kamendulis, 2004]. In addition, the integration

of mtDNA fragments into nuclear DNA may lead to nuclear DNA mutation [Penta et al., 2001].

The aging process in humans was shown to be associated with reduced levels of mtDNA transcripts, increased mtDNA content in brain, and increased mtDNA copy number in the lung, skeletal muscle, and liver [Lee et al., 2000; Barrientos et al., 1997; Barazzoni et al., 2000]. These results support the notion that as the respiratory function of the mitochondria harboring mtDNA with oxidative damage or mutation in aging tissues declines, the tissue cells manage to compensate for the reduced ATP synthesis by increasing mtDNA content. However, the hypothesis concerning the age-dependent upregulation of mtDNA copy number was not supported by another experiment, which indicated that the maintenance of mitochondrial function was controlled on the transcriptional level and not adjusted by mtDNA copy number [Frahm et al., 2005].

To the best of our knowledge, this is the first cross-sectional study of the relationship between mtDNA copy number and occupational benzene exposure. Although our key findings were statistically significant, the magnitude of mtDNA amplification is not substantial. It is possible that the association was due to confounding or chance. We tried to control confounding by adjusting for different covariates in difference scales but adjustment for none of them except infection changed the association substantially. In addition, this marker as oxidative stress or benzene toxicity was not validated adequately. As such, replication of key findings in other benzene-exposed populations is critical.

Acknowledgments

Grant sponsor: National Cancer Institute; Grant sponsor: NIH; Grant numbers: R01ES06721, P42ES04705, P30ES01896, P42ES05948, P30ES10126.

REFERENCES

- Barazzoni R, Short KR, Nair KS. Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *J Biol Chem* 2000;275:3343–3347. [PubMed: 10652323]
- Barrientos A, Casademont J, Cardellach F, Estivill X, Urbano-Marquez A, Nunes V. Reduced steady-state levels of mitochondrial RNA, increased mitochondrial DNA amount in human brain with aging. *Brain Res Mol Brain Res* 1997;52:284–289. [PubMed: 9495550]
- Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN. Molecular analysis of H₂O₂-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J* 1998;332(Part 1):43–50. [PubMed: 9576849]
- Eliseev RA, Gunter KK, Gunter TE. Bcl-2 prevents abnormal mitochondrial proliferation during etoposide-induced apoptosis. *Exp Cell Res* 2003;289:275–281. [PubMed: 14499628]
- Frahm T, Mohamed SA, Bruse P, Gemund C, Oehmichen M, Meissner C. Lack of age-related increase of mitochondrial DNA amount in brain, skeletal muscle and human heart. *Mech Ageing Dev* 2005;126:1192–1200. [PubMed: 16099018]
- Goldstein BD. Benzene toxicity. *Occup Med* 1988;3:541–554. [PubMed: 3043738]
- Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–1312. [PubMed: 9721092]
- Inayat-Hussain SH, Ross D. Intrinsic pathway of hydroquinone induced apoptosis occurs via both caspase-dependent and caspase-independent mechanisms. *Chem Res Toxicol* 2005;18:420–427. [PubMed: 15777082]
- Klaunig JE, Kamendulis LM. The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol* 2004;44:239–267. [PubMed: 14744246]
- Kolachana P, Subrahmanyam VV, Meyer KB, Zhang L, Smith MT. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. *Cancer Res* 1993;53:1023–1026. [PubMed: 8439949]

- Lan Q, Zhang L, Li G, Vermeulen R, Weinberg RS, Dosemeci M, Rappaport SM, Shen M, Alter BP, Wu Y, Kopp W, Waidyanatha S, Rabkin C, Guo W, Chanock S, Hayes RB, Linet M, Kim S, Yin S, Rothman N, Smith MT. Hematotoxicity in workers exposed to low levels of benzene. *Science* 2004;306:1774–1776. [PubMed: 15576619]
- Lee CF, Chen YC, Liu CY, Wei YH. Involvement of protein kinase C delta in the alteration of mitochondrial mass in human cells under oxidative stress. *Free Radic Biol Med* 2006;40:2136–2146. [PubMed: 16785027]
- Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *Int J Biochem Cell Biol* 2005;37:822–834. [PubMed: 15694841]
- Lee HC, Yin PH, Lu CY, Chi CW, Wei YH. Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. *Biochem J* 2000;348(Part 2):425–432. [PubMed: 10816438]
- Liu CS, Tsai CS, Kuo CL, Chen HW, Lii CK, Ma YS, Wei YH. Oxidative stress-related alteration of the copy number of mitochondrial DNA in human leukocytes. *Free Radic Res* 2003;37:1307–1317. [PubMed: 14753755]
- Moran JL, Siegel D, Sun XM, Ross D. Induction of apoptosis by benzene metabolites in HL60 and CD34 + human bone marrow progenitor cells. *Mol Pharmacol* 1996;50:610–615. [PubMed: 8794901]
- Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. *Mutat Res* 2001;488:119–133. [PubMed: 11344040]
- Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci USA* 1988;85:6465–6467. [PubMed: 3413108]
- Wallace L. Environmental exposure to benzene: an update. *Environ Health Perspect* 1996;104(Suppl 6): 1129–1136. [PubMed: 9118882]
- Yoon BI, Hirabayashi Y, Kawasaki Y, Kodama Y, Kaneko T, Kim DY, Inoue T. Mechanism of action of benzene toxicity: cell cycle suppression in hemopoietic progenitor cells (CFU-GM). *Exp Hematol* 2001;29:278–285. [PubMed: 11274754]
- Zeger SL, Liang KY. Longitudinal data analysis for discrete and continuous outcomes. *Biometrics* 1986;42:121–130. [PubMed: 3719049]

TABLE I

Demographic and Benzene Exposure Levels by Benzene Exposure of the Study Population

	Controls (n = 40)	1–10 ppm (n = 20)	>10 ppm (n = 20)	P value
Sex				
Male	12 (30)	4 (20)	8 (40)	
Female	28 (70)	16 (80)	12 (60)	0.41 ^a
Current smoking				
Yes	14 (35)	3 (15)	6 (30)	
No	26 (65)	17 (85)	14 (70)	0.29 ^a
Current alcohol use				
Yes	13 (32)	3 (15)	9 (45)	
No	27 (68)	17 (85)	11 (55)	0.13 ^a
Recent infection				
Yes	2 (5)	.	3 (15)	
No	38 (95)	20 (100)	17 (85)	0.14 ^a
Age	32.23 ± 9.30	32.90 ± 9.82	32.75 ± 9.12	
BMI	22.96 ± 3.46	22.96 ± 3.83	22.08 ± 2.65	
Benzene air level in the previous month ^b (ppm)	<0.04 ^c	2.48 ± 1.85	26.15 ± 23.54	
Benzene air level in the previous year ^b (ppm)	<0.04 ^c	5.06 ± 2.97	26.30 ± 19.16	
Benzene urine ^b (µg/L)	0.32 ± 0.50	99.76 ± 156.10	820.82 ± 1253.27	

^a Fisher's exact test.^b Unadjusted mean ± standard deviation.^c Limit of detection.

TABLE II

Hematologic Characteristics and mtDNA Copy Number by Benzene Exposure of the Study Population

	Controls (<i>n</i> = 40)	1–10 ppm (<i>n</i> = 20)	>10 ppm (<i>n</i> = 20)	<i>P</i> value _{trend}
Peripheral blood cell counts ^{ab}				
Total white blood cells	6340 ± 1550	6170 ± 1190	5050 ± 1080	0.0022
Granulocytes	4020 ± 1130	3790 ± 1160	3010 ± 1000	0.0017
Lymphocytes	2080 ± 683	2135 ± 487	1830 ± 396	0.41
CD4 ⁺ -T cells	732 ± 279	704 ± 137	565 ± 209	0.052
CD8 ⁺ -T cells	570 ± 268	645 ± 261	581 ± 182	0.14
CD4 ⁺ /CD8 ⁺ ratio	1.41 ± 0.54	1.22 ± 0.42	1.02 ± 0.38	0.0008
B cells	211 ± 72	219 ± 152	125 ± 78	0.0004
NK cells	536 ± 359	573 ± 244	446 ± 189	0.78
Monocytes	222 ± 102	245 ± 110	205 ± 76	0.96
Platelets	230 ± 70.0 × 10 ³	217 ± 47 × 10 ³	179 ± 319 × 10 ³	0.0039
Hgb (g/dL)	14.3 ± 1.7	14.3 ± 1.4	14.1 ± 1.4	0.28
mtDNA copy number ^c				
mtDNA copy number	116.76 ± 32.56	121.73 ± 40.46	134.70 ± 35.84	
	Ref.	β = 3.3, <i>P</i> = 0.74	β = 21.3, <i>P</i> = 0.016	0.024

^aUnadjusted mean ± standard deviation.^bLinear trend was estimated in a GEE model, adjusting for age, sex, and BMI.^cGEE model was used, adjusting for recent infection (β = 33.4, *P* < 0.0001).