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Research Article

The Influence of Genetic Polymorphisms in *XRCC3* and *ADH5* GENES on the Frequency of Genotoxicity Biomarkers in Workers Exposed to Formaldehyde

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The International Agency for Research on Cancer classified formaldehyde as carcinogenic to humans because there is ''sufficient epidemiological evidence that it causes nasopharyngeal cancer in humans''. Genes involved in DNA repair and maintenance of genome integrity are critically involved in protecting against mutations that lead to cancer and/or inherited genetic disease. Association studies have recently provided evidence for a link between DNA repair polymorphisms and micronucleus (MN) induction. We used the cytokinesis-block micronucleus (CBMN assay) in peripheral lymphocytes and MN test in buccal cells to investigate the effects of *XRCC3* Thr241Met, *ADH5* Val309Ile, and Asp353Glu polymorphisms on the frequency of genotoxicity biomarkers in individuals occupationally exposed to formaldehyde ($n = 54$) and unexposed workers ($n = 82$). *XRCC3* participates in DNA double-strand break/recombination repair, while *ADH5* is an important component of cellular metabolism for the elimination of formaldehyde. Exposed workers had significantly higher frequencies ($P < 0.01$) than controls for all genotoxicity biomarkers evaluated in this study. Moreover, there were significant associations between *XRCC3* genotypes and nuclear buds, namely *XRCC3* Met/Met (OR = 3.975, CI 1.053–14.998, $P = 0.042$) and *XRCC3* Thr/Met (OR = 5.632, CI 1.673–18.961, $P = 0.005$) in comparison with *XRCC3* Thr/Thr. *ADH5* polymorphisms did not show significant effects. This study highlights the importance of integrating genotoxicity biomarkers and genetic polymorphisms in human biomonitoring studies. Environ. Mol. Mutagen. 54:213–221, 2013.

Key words: genetic susceptibility; genotoxicity biomarkers; occupational exposure

INTRODUCTION

In June 2004, the International Agency for Research on Cancer (IARC) classified formaldehyde as carcinogenic to humans (Group 1) because there is ''sufficient epidemiological evidence that formaldehyde causes nasopharyngeal cancer in humans'' and also concluded that there was ''strong but not sufficient evidence for a causal association between leukaemia and occupational exposure to formaldehyde'' [IARC, 2006; Zhang et al., 2009].

Epidemiological studies have provided strong evidence for a causal relationship between exposure to formaldehyde and cancer in humans. Causality is indicated by consistent findings of increased risks of nasopharyngeal cancer, sinonasal cancer, and lymphohematopoietic cancer, specifically myeloid leukemia [Zhang et al., 2010a], among individuals with higher measures of exposure to formaldehyde (exposure level or duration), which cannot be explained by chance, bias, or confounding alone [National Toxicology Program, 2011]. However, some studies led to mixed results and inconclusive evidence [Franks, 2005] prompting a re-evaluation of former studies that had suggested a causal association between form-

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aldehyde exposure and mortality from leukemia, myeloid leukemia and nasopharyngeal cancer [Marsh and Youk, 2004; Marsh et al., 2010; Rhomberg et al., 2011].

In spite of some controversy, studies in humans have demonstrated that inhaled formaldehyde can cause genotoxicity in lymphocytes, including DNA-protein cross-links, DNA strand breaks, micronucleus formation, and chromosomal aberrations [National Toxicology Program, 2011]. The cytokinesis-block micronucleus (CBMN) assay is frequently used in biomonitoring studies and can be considered as a ''cytome'' assay covering chromosomal changes, such as micronuclei (MN), nucleoplasmic bridges, and nuclear buds. The detection of MN is extensively used in molecular epidemiology as a biomarker of chromosomal damage, genome instability, and increased cancer risk. The occurrence of MN represents an integrated response to chromosome-instability and altered cellular viabilities caused by genetic defects and/or exogenous exposures to genotoxic agents [Hedberg, 2001]. MN contain either acentric chromosomal fragments formed by unrepaired double-strand breaks, or lagged chromosomes that have failed to segregate into a daughter macronucleus during mitosis [Fenech et al., 1999; Fenech, 2002; Mateuca et al., 2006; Iarmarcovai et al., 2006, 2008]. Nucleoplasmic bridges (NBP) are biomarkers of dicentric chromosomes resulting in telomere end-fusions or DNA misrepair [Fenech et al., 2002; Thomas et al, 2003; Fenech, 2005, 2006]. These events occur when centromeres of dicentric chromosomes are pulled to opposite poles of the spindle during anaphase. Nuclear buds (NBUD) are characterized by the same morphology as MN, except that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmatic material depending on the stage of the nuclear budding process. They are considered as biomarkers of the elimination of amplified DNA and/or DNA repair complexes [Tolbert et al., 1991; Fenech et al., 2002; Thomas et al., 2003; Fenech, 2006]. Our previous studies reported evidence that long-term exposures to formaldehyde and high peak formaldehyde concentrations are associated with an increase in the frequency of MN in lymphocytes and exfoliated buccal mucosa cells in workers at anatomy and pathology laboratories and at formaldehyde-resins production factories [Viegas et al., 2010] and with an increase in MN, NPB and NBUD in workers at histopathology laboratories [Ladeira et al., 2011].

Recently, association studies have linked genotypes, which account for interindividual differences in the response to genotoxic exposure, to the occurrence of MN as a measure of genetic damage due to environmental exposures [Dhillon et al., 2011]. Polymorphisms in various genes involved in DNA repair, activation/deactivation of carcinogens/chemicals/drugs/alcohol, folate metabolism pathway, and micronutrient transport have all been shown to affect MN formation [Dhillon et al., 2011]. Genes involved in DNA repair and maintenance of genome integrity are critically involved in protecting against DNA lesions that lead to cancer and/or inherited genetic disease [Matullo et al., 2001]. Single-nucleotide polymorphisms (SNPs) in these genes are recognized as potential cancer susceptibility factors [Figueiredo et al., 2004]. Molecular epidemiology studies have shown that the inheritance of certain genetic variants at one or more loci results in a reduced DNA repair capacity and an increase in the individual risk of cancer [Winsey et al., 2000; Matullo et al., 2001].

The X-ray repair cross-complementing gene 3 (*XRCC3*) participates in DNA double-strand break/recombination repair and is a member of an emerging family of Rad-51-related proteins that participate in the homologous recombination (HR) pathway to maintain chromosome stability, repair DNA damage, and correct chromosome segregation in mammalian cells [Bolognesi et al., 1999; Catalán et al., 2000; Matullo et al., 2001; Bonassi et al., 2003; El-Zein et al., 2006; Kirsch-Volders et al., 2006; Iarmarcovai et al., 2006; Battershill et al., 2008; Mateuca et al., 2008]. This pathway is of great importance in preventing chromosomal fragmentation, translocations, and deletions, which can lead to carcinogenesis [Winsey et al., 2000]. The Rad 51 paralogue XRCC3 promotes the HR repair of double strand breaks induced either directly or indirectly following replication of closely spaced single strand breaks [Mateuca et al., 2008]. XRCC3 is required for the assembly and stabilization of Rad51 [Winsey et al., 2000; Shen et al., 2002]. In addition to repairing double strand-breaks, XRCC3 also plays a role in the repair of more global DNA damage arising from carcinogen treatment [Araujo et al., 2002].

XRCC3 is located on chromosome 14 (14q32.3) and its most studied polymorphism is a transition between cytosine and thymine in exon 7 (*XRCC3*-18067C> T) at codon 241 that results in the substitution of a threonine by a methionine [Bonassi et al., 2003; Wang et al, 2003; Battershill et al., 2008; El-Zein et al., 2008]. The XRCC3 Thr241Met variation does not reside in the adenosine triphosphate-binding domain, the only functional domain identified in the protein [Manuguerra et al., 2006]; however, conversion from a hydroxyl amino acid to one with a sulfhydryl group represents a substantial change in protein functional characteristics [Winsey et al., 2000]. This polymorphism has been proposed as an allele of low penetrance associated with breast and lung cancer, acute myeloid leukemia, risk of upper aerodigestive tract cancer [Stich and Rosin, 1983; Ramirez and Saldanha, 2002; El-Zein et al., 2008] and risk for melanoma skin cancer and bladder carcinoma [Wang et al., 2003]. Mammalian alcohol dehydrogenases (ADH; EC 1.1.1.1.) are zinc-containing dimeric enzymes that catalyze the reversible oxidation of a wide variety of alcohols, using NAD^+ as the preferred coenzyme. They form a gene family divided into at least five distinct classes with about 60% amino acids in common as identified in interclass comparisons [Hur et al., 1992]. Alcohol dehydrogenase 5 (ADH5), originally known as formaldehyde dehydrogenase (FDH), differs in cat-

alytic profile from all other alcohol dehydrogenases because it appears to have no ethanol oxidation activity and its best known substrate is S-nitrosoglutathione [Wu et al., 2007]. ADH5 is composed of nine exons and eight introns [Hur et al., 1992] and is located on chromosome 4 (4q23) [Just et al., 2001] and has been detected in all human tissues and at all stages of development. This is the only ADH identified thus far that is capable of oxidizing formaldehyde in a glutathione dependent reaction [Kaiser et al., 1991; Engeland et al., 1993; Lee et al., 2003]. ADH5 is an important component of cellular metabolism for the elimination of formaldehyde serving as the prime guardian against formaldehyde [Hedberg, 2001] and offering enzymatic defence against both formaldehyde and nitrosative stress in human oral tissue and in epithelial cell lines. Although formaldehyde is rapidly metabolized, it is an electrophile that reacts with a variety of endogenous molecules, including glutathione, proteins, nuclei acids, and folic acid [National Toxicology Program, 2011].

Two ADH5 polymorphisms are known: *ADH5* Val309Ile, a transition of a cytosine to a thiamine in codon 309 that consists in the substitution of a valine by an isoleucine; and *ADH5* Asp353Glu, a transversion of an adenine to a cytosine in codon 353 that results in the substitution of an aspargine by a glutamine. To our knowledge, no association has been found between ADH5 polymorphisms and disease [Wang et al., 2010].

The CBMN assay was extensively used over the past decade in molecular epidemiology studies [El-Zein et al., 2006; Fenech, 2006; Battershill et al., 2008]. It was based upon this technique that Dhillon et al. [2011] suggested that the genotype might influence the frequency of MN in lymphocytes and that NPB and NUBD measurements should be investigated with regard to the impact of genotype on these biomarkers. In previous reports, we have provided evidence for an association between exposure to formaldehyde and genotoxicity biomarkers [Viegas et al., 2010; Ladeira et al., 2011]. In this study we focus upon the association between genotoxicity biomarkers and genetic polymorphisms in key genes involved in DNA repair and formaldehyde metabolism. The goal of this study is to compare individuals occupationally exposed to formaldehyde and matched controls with regard to the effects of *XRCC3* Thr241Met, *ADH5* Val309Ile and Asp353Glu polymorphisms on the frequency of genotoxicity biomarkers detected by the CBMN assay in peripheral lymphocytes and the MN test in buccal cells.

MATERIALS AND METHODS

Subjects

This study was conducted with a group of 54 workers occupationally exposed to formaldehyde at six histopathology hospital laboratories in Portugal (Lisbon and Tagus Valley region), and a group of 82 administrative staff members with no known exposure to formaldehyde.

Ethical approval was obtained from the Institutional Ethical Board and Service Director of the hospitals, and all subjects gave informed consent to participate. Each person answered a questionnaire aimed at identifying exclusion criteria such as a history of cancer, radio or chemotherapy, use of therapeutic drugs, exposure to diagnostic X-rays in the past six months, intake of vitamins or supplements like folic acid, as well as information related to working practices, such as years of employment and the use of protective measures.

Environmental Monitoring of Formaldehyde Exposure

Exposure assessment was based on two techniques of air monitoring conducted simultaneously [Viegas et al., 2010]. First, environmental samples were obtained by air sampling with low flow pumps for 6–8 hr during a typical working day. Formaldehyde levels were measured by gas chromatography analysis and time-weighted average (TWA_8) was estimated according to the National Institute of Occupational Safety and Health method - NIOSH 2541 [NIOSH, 1994].

The second method was aimed at measuring ceiling values of formaldehyde using Photo Ionization Detection (PID) equipment (11.7 eV lamps) with simultaneous video recording [McGlothlin, 2005]. Instantaneous values of FA concentration were obtained on a per second basis. A relationship could thus be established between worker activities and ceiling values and the main sources of exposure could be identified.

Genotoxic Effects Evaluation

Evaluation of genotoxic effects was conducted by applying the CBMN assay in peripheral blood lymphocytes and the MN test in exfoliated cells from the buccal mucosa. Whole blood and exfoliated cells (buccal mucosa cells) were collected from each subject between 10 A.M. and 12 P.M. and were processed for testing. All samples were coded and analyzed under blind conditions. The criteria for scoring the nuclear abnormalities in lymphocytes and MN in the buccal cells were the ones described by Fenech et al. [1999] and Tolbert et al. [1991], respectively.

Cytokinesis-Block Micronucleus Assay

Heparinized blood samples were obtained by venipuncture from all subjects and freshly collected peripheral blood was used for the CBMN assay. Lymphocytes were isolated using a Ficoll-Paque gradient and placed in RPMI 1640 culture medium with L-glutamine and phenol red added with 10% inactivated fetal calf serum, 50 μg/ml streptomycin + 50U/mL penicillin, and 10 μg/mL of phytohaemagglutinin. Duplicate cultures from each subject were incubated at 37°C in a humidified 5% CO_2 incubator for 44 h, and 6 μg/mL cytochalasin B was added to the cultures to prevent cytokinesis. After 28 h incubation, cells were spun onto microscope slides using a cytocentrifuge. Smears were air-dried and double stained with May-Grünwald-Giemsa and mounted with Entellan®. One thousand cells were scored from each individual by two independent observers on two slides. Each observer visualized 500 cells/individual.

Buccal Mucosa Cells

Cells from the buccal mucosa were collected with an endobrush swab. Exfoliated cells were smeared onto slides and fixed with Mercofix®. The Feulgen staining technique without counterstain was used. Two thousand cells were scored from each individual by two independent observers on two slides. Each observer visualized 1,000 cells/individual. Only cells that were neither clumped nor overlapped and contained intact nuclei were included in the analysis.

Polymorphisms Analysis

Whole blood samples were collected and stored at −20°C until total white blood cell DNA was extracted using the standard protocol of phenol-chloroform. The *XRCC3* Thr241Met (rs861539), ADH5 Val309Ile (rs28730628), and *ADH5* Asp353Glu (rs16996593) polymorphisms were

TABLE I. Comparison of the Two Samples

	Control group	Exposed group	*P*-value
Number of subjects	82	54	
Sex			
Female	53 (64.6%)	35 (64.8%)	0.983
Male	29 (35.4%)	19 (35.2%)	
Age			
(mean ±standard deviation, in years)	32.79±8.03	39.80±11.56	<0.001
Range	20-53	20-61	
Tobacco consumption			
Non-smokers	57 (69.5%)	43 (79.6%)	0.191
Smokers	23 (30.5%)	11 (20.4%)	
Alcohol consumption			
Non-drinkers	18 (22%)	18 (33.3%)	0.141
Drinkers	64 (78%)	36 (66.7%)	

Descriptive statistics and *P*-value of Qui-square test for sex, tobacco and alcohol consumption and *t*-test of independent samples for age.

determined using the TaqMan SNP genotyping assay with Real Time PCR (Applied Biosystems).

To perform the genotype analysis of *XRCC3* and *ADH5* polymorphisms the target fragments were amplified in 20 µl reaction mixture containing 10 µl *TaqMan Universal PCR Master Mix*, 1 µl primers, 5 µl Miliq water, and 4 µl DNA. Real Time PCR, *iCycler iQ® Multicolor Real-Time PCR Detection System* (BIO-RAD), was then conducted as follows: 2 min of the initial step at 50°C, 30 sec and 10 min at 95°C, 50 cycles of 15 sec and 1 min at 92°C and 60°C, respectively, and a final temperature stay at 4°C. All inconclusive samples were reanalyzed.

Statistical Analysis

Variables were compared with the Normal distribution using the Shapiro-Wilk test. Rejection of the null hypothesis of underlying normality led us to proceed with nonparametric procedures to compare means and check associations. The association between each of the genotoxicity biomarkers, occupational exposure to formaldehyde, and *XRCC3* and *ADH5* polymorphisms was evaluated by binary logistic regressions. The biomarkers were dichotomized (absent/present) and considered the dependent variable, taking absence as the reference. Occupational exposure, genetic polymorphisms of XRCC3, and ADH5 were considered independent variables.

Each genetic polymorphism has three possible genotypes. Two dummy variables were considered for each polymorphism and the last category was taken as the reference, namely, the Thr/Thr genotype for *XRCC3* Thr241Met; the Val/Ile genotype for *ADH5* Val309Ile; and the Asp/Glu genotype for *ADH5* Asp353Glu.

The nonparametric Mann-Whitney and Kruskal-Wallis tests were also used to compare the groups. Statistical analyses were performed using the SPSS package for Windows, version 19.0. The analysis of genotype and allele frequency and Fisher-exact test was made with the GenPop program.

RESULTS

Population characteristics such as sex, age, tobacco habits, and alcohol consumption for the control and exposed groups are shown in Table I. The frequencies of genotypes and alleles of the studied polymorphisms in the two study populations are shown in Table II. No significant differences were observed in genotype and allele frequencies for the three polymorphisms analysed (Fisher's exact test, $P > 0.05$).

TABLE II. Frequency of Genotypes and Alleles in the Study Samples (P-Value of Fisher-Exact Test)

Genes	Genotypes	All	Exposed	Controls	*P*-value
XRCC3	Met/Met	33 (24.3%)	13 (24.1%)	20 (24.4%)	0.669
Met 241Thr	Met/Thr	49 (36.0%)	22 (40.7%)	27 (32.9%)	
	Thr/Thr	54 (39.7%)	19 (35.2%)	35 (42.7%)	
	Met	115 (0.423)	48 (0.44)	67 (0.409)	0.621
	Thr	157 (0.577)	60 (0.56)	97 (0.591)	
ADH5	Val/Val	50 (36.8%)	21 (38.9%)	29 (35.4%)	0.719
Val309Ile	Val/Ile	86 (63.2%)	33 (61.1%)	53 (64.6%)	
	Val Ile	186 (0.684)	75 (0,694)	111 (0,677)	0.795
		86 (0.316)	33 (0,306)	53 (0,323)	
ADH5	Asp/Asp	59 (43.4%)	24 (44.4%)	35 (42.7%)	0.863
Asp353Glu	Asp/Glu	77 (56.6%)	30 (55.6%)	47 (57.3%)	
	Asp	195 (0.717)	78 (0,722)	117 (0,713)	
	Glu	77 (0.283)	30 (0,278)	47 (0,287)	0.892

Formaldehyde Exposure

The mean level of formaldehyde exposure of the 54 exposed individuals was 0.16 ppm (min–max: 0.04–0.51 ppm), a value below the Occupational Safety and Health Administration (OSHA) reference of 0.75 ppm. The mean ceiling concentration found in the laboratories was 1.14 ppm (min–max: 0.18–2.93 ppm), a value well above the 0.3 ppm reference of the American Conference of Governmental Industrial Hygienists (ACGIH) for ceiling concentrations. The highest formaldehyde concentration was observed during macroscopic examination of biological samples by the exposed workers.

The effect of formaldehyde exposure on the frequencies of genotoxicity biomarkers is shown in Table III. Significant increases ($P < 0.001$ for lymphocytes and $P = 0.006$ for buccal mucosa cells) were found in the exposed workers relative to controls for all the genotoxicity biomarkers examined.

XRCC3 Polymorphisms

Results of binary logistic regression provided evidence for a statistically significant association between *XRCC3* polymorphisms and NBUD. Specifically, *XRCC3* Met/Met (OR = 3.975, $CI_{95\%}$ 1.053–14.998, $P = 0.042$) and *XRCC3* Thr/Met (OR = 5.632, $CI_{95\%}$ 1.673–18.961, $P = 0.005$) are risk factors for NBUD in comparison with *XRCC3* Thr/Thr. As shown in Table IV, lower means of NBUD were found in carriers of Thr/Thr polymorphism for both in exposed and controls. All the other biomarkers showed higher means in exposed workers, however, no increase was statistically significant.

TABLE III. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Two Samples (Mean ± Mean Standard Error, Range, and P-Value of Mann-Whitney Test)

	Mean, MN lymphocytes ± S.E. (range)	Mean, NPB±S.E. (range)	Mean, NBUD±S.E. (range)	Mean, MN buccal cells ±S.E. (range)
Controls	0.83 ± 0.18 (0–7)	0.18 ± 0.06 (0–3)	0.07 ± 0.03 (0–1)	0.17 ± 0.06 (0–2)
Exposed	4.00 ± 0.52 (0–14)	3.1 ± 0.54 (0–13)	0.79 ± 0.3 (0–5)	1.0 ± 0.267 (0–9)
P-value	<0.001	<0.001	<0.001	0.006

TABLE IV. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by XRCC3 Met241Thr Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	XRCC3	*N*	Mean MN lymphocytes ± S.E.	Mean NPB± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
	Met/Met	13	2.92 ± 0.93 (0–12)	2.00 ± 1.14 (0–15)	0.38 ± 0.18 (0–2)	1.00 ± 0.71 (0–9)
Exposed	Thr/Met	22	5.05 ± 0.98 (0–14)	3.91 ± 0.84 (0–13)	1.50 ± 0.33 (0–2)	1.05 ± 0.38 (0–5)
	Thr/Thr	19	3.53 ± 0.80(0–12)	2.95 ± 0.90(0–13)	0.21 ± 0.12(0–2)	0.95 ± 0.51(0–8)
	P–value		0.372	0.156	0.002	0.733
	Met/Met	20	1.15 ± 0.46 (0–7)	0.25 ± 0.12 (0–2)	0.2 ± 0.09 (0–1)	0.25 ± 0.14 (0–2)
Controls	Thr/Met	27	0.70 ± 0.30 (0–6)	0.15 ± 0.12 (0–3)	0.04 ± 0.04 (0–1)	0.11 ± 0.82 (0–2)
	Thr/Thr	35	0.74 ± 0.23 (0–6)	0.14 ± 0.07 (0–2)	0.03 ± 0.29 (0–1)	0.17 ± 0.10 (0–2)
	p-value		0.621	0.450	0.045	0.664

TABLE V. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by ADH5 Val309Ile Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	ADH5	*N*	Mean MN lymphocytes ± S.E.	Mean NPB± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
Exposed	Val/Val	21	2.57 ± 0.65 (0–11)	3.19 ± 0.89 (0–14)	0.62 ± 0.28 (0–5)	0.95 ± 0.41 (0–6)
	Val/Ile	33	4.91 ± 0.75 (0–14)	3.06 ± 0.69 (0–15)	0.88 ± 0.21 (0–5)	1.03 ± 0.39 (0–9)
Controls	*P*–value		0.024	0.957	0.274	0.713
	Val/Val	29	0.97 ± 0.28 (0–6)	0.17 ± 0.07 (0–1)	0.00 ± 0.00 (0)	0.14 ± 0.10 (0–2)
	Val/Ile	53	0.75 ± 0.23 (0–7)	0.17 ± 0.08 (0–3)	0.11 ± 0.04 (0–1)	0.19 ± 0.08 (0–2)
	P-value		0.176	0.370	0.061	0.546

TABLE VI. Descriptive Statistics of MN in Lymphocytes and Buccal Cells, NPB, and NBUD Means in the Studied Population (Mean ± Standard Error, Range) by ADH5 Asp353Glu Polymorphisms and Exposure to Formaldehyde (P-Value of Kruskal-Wallis Test)

Groups	ADH5	*N*	Mean MN lymphocytes ± S.E.	Mean NPB± S.E.	Mean NBUD± S.E.	Mean MN buccal cells ± S.E.
Exposed	Asp/Asp	24	4.08 ± 0.91 (0–14)	4.21 ± 0.96 (0–15)	0.71 ± 0.23 (0–3)	0.92 ± 0.37 (0–6)
	Asp/Glu	30	3.93 ± 0.67 (0–12)	2.23 ± 0.57 (0–14)	0.83 ± 0.25 (0–5)	1.07 ± 0.43 (0–9)
	P-value		0.700	0.217	0.740	0.983
Controls	Asp/Asp	35	0.86 ± 0.23 (0–6)	0.29 ± 0.12 (0–3)	0.06 ± 0.04 (0–1)	0.29 ± 0.12 (0–2)
	Asp/Glu	47	0.81 ± 0.26 (0–7)	0.09 ± 0.04 (0–1)	0.09 ± 0.04 (0–1)	0.09 ± 0.05 (0–2)
	P-value		0.211	0.204	0.633	0.202

ADH5 Polymorphisms

The descriptive statistics concerning the relationship between genotoxicity biomarkers and the two *ADH5* polymorphisms studied is shown in Tables V and VI. In this study, we did not find any individuals homozygous for the variant allele of the two *ADH5* polymorphisms investigated. Results of binary logistic regression did not show statistically significant associations between *ADH5* polymorphisms and the genotoxicity biomarkers studied. However, a borderline significant association ($P = 0.06$) was found with NBUD, as the Asp/Asp genotype had lower means than the Asp/Glu genotype. As shown in Table V, there was a statistically significant difference between Val/Val and Val/Ile genotypes for the *ADH5* Val309Ile polymorphism in the exposed group (Kruskal-Wallis, $P =$

0.024) with carriers of the heterozygote genotype having higher mean values than the homozygotes.

DISCUSSION

Exposure to formaldehyde in occupational settings is often prolonged enough to lead to the accumulation of DNA damage and increase in mutation risk [Mateuca et al., 2006]. Previous studies have suggested that genetic polymorphisms in specific genes affect chromosome damage levels associated with environmental exposures to genotoxic agents [Umegaki et al., 2000]. Genetic polymorphisms are potentially important in MN formation, depending on level of exposure, biological matrix studied and ethnicity of the studied population [Umegaki et al., 2000]. Chromosomal instability and impaired cell viability have been correlated with XRCC3 mutations and several other genes known or thought to be involved in HR [Bolognesi et al., 1999; Brenneman et al., 2000]. Previous studies have revealed a requirement for the HR pathway in processing DNA damage induced by formaldehyde [Zhang et al., 2010b].

In this study, we report a statistically significant association between XRCC3 Thr241Met polymorphism and NBUD. The carriers of the *XRCC3* Met/Met and Thr/Met genotypes had higher NBUD frequencies than their Thr/Thr genotype counterparts. Gene amplification plays a crucial role on the malignant transformation of human cells as it mediates the activation of oncogenes or the acquisition of drug resistance [Utani et al., 2007]. Excess DNA may be expelled from the nucleus by the formation of NBUD and subsequent micronucleation [Lindberg et al., 2007]. Studies have described in vivo budding of nuclear material in cell lines where changes in chromosomal numbers were occurring, and the spontaneous formation of NBUD structures was seen as a possible mechanism for the loss of chromosomes and for the generation of MN [Fenech et al. 2011]. Therefore, NBUD should also be considered genotoxic biomarkers with an origin comparable with that of MN [Serrano-García and Montero-Montoya, 2001].

Previous studies have shown that carriers of the XRCC3 heterozygous genotype had increased levels of chromatid breaks and sister-chromatid exchanges in smokers and increased DNA adducts in lymphocytes [Fenech et al., 1999] suggesting that this polymorphism is associated with low DNA repair capacity and may increase the risk of many types of cancer [Benhamou et al., 2004; Han et al., 2006; Batershill et al., 2008]. Studies from Yoshihara et al. [2004] and Lindh et al. [2006] suggested that XRCC3 Thr241Met variants contribute to the induction of MN arising from chromosome loss. Carriers of the Met/Met alleles would present higher MN frequencies than their wild-type Thr/Thr allele counterparts [Mateuca et al., 2008]. A significant increase of MN frequency in the Thr/Met genotype of XRCC3 was reported in workers exposed to oil from the Prestige accident, indicating that this polymorphism must be taken into account in chronic exposure scenarios [Pérez-Cadahía et al., 2008]. Shen et al. [2002] suggested that the Met/Met genotype may contribute to a subset of squamous cell carcinoma of the head and neck and Figueiredo et al. [2004] found that both carriers of Met/Met and Thr/Met genotypes have an increased risk for breast cancer. The Met/Met genotype may cause genetic instability and lead to an increased susceptibility to various cancers due to the inability of genotype carriers to complement the centrosome amplification defect and to a decrease of apoptotic rates [Lindt et al. 2006], factors that may prevent aberrant cells from entering apoptosis. However, other studies did not find evidence for the influence of XRCC3 genotype in the MN basal frequency [Iarmarcovai et al., 2006].

The functional differences between the XRCC3 alleles are not entirely understood. The amino acid substitution of a threonine by a methionine has the potential to affect protein structure and integrity [Dhillon et al., 2011]. Variants leading to diminished XRCC3 function may be predicted to confer an increased risk of cancer due to accumulated levels of DNA damage. As many genes are involved in the repair of DNA damage, there is also the possibility that these polymorphisms might be in linkage disequilibrium with other causative factors [Figueiredo et al., 2004].

Our study did not provide conclusive evidence that some *ADH5* polymorphisms may influence the carrier's capacity to protect against DNA damage. A borderline association ($P = 0.06$) was found between the frequency of NBUD and the homozygous Asp/Asp genotype, as compared to the Asp/Glu heterozygous genotype. These individuals may be more prone to nuclear alterations following a possible alteration in formaldehyde metabolism and adduct formation. Another interesting result was the statistically significant difference in carriers of the Val/Ile genotype in comparison with Val/Val genotype of the ADH5 Val309Ile polymorphism in MN in lymphocytes in the exposed group. The carriers of the heterozygous genotype showed higher means of MN in lymphocytes in the exposed group but not in the control group suggesting that the carriers of Val/Ile genotype metabolize poorly formaldehyde and present more DNA damage. Our results are in agreement with the findings of Just et al. [2011], who investigated three different polymorphisms in the transcribed regions of *ADH5* for inter-individual differences against the genotoxicity of formaldehyde in the German population and found no biologically relevant variants. The biological significance of ADH5 polymorphisms in relation to disease remains uncertain.

A better understanding of MN induction driven by genetic polymorphism affecting DNA repair and/or genome stability, in particular XRCC3 Thr241Met, requires larger scale studies and the assessment of other relevant polymorphism interacting with individual DNA repair capacity [Mateuca et al., 2008]. The association between

SNPs in relevant genes and the frequency of MN in lymphocytes is a valuable tool for this purpose, as the latter is one of the best validated DNA damage biomarker known to be sensitive to a wide range of endogenous, environmental, and lifestyle factors that can harm the genome [Dhillon et al., 2011]. Some genetic polymorphisms of xenobiotic-metabolizing enzymes have been observed to influence the level of genotoxic damage in humans. This may facilitate the identification of risk groups and increase the sensitivity of biomarkers in biomonitoring [Norppa, 2001]. However, studies that report an association between genotypes and biomarkers, such as MN, have some limitations in design and analysis. Common limitations are group sample size, usually too small to evaluate rare polymorphisms, and the wide range of allele frequency variation for each genotype in different ethnic populations. The statistical analysis is often plagued with problems of lack of power (due to insufficient sample size) and confounding can seldom be precluded given the amount of potential factors involved that have not been measured [Chung et al., 2010; Hunter, 2005].

In conclusion, this study showed that occupational exposure to formaldehyde increased the frequencies of genotoxicity biomarkers. Our results showed a significant statistical association between XRCC3 Thr241Met polymorphism and NBUD. ADH5 polymorphisms did not show significant association with the genotoxicity biomarkers studied. Several association studies have recently addressed the link between DNA repair polymorphism and MN induction, but the evidence that DNA repair polymorphisms influence MN frequencies remains limited [Mateuca et al., 2008]. This study highlights the importance of applying biomarkers of effect, such as genotoxicity biomarkers, and individual susceptibility biomarkers, such as genetic polymorphisms, to human biomonitoring studies in occupational exposure settings.

AUTHOR CONTRIBUTIONS

C. Ladeira, M.C. Gomes, and M. Brito conceived the idea and designed the study. C. Ladeira performed de CBMN assay and MN test for the assessment of the genotoxicity biomarkers and Real Time PCR analysis for genetic polymorphisms analysis. S. Viegas performed the exposure assessment. E. Carolino performed the statistical data analysis. All authors have read and approved the final manuscript.

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