Influence of vitamin intake and *MTHFR* polymorphism on the levels of DNA damage in tobacco farmers

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ABSTRACT

Background: Genetic damage may occur spontaneously under normal metabolic circumstances, inadequate intake of nutrients, and excessive exposure to environmental mutagens. **Objectives:** To evaluate the influence of the intake of micronutrients vitamin B₁₂, vitamin B₆, and folate and of the polymorphism methylenetetrahydrofolate reductase (*MTHFR*) C677T on the induction of DNA damage in tobacco farmers. **Methods:** The study involved 66 men and 44 women engaged in tobacco cultivation in the region of Venâncio Aires (Rio Grande do Sul state, Brazil). Peripheral blood samples were collected to analyze DNA damage using the Comet assay, the micronucleus (MN) test and *MTHFR* C677T polymorphism. Dietary intake was evaluated based on the mean values obtained from three 24-h diet recall questionnaires, and nutrient intake data were computerized and estimated in the Food Processor SQL 10.9 program. The statistical tests used to generate the stated results were Kruskal–Wallis test, Exact Fisher's test, and multivariate linear regression analysis. **Results:** DNA damage was significantly higher in individuals who had an inadequate intake of folate, vitamin B₁₂, and vitamin B₆ ($P < 0.01$) assessed by Comet assay. In relation to MN test results, buccal cells showed MN frequency higher in individuals with inadequate intake of vitamin B₆ ($P < 0.01$). No difference was observed in MN lymphocytes frequency. No significant association was detected between *MTHFR* C677T polymorphism and DNA damage in tobacco farmers. **Conclusion:** Our results suggest that folate, vitamin B₁₂, and vitamin B₆ deficiency may be associated with genotoxic effect in individuals exposed to pesticides.

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Introduction

As research areas that recently attracted the attention of the scientific community, nutrigenomics and nutri-genetics seek to understand the effects of diet and food as genetic response modulators. The effects of deficiency and the imbalance of certain nutrients as well as the toxic concentrations of dietary compounds have been the subject of research on nutrition. In this scenario, gene function and expression have been explored in studies with different diets to understand these effects based on research on interventions and the use of biomarkers [1,2].

Folate and other B-complex vitamins perform key functions in the biological processes essential to health. Dark green vegetables, cereals, fruit, pulses, and meat contribute to folate intake [3,4]. Besides dietary intake, the levels of folate in the organism are influenced by dietary supplementation and by the variation of genes encoding enzymes involved in folate metabolism [5,6].

The role of micronutrients, especially B-complex vitamins, in maintaining genomic stability has been discussed in numerous studies. It has been established that micronutrients maintain genome stability by regulating DNA synthesis, repair, and methylation [7]. Folate deficiency is associated with increased DNA damage, i.e. chromosomal aberrations and micronucleated cells. However, the role of the genetic susceptibility as well as the inadequate consumption of these micronutrients and/or their cofactors in the DNA damage observed is surrounded by controversies [8–10].

The enzyme methylenetetrahydrofolate reductase (*MTHFR*), encoded by gene *MTHFR*, converts 5,10-methylene-THF into 5-methyl-THF, generating the methyl groups that will be used for DNA methylation and nucleotide synthesis. By comparison with the homozygous CC genotype, the activity of enzyme *MTHFR* is 70% and 35%, lower in individuals who are homozygous for the T allele and heterozygous individuals, respectively [11]. Significant association between genomic instability and polymorphism of

MTHFR, folic acid levels, and the riboflavin cofactor has been demonstrated [12].

Chromosome instability has been observed in individuals exposed to occupational risk. Chromosomal aberrations, sister-chromatid exchanges, and micronuclei (MN) were described in most studies about exposure to pesticides [13]. Exposure to pesticides and other chemical compounds used in agriculture is acknowledged as toxic to human health. The effects of short- or long-term exposure may be mild or severe [14]. The amounts used and the frequency pesticides are applied to protect plants against insects and diseases may cause poisoning, skin diseases, irritation of the skin and eyes, kidney damage, as well as nervous and respiratory system problems [15,16]. Studies using the Comet assay and the MN test have demonstrated the genotoxic and mutagenic effects of pesticides on populations exposed to the variables associated with tobacco cultivation [17–20]. Tobacco farmers are routinely exposed to a complex mixture of organic and inorganic pesticides and to nicotine present in the tobacco leaf. These mixtures, including dust and liquids containing toxic and carcinogenic substances, enter the organism via various exposure pathways, such as inhalation and dermal absorption [17,18].

Therefore, environmental exposure levels have to be assessed considering the effects of inadequate intake of micronutrients on the diet of occupationally exposed populations. Thus, the purpose of our study was to evaluate the influence of an adequate intake of micronutrients like folate, vitamin B₆, and vitamin B₁₂, and the role of *MTHFR C677T* polymorphism in the incidence of DNA damage in a population occupationally exposed to pesticides.

Materials and methods

Subjects

The sample was composed of 110 individuals (66 males and 44 females) living in the city of Venâncio Aires, south-central state of Rio Grande do Sul, Brazil. These individuals presented increased DNA damage in previous research, which confirmed occupational risk due to activities in tobacco production [17–20]. The individuals participated voluntarily and could drop out of the study at any time. The study was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, and written informed consent was obtained from each individual before the research started. Blood samples were collected by venipuncture using vacutainers with heparin or ethylenediaminetetraacetic acid and processed as quickly as possible in order to prevent damage associated with storage; blood cell samples were transported to the laboratories at or below 8°C and processed

(DNA extraction) within 24 h of collection. Buccal cell samples were obtained by gently rubbing the inside of the cheeks (right and left sides) with a cytobrush. The cytobrush was immersed in 5 mL of cold saline (0.9% (w/v) aqueous NaCl) in a conical tube and transported under refrigeration to the laboratory. The samples were collected during pesticide application on tobacco crops from July 2008 to February 2010.

Anthropometric measurement and questionnaires, and assessment of vitamin intake

A form was used to collect data about age, gender, smoking habit, dietary restrictions, alcohol consumption, and use of medication. Individuals who consumed at least 1.0 g/kg/day, between 1.0 and 3.0 g/kg/day, and more than 3.0 g/kg/day of alcohol were considered light, moderate, and heavy drinkers, respectively. Individuals who smoked at least 3 cigarettes/day were considered smokers. Some individuals reported having stopped smoking, and did not smoke any cigarettes in the past 5 years. In addition, dietary consumption was obtained using questionnaires. Body fat was determined based on the body mass index (BMI) calculated using the equation:

$$\text{BMI} = \frac{W(\text{kg})}{E^2(\text{m})}, \quad (1)$$

where *W* is the weight in kilogram and *E* is the square of the body height (in square meters).

The values were classified according to the criteria defined by the World Health Organization for adults and the elderly [21]. Three 24-h dietary recalls (R24h) were used at 4-month intervals on different days of the week to determine consumption of sources of folate, vitamin B₁₂, and vitamin B₆. The R24h data were converted into nutrient information using the Food Processor SQL 10.9 program (ESHA Research, Salem, OR) program, whose main database is the North American table of the United States Department of Agriculture. The nutritional value of Brazilian foods that were not in the program was included according to official information published in Brazil [22]. The present study included only the micronutrients folate, vitamin B₁₂, and vitamin B₆, and the mean of the three recalls was calculated considering the daily intake of each individual. To standardize and support the identification of the portions, domestic measuring utensils were used (serving spoon, skimmer, ladle, soup spoon, dessert spoon, tea spoon, cup of coffee, and a 190-mL tumbler) besides pictures obtained from the photographic record for dietary enquiries. According to the estimated average requirement, adequate consumption values of folate, vitamin B₁₂, and vitamin B₆ were ≥320 µg/day,

2.0 µg/day, and 1.1 µg/day for adults over 19 years [23].

Genotyping

MTHFR C677T polymorphism was genotyped using the primers 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTGC-3' under the PCR conditions described by Frosst et al. [24]. The wild and variant alleles were detected after digestion with enzyme *HinfI*.

Micronucleus test

MN frequency in lymphocyte cells was analyzed using the cytokinesis-block method with 5% (v/v) Giemsa staining. MN was scored in 2000 binucleated lymphocyte cells according to published criteria [25]. Similarly, 2000 buccal cells (1000 from each of the duplicate slides) were analyzed by two different scorers using identical microscopes (Axiophot, Zeiss, Carl Zeiss, Oberkochen, Germany) (no significant variability was observed between the scorers). All slides were coded for blind analysis.

Comet assay

The Comet assay was performed as described previously [26] with modifications [27]. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed by the same person using bright-field optical microscopy. Two parameters were evaluated: (1) damage index (DI), when each cell was allocated to one of five classes (from no damage = 0 to maximum damage = 4) according to tail size and shape and the values obtained for each volunteer could range from 0 (0×100) to 400 (4×100); and (2) damage frequency (DF), calculated as the percent number of damaged cells. International guidelines and recommendations for the Comet assay consider that visual scoring of comets is a well-validated evaluation method. Although DI is subjective, it is highly correlated with computer-based damage analysis [27,28]. All the slides were scored blind.

Statistical methods

The mean significance of MN frequency, *MTHFR* polymorphism, and adequacy of intake of folate, vitamin B₆, and vitamin B₁₂ was estimated using the Kruskal–Wallis test. The Exact Fisher's test was used to calculate the Hardy–Weinberg equilibrium using the software INSTAT (GraphPad Software Inc., La Jolle, CA, USA). Multivariate linear regression analysis was used to assess the influence of age, gender, smoking habit, drinking status, and intake of micronutrients (independent variables) on DNA damage

(dependent variable) in tobacco farmers. After adjustment, residual analysis was performed to find out whether the adjustment was appropriate. All data are expressed as mean $\pm$ standard deviation, and statistical analyses were calculated using the program SPSS 15.0 (IBM Corporation, Chicago, IL, USA) and the level of significance considered was a *P* value below 0.05.

Results

Analyses included 110 individuals (66 men and 44 women; mean age: 42.3 ± 13.32 years). The reported time of exposure was 30.34 ± 15.35 years. The characteristics of the studied population are shown in Table 1. No worker presented dietary restrictions.

The relationships between DNA damage and adequate dietary intake of micronutrients folate, vitamin B₆, and vitamin B₁₂ are shown in Table 2. The results of the analyses showed that DNA damage was higher in individuals with inadequate consumption of folate, vitamin B₁₂, and vitamin B₆ ($P < 0.01$). When we divided the sample based on adequate consumption (considering folate, vitamin B₁₂, and vitamin B₆ simultaneously), it was observed that subjects with adequate micronutrient intake had lower levels of DI (7.92 ± 4.59 ; mean $\pm$ standard deviation), DF (5.38 ± 2.76), and MN buccal cells (8.00 ± 2.52), when compared to individuals with inadequate micronutrient intake (DI: 18.73 ± 14.84 , $P < 0.001$; DF: 12.55 ± 9.25 , $P < 0.001$; and MN buccal cells:

Table 1. Characteristics of the studied population with respect to body mass index (BMI), personal protective equipment (PPE) utilization, medicine intake, smoking, and alcohol ingestion.

Parameters	No. of subjects (%)
BMI	
Underweight = < 18.5	01 (0.9)
Normal weight = $18.5\text{--}24.9$	42 (38.2)
Overweight = $25\text{--}29.9$	51 (46.4)
Obesity (Class 1) = $30\text{--}34.99$	15 (13.6)
Obesity (Class 2) = $35\text{--}39.99$	01 (0.9)
PPE	
Do not	31 (32.6)
Yes	37 (38.9)
Some	27 (28.4)
Medication	
No medication	85 (77.3)
Vitamins and supplements	04 (3.6)
Antihypertensive drugs	05 (4.5)
Antibiotics	01 (0.9)
Tranquilizing drugs	06 (5.5)
Muscle relaxant drugs	05 (4.5)
Other	04 (3.6)
Smoking	
Never smoked	71 (64.6)
Have stopped smoked	25 (22.7)
Current smoking	14 (12.7)
Frequency alcohol ingestion	
No drinker	45 (41.3)
Little <1.0 g/kg/day	10 (9.2)
Moderately >1.0 to <3.0 g/kg/day	47 (43.1)
Heavy >3.0 g/kg/day	07 (6.4)

Table 2. Distribution of adequacy/inadequacy of folate, B₁₂ and B₆ according to DNA damage (mean $\pm$ standard deviation (n)).

DNA damage	Folate intake			B ₁₂ intake			B ₆ consumption		
	Inadequate (n)	Adequate (n)	P ^a	Inadequate (n)	Adequate (n)	P ^a	Inadequate (n)	Adequate (n)	P ^a
Damage frequency	15.30 $\pm$ 9.02 (67)	4.63 $\pm$ 2.57 (35)	<0.001	16.15 $\pm$ 8.79 (62)	4.65 $\pm$ 2.68 (40)	<0.001	12.81 $\pm$ 9.04 (80)	7.36 $\pm$ 7.69 (22)	0.003
Damage index	23.18 $\pm$ 14.46 (67)	6.20 $\pm$ 3.93 (35)	<0.001	24.47 $\pm$ 14.23 (62)	6.33 $\pm$ 3.90 (40)	<0.001	19.20 $\pm$ 14.87 (80)	10.64 $\pm$ 10.30 (22)	0.005
MN lymphocytes	6.10 $\pm$ 3.53 (58)	8.19 $\pm$ 4.70 (31)	0.060	6.96 $\pm$ 3.87 (56)	6.61 $\pm$ 4.46 (33)	0.389	6.72 $\pm$ 3.80 (71)	7.28 $\pm$ 5.11 (18)	0.906
MN buccal cells	10.13 $\pm$ 4.95 (63)	8.90 $\pm$ 3.65 (29)	0.182	10.13 $\pm$ 4.80 (60)	9.00 $\pm$ 4.18 (32)	0.349	10.40 $\pm$ 4.65 (73)	7.21 $\pm$ 3.41 (19)	0.008

^aTested by non-parametric Mann-Whitney test; n = number of individuals.

10.00 $\pm$ 4.79, $P = 0.036$). The MN frequency and Comet assay parameters did not show any significant correlation with age, gender, smoking habit, years of exposure, and BMI (data not shown).

Corroborating the results above, the multivariate analysis of lifestyle characteristics, micronutrient intake, and effects of exposure on DNA damage revealed the influence of micronutrient intake and alcohol consumption on damage levels. In the Comet assay, the ID is inversely influenced by folate and vitamin B₁₂ intake ($\beta = -.269$, $P = 0.014$ and $\beta = -.425$, $P < 0.001$, respectively), and directly influenced by alcohol consumption ($\beta = .309$, $P = 0.004$). In addition, only vitamin B₆ consumption was inversely correlated with MN buccal frequencies ($\beta = -.297$, $P = 0.016$).

The genotypic distribution of the polymorphism *MTHFR* C677T in tobacco farmers was 36.4% (CC), 45.5% (CT), and 18.2% (TT). The T allelic frequency was 0.41. Genotypic distribution was consistent with the Hardy-Weinberg equilibrium ($P_{HWE} = 0.53$). No significant associations were found between DNA damage and polymorphism *MTHFR* C677T (Table 3).

Discussion

The relationship between nutrition, exposure to xenobiotics, and genetic response modulators is complex and may affect similar biological paths. Interindividual differences in the ability to activate and detoxify substances and repair damage to DNA may explain the individual susceptibility to DNA damage after exposure to xenobiotics [16,29]. Furthermore, nutrition is also an important factor that may modulate the toxicity of different agents [30,31].

Our findings highlight the difference between the consumption of micronutrients folate, vitamin B₆, and vitamin B₁₂ and recommended current dietary intake reported in population studies based on age and gender [32]. Consuming adequate portions of various food groups according to recommendations for age and gender appears to reflect the diet pattern of the population studied (tobacco workers in the rural area of Venâncio Aires, RS, Brazil). Data published in the study carried out by the Third National Health and Nutrition Examination Survey indicated that 50% of the American population consumes less folate than the daily recommended intake [33]. In this study, the mean value obtained for women was 352.8 μ g/day, which is less than the value described by Ferreira and Giugliani [34] (404.7 μ g/day) in an evaluation of the consumption of folate by women in the city of Porto Alegre, RS, southern Brazil. The scarcity of data as well as the use of different methodologies and cutoff values found in the literature make it difficult to compare data about the frequency

Table 3. Distribution of *MTHFR* C677T polymorphism in according to DNA damage (mean $\pm$ standard deviation (n)).

MTHFR genotypes	Damage frequency (n)	<i>P</i> ^a	Damage index (n)	<i>P</i> ^a	MN lymphocytes (n)	<i>P</i> ^a	MN buccal cells (n)	<i>P</i> ^a
CC	11.95 $\pm$ 8.47 (40)	0.644	17.82 $\pm$ 13.60 (40)	0.647	6.68 $\pm$ 3.89 (31)	0.981	10.58 $\pm$ 5.37 (38)	0.442
CT	11.66 $\pm$ 9.16 (44)		17.77 $\pm$ 15.55 (44)		6.91 $\pm$ 4.30 (43)		9.44 $\pm$ 3.63 (39)	
TT	10.89 $\pm$ 10.27 (18)		15.28 $\pm$ 13.83 (18)		6.93 $\pm$ 4.04 (15)		8.40 $\pm$ 4.61 (15)	

^aTested by non-parametric Wilcoxon test for related samples.

of women whose folate intake is below or above the DRI [17,34].

Concerning folate and vitamin B₁₂ intake and the influence thereof on DNA damage detected in this study, Beetstra et al. [35] showed that folic acid deficiency increases chromosomal instability. Vitamin B₁₂ is a major cofactor in folate metabolism, and all clinical manifestations resulting from the inadequate intake of this vitamin may prompt changes in DNA. Folic acid is an essential component to prevent chromosome breaks and hypomethylation of DNA; therefore, this chromosome repair mechanism may also be affected when the concentration of vitamin B₁₂ is low [36]. Tuimala et al. [37] demonstrated that MN formation is influenced by both DNA repair and genes with a role in folate metabolism, such as *MTHFR*.

Studies with human subjects showed that hypomethylation of DNA, chromosome breaks, incorporation of uracyl, and formation of MN are minimized when folate concentration in red cells is higher than 700 nmol/L. Research also proved that the formation of MN is minimized when the plasma concentration of vitamin B₁₂ is higher than 300 pmol/L and lower than 7.5 μ mol/L. These concentrations are measured after intake levels equal to or higher than DRIs (320 μ g/day folate and 2 μ g/day vitamin B₁₂), and depend on an individual's capacity to absorb and metabolize these vitamins and genetic and epigenetic differences [7]. In our study, individuals with a deficiency of folic acid, vitamin B₁₂, and vitamin B₆ presented a significant increase in DNA damage, compared to individuals with an adequate intake of these micronutrients, indicating that adequate levels of these compounds protect the organism from the genotoxic and mutagenic action of pesticides. A diet based on adequate levels of both folate and vitamin B₁₂ could play a favorable role in DNA repair. *In vivo* studies demonstrated that vitamin B₁₂ deficiency and high levels of homocysteine are strongly correlated with increased formation of MN [38].

Research has shown the importance of adjusting diet as a protection strategy against DNA damage in populations exposed to pesticides. *In vitro* studies suggest that folate deficiency increases DNA damage levels, thus increasing the genotoxicity of chemical agents. Others point out that polymorphism in the genes that encode metabolizing enzymes of xenobiotics, DNA repair, or folate metabolism are associated with a higher risk of cancer, depending on exposure to carcinogens, type of tumor, and ethnicity [39,40].

Studies have discussed the metabolic importance of the gene *MTHFR* C677T and its impact on required folate intake, showing that the need for this micronutrient may vary in mutant homozygous individuals (TT) compared to heterozygous (CT) and wild homozygous (CC) [8,41–45]. By comparison with other Brazilian populations, the genotypic distribution of the gene *MTHFR* and the allelic frequency in the population of tobacco farmers who took part in the present study are similar to the frequencies reported for the populations of the states of Rio Grande do Sul and São Paulo. When these populations are compared to the inhabitants of other regions in our country, they are shown to be more closely related with European populations, probably because these states were colonized mainly by European settlers [45].

The results of the present study did not indicate any association between *MTHFR* polymorphism and the results obtained in the Comet assay and the MN tests, corroborating studies of Crott et al. [46] and Smolkova et al. [47], who showed that *MTHFR* 677C>T does not influence MN frequency. Folate and vitamin B₁₂ are important to prevent DNA damage, but the polymorphisms of genes involved in the uptake, transport, and metabolism of these micronutrients are also important. *MTHFR* C677T is one of the polymorphisms involved, but other polymorphisms such as *MTHFR* A1298T, *MTR*A2756G, *MTR* (RS-1805087), *MTRR* (RS-1801394), and *SHMT* (RS-1979277) have been described to influence the individual metabolic response of these vitamins [48].

Besides polymorphism, parameters like age, gender, smoking habit, years of exposure, and BMI did not influence the high levels of DNA damage in tobacco farmers. Our result was similar to that observed by Paiva et al. [15] in a study that showed that the levels of DNA damage for exposure times did not vary between individuals in rural communities. Age and gender are the other confounding factors, which may possibly modulate the level of DNA damage. Fenech and Bonassi [49] stated that the damage to DNA increases because of age, probably due to the combination of several factors: inadequate nutrition, occupational, or environmental exposure to genotoxins, and a wide variety of unhealthy lifestyle factors. Studies found a significant correlation between age and the level of DNA damage [50].

No significant differences between men and women regarding adequate mean consumption of micronutrients and DNA damage were found. However, when mean folate consumption by women is compared with

values observed for men, our results indicated that women consumed more folate than men. When our research group compared the frequency of MN in men and women for this population, higher values were noted in men [19], differently from previous studies [49,51,52]. However, in Da Silva [19], this higher level of DNA damage in men was associated with occupational activities, where men work with pesticides more often than women.

Alcoholism is associated with significantly low levels of folate, vitamin B₁₂, and vitamin B₆ and with the promotion of DNA lymphocyte hypomethylation. Concerning the participants of this study, 40% of the individuals were revealed to consume alcoholic beverages and about 84% of these were moderate or light drinkers, consuming less than 3.0 g/kg/day of alcohol, which suggests that there was no interference from this association [36].

The fact that the forms were completed by nutritionists also helped ensure greater clarity of records due to the detailed information collected on food consumed, such as details about how food is prepared and the ingredients used. Supporting material, such as photographs and home measuring utensils, also proved useful and helped standardize the data. The results of our study shed light on an association of inadequate consumption of folate and vitamin B₁₂ with genomic instability in human lymphocytes, showing diet to be a protective factor in a population exposed to pesticides.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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