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The evaluation of maté (*Ilex paraguariensis*) genetic toxicity in human lymphocytes by the cytokinesis-block in the micronucleus assay

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Abstract

The present study evaluates the clastogenic and/or aneugenic potential of maté (*Ilex paraguariensis*) – previously tested for the presence of 48 organophosphorous pesticides – in the culture of human lymphocytes in the absence of exogenous metabolic activation. Peripheral blood was obtained once from three healthy female donors for lymphocyte cell cultures. The cultures were treated with maté infusion (filtered in sterilized sartorius filter with a 0.22 mm pore membrane), distilled water (negative control), and 6 μg/ml bleomycin (positive control). For each experimental person, 3000 binucleated cells (BN) from two independent cultures (1000 cells from replicate cultures) were scored for the presence of micronuclei (MN). No statistical differences between maté infusion concentrations were observed: 1400 μg/ml (0.001 ± 0.002), 700 μg/ml (0.0006 ± 0.0015), 350 μg/ml (0.002 ± 0.002), 175 μg/ml (0.002 ± 0.003) and negative control (0.001 ± 0.001). The present findings show that there is no clastogenic or/and aneugenic basis underlying maté action in the CBMN assay.
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Keywords: Maté; *Ilex paraguariensis*; Micronuclei; Cancer

1. Introduction

Maté (MT) is a tea-like beverage habitually consumed in South America and by South Americans across the world. It is brewed from the dried leaves and stemlets of the perennial tree *Ilex paraguariensis* (maté), a species that belongs to the *Aquifoliaceae* family. Chronic maté consumption has been associated with an increased rate of oral, oropharyngeal, esophageal, and laryngeal cancers. However, these data were based on case control studies carried out in maté-drinking populations that also used alcohol and tobacco products. Thus, the influence of maté as an independent risk factor was masked (Goldenberg et al., 2003). Although the exact carcinogenesis mechanism is still unknown, the information currently available suggests that maté infusion should be considered one of the risk factors for oral and oropharyngeal cancer. The beverage maté is a risk factor for head and neck cancer (Goldenberg, 2002).

The genotoxicity of instant water-soluble maté preparation and maté extracts has been associated to lysogenic induction in *Escherichia coli* (at concentrations of 20–50 mg/plate), as well as to point mutation in *Salmonella typhimurium* (10 mg/plate). Additionally, maté extracts (range doses 100–750 μg/ml) increased the frequency of chromosomal aberrations in cultured human peripheral

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lymphocytes, but did not cause increments in this endpoint when rat bone marrow cells were used (at oral doses up to 2 g/kg) (Fonseca et al., 2000; Leitao and Braga, 1994).

The cytokinesis-block micronucleus (CBMN) assay is the most extensively used method for measuring MN in human lymphocytes, and can be considered as a "cytome" assay covering cell proliferation, cell death and chromosomal changes. The key advantages of the CBMN assay lie in its ability to detect both clastogenic and aneugenic events and to identify cells which divided once in culture. A number of findings support the hypothesis of a predictive association between the frequency of MN in cytokinesis-blocked lymphocytes and cancer development (Mateuca et al., 2006).

No data concerning the genetic toxicity of aqueous infusion prepared with naturally dried maté leaves – representing the way maté is consumed – are available in literature. This study focused on the genotoxic assessment of maté aqueous infusion by means of CBMN assay, which was demonstrated to be a reliable method for assessing *in vitro*, maté extract-induced chromosome damage in peripheral blood lymphocytes.

2. Material and methods

2.1. Sampling preparation

Maté leaves were bought in a market in the city of Porto Alegre (south of Brazil, Rio Grande do Sul). The dried leaves were boiled in distilled water (200 g/L) for 10 min. The infusion was then filtered in a sterilized sartorius filter with a 0.22-mm pore membrane and immediately used for the preparation of the different concentrations applied. We used a similar range doses proposed by Fonseca et al. but a different method of sample preparation (maté infusion), which is the usual intake of human consume (Fonseca et al., 2000). Bleomycin sulfate (BLEO; 9041-93-4) was obtained from Biossintética (São Paulo, Brazil).

2.2. Cytokinesis-block micronucleus assay (CBMN)

2.2.1. Donors, cell cultures, treatment and cell harvesting

Peripheral blood was obtained once from three healthy female donors who had no smoking history and no recent consumption of drugs (aged 26, 28 and 32 years, referred to as donor 1, 2 and 3, respectively) with normal karyotypes. For each donor, one series of cultures was prepared with two parallel cultures for every sample tested. We used whole blood cultures as recommended by Migliore et al. (1989). For each culture, 0.8 ml heparinized blood was added to 8 ml RPMI-1640 medium (Sigma), containing 10% fetal calf serum, 1% penicillin/streptomycin (Ceme) and 80 μl phytohemaglutinin (10 μl/ml) (PHA, Gibco). Twenty-four hours after PHA stimulation, cultures were supplemented with maté infusion. The negative control was sterile distilled water and the positive control was 6 μg/ml bleomycin (Blenoxane, Bristol) dissolved in sterile water immediately before use. Forty-four hours after PHA stimulation, 6 μg/ml cytochalasin B (Sigma) was added to the cultures to prevent cells that had completed one nuclear division from performing cytokinesis. The use of Cyt-B enables the accumulation of virtually all-dividing cells at the binucleate stage as dividing cell populations, regardless of their degree of synchrony and the proportion of dividing cells (Fenech, 2000). Blood cultures were incubated for a total of 72 h at 37 °C. The binucleated lymphocytes were harvested for 28 h after adding Cyt-B, and hypotonic treatment was performed with 0.075 M KCl to lyse red blood cells. Cells were fixed with (3:1) methanol:acetic acid prior to transference to slides and then stained with Giemsa (Fenech et al., 2003; Kirsch-Volders et al., 2002).

2.2.2. Slides analysis

To avoid scorer bias, slides were coded so as to blind the scorer to the sample. Cells were scored for MN according to the criteria proposed by Fenech et al. (2003). MN frequencies were scored from controls and treated (Table 1) cultures and from each of their duplicates. For each culture, 1000 binucleate cells were studied. Since the data of duplicate cultures showed no statistical differences they were pooled – totaling 3000 for both cultures studied for each donor and concentration.

As a parameter for cytotoxicity, the nuclear division index (NDI) was studied by screening 1000 cells per donor at 400× magnification for the frequency of cells with one, two, three or four nuclei. From these data, NDI was calculated according to the formula

$$\mathrm{NDI} = [M1 + 2(M2) + 3(M3) + 4(M4)]/N \qquad (1)$$

where $M1$–$M4$ represents the number of cells with 1–4 nuclei and N is the total number of cells scored. Since nuclear divisions are asynchronous in CBMN blocked lymphocytes, cells with three nuclei may arise (Eastmondand and Tucker, 1989; Rosefort et al., 2004).

One-way analysis of variance (ANOVA) was performed in order to compare the different maté infusion concentrations to the negative control (NC). The Tukey test was used when significant results were obtained in the ANOVA. The Mann–Whitney test was performed in order to compare the frequency of micronucleus cells between our negative

Table 1
Results of micronucleus frequencies analysis

Maté concentration	Cells scored	MM	FMM
Negative control (distilled water)	2542	3	0.001 ± 0.001
Positive control bleomycin at 6 μg/ml	3000	19	0.038 ± 0.002*
175 μg/ml	2776	7	0.002 ± 0.003
350 μg/ml	3000	6	0.002 ± 0.002
700 μg/ml	3000	2	0.0006 ± 0.0015
1400 μg/ml	2662	4	0.001 ± 0.002

Mean ± SD, MM = number of micronucleus cells and FMM = MM/number of binucleated cells.

* $p < 0.0001$.

and positive control. In all tests, the normality was tested and the significance level chosen was 0.05.

2.2.3. Determination of organophosphorous residues

A multiresidue method for the analysis of 48 organophosphorous pesticides was used. The maté sample (10 g) was extracted with 100 ml ethyl acetate in Ultra-Turrax for 5 min at 3000 rpm and vacuum-filtered. After rinsing, the whole extract was percolated in a 30-g sodium sulfate column and concentrated in a rota-vapor at 40 °C down to 2 ml. After concentration, the extract was analyzed by gas chromatography (HP-5890 series II and automatic sampler HP 7673 A).

3. Results and discussion

3.1. Organophosphorous residues

To elucidate the presence of compounds with genotoxic potential we investigated 48 organophosphorous residues in maté extract. Only one compound was detected in the maté extract – phosphamidon in 0.12 mg/kg concentration. This pesticide has short persistence in the environment and presents dislocation to/from nearby regions. In Brazil Phosphamidon is registered in cotton, rice, potato, sugar cane, peanut, bean, corn, soy, tomato and wheat cultures. Phosphamidon caused considerable genetic damage in human *in vitro* system using lymphocytes from peripheral blood and nonhuman *in vivo* systems using bone marrow from mice (Patankar and Vaidya, 1980).

3.2. CBMN data

Our *in vitro* experiments revealed that the nuclear division index (NDI) obtained for negative controls and for the maté concentrations applied are not statistically significant, showing that maté is neither cytotoxic nor cytostatic to human lymphocytes. However, NDI values were significantly lowered by bleomycin when compared with negative control. Moreover, baseline MN frequencies for negative control (distilled water) and MN frequencies for positive control (bleomycin) are in accordance with previously published literature data (Fenech et al., 2003).

Table 1 shows that the micronucleus frequencies observed in the different maté infusion concentrations used ($p = 0.479$) are not statistically different from the negative control, which means that, in the present experimental condition, maté does not induce chromosome breakage and/or loss in binucleated cells. It is important to emphasize the fact that previous studies focused on samples obtained from instant water-soluble maté ascribed mutagenic and genotoxic properties to maté (Rosefort et al., 2004). A similar observation concerning lysogenic induction in *E. coli*, as well as induction of mutagenic events – base pair substitutions – in *Salmonella thyphimurium*, was observed for lyophilized aqueous extracts of maté. These last samples were also able to cause significant increments in the incidence of chromosomal mutation in cultured human peripheral lymphocytes, but not in rat bone marrow cells (Fonseca et al., 2000). Two points may justify the differences from our negative data and the mutagenic response previously reported. First of all, Leitao and Braga (1994) used a form of instant maté beverage, Fonseca et al. (2000) tested a lyophilized aqueous extracts of maté, and our group used a maté infusion, which is similar to the way it is used in human consumption. Secondly, the data from the three investigations are related to different maté commercial suppliers.

In fact, there are some confusing variables in relation to the use of alcohol and tobacco products persist, as well as the role of thermal injury in the genotoxic and carcinogenis activities ascribed to maté. Thermal injury was studied in Paraguay (Prolla et al., 1993), Japan (Segi, 1975), Iran (IARC/Iran Study Group, 1977), Russia (Kolycheva, 1969), Puerto Rico (Martinez, 1969), and in Brazil (de Barros et al., 2000), suggesting an association between hot beverage intake and the development of esophagus cancer. These studies support the hypothesis that the high temperature used in MT could increase the risk of carcinogenesis, especially when associated with alcohol and tobacco (Rolon et al., 1995).

Moreover, when adult rats that had ingested maté from the same source as used in the present study – on a daily basis for nine weeks – were analyzed for the development of tongue cancer, no association in both anatomical–pathological and immunohistochemical evaluations was observed (Vargas et al., 2007). These same samples were used to analyze the histology of the aero-digestive tract of rats that consumed maté at room temperature for five months, but no cancer evidence was obtained (Jotz et al., 2006). Rats treated with maté at the room temperature for five months were also analyzed for the presence of: (i) tumor necrosis factor-α (TNF-α) – a valuable tool for oral cancer physio-pathology diagnosis and (ii) heat shock protein 70 (Hsp70), which is associated with oral epithelium carcinogenesis (Kurokawa et al., 1998; Ciocca and Calderwood, 2005). Overall, both control and treated groups demonstrated equivalent basal TNF-α and Hsp70 release distributions (Vargas et al., 2005). In all our experiments, the confusing variables were excluded – alcohol, tobacco products and hot water – and maté from the same source were used. For this same maté source no mutagenic action – expressed as aneugenic and/or clastogenic events – immunohistochemical alterations, cancer evidence in the histological studies or increments in TNF-α and Hsp70 release were observed. All in all, the present findings show that there is no clastogenic and/or aneugenic basis underlying maté action on CBMN assay. Probably, MT itself is not able (*in vivo*) to induce molecular alterations that will develop a neoplasia.

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