

The anticancer homeopathic composite “Canova Method” is not genotoxic for human lymphocytes *in vitro*

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Genet. Mol. Res. 2 (2): 223-228 (2003)

Received November 05, 2002

Accepted May 23, 2003

Published June 30, 2003

ABSTRACT. The Canova Method® (CM) is a homeopathic medicine indicated for the treatment of patients with cancer and for pathologies that involve a depressed immune system, such as AIDS. This product is composed of homeopathic dilutions of *Aconitum napellus*, *Arsenicum album* (arsenic trioxide), *Bryonia alba*, *Lachesis muta* venom and *Thuya occidentalis*. It stimulates the immune system by activating macrophages. Activated macrophages stimulate the lymphocytes so that they increase their cytotoxic action in response to tumoral growth or infection. Given that the CM stimulates and accelerates the activity of macrophages and lymphocytes, we evaluated genotoxic effects induced in human lymphocytes treated with this homeopathic medication *in vitro*. Structural and numerical chromosomal aberrations were scored for the assessment of induced genotoxic effects, while the variation in mitotic index was considered as a monitor for induced cellular toxicity. The lymphocytes were cultivated for 24, 48 or 72 h in the following final concentrations of the medicinal composite CM: 4, 8 and 12%. Treatments with the CM

did not affect mitotic indexes, nor did they provoke chromosomal aberrations, when compared with untreated controls. There was no cytotoxicity or genotoxicity at the chromosomal level.

Key words: Mutagenesis, Canova Method, Mitotic index, Cytogenetics

INTRODUCTION

The development of new drugs that have chemotherapeutic activity against cancer is a great medical challenge. The discovery of these drugs is very difficult, because they must be able to destroy the tumor cells without causing excessive side effects. Most anti-tumor drugs cause hepatotoxicity, heart alterations and nephrotoxicity (Navis et al., 1999). A therapy that does not provoke the adverse effects of the classic anti-tumor drugs or which can be used as an adjuvant with such drugs, without the loss of activity against the tumor cells, would be useful for treating cancer patients.

The Canova Method® (CM) is a medicine composed of five traditional homeopathic substances. It is found in homeopathic drugstores in Brazil and Argentina and is indicated for patients with a depressed immunological system. This medicine was developed in the Canova Laboratory in Argentina, and today it is produced in Curitiba, Brazil (Piraíno, 2003).

The CM acts mainly by increasing the immune response through the activation of macrophages, and is indicated for the treatment of cancer or of pathologies that provoke a depressed immune system, such as AIDS. This medicinal composite has diminished tumor size in the treatment of cancer and has caused cancer regression, in many cases. Numerous tests have been made, confirming activity, both *in vivo* and *in vitro* (De Freitas Buchi and Del Vecchio, 2002; Piraíno, 2003). Immune response activation has been observed at both the cellular and molecular levels (Da Rocha Piemonte and De Freitas Buchi, 2002). Activated macrophages stimulate T-cells and cause them to increase their cytotoxic effect in response to tumoral growth or infections (Roitt et al., 1999). In clinical observations of patients treated with the CM, there was a decrease in infection and a concomitant reduction of inflammation (De Freitas Buchi and Del Vecchio, 2002; Piraíno, 2003). Since the CM is effective in stimulating and accelerating macrophage effectiveness, and it indirectly stimulates and accelerates T-cell action, we studied the genotoxic effects of this medicinal composite in human T-cells that were stimulated by the mitogenic action of phytohemagglutinin (PHA) (Janossy and Greaves, 1972; Preston et al., 1987).

MATERIAL AND METHODS

The CM material was obtained from homeopathic pharmacies in Brazil. It is formulated in 70% ethanol. The Hahnemannian Homeopathic method used to prepare the Canova medicine is described in the Farmacopéia Homeopática Brasileira (Lacerda, 1998; CPRFB, 1997). DH units were used; one DH is a decimal dilution. The number before the DH is the number of times the decimal dilution is made; 10 DH would be 1×10^{-10} . The Canova formula is composed of: 19DH *Thuya occidentalis* (Cupresaceae), made from the bark; 18DH *Bryonia alba* (Curcubitaceae), made from fresh roots; 11DH *Aconitum*

napellus (Ranunculaceae), made from fresh preparations of the whole plant, including the roots, at the beginning of flowering; 19 DH *Arsenicum album* (arsenic trioxide) and 18DH *Lachesis muta* (Viperidae) venom. The active ingredients were all extracted or diluted in 70% alcohol, in equal parts.

Mutagenic treatments

Cultures of human lymphocytes were set up for each experiment; they were prepared from 0.5 ml heparinized blood obtained by venipuncture from six 20- to 30-year-old healthy adult donors (three males and three females). The protocol of the present study was approved by the Ethics Committee of the Hospital das Clínicas de Curitiba, PR. The Ham-F10 medium (Sigma) was supplemented with 20% fetal bovine serum and antibiotics. The cells were stimulated with 2% PHA (Gibco). We used three final concentrations of the CM solution: 4, 8 and 12%; the control cultures received only water.

Experiment 1: Lymphocytes maintained in culture for 72 h with CM and PHA.

Experiment 2: Twenty-four hours after setting up the cultures (with PHA stimulation) the lymphocytes was treated with CM for 48 h (two cell cycles).

Experiment 3: Twenty-four hours after setting up the cultures (with PHA stimulation), the lymphocytes were treated with CM for 24 h, allowing the cells to be in contact with CM for just one cell cycle. These lymphocytes remained in culture for only 48 h.

Cytogenetic studies

Colchicine 4% (10^{-5} M) was added to the cultures 2 h before harvesting in all treatments. Hypotonic treatment, fixation, slide preparation and staining were performed according to standard procedures. Briefly, cells were centrifuged (1000 rpm for 10 min), the supernatant was removed and a hypotonic treatment with 0.075 M KCl was initiated at 37°C for 20 min. Cells were then centrifuged and fixed three times with fresh fixative (3:1 methanol/glacial acetic acid). Metaphase preparations were obtained by the technique of Moorhead et al. (1960). Cell suspensions were dropped onto clean slides. After drying the slides, they were stained with 4% Giemsa solution (pH 6.8) for 10 min and rinsed with distilled water. One hundred clear metaphases from each culture were analyzed in coded slides for structural and numerical (euploidy type) chromosome aberrations. One thousand and eight hundred metaphases were analyzed per person in the three treatments, giving a total of 10,800 metaphases. To assess the mitotic index (MI) 2,000 cells were analyzed from each individual/experiment (24,000 cells per individual), giving a total of 144,000 cells. Statistical analyses were carried out by the Friedman test or the Fisher exact test to assess the effects of treatment (Ayres et al., 2000).

RESULTS AND DISCUSSION

Table 1 presents the cytogenetic data observed in lymphocytes treated with CM in the G0 phase of the cell cycle; this treatment was maintained for 72 h after PHA stimulation. The effect of CM on lymphoblast (PHA-stimulated lymphocytes) was investigated after two and one cell cycles (Tables 2 and 3, respectively).

Cells treated with CM did not have a significant increase in chromosomal aberrations

Table 1. Mitotic index, frequency of chromosomal aberrations and numerical alterations in human peripheral blood lymphocytes treated with Canova Method (plus phytohemagglutinin) for 72 h; 600 metaphases/concentration.

CM (%)	MI (%)	CA		POL	END
		Gaps	Breaks		
0 (water)	3.7	3	1	0	0
4	3.3	3	0	0	0
8	3.9	4	1	0	0
12	3.5	3	0	0	0

CM = Canova Method treatment concentrations; MI = mitotic index; CA = frequency of chromosomal aberrations; POL = polyploid cells; END = endoreduplicate cells.

Table 2. Mitotic index, frequency of chromosomal aberrations and numerical alterations in human peripheral blood lymphocytes treated with the Canova Method after 24 h of stimulation with phytohemagglutinin (72-h culture); 600 metaphases/concentration.

CM (%)	MI (%)	CA		POL	END
		Gaps	Breaks		
0 (water)	3.5	2	0	0	0
4	3.5	0	0	0	0
8	3.8	3	0	0	0
12	3.4	2	0	0	0

For abbreviations, see legend to Table 1.

Table 3. Mitotic index, frequency of chromosomal aberrations and numerical alterations in human peripheral blood lymphocytes in cultures treated with the Canova Method after 24 h of stimulation with phytohemagglutinin (48-h culture); 600 metaphases/concentration.

CM (%)	MI (%)	CA		POL	END
		Gaps	Breaks		
0 (water)	2.3	1	0	0	0
4	2.4	2	0	0	0
8	2.2	2	1	0	0
12	2.2	3	0	1	0

For abbreviations, see legend to Table 1.

($P > 0.5$) when compared to untreated controls. The doses used here did not produce toxicity in the MI assessment, as no significant effect was detected in any of the treatments ($P > 0.5$).

The MI represents the proportion of cells in the M-phase of the cell cycle. A decreased MI reflects an inhibition of cell-cycle progression and/or loss of proliferative capacity. During *in vitro* experiments on chromosomal aberrations, the MI is used to monitor induced cellular toxicity. Information on the degree of cytotoxicity is essential to adequately select harvest times

and test concentrations, and is especially important when the results are used for risk assessment of compounds to which humans may be exposed (Amorim et al., 2000). We tested three treatment regimes with CM; in the first one the lymphocytes were treated with CM and the PHA simultaneously. The objective of this experiment was to determine if CM increases the mitogenic action of PHA, because CM by itself does not present this property (data not shown). We found that CM did not increase the MI of the lymphocytes; therefore, CM is not synergistic with PHA. In the second experiment, we treated the 24-h lymphocytes after stimulation with PHA; the cells were treated for 48 h with CM (two cell cycles). In the third treatment, CM was also applied at 24 h, but the cells remained in culture for only 24 h more (one cell cycle). The CM did not provoke cytotoxicity in the human lymphocytes; the MI found for the cultures of cells treated with the three concentrations of CM (4, 8 and 12%) were not different from those found in the control cultures. The third treatment had a MI lower than that found for treatments 1 and 2; this is because most of the cells went through only one cell division in the 48-h cultures, while the 72-h cultures gave 70 to 75% of the cells in the second or third division (Crossen and Morgan, 1977; Beek and Obe, 1979; Gebhart et al., 1980). Because CM did not provoke cytotoxicity in these experiments, there was no need to study the effect of CM separately in each phase of the cell cycle. The absence of toxicity of Canova Method was also observed by Laguens (1995); guinea pigs treated with CM did not have increased morbidity or mortality.

The CM is obtained by combining some active principles, most of them being medicinal botanical extracts. The active principles of the compounds that are formulated in CM are well known, as are their biological effects (Laguens, 1995; Evans, 2002). To our knowledge this is the first evaluation of the genotoxicity of this composite. Chromosomal aberrations constitute an important fraction of the damage caused by chemical, physical and biological agents to genetic material. Many composites that provoke gene mutations also cause chromosomal aberrations (Rabello-Gay et al., 1991). *In vitro* experiments have been shown adequate in routine cytotoxic research of different composites used in the treatment of human disease (WHO, 1980). For this reason we analyzed the possible genotoxic effects of the medicinal composite CM in human T-cell cultures. There was no indication of a toxic or clastogenic effect on the cultured cells, even when concentrations as high as 12% were used. Seventy-two-hour cultures allow for two cell cycles and alterations generated in the first cycle can undergo cellular repair in the second cycle (Natarajan and Obe, 1980). Therefore, the analysis of 72-h cultures can underestimate aberration frequency; that is why we exposed the cells to CM for only one cell cycle in experiment 3, to avoid the action of the repair system *in vitro*. There was no indication of a genotoxic effect of CM.

The choice of T-cells as the target for the study of possible toxic effects of the CM on cells was deemed adequate, since CM indirectly increases the potential for toxicity in T-cells (Da Rocha Piemonte and De Freitas Buchi, 2002). The presence of PHA was fundamental to this experiment as it stimulates the proliferation of T-cells (Janossy and Greaves, 1972; Preston et al., 1987). The fact that MI did not differ significantly from that found in the control treatment strengthens the hypothesis that CM does not exercise a direct effect on normal T-cells, even though it strongly affects macrophages. In order to determine exactly how the CM works, other studies will have to be made. One such experiment might feature the concomitant use of macrophage and lymphocyte cultures. We conclude that the CM, an immunization modulator, has no toxic effects at the chromosomal level, which enhances the usefulness of this low-cost homeopathic medicine.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Roberto Piraíno, Dr. Narciso Da Lozzo Neto, José Angelo Garcia and Maria da Glória Teixeira de Melo for their support of this work by kindly providing the samples of the “Canova Method”. Research partially supported by UFPA and CAPES.

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