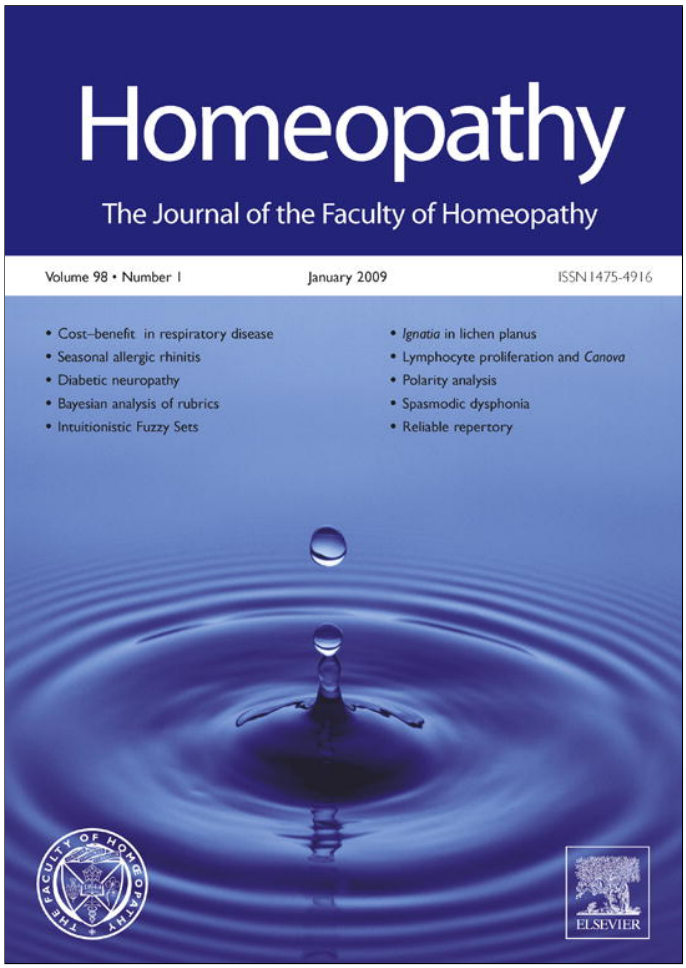

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ORIGINAL PAPER

Lymphocyte proliferation stimulated by activated human macrophages treated with *Canova*

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Introduction: ***Canova*** **(CA) is a homeopathic medication with immunomodulatory properties, recommended for patients with a depressed immune system. CA has been reported to increase in leukocyte numbers, cellular differentiation and reduction in tumor size.**
Aim and method: **Since CA may stimulate lymphocyte differentiation, proliferation, and/or survival, the aim of the present study was to compare the mitotic index (MI) of phytohemagglutinin-stimulated human lymphocytes cultured in a medium supplemented with human macrophages activated by CA, with lymphocytes cultured in a medium without CA-treated macrophages.**
Results: **In this study, the MI of lymphocyte cultured received the medium containing CA-stimulated macrophages showed a higher proliferation index ($p < 0.01$) than the lymphocytes cultured in a medium without CA-treated macrophages. Our results suggest that CA treatment, in addition to activating macrophages, indirectly induces lymphocyte proliferation and has potential as a new adjuvant therapeutic approach.** *Homeopathy* (2009) **98**, 45–48.

Keywords: *Canova*; Activated macrophages; Lymphocyte proliferation; Mitotic index; Homeopathy

Introduction

Canova (CA) is a complex homeopathic medicine containing *Aconitum napellus*, *Thuya occidentalis*, *Bryonia alba*, *Lachesis muta* and *Arsenicum album*. CA is indicated for patients whose immune system is depressed. CA treatment seems to enhance an individual's ability to trigger a specific immunologic response against several pathological conditions.[1]

CA activates macrophages both *in vivo* and *in vitro*. *In vitro* production of tumor necrosis factor-alpha by macrophages is significantly diminished by CA.[2] NADPH oxidase activity was increased as was that of inducible nitric oxide synthase (iNOS), producing reactive oxygen species (ROS) and nitric oxide (NO), respectively.[3] Cesar *et al.*[1] reported the effect of *in vitro* administration of the medication on the mononuclear differentiation of the bone marrow cells. Our research group has previously demonstrated that CA treatment does not induce cytotoxic or genotoxic effects at the chromosomal level.[4]

Since CA may lead to lymphocyte differentiation, proliferation, and/or increased survival, the aim of the present study was to evaluate the mitotic index (MI) of human lymphocytes stimulated by the mitogenic action of phytohemagglutinin (PHA)[5,6] cultured in a medium supplemented with human macrophages activated by CA compared with the MI of lymphocytes cultured in a medium without macrophages treated with CA. The MI represents the proportion of cells in the M-phase of the cell cycle.[7] Thus, an increased MI reflects a stimulation of cell-cycle progression and/or gain of proliferative capacity.

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Material and methods

Canova

'*Canova* do Brasil', a Brazilian company, holds the international patent of this medicine (www.Canovadobrasil.com.br). CA is produced in drops, inhalant and intravenous forms, and sold only in authorized pharmacies and laboratories. CA is standardized and authorized by competent agencies for medicinal application. It is currently registered as a magistral formula, according to Law No 5991/73. The preparation of this commercial medicament follows Hahnemannian homeopathic techniques. Mother tinctures are purchased from suppliers authorized by the Brazilian Health Ministry. The final product contains *A. napellus* (Ranunculaceae) 11dH, *B. alba* (Curcubitaceae) 18dH, *Thuja occidentalis* (Cupresaceae) 19dH, *A. album* (arsenious trioxide) 19dH and *L. muta* (Viperidae) 18dH and less than 1% ethanol in distilled water. It is an aqueous, colourless and odourless solution. Experiments were performed with commercial CA donated by '*Canova* do Brasil'.

Samples

Intraperitoneal fluid and peripheral blood samples were obtained from 10 patients, two women and eight men, aged 50–67 years, with primary gastric adenocarcinomas. Patients were noncirrhotic and without renal failure or evidence of breast, lung, liver or bone metastases. All ascitic fluid cytology was positive for adenocarcinoma cells. Patients supplying cells for this study had never had cancer chemotherapy or radiotherapy prior to biopsy or any other diagnosed cancer. Cytogenetic study of samples was approved by the Ethics Committee of Hospital University Joao de Barros Barreto.

Human macrophages culture

Human macrophages in ascitic fluid were counted in a Neubauer chamber. Macrophages were incubated at 37°C under 5% CO_2 for 15 min and nonadherent cells were removed by washing with PBS. The culture medium consisted of 80% HAM-F10 (Cultilab Mat. Cult. Cel Ltda, Campinas, SP, Brazil), supplemented with 20% heat-inactivated fetal calf serum (GIBCO laboratories, Grand Island, NY, USA), 0.01 mg/mL penicillin (Sigma Aldrich Co, St. Louis, MO, USA) and 0.005 mg/mL streptomycin (USB, Cleveland, OH, USA). More than 90% of adherent cells were macrophages[2] and the preparation was not further purified.

After 3 h of culture, CA was added at 10% of total medium volume in CA-treated samples. All experiments were performed at least three times in quadruplicate and with three control groups: (1) cells with no treatment, (2) cells treated with 10% PBS and (3) cells treated with a 10% of 0.1% v/v ethanol solution.

After 24 h, a fresh dose of 1% CA, or control solutions in the control groups, was added without replacing the medium. Treatment was carried out for 48 h *in vitro*. CA was succussed before administration.

Human lymphocytes proliferation in supplied medium

Short-term lymphocyte cultures were initiated according to a standard protocol.[6] Human lymphocytes were cultured in the supplemented medium (SM) used for macrophage activation by CA treatment, with the addition of 2% PHA. This culture medium was adjusted to 78% HAM-F10 and 20% heat-inactivated fetal calf serum. Lymphocytes were cultured for 72 h (37°C, humidified atmosphere with 5% CO_2).

Lymphocyte cultures with the medium used for macrophage activation control groups 1, 2 and 3 (control medium – CM) were also performed. In, a 4th control group, lymphocytes were cultured as for standard lymphocyte culture (new medium – NM).[6]

Metaphase preparations were obtained as described by Moorhead *et al*.[8] In order to obtain a sufficient number of metaphases, colchicine at a final concentration of 0.0016% was added 2 h prior to harvest. Cells were harvested by centrifugation and treated with 0.075 M KCl at 37°C for 20 min, the hypotonic solution caused cells to swell and increased metaphase spreading. Cells were then centrifuged and fixed in 1:3 (v/v) acetic acid:methanol. Finally, air-dried slides were prepared and stained with 3% Giemsa solution (pH 6.8) for 8 min.

Slides were analyzed with an optical microscope and MI (number of metaphases per 2000 lymphoblasts per culture) was determined in CA-treated cultures and controls. All MIs were counted twice by two different researchers (Figure 1).

Statistic analysis

F test (ANOVA) and χ^2 test were used to assess differences between MI values and between activation index of CA-treated and non-treated macrophages (activation ratios), respectively. In both analyses, the confidence interval was of 99% and *p* values less than 0.01 were considered significant.

Results

Human macrophage culture

Cells in the control groups were mainly resting macrophages, however activated macrophages were also present.

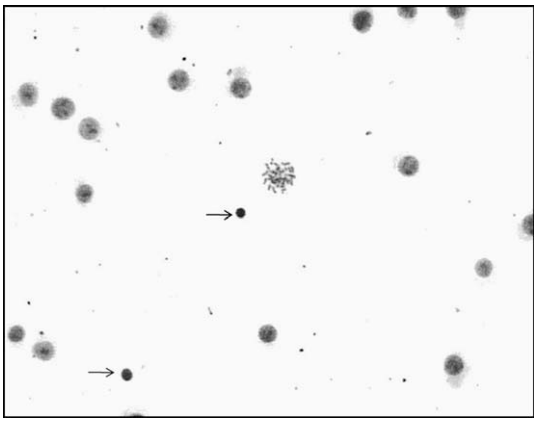

Figure 1 Lymphoblasts and one metaphase cell. Arrows mark picnotic nuclei, which were not counted in the evaluation of MI.

The index of macrophage activation in control group 1 (no treatment) was 41.5%, in control group 2 ((10% PBS), 46.9% and in control group 3 (10% 0.1% ethanol), 45.2%. Almost all cells (88%) from the CA-treated group were activated, as defined by morphological alterations.[2] There were no statistical differences among the control groups. The CA-treated group was significantly different from the control groups ($p < 0.01$) (Table 1).

Human lymphocytes proliferation in supplied medium

The MI of lymphocytes that were cultured in supplemented medium was significantly higher than the MI of groups cultured in control medium ($p < 0.01$) and the NM group ($p < 0.01$). No significant difference was observed between CM groups 2 and 3. However, the MI of CM group 1 was significantly higher than the MI of CM groups 2 and 3. The MI of NM group was also significantly higher than the CM groups ($p < 0.01$) (Table 2).

Discussion

Macrophages play an essential role in host defence against infection and tumoral cells. A large body of data indicates that macrophages must be activated in order to be effective. In this study, almost all cells from the treated group were activated (88%). This activation ratio was similar to the ratio observed in CA-treated mice macrophages.[2]

Cells from our control groups were mainly resting macrophages, but activated macrophages were also present. The presence of activated macrophages in these samples may be due to the low grade of inflammation generally observed in ascites patients.[9] The index of macrophages activation in control groups was about 41.5%. The macrophage activation index in the treated group was significantly different from control groups. Previous studies demonstrated that CA activates macrophages both *in vivo* and *in vitro*.[1,2] Improvement in immune response of CA-treated mice was demonstrated in studies with Sarcoma cell line 180. A reduction in sarcoma size was observed and a significant infiltration of lymphoid cells, granulation tissue and fibrosis occurred, surrounding the tumor. All animals from the treated group survived, and in 30% of them a total regression of the tumor was observed. Treatment with CA increased total numbers of leukocytes, specifically T, CD4, B and NK cells increased.[10] These results suggested a direct or indirect action of the CA on hematopoiesis. So the bone marrow cells were treated and analyzed by light, transmission and scanning electron, and bifocal microscopy, and flow cytometry. All microscopy techniques showed that monocytic lineage (CD11b) and stromal cells (adherent cells) were activated by treatment. CA also increased cell clusters over adherent cells, suggesting areas of proliferation and differentiation.[11]

Activated macrophages stimulate T-cells and lead to an increased cytotoxic effect in response to tumoral growth or infections.[12] Clinical observations include decreased in infection and concomitant reduction of inflammation in patients treated with CA.[1]

Since CA stimulates macrophages, and indirectly stimulates and accelerates T-cell action, we evaluated the index of lymphocyte proliferation in a culture supplemented with macrophages activated by CA. Our results suggest cytokines and growth factors secreted by macrophages activated with CA treatment lead to an increased T-lymphocyte proliferation stimulated by PHA. The presence of PHA was fundamental in this study to stimulate T-cell proliferation.[6] CA does not itself induce lymphocyte proliferation *in vitro* (data not shown).

The MI of lymphocytes cultured in SM was higher than the MI of lymphocytes cultured in CM (control 1–3) and in NM (control 4). Control 1 showed a higher MI than controls 2 and 3. These findings suggest some interference of PBS and ethanol in lymphocyte cultures and emphasize that CA treatment, despite its ethanol content, can indirectly lead to lymphocyte proliferation.

Control 4 (new medium) showed a higher MI than controls 1–3. This finding may be due to nutrient consumption by macrophages and alterations generated during culture time (122 h) in CM groups.

Other lymphocyte cultures performed by our research group showed a higher MI (5.4) than that observed in control 4 (median 4.0) using peripheral bleed lymphocytes.[13] This difference may be due to the fact that the patients of

Table 1 Macrophage activation index of human samples cultured in different media

Sample	*CA treatment*	*Control 1**	*Control 2†*	*Control 3‡*
1	92.4	3.1	2.5	2.3
2	91.2	3.0	2.4	2.4
3	85.4	3.2	2.7	2.5
4	86.4	3.1	2.6	2.5
5	87.5	3.0	2.5	2.5
6	89.2	3.3	2.6	2.6
7	87.5	3.3	2.8	2.7
8	87.8	2.9	2.6	2.6
9	86.8	3.1	2.4	2.2
10	85.8	3.2	2.7	2.7
Mean (SD)	88 (2.29)	41.5 (2.59)	46.9 (1.42)	45.2 (2.16)

* Cells with no treatment.
† Cells treated with 10% PBS.
‡ Cells treated with 10% of 0.1% v/v ethanol solution.

Table 2 MI of human lymphocytes cultured in different media

Sample	*CA treatment*	*Control 1**	*Control 2†*	*Control 3‡*	*Control 4§*
1	6.8	3.1	2.5	2.3	4.0
2	6.5	3.0	2.4	2.4	3.8
3	6.7	3.2	2.7	2.5	3.7
4	6.8	3.1	2.6	2.5	3.9
5	6.2	3.0	2.5	2.5	3.8
6	6.9	3.3	2.6	2.6	4.1
7	7.0	3.3	2.8	2.7	4.3
8	6.4	2.9	2.6	2.6	3.8
9	6.4	3.1	2.4	2.2	3.7
10	6.5	3.2	2.7	2.7	3.9
Mean (SD)	6.62 (0.26)	3.12 (0.13)	2.58 (0.13)	2.5 (0.16)	3.9 (0.19)

* Cells with no treatment.
† Cells treated with 10% PBS.
‡ Cells treated with 10% of 0.1% v/v ethanol solution.
§ Cells cultured in a NM.

this study, who, although they had not had cancer chemo- or radiotherapy, were seriously ill and had ascites. Peripheral blood of controls used in mutagenesis studies are usually collected from healthy donors up to 30 years old and without history of smoking/drinking or chronic drug use.

In conclusion, CA treatment indirectly induced lymphocyte proliferation through activated macrophages. CA has potential as a new adjuvant therapeutic approach to known therapies.

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Conflict of interest

All authors declare that they have no conflicts of interest.

Role of funding source

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