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ORIGINAL PAPER

In vitro assessment of anticytotoxic and antigenotoxic effects of CANOVA®

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Background: **CANOVA® (CA) is a homeopathic immunomodulator. It contains several homeopathic medicines prepares according to the Brazilian Pharmacopoeia. CA is indicated in clinical conditions in which the immune system is impaired and against tumors. N-methyl-N-nitrosourea (NMU) is an N-nitroso compound, with genotoxic/mutagenic properties. Although several studies have shown promising results in the use of CA, there are no studies reporting possible antigenotoxic effects.**
Method: **This study evaluated the *in vitro* antigenotoxic and anticytotoxic effects of CA in human lymphocytes exposed to NMU. Samples of human lymphocytes that were subjected to different concentrations of a mixture containing CA and NMU were used. The genotoxicity/antigenotoxicity of CA was evaluated by the comet assay, anticytotoxicity was assessed by quantification of apoptosis and necrosis using acridine orange/ethidium bromide.**
Results: **CA significantly reduced DNA damage induced by NMU and reduced significantly the frequency of NMU-induced apoptosis after 24 h of treatment.**
Conclusion: **CA has an important cytoprotective effect significantly reducing the DNA damage and apoptosis induced by the carcinogen NMU.** *Homeopathy* (2016) **105**, 265–269.

Keywords: CANOVA; NMU; Anticytotoxicity; Antigenotoxicity; Cytoprotection

Introduction

CANOVA® (CA) is a homeopathic immunomodulator described in the Brazilian Homeopathic Pharmacopoeia and prepared by the Hahnemannian method.[1,2] Mother tinctures are purchased from authorized agencies approved by the Brazilian Health Ministry. Its final composition is *Aconitum napellus* (Ranunculaceae) 11dH, *Bryonia alba* (Cucurbitaceae) 18dH, *Thuja occidentalis* (Cupressaceae) 19dH, *Arsenicum album* (arsenious trioxide) 19dH, *Lachesis muta* (Viperidae) 18dH, in 1% of ethanol in distilled water. CA is regulated by the decree n° 79.094/77 as a magistral formula and is coded as 'product NDC 58088-001' by the Food and Drug Administration – FDA (USA).[3]

Observation in immunosuppressed patients treated with CA has shown success of this compound in the treatment of this clinical feature[4]; CA increases the immune response against several serious diseases through the activation of macrophages, which stimulate the action of T cells leading to increased cytotoxic effect in response to the growth of infections or tumors.[5] Mice with Sarcoma 180 treated with CA had an improvement in the immune response together with a complete regression of the tumor in 30% of the animals.[6] Ribeiro[7] found that the homeopathic compound was able to decrease the expression of MYC oncogene in PG100 cells, a cell line established from a

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primary gastric adenocarcinoma, which shows amplification of this gene.

The precise mechanism by which CA acts is still unknown. The majority of studies takes into account their role in macrophages because these cells, when treated with CA, increase their NAD(P)H oxidase and iNOS activities, which induce the production of reactive oxygen species (ROS) and nitric oxide, respectively. Such effects induce macrophage activation, which promotes changes that result in an increase of the immune response of the individual.[8]

Although several studies have shown promising results in the use of CA, there are no studies reporting possible antigenotoxic effects of this medicine, despite its anticancer potential. Therefore, studies that characterize their effects on DNA have an important impact, as they may collaborate to create new therapeutic strategies to intensify its use as a cytoprotective agent. Thus, the present study evaluated *in vitro* the antigenotoxic and anticytotoxic effects of the drug CA in human lymphocytes exposed to N-methyl-N-nitrosourea (NMU), a N-nitroso carcinogenic alkylating agent used as an experimental model for inducing carcinogenesis in rodents and monkeys and which also shows genotoxic/mutagenic effects.

Materials and methods

Lymphocytes cultures preparation

The peripheral blood samples were collected from three individuals, two men and a woman, who fulfilled the standards required for genotoxic testing: age between 18 and 35 years; nonsmokers; and without recent exposure to chemicals, radiation, and genotoxic agents.[9] The volunteers were interviewed and signed the consent form for participation in the study after being fully informed about the objectives, nature, and risks of all procedures performed. This work was carried out in accordance with the guidelines of our institute and with the Declaration of Helsinki (2013) of the World Medical Association.

Blood was collected with syringes of 20 mL properly heparinized to prevent coagulation and then subjected to the lymphocyte isolation procedure for the set up of short-term cultures, as described by Fenech[10] with some adjustments. For the experiments, the cultures were incubated at 5% CO_2 at a temperature of 37°C.

Cell treatment

CA was donated by 'Canova do Brasil', the Brazilian company that holds the international patent, and NMU was obtained from Sigma Chemical Co., St. Louis. The following experimental groups were used:

a) Negative control (NC): cells grown only in the presence of RPMI 1640 medium (Cultilab, Campinas, Brazil) supplemented with 20% FBS, 4% phytohemagglutinin A (Gibco-Invitrogen, Carlsberg, CA), 1% streptomycin, and 1% kanamycin;
b) NMU (positive control − PC): cells treated with a single concentration of NMU (125 μg/mL);
c) CA: cells treated with three CA concentrations (4%, 8%, and 16%) added to the culture medium;
d) CA + NMU: cells treated simultaneously with three CA concentrations and a single NMU concentration (NMU + CA 4%, NMU + CA 8%, and NMU + CA 16%).

The single NMU concentration was defined according to Stephanou et al.[11] and in previous tests performed in our laboratory. CA concentrations were defined according to Seligmann et al.[12] All experiments were performed in triplicate and as a controlled trial.

Comet assay

For the comet assay, 1×10^6 cells were seeded in 12-well culture plates (Corning) with 1 mL of complete medium for 20 h. Lymphocytes were afterward treated for 3 h according to the experimental groups cited in the 'cell treatment' section. After treatment, an aliquot of 450 μL from each culture was taken for the alkaline version of the comet assay as described by Singh et al.[13] Briefly, the aliquot was taken and centrifuged at 1000 rpm for 5 min in a microcentrifuge (Eppendorf). The resulting pellet was homogenized with 300 μL of a low melting point agarose (0.8%), spread onto microscope slides precoated with a normal melting point agarose (1.5%), and covered with a coverslip. After 5 min at 4°C, the coverslip was removed, and the slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, and 1% Triton-X, pH: 10) for one week. According to Tice et al.,[14] the lysis duration used by different investigators varies considerably. One week was chosen because such period was suitable to our laboratory routine. After lysis, the slides were placed in an electrophoresis chamber and covered with freshly made electrophoresis buffer (300 mM NaOH; 1 mM EDTA, pH > 13). The electrophoresis was run for 25 min (34 V and 300 mA). Afterward, the slides were neutralized by submersion in distilled water (4°C) for 5 min and fixed in 100% ethanol for 3 min. Staining of the slides was performed immediately before the analyses using ethidium bromide (20 μg/mL). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each slide) using a fluorescent microscope (Olympus BX41) at ×40 magnification. The damage index (DI) was visually determined based on the size and intensity of the comet's tail. The following five categories (0−4) were used: class 0 (no damage), class 1 (little damage with a short tail length smaller than the diameter of the nucleus), class 2 (medium damage with a tail length one or two times the diameter of the nucleus), class 3 (significant damage with a tail length between two-and-a-half to three times the diameter of the nucleus), and class 4 (significant damage with a long tail of damage more than three times the diameter of the nucleus). Categories were used according to Collins et al.[15]