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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]

Evaluation of necrosis and apoptosis by fluorescent differential staining with acridine orange−ethidium bromide (LA/BE)

To evaluate apoptosis and necrosis of lymphocyte culture, 1×10^6 cells were seeded in 12-well culture plates (Corning) with 1 mL of complete medium. After 24 h, the cells were treated according to the group treatments described above in the 'cell treatment' section for 24 and 48 h. Afterward, a solution of orange acridine/ethidium bromide (100 μg/mL) was added to the cells that were visualized in a fluorescence microscope Olympus BX41 with FITC filter. Three hundred cells were analyzed for each treatment group according to the criteria used by Montenegro et al.[16]

Statistical analysis

To compare the frequencies of the various parameters, the results were submitted to analysis of variance (ANOVA), followed by Tukey post test, using the BioStat 5.0 software.[17] In all analyses, the significance level was 5%.

Results

The results of the comet assay show that CA significantly reduced ($p < 0.05$) the NMU-induced DI in all the NMU + CA combinations tested. The DI of NMU was 3.22, whereas the DI of the NMU + CA combinations were 1.20, 0.87, and 0.69 at the NMU + CA 4%, NMU + CA 8%, and NMU + CA 16% concentrations, respectively. There was also a significant difference ($p < 0.05$) between the NMU DI (3.22) and the DI of the NC (0.81). Cells treated only with CA did not show an increase in DI after treatment (Figure 1).

CA anticytotoxicity was assessed 24 and 48 h after treatment. Overall, there was a decrease in both apoptotic and necrotic cells in the combined treatments (NMU + CA) when compared with treatment only with NMU in the two harvest moments; however, a significant decrease ($p < 0.05$) was observed only in the percentage of apoptotic cells harvested 24 h after treatment in the NMU + CA 16% (5.22%) treatment when compared with treatment with NMU only (8.89%). There was also a significant difference ($p < 0.05$) between the percentage of apoptotic cells induced by NMU (8.89%) after 24 h of treatment compared with its NC (3.56%) and between the NMU (10%) and NC (2.44%) after 48 h of treatment (Figure 2).

Discussion

In the present study, CA alone was neither genotoxic nor cytotoxic to lymphocytes (Figure 1). The only work available in the literature assessing the genotoxic effects of the CA was carried out by Seligmann et al.[12] These authors demonstrated that CA is not genotoxic to lymphocytes from peripheral blood by evaluation of numerical and structural chromosomal aberrations. In addition, the authors demonstrated that CA was not cytotoxic by the mitotic index.

In a 2011 work, Oliveira et al.[18] performed tests with highly diluted mixtures of compounds containing, among others, *Thuja occidentalis, Aconitum napellus,* and *Arsenicum album* (present in the formulation of the CA). The authors used the neoplastic cell lines HT29 (colorectal cancer) and K562 (myeloid leukemia) as well as monocytes and macrophages, to evaluate the induction of apoptosis by Annexin V. The results show that none of the blends induced cytotoxicity for all cells tested. These results along with the results of Seligmann et al.[12] confirm our data of absence of apoptosis after treatment with CA (Figure 2).

The genotoxic effects of NMU were significantly reduced when cells were treated simultaneously with CA (CA + NMU) (Figure 1). Similar results were observed

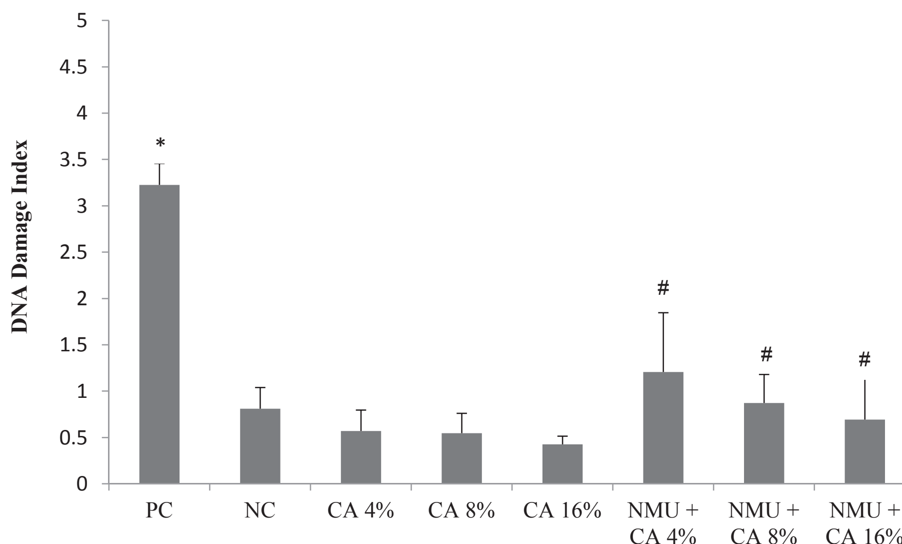

Figure 1 DNA damage index observed in the lymphocyte culture after the treatment with CA and CA + NMU. Media of the three experiments. *($p < 0.05$) when compared to the negative control. # ($p < 0.05$) compared to the positive control (NMU − 125 μg/mL). ANOVA/Tukey post test. NC (negative control), CA (CANOVA); PC (positive control − NMU).

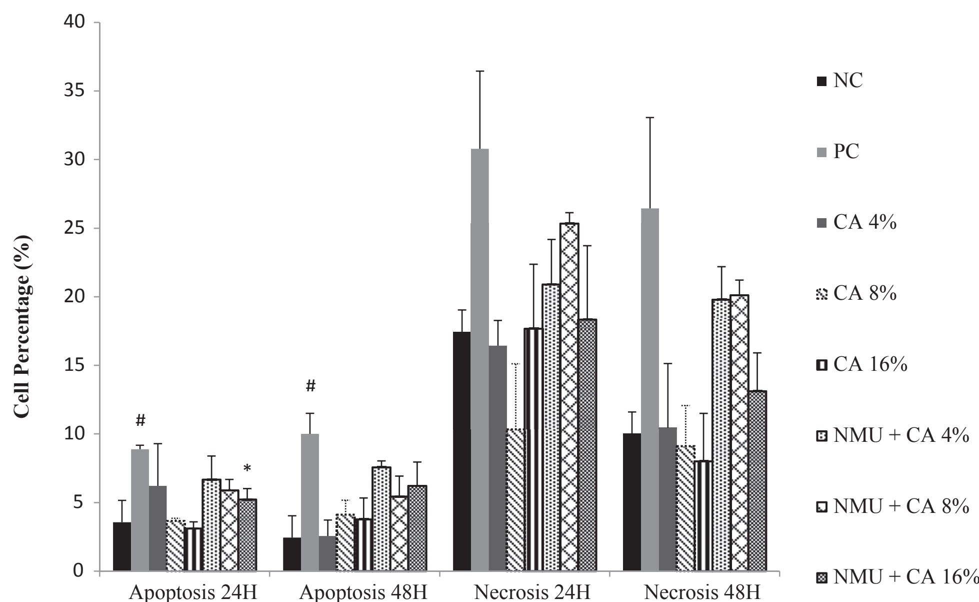

Figure 2 Induction of apoptosis and necrosis in lymphocytes submitted to different concentrations of CA together with NMU. 300 cells analyzed.*($p < 0.05$) when compared to NMU (positive control). #($p < 0.05$) when compared to negative control. ANOVA/Tukey post test. PC (positive control − NMU); NC (negative control); CA (CANOVA).

by Matos.[19] This author showed that CA was able to decrease the DI and the frequency of micronuclei induced by NMU in *Cebus apella* treated *in vivo* with such compounds. The characterization of the antigenotoxic and anticytotoxic effects of CA is difficult because there are few studies that demonstrate the effects of this medicine in lymphocytes. However, some inferences can be made based on the work carried out by Oliveira et al.[18] Using microarray technology, these authors evaluated the differential gene expression of peritoneal leukocytes in mice treated with CA; 147 genes were differentially expressed in the group treated with CA. These genes are mainly involved in transcription/translation, dynamic cell structure, immune response, cytoprotection, enzymatic processes, and receptors/ligands.

Among the cytoprotective genes induced in Oliveira et al.[18] experiments, the Hsp70-1 gene, which belongs to the family of heat shock proteins, was found. This group of genes is expressed in response to extreme heat and a variety of other stress stimuli, which can be physiological, physical, and chemical.[20] Some studies, such as the one by Calini et al.[21] have demonstrated the role of this family in the process of DNA repair. In their work, these authors demonstrated through the comet assay that C3H 10T1/2 cells (mouse embryo fibroblasts) showed a significant decrease in DNA damage induced by ionizing radiation, when they were pretreated with water at 43°C for 1 h. Hsp70 protein expression was confirmed by western blotting.

The mechanisms that induce Hsp70-induced antigenotoxic effects are not fully known. One hypothesis would be that translocation of Hsp70 to the nucleus during stress may contribute to the stabilization of the chromatin structure, preventing chromatin damage.[21] It is tempting to assume that a similar mechanism may have occurred so that there was a decrease in DI observed in lymphocytes treated simultaneously with CA and NMU, in the present work.

Another gene induced by CA in the experiments of Oliveira et al.[18] was GADD45β. This gene belongs to a class that has been implicated in stress signaling in response to environmental or physiological stimuli, resulting in cell cycle arrest, DNA repair, cell survival, and senescence or apoptosis. The exact role of GADD45 proteins in apoptosis has not been fully clarified. There are studies indicating that these proteins act as proapoptotics or antiapoptotics.[22]

Gupta et al.[23] in an elegant study demonstrated the antiapoptotic role of GADD45β. The authors demonstrated that bone marrow cells of knockout mice for this gene had a greater sensitivity to apoptosis induced by ultraviolet radiation and the chemotherapeutics daunorubicin and VP-16 when compared with wild-type cells. The reintroduction of the gene in the knockout cells restored its wild-type phenotype to apoptosis. This experiment clearly shows that the protein GADD45β protects hematopoietic cells from genotoxic stress induced by chemotherapeutic agents, although the mechanisms by which this occurs is not known. However, its antiapoptotic function in this context is clear. The antiapoptotic effects of CA observed in this study (Figure 2) may also have been mediated possibly by induction of GADD45β in lymphocytes simultaneously treated with NMU + CA. However, both this hypothesis, as the involvement of Hsp70 in the CA antigenotoxicity requires verification.

The results of this study allow new perspectives to the use of CA, for demonstrating that, besides acting as an excellent immunomodulator, CA has an important cytoprotective role because it significantly reduces the DNA damage induced by the carcinogen NMU.

Conclusion

CA showed both an antigenotoxic and anticytotoxic effect significantly reducing the DNA damage and apoptosis induced by the carcinogen NMU in our experimental conditions.

Conflict of interest

The authors declare that there are no conflicts of interest. The authors did not receive any support from Canova do Brasil beyond donation of CA.

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