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Radio-protective effect of cinnamic acid, a phenolic phytochemical, on genomic instability induced by X-rays in human blood lymphocytes *in vitro*

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ABSTRACT

The present study was designed to determine the protective activity of cinnamic acid against induction by X-rays of genomic instability in normal human blood lymphocytes. This radio-protective activity was assessed by use of the cytokinesis-block micronucleus test and the alkaline comet assay, with human blood lymphocytes isolated from two healthy donors. A Siemens Mevatron MD2 (Siemens AG, USA, 1994) linear accelerator was used for the irradiation with 1 or 2 Gy. Treatment of the lymphocytes with cinnamic acid prior to irradiation reduced the number of micronuclei when compared with that in control samples. Treatment with cinnamic acid without irradiation did not increase the number of micronuclei and did not show a cytostatic effect in the lymphocytes. The results of the alkaline comet assay revealed that cinnamic acid reduces the DNA damage induced by X-rays, showing a significant radio-protective effect. Cinnamic acid decreased the frequency of irradiation-induced micronuclei by 16–55% and reduced DNA breakage by 17–50%, as determined by the alkaline comet assay. Cinnamic acid may thus act as a radio-protective compound, and future studies may focus on elucidating the mechanism by which cinnamic acid offers radioprotection.

1. Introduction

Radiotherapy is an effective treatment for cancer in general, based on the observation that exposure to a sufficient quantity of ionizing radiation kills or sterilizes cells [1]. Radiation induces DNA damage within cancer cells, rendering them unable to further divide and grow. Radiation is often given with the intent of destroying the tumour and curing the disease. However, although radiation is directed at the tumour, it is inevitable that the normal, non-cancerous surrounding tissue will also be affected and damaged by the radiation [2]. Therefore, several attempts have been made to minimize damage to normal tissue with synthetic compounds such as cysteine, cysteamine and amifostine [3]. Although the use of these radioprotectants seems promising in medical practice, inherent systemic toxicity in addition to their short biological half-lives are limiting factors to their efficacy [3]. Based on the limitations to current radio-protective substances, a large effort has been made to identify less toxic compounds that offer radioprotection. The potential use of natural phenolic compounds as radioprotectants is of increasing interest [4,5] in view of their proven activities as antioxidants and free-radical scavengers [3,5–8]. Thus, it is necessary to assess the potential of common phytochemicals and phenolics to serve as non-toxic radioprotectants for cancer patients undergoing radiation therapy. In several reports, *in-vitro* irradiation experiments with blood lymphocytes have been generally preferred as a model to understand the harmful effects of radiation in healthy human cells [9,10].

Cinnamic acid (CA, *trans*-cinnamic acid, Fig. 1) is a natural phenolic substance that is obtained from cinnamon oil or from balsams such as storax. However, CA can also be produced synthetically. CA has a long history of human usage as a plant-derived scent and flavour [11], it is used in a wide range of products including baked goods, confections and beverages as well as in toothpaste, mouthwash, and chewing gum [12]. CA is an important constituent in the biochemistry of lignin production in certain plants [13]. Natural hydroxylated cinnamates have been shown to possess antitumour and antioxidant properties [14–16], but there are no

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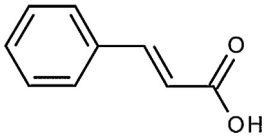

Fig. 1. The chemical structure of cinnamic acid.

studies investigating the use of these compounds in the defense against radiation-induced cellular damage. Thus, the present study attempts to evaluate the effect of CA as a protective agent against X-ray-mediated genomic damage in human blood lymphocytes by use of the cytokinesis-block micronucleus (CBMN) test and the alkaline comet assay. We also measured intracellular reactive oxygen species (ROS) by means of 2′,7′-dichlorofluorescein diacetate (DCFH-DA), in order to assess the mechanism of radioprotection by CA.

2. Materials and methods

2.1. Chemicals

Cytochalasin-B, heat-inactivated fetal calf serum (FCS), colcemid, ethidium bromide, Ficoll-Histopaque, low-melting agarose (LMA), normal-melting agarose (NMA), cinnamic acid (Fig. 1), RPMI-1640 medium, penicillin-streptomycin, dimethyl sulfoxide (DMSO), L-glutamine, Triton X-100, ethylene diammine tetraacetic acid (EDTA), trypan blue dye, sodium sarcosinate, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich®, Germany. Standards of 2′,7′-dichloro-dihydrofluorescein (DCF) were obtained from Cell Biolabs', CA, USA. All other chemicals were purchased from GIBCO-BRL, MN, USA. Giemsa stain was obtained from Merck KGaA, Darmstadt, Germany.

2.2. Lymphocyte isolation and culture

The experimental design was approved by the Uludag University Faculty of Medicine Human Ethics Committee. Blood samples were collected from two healthy donors, one male and one female. The donors were 25–35-year-old non-smokers with no history of radiotherapy, no alcohol consumption or use of medication, and no disease at the time of blood collection. For the cytogenetic studies, preservative-free heparin was used as an anticoagulant. Written consent was obtained from each blood donor. Lymphocytes were isolated from the blood by means of a Ficoll–Histopaque gradient, and were cultured in duplicate as previously described [17]. Briefly, blood samples were diluted 1:1 with phosphate-buffered saline (PBS) and layered onto the Ficoll–Histopaque with a 4:3 ratio of blood-PBS/Histopaque. The samples were centrifuged at 400 × *g* for 35 min, and the lymphocyte-enriched layer was removed, washed twice with PBS by centrifugation at 350 × *g* for 10 min and finally washed with RPMI-1640 medium. The number of viable cells was assessed by staining with trypan blue and counting the cells with a haemocytometer. Viable cells were suspended in RPMI-1640 supplemented with 15% FCS, 200 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). Phytohaemagglutinin (0.2 ml) was added to the lymphocyte culture to initiate cell division. Cells were incubated at 37 °C in a humidified incubator maintained with 5% CO_2.

2.3. Study design

Cultured lymphocytes were divided into 13 treatment groups (Scheme 1).

Treatment groups

1. Untreated control
2. 0.02% DMSO control
3. Cinnamic acid (6.75 μM)
4. Cinnamic acid (13.5 μM)
5. Cinnamic acid (27 μM)
6. 1 Gy irradiation group
7. Cinnamic acid (6.75 μM)+ 1 Gy irradiation group
8. Cinnamic acid (13.5 μM)+ 1 Gy irradiation group
9. Cinnamic acid (27 μM)+ 1 Gy irradiation group
10. 2 Gy irradiation group
11. Cinnamic acid (6.75 μM)+ 2 Gy irradiation group
12. Cinnamic acid (13.5 μM)+ 2 Gy irradiation group
13. Cinnamic acid (27 μM)+ 2 Gy irradiation group

DMSO, dimethyl sulfoxide

Scheme 1. Treatment groups.
DMSO, dimethyl sulfoxide.

2.4. Cell treatment

Lymphocytes were exposed to phytohaemagglutinin for 24 h before irradiation. Thirty minutes prior to irradiation of the cells, three test doses (6.75, 13.5 and 27 μM) of CA dissolved in 0.02% DMSO were added to the lymphocyte cultures. Preliminary studies were conducted to determine whether these concentrations showed toxicity by trypan-blue dye-exclusion prior to X-ray irradiation: the treatment with CA did not affect lymphocyte viability, which was in all samples >85%.

6-MV X-rays were used to irradiate the cells in 40-mm covered Petri dishes. The treatment doses were administered at a dose rate of 200 cGy per min with a linear accelerator (Siemens Mevatron MD2, Erlangen-Germany) in the Uludag University, Faculty of Medicine, Radiotherapy Center Bursa-Turkey. The given dose was calculated by means of a radiotherapy-planning system (CMS-XiO, Freiburg, Germany) with computerized tomography images of the petri dishes in the treatment position. A gel bolus had been used to eliminate the dose reduction due to the build-up region of 6-MV X-rays.

After irradiation, the samples were incubated for 1 h at 37 °C to permit DNA repair as it happens under in-vivo conditions and then transported on ice to the laboratory. Cultures were equilibrated at room temperature and then subjected to the alkaline comet assay and the cytokinesis-block micronucleus (CBMN) test.

2.5. CBMN assay and scoring methodology

The presence of micronuclei (MN) in bi-nucleated cells was assayed by blocking the cells at cytokinesis as described by Fenech and Morley [18]. The frequency of MN induction was evaluated by scoring 1000 bi-nucleated lymphocytes per sample. The slides were analysed at 1000× magnification by a blinded observer. Cells were scored according to criteria outlined by Fenech et al. [19]. Cytochalasin-B (6 μg/ml) was added to the culture at 44 h. The cells then were incubated at 37 °C for another 28 h, after which they were harvested, cast on a pre-cooled slide and stained with Giemsa. Bi-nucleated cells surrounded by cytoplasm were scored for the presence of MN according to established criteria [19]. Cells with two distinct nuclei of approximately equal size that were not connected by a nucleoplasmic bridge, touched each other at the edges, or overlapped only slightly were selected. To be scored, a MN was required to be located within the cytoplasm of a bi-nucleated cell and could not be refractive. The MN was also required to be morphologically identical to the main nucleus but smaller and could not be connected to the main nucleus via a nucleoplasmic bridge. The data are expressed as the number of MN per 1000 bi-nucleated cells and as the frequency of bi-nucleated cells containing one or more MN. To provide data regarding proliferation kinetics, the frequencies of mono-, bi-, tri- and tetra-nucleated cells were determined for each treatment group. A nuclear