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Effect of blueberries (BB) on micronuclei induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and 7,12-dimethylbenz(*a*)anthracene (DMBA) in mammalian cells, assessed in *in vitro* and *in vivo* assays

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ABSTRACT

The protective effect of blueberry (BB) on the clastogenic effects of MNNG and DMBA was evaluated with the induced micronucleus (MN) frequency as a biomarker, both *in vitro* and *in vivo*. Human hepatoma HepG2 cells, which contain most of the metabolic activating enzymes was used for the *in vitro* test. MN frequencies were determined in binucleated cells generated by blocking cytokinesis by use of cytochalasin-B. The MN frequency *in vivo* was determined in polychromatic erythrocytes (PCEs) from the bone marrow of treated mice. BB by itself was not toxic both *in vivo* and *in vitro*. There was no evidence of a potential physico-chemical interaction between BB and the test carcinogens *in vitro*. Pre-treatment with BB reduced the MN frequency induced by MNNG. But, simultaneous treatment and post-treatment with BB did not affect the frequency of MNNG-induced MN. BB did not affect the frequency of DMBA-induced MN *in vitro* under any test condition. Under *in vivo* conditions, BB reduced the frequencies of MNNG- and DMBA-induced MN in PCEs, but in the case of the protective effect of BB against DMBA a dramatic reduction in the percentage of PCEs was observed, suggesting increased cytotoxicity.

1. Introduction

Several studies have demonstrated that a diet rich in fruits and vegetables exerts protective effects against infectious diseases and age-related diseases such as neural degeneration, diabetes, and cancer [1–3]. One such dietary agent with a beneficial impact is the blueberry and its constituent phytochemicals. Blueberry is a species of the genus *Vaccinium* belonging to the family *Ericaceae*. Blueberries are sold fresh or processed as individually quick frozen (IQF) fruit, juice or dried or infused berries, which in turn may be used in a variety of consumer products such as jellies, jams, pies, muffins, snack foods and cereals. Blueberries have been shown to reduce the risk of artherosclerosis in apolipoprotein-E deficient mice, which are highly prone to oxidative stress and antioxidant deficiencies in situations of high blood cholesterol [4]. Blueberry extract was found to be effective in inhibiting proliferation of HL60 human leukemia and HCT116 human colon carcinoma cells *in vitro*. Blueberry extracts and anthocyanins purified from these extracts have been shown to induce apoptosis in HL60 cells, suggesting a role of growth inhibitory and apoptosis-inducing effects of blueberry in cancer prevention [5]. Wild blueberry was found to decrease H_2O_2-induced DNA damage in Sprague-Dawley (SD) rats as evaluated in lymphocytes by means of the comet assay [6]. Extracts of berries of the *Vaccinium* species inhibit the induction of ornithine decarboxylase activity by the tumor promoter phorbol-12-myristate-13-acetate (TPA) [7]. Blueberry extracts have been shown to inhibit the growth of HT29 cells *in vitro* even at a concentration of 10 mg/ml whereas other berries like black currant, strawberry, raspberry and lingonberry show similar inhibitory effects only at higher concentrations (30, 40 and 60 mg/ml). Low bush "wild" blueberries (*Vaccinium angustifolium*) possess a high antioxidant potential due to their high concentration of polyphenolic anthocyanins [8–12]. This anthocyanin content gives the blueberry a high Trolox-equivalent antioxidant capacity (TEAC) of 27.60 μM/g of fresh weight [13], however, these important mechanisms of protection are strictly dependent on the bio-availability of the compounds. There is no significant difference between the transport and absorption efficiency of anthocyanins in blueberry extracts. It is well known that anthocyanins are rapidly absorbed,

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metabolized and then excreted in urine and feces in humans [14,15]. Thus, blueberry extract with anthocyanins and other phytochemicals are thought to be promising ingredients in functional foods for reducing the risk of cancer [16]. Likewise the positively acting foodstuffs, there are also food ingredients that adversely affect human health through intake of food mutagens that affect the whole cellular control directly or indirectly. Some of the clearly identified and suspected food mutagens are *N*-nitrosamines, polycyclic aromatic hydrocarbons or heterocyclic amines [17]. Food mutagens cause different types of DNA damage, *i.e.* nucleotide alterations and gross chromosomal aberrations. Most mutagens begin their action at the DNA level by forming carcinogen-DNA adducts [18], which result from the covalent binding of a carcinogen or part of a carcinogen to a nucleotide. For assessing the beneficial activities of food components, it is necessary to have a marker. For manipulating genotoxicity induced by food mutagens and its prevention by functional foods, chromosomal damage can be used as a biomarker [19]. In this study, we used induction of micronuclei as a biomarker for the validation of the effect of blueberry extract on the two genotoxic carcinogens, 7,12-dimethylbenz[*a*]anthracene (DMBA) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). The latter compound decomposes into short-lived highly reactive electrophiles, of which the alkonium ion is probably the ultimate mutagen, and its electrophilic attack on nucleophilic sites in DNA leads to altered bases [20]. Unlike MNNG, DMBA requires metabolic activation by the endogeneous enzymes for its carcinogenic and mutagenic action. The metabolic derivatives of DMBA, DMBA-3,4-diol-1,2-epoxides (DMBADEs) can bind to the amino groups of deoxyguanosine and deoxyadenosine bases and lead to the formation of DNA adducts. This may then create apurinic sites (AP sites) in the DNA as a result of depurination. These DNA damages can cause strand breaks, which in turn lead to micronucleus formation [21–23]. In our study an *in vivo* micronucleus assay was performed with bone-marrow cells of CD1 Swiss albino mice and validated against the cytochalasin-blocked micronucleus (CBMN) assay *in vitro* in HepG2 (human hepatoma) cell line that has retained most of the endogenous metabolic activity. This cell line has been widely used for the detection of mutagens and antimutagens and it has been shown to express phase-I and phase-II drug-metabolizing enzymes [24,25] and particularly to be able to metabolize DMBA [26].

2. Materials and methods

2.1. Cells and culture conditions

From deep-frozen aliquots of human hepatoma (HepG2) cells [27], cultures were set-up and used for the experiments up to a maximum of seven passages after thawing. Cells were grown as monolayer cultures in Dulbecco's minimal essential medium (DMEM, Gibco) supplemented with 15% fetal bovine serum (Gibco), 2 mM L-glutamine (Lonza) and antibiotics (Lonza) – penicillin (100 U/ml) and streptomycin (0.1 mg/ml). The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Chemicals

2.2.1. MNNG

For *in vitro* experiments, MNNG (TCI Europe, CAS: 70-25-7) was first dissolved in dimethyl sulfoxide (DMSO) (Sigma, CAS: 67-68-5) at a concentration of 15 mM. To obtain the solution utilized for the experiments, this stock solution was diluted in distilled water to a concentration of 2.5 mM, kept at −20 °C in dark, and used for the experiments at a final MNNG concentration of 25 μM. For *in vivo* experiments, MNNG was first dissolved in 2% DMSO and then diluted in water in order to treat the mice with a final concentration of 40 mg/kg body weight (bw) by intra-peritoneal injection, in a volume of 320–390 μl according to the body weight of the animal.

2.2.2. DMBA

For *in vitro* experiments, DMBA (Sigma, CAS: 57-97-6) was dissolved in DMSO at a concentration of 20 mM. This stock solution was further diluted in DMSO to a concentration of 0.4 mM. This solution was kept at −20 °C in dark and utilized for experiments at a final DMBA concentration of 2 μM. For *in vivo* experiments, DMBA was dissolved in 2% DMSO in corn oil to reach a final concentration of 25 mg/kg bw/10 ml, and administrated by intra-peritoneal injection in a volume of 320–390 μl according to the body weight of the animal.

2.2.3. Blueberry

Blueberry extract (BE) in capsules were purchased from Bouroff Ltd. (manufacturer: Burov, Sofia; factory: Renipharma, Dupnica). Each capsule contains 210 mg extract from Blueberry (extr. *Vaccinium myrtillus*) and 40 mg lactose. For *in vitro* experiments, it was dissolved in distilled water at a concentration of 5 mg/ml as a stock solution. It was kept at room temperature in the dark and utilized for experiments at a final BE concentration of 100 μg/ml of the culture medium. For *in vivo* experiments instead, a 100% naturally freeze-dried blueberries powder (BP) (90 g, corresponding to 630 g fresh blueberries) was purchased at SuperfruitStore.com. The daily recommended dose of one scoop corresponds to about 3 g of the powder (5 kcal), containing 3.6 mg vitamin C (4.5%), and 102 mg anthocyanins. The powder was dissolved in water to make a final concentration of 128 or 700 mg/kg bw/10 ml and given by oral gavages of about 320–390 μl depending on the body weight of the animal. Neither for BE nor for BP it was verified whether the product contained what it was purported to contain, or that it was not adulterated. Furthermore, two different sources of blueberry were used as the BP, which is a naturally freeze-dried powder, cannot be dissolved in order to treat the cells in *in vitro* experiments.

2.2.4. Cytochalasin-B

Cytochalasin-B (cyto-B, Sigma, CAS: 14930-96-2) was diluted in DMSO (Sigma, CAS: 67-68-5) to obtain a stock solution of 1.2 mg/ml and kept at −20 °C until use at a final cyto-B concentration of 6 μg/ml. Acridine orange (Sigma, CAS: 65-61-2) was dissolved in distilled water at a concentration of 40 μg/ml and kept at +4 °C until use for staining of HepG2 cells.

2.3. In vitro experiments

2.3.1. Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was carried out with the standard technique proposed by Fenech [28], with minor modifications. After completion of the treatments, cells were washed twice with 5 ml phosphate-buffered saline (PBS) (Lonza) and trypsinized with 0.5 ml trypsin–EDTA (0.25%) (Lonza). They were centrifuged at 225 × g for 5 min and the pellet was then re-suspended in cold hypotonic solution (1% sodium citrate). The cell suspension was centrifuged again at 125 × g for 5 min and fixed with freshly mixed methanol:acetic acid (5:1). Air-dried preparations were made and coded. The slides were stained with 40 μg/ml acridine orange solution. A total of 1000 binucleated cells with intact cytoplasm were scored per culture for the presence of micronuclei (MN) for each experimental point. For the analysis of cell-cycle progression, 1000 cells per treatment were scored for the presence of one, two or more than two nuclei and the cytokinesis-block proliferation index (CBPI) was calculated as follows: $CBPI = [1N + (2 \times 2N) + (3 \times >2N)]/TC$ where 1N is number of cells with one nucleus, 2N with two nuclei, >2N with more than two nuclei and TC is the number of cells examined. On the basis of this parameter, the percentage of cytostasis was calculated with the formula: $\%\ \text{cytostasis} = 100 - 100[CBPI_t - 1/CBPI_c - 1]$ where t and c are treated and control samples, respectively [29].

2.3.2. Statistical analysis in vitro

The statistical significance in the yield of micronuclei per cell between the control and treated samples was evaluated by Student's *t*-test and with the Chi-squared test for the cytostatic effect. $P < 0.01$ or <0.05 was considered to correspond with statistical significance.

2.4. Protocol for the in vitro CBMN assay

The effect of Blueberry extract on MNNG- and DMBA-induced chromosomal damage (MN) was tested. The experiments were repeated twice. The doses of MNNG, DMBA and blueberry extract were selected on the basis of pilot studies conducted in order to evaluate their cytotoxicity in HepG2 cells, measured in terms of a decrease in the CBPI (cytokinesis-block proliferation index). The blueberry extract dose of 100 μg/ml was selected to test its chemo-preventive effect in a 24-h treatment (Table 1) as at higher doses there was a significant effect on cell proliferation. In order to exclude a potential physico-chemical interaction between BE and the carcinogens, BE alone (solution 1), MNNG alone (solution 2), DMBA alone (solution 3), MNNG plus BE (solution 4), and DMBA plus BE (solution 5) were added at the respective doses in 5 ml of complete medium (HBSS in the case of MNNG) and incubated at 37 °C in the dark for 1 h (in the case of MNNG) or 24 h (in the case of DMBA). Afterwards, the cells were treated for 1 h (in the case of MNNG) or 24 h (in the case of DMBA) with these solutions and the CBMN assay was performed to determine the MN frequency and the percentage (%) of cytostasis. Treatments with MNNG were performed in HBSS to avoid loss of activity of MNNG by extracellular SH-groups [30]. The final DMSO concentration in the medium did not exceed 1%, and it was verified in pilot experiments that the solvent had no effect on the spontaneous MN frequencies. The treatment protocols for CBMN were as follows:

Pre-treatment: Twenty-four hours after seeding, the cells were treated with blueberry extract (100 μg/ml) for 24 h. Then, the cells were washed twice with PBS and treated with MNNG (25 μM) for 1 h. After this treatment, cells were washed twice