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SCIENCE DIRECT
Toxicology in Vitro 20 (2006) 1168–1172
Toxicology in Vitro
www.elsevier.com/locate/toxinvit

Evaluation of apigenin using in vitro cytochalasin blocked micronucleus assay

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Received 2 November 2005; accepted 27 March 2006
Available online 4 April 2006

Abstract

High doses of flavonoids are reported to be clastogenic in contrast to their potential to reduce oxidative DNA damage, retard growth of leukemia cells, obstruct cell signal transduction and induce cellular differentiation in cancers. In the present study, we evaluated apigenin, a plant-derived flavonoid in doses of 10, 33, and 100 μM per 5 ml culture using cytochalasin-B blocked micronucleus (CBMN) assay in peripheral human lymphocytes. Apigenin was found to induce micronuclei in a dose dependent manner indicating potential genotoxic hazard in humans. Hence, flavonoids may act as mutagen, pro-oxidant or as inhibitor of key enzymes to produce clastogenic effects depending upon the levels consumed as well as the physiological parameters.

Keywords: Apigenin; CBMN assay; Cytochalasin-B; Genotoxicity; Human peripheral lymphocytes; Micronucleus

1. Introduction

Apigenin is a natural flavonoid found in high amounts in parsley, peppermint, lemon, perilla, berries, and fruits (Peterson and Dwyer, 1998). In vitro studies show that it can inhibit growth of several types of cancer cells such as breast (Way et al., 2004; Hirano et al., 1989; Rosenberg et al., 1998) and prostate (Kobayashi et al., 2002). Such anti-proliferative activities exhibited by apigenin involve inhibition of cancer cell signal transduction and apoptosis, which consequently leads to reduced DNA oxidative damage. Topical applications of apigenin were reported to significantly reduce the number of UV induced benign carcinomas in mice (Wei et al., 1990). Apigenin effectively blocks tumor necrosis factor (TNF) induced intercellular adhesion molecule-1 (ICAM-1) up regulation and DNA binding activity of NF-B in response to cytokines in vitro. Apart from these anti-proliferative properties, flavonoids in general exhibit anti-inflammatory and antispasmodic properties. Flavonoids have also been shown to possess anti-mutagenic properties in bacterial assays conducted in the presence of mammalian metabolic enzymes (Choi et al., 1994; Miyazawa and Hisama, 2003). However, effects of flavonoids at high doses remain highly speculative (Stopper et al., 2005). This inconsistency regarding the biological activity of flavonoids, especially as pro-oxidants resulting in DNA damage, has led many investigators to ascertain the exact nature of flavonoids (Snyder and Gillies, 2002). A number of studies provide evidence on dual biological activities of flavonoids in mutagenesis and carcinogenesis. The anti-mutagenic/pro-mutagenic and anti-oxidant/pro-oxidant activity largely depends upon the levels consumed as well as the physiological conditions of the body (Christine and Smith, 2000). Unfortunately, the potential toxic effects of excessive flavonoid intake are largely ignored.

Thus far genotoxic potential of apigenin has not been monitored in a dose dependent manner inspite of the speculative behavior shown by most of the flavonoids specially when consumed at high doses. In the present work we have employed the cytochalasin-B blocked micronucleus

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doi:10.1016/j.tiv.2006.03.007

(CBMN) assay for evaluating the genotoxicity of apigenin in normal human peripheral lymphocytes. The CBMN assay was used because of its sensitivity and simplicity by which affected cells can be scored quickly to measure the extent of clastogenicity and aneugenicity of xenobiotics (Miller et al., 1997). The assay also provides valuable information to supplement the results of other in vitro genotoxicity assays (Stopper et al., 1997). Additionally, the information obtained from CBMN assay can be extrapolated to predict health hazards in humans.

2. Material and methods

2.1. Lymphocyte culture, apigenin dosing and slide preparation

Blood was withdrawn by venipuncture from healthy volunteers and immediately transferred to a tube containing heparin (Sigma) at concentration of 100 U/ml of blood. All the cultures were performed in triplicates each of 5 ml described briefly as follows. 500 µl of heparinized blood was added per culture tube containing 4.5 ml of RPMI 1640 (Sigma) [with 15% fetal calf serum (Himedia)]. Streptomycin (0.25 mg/ml), ampicillin (0.05 mg/ml) and amphotericin B (0.001 µg/ml) were used as antibacterial and antifungal agents to hamper the undesirable growth of bacteria and fungi. Cell division was stimulated by the addition of 4.5 µg/ml phytohaemaglutinin-B (Sigma) and culture tubes (30 ml) were incubated at 37 °C and 5% CO_2 concentration for 40 h. Mitomycin C (6 µM/culture) was added as positive control whereas sterile triple distilled water (TDW) was used as a negative control after 40 h incubation. Similarly, apigenin was added in three different concentrations of 10 µM, 33 µM and 100 µM per culture tube. After incubating the culture for 4 h, 4.5 µg cytochalasin-B (Sigma) was added per ml of culture to restrict cells in binucleated stage. The culture was incubated again for 28 h before harvesting the lymphocytes. The culture was transferred to centrifuge tubes with 10 ml freshly prepared 0.075 M KCl (pH 7.2) and maintained at 37 °C for 10 min with occasional agitation. The sample was then centrifuged at 2000 rpm for 5 min and pallet resuspended in freshly prepared chilled methanol: acetic acid (3:1) fixative, maintained at 37 °C for 10 min before centrifugation at 2000 rpm. This step was repeated till the haem portion of the blood was substantially removed. The final lymphocyte pallet was resuspended in small volume of fixative and 3–4 drops were carefully placed on chilled slides so as, not to burst the swollen cells. The slides were coded and dried immediately on a hot plate and stored in a dust free place. After maturation cells were fixed on slides with methanol and stained with 10% geimsa (Qualigens) for 1 h. The slides were washed directly under tap water followed by single distilled water and dried before counterstaining with 1% eosin (Qualigens) for 1–2 min. The slides were washed finally with methanol, dried, mounted in DPX (Qualigens) and observed under the microscope.

2.2. Cytokinesis-block proliferation index (CBPI)

The effect of apigenin on lymphocyte proliferation was assessed by determining the cytokinesis-block proliferation index. We Scored 1000 cells per treatment group for the presence of one, two or more nuclei. The CBPI was calculated as follows: CBPI = [number of binucleated cells + 2(number of multinucleated cells)]/total number of cells (www.swan.ac.uk/cget/ejgt/article1.htm).

2.3. Lymphocyte viability at different concentration of apigenin

The viability of the cells in the culture was assessed to monitor the cytotoxicity of apigenin. Cells were stained with 0.4% trypan blue (Sigma) for 5 min and percent cell viability was calculated as: Cell viability (%) = (Number of viable cells/Total no. of cells) × 100 (Fernandez-Botlan and Vetvick, 1995).

2.4. Assessment of slides and statistical analysis

Slides were observed using Olympus BH2 oil immersion lens (100 X). Triplicate cultures of control, positive control and apigenin treated cells were made to prepare slides from each culture. Four slides for each concentration were prepared and each was scored for 1000 binucleated (BN) lymphocytes. Observations were made for BN cells with micronuclei (MN), multiple micronuclei, nucleoplasmic bridges and mononucleated cells with micronuclei. Data of controls (both positive and negative) and the apigenin treated samples were analyzed statistically by one way ANOVA with Newman-Keuls post analysis test using GraphPad Prism version 3.00 for Windows to find significant differences in the number of MN and CBPI.

3. Results

The number of micronuclei scored per 1000 BN cells increased in a dose dependent manner and were comparable to Mitomycin C (positive control) at highest concentration. Similar increase in number was also observed for cells with multiple micronuclei per 1000 BN cells scored. The trend in increase of BN cells with MN ($P < 0.001$) and

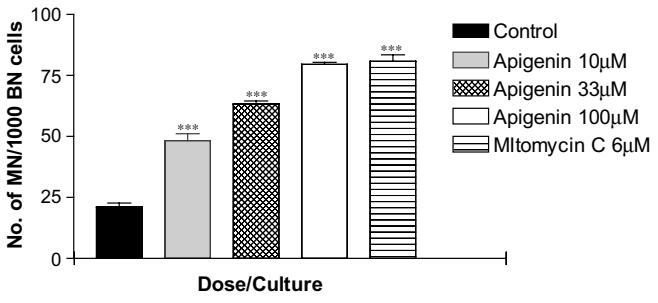

Fig. 1. Number of cells with MN/1000BN cells at different concentrations of apigenin.