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Anticlastogenic effect of apigenin in human lymphocytes treated with ethinylestradiol

Yasir Hasan Siddique *, Gulshan Ara, Tanveer Beg, Mohammad Afzal

Human Genetics and Toxicology Lab, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, U.P. 202002, India

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ABSTRACT

In the present study the antigenotoxic effect of apigenin was studied against a genotoxic dose of ethinylestradiol using the damage parameters of chromosomal aberrations (CAs), sister chromatid exchanges (SCEs) and cell cycle kinetics (CCK). Human peripheral blood lymphocytes were cultured and treated with 10 μM of ethinylestradiol along with doses of 5, 10, 15 and 20 μM of apigenin. A clear decrease in the genotoxic damage induced by ethinylestradiol was observed with increasing doses of apigenin, suggesting a protective role for apigenin during ethinylestradiol therapy.

1. Introduction

Estrogens are labeled as human carcinogens in the fourth-annual report on carcinogens of U.S. DHHS, National Toxicology Program [1] and there is sufficient evidence for their carcinogenicity [2]. Ethinylestradiol is a white, odorless crystalline powder, with a melting point of 182–184 °C. Ethinylestradiol is used to treat hypoestrogenism due to hypogonadism, castration, or primary ovarian failure [3]. Currently, many of the oral contraceptives used in United States contain either 30 or 35 μg of ethinylestradiol. This dose level is used because of its contraceptive efficacy, because it is tolerated well and because of low risk of adverse effects such as breakthrough bleeding [4]. However, several investigators have reported unscheduled DNA synthesis (UDS) and adduct formation by ethinylestradiol in rat and human hepatocytes *in vitro* [5–7]. The compound has also been associated with aneuploidy in Chinese Hamster DON cells *in vitro* [8], as well as both aneuploidy and polyploidy in V79 cells *in vitro* [9]. Ethinylestradiol has also been associated with chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in both cultured human lymphocytes [10,11], and in mouse bone marrow cells [11]. Furthermore the compound has caused SCEs in CHO cells *in vitro* [12] and CAs in rat bone marrow cells [13]. Oral contraception with a combination of 20 μg of ethinylestradiol and 75 μg of gestodene during six consecutive menstrual cycles does not induce micronuclei in peripheral blood lymphocytes of women [14]. The induction of chromosomal abnormalities, such as aberrant chromosomal number, gross deletions, translocations and gene amplifications gives an indication of the neoplastic development of certain cancers. Genetic toxicology tests are *in vivo* and *in vitro* assays designed to detect compounds that induce genetic damage directly or indirectly [15]. There are multiple pathways of genetic damage such as the inhibition of DNA repair, methylation status and co-carcinogenesis with other environment toxicants that may be responsible for the genetic damage [16]. An increase in the frequency of CAs in peripheral blood lymphocytes is associated with an increase in overall risk of cancer [17,18]. The majority of the CAs observed in metaphases are lethal, although some aberrations may be viable and can cause genetic effects, either somatic or heritable genetic effects [19]. Because detection of SCEs resulting from toxicant-DNA damage is sensitive and can be quantified, this end point is typically used as an indicator of DNA damage in cultured human blood lymphocytes of individuals exposed to genotoxic carcinogens [20]. Thus, the above genotoxic end points are well

* Corresponding author.
E-mail address: yasir_hasansiddique@rediffmail.com (Y.H. Siddique).

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established markers of compound genotoxicity. Any reduction in the frequency of these above mentioned genotoxic end points gives an indication of the antigenotoxicity of a particular compound [20]. The prolonged use of oral contraceptives has also been reported to cause development of various types of cancers [1,21], hence we decided to study the action of ethinylestradiol in combination with apigenin. Natural plant products and antioxidants have been reported to reduce the genotoxicity of estrogens and synthetic progestins [22–28], thereby reducing the possibility of tumor development during therapy. Apigenin (Fig. 1), a member of the flavone family of the flavonoids, is an active ingredient in many fruits and vegetables [29]. It has been recognized as an alternative medicine for its pharmacological activity [30], free radical scavenging and anticarcinogenic effects [31], tumor inhibition [32] and anti-genotoxic properties [33]. The focus of the present study was to assess the antigenotoxic effects of apigenin against the genotoxic effects of ethinylestradiol.

2. Materials and method

2.1. Chemicals

Apigenin (CAS: 520-36-5, 4',5,7-trihydroxyflavone), ethinyl-estradiol (CAS: 57-63-6, 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol) and cyclophosphamide (CAS No: 6055-19-2) were purchased from Sigma-Aldrich Co., USA. RPMI 1640, fetal calf serum (FCS), Phytohaemagglutinin-M (PHA) and antibiotic-antimycotic mixture were purchased from In vitrogen (Gibco), USA. Dimethylsulphoxide (DMSO), 5-bromo-2-deoxyuridine (BrdU) and colchicine were purchased from SRL, India. Giemsa stain was purchased from E. Merck, India.

2.2. Human lymphocyte culture

Duplicate peripheral blood cultures of two healthy, non smoker female donors (24 and 26 years of age) were treated according to Carballo et al. [34]. Two cultures were performed per donor for each dose level. Briefly, a heparinized, blood sample (0.5 ml), was obtained from the healthy female donor and was placed in a sterile culture tube containing 7 ml of RPMI 1640 medium, supplemented with fetal calf serum (10%), antibiotic-antimycotic mixture (10%) and phytohemagglutinin (1%). The cultures tubes were then placed in an incubator at 37 °C for 24 h.

2.3. Chromosomal aberration analysis

Following a 24 h, incubation 10 μM of ethinylestradiol (dissolved in DMSO, 5 μl/ml) was given with 5, 10, 15 and 20 μM of apigenin, along with 0.5 ml of S9 mix at final dose of culture. S9 mix was prepared according to the standard protocol of Maron and Ames [35] and the cells were incubated with the S9 mix for 6 h. The cells were then collected by centrifugation and washed in prewarmed medium to remove the excess traces of the S9 mix and added drugs and were further incubated for the remaining 42 h. Treatment of 0.2 ml of colchicine (0.2 μg/ml) was given to the culture tubes, prior to 1 h of the harvesting. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 8 ml of prewarmed (37 °C) 0.075 M KCl (hypotonic solution) was added. Cells were resuspended and incubated at 37 °C for 15 min. The supernatant was removed after centrifugation at 1000 rpm for 10 min, and subsequently 5 ml of chilled fixative was added. The fixative was removed by centrifugation and the procedure was repeated twice. To prepare slides, 3–5 drops of the fixed cell suspension were dropped on a clean slide and air-dried. The slides were stained in a 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. Slides were coded before scoring and approximately 75 cells were scored per culture. Three hundred metaphases were examined for the occurrence of genetic abnormalities. Criteria to classify different types of genetic aberrations were in accordance with the recommendation of Environmental Health Criteria 48 for Environmental Monitoring of Human Population [36].

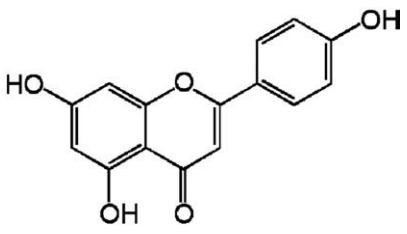

Fig. 1. Structure of Apigenin ($C_{15}H_{10}O_5$).

2.4. Sister chromatid exchange analysis

To study sister chromatid exchange analysis, bromodeoxyuridine (BrdU) (10 μg/ml) was added at the beginning of the culture. After 24 h, 10 μM of ethinylestradiol (dissolved in DMSO, 5 μl/ml) was given with 5, 10, 15 and 20 μM of apigenin, along with 0.5 ml of S9 mix at final dose of culture. The cells were then collected by centrifugation and washed in pre-warmed media to remove traces of the S9 mix and drugs. Mitotic arrest was subsequently triggered by the addition 0.2 ml of colchicine (0.2 μg/ml). Hypotonic treatment and fixation were performed in the same manner as previously described for chromosomal aberration analysis. The slides were coded before scoring and the sister chromatid exchange average was calculated from an analysis of metaphase during the second cycle of division [37]. Fifty metaphases per culture were scored for SCEs.

2.5. Cell cycle kinetics

During cell cycle analysis, 50 metaphases per culture were examined. Each metaphase was classified as being in the first (M_1), second (M_2) and third (M_3) metaphase divisions. Metaphase divisions were detected by the with BrdU-Harlequin technique for differential staining of metaphase chromosomes [38,39]. Treatments were similar as described earlier in the text. The replication index (RI), an indirect measure of studying cell cycle progression was calculated by applying the following formula [40].

$$\mathrm{RI} = \frac{M_1 + 2M_2 + 3M_3}{100}$$

where M_1, M_2 and M_3 denote the number of metaphases in the first, second and third cycle, respectively.