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Radioprotective activity and cytogenetic effect of resveratrol in human lymphocytes: An *in vitro* evaluation

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ABSTRACT

Trans-resveratrol is a natural occurring polyphenol, obtained from grapes and other berries. This compound has shown antioxidant, anti-inflammatory, immunostimulant or anti-carcinogenic properties. Our aim was to evaluate the radioprotective efficacy, *in vitro*, of *trans*-resveratrol against radiation-induced chromosomal damage and to study the genotoxicity and cytotoxicity of this polyphenol in cell cultures without irradiation. The study was carried out by the pre-treatment of human lymphocytes at concentrations from 0 to 219 μM of *trans*-resveratrol. The results showed that all concentrations tested reduced radiation-induced chromosomal damage compared with cells with any treatment. Maximum damage protection was observed at the concentration of 2.19 μM. Concerning genotoxic results, all tested *trans*-resveratrol concentrations increased the sister chromatid exchange (SCE) index compared with no *trans*-resveratrol treatment. Cytotoxic indexes (Mitotic and Proliferation Index) showed that the lowest concentrations could enhance the cell proliferation rates and the highest ones could negatively affect to human peripheral lymphocytes growth.

1. Introduction

The use of radiation in diagnosis and treatment have been extended so rapidly in recent years that one or another form of radiation is now indispensable in virtually every branch of medicine. Unfortunately, planned (such as diagnosis with ionizing radiation and cancer radiotherapy) or unplanned radiation exposures (such as nuclear accidents) can cause damage to normal cells. Hence, the interest to find several agents to protect healthy tissues from ionizing radiation has been increased in last decades. These agents, known as radioprotectors, should have characteristics such as being able to mitigate injuries caused by radiation-induced highly reactive free radicals and they should not produce cumulative or irreversible toxicity (Arora et al., 2005).

Many synthetic compounds have been investigated in the recent past; nonetheless the currently available ones have many drawbacks including high cost, side effects and toxicity (Maurya et al., 2006). Nowadays, attention has been focused in natural compounds which have lower toxicity than synthetic radioprotectors (Weiss and Landauer, 2003), more favorable administration routes and improved pharmacokinetics. Many natural compounds as lycopene (Cavusoglu and Yalcin, 2009; Srinivasan et al., 2009), ferulic acid (Prasad et al., 2006) and melatonin (Vijayalaxmi et al., 1995) have been already tested as radioprotectors. Extracts of natural substances have been also evaluated, i.e., ethanolic extract of propolis (Montoro et al., 2005), ginseng (Lee et al., 2005) or water-soluble and alcohol-soluble extract of spearmint (Baliga and Suresh, 2010); nevertheless extracts of natural substances contain a high number of compounds so that is difficult to assess which one or which combination act as radioprotector.

Resveratrol is a phytoalexin, from the group of stilbenes, found in grapes and other berries as *cis* and *trans* form being *trans* isomer by far more naturally found (Signorelli and Ghidoni, 2005). According to the literature, many health benefits have been attributed to this molecule (anticarcinogenic, anti-inflammatory, neuroprotective and antidiabetic properties, among others) (Bisht et al., 2010; Chakraborty et al., 2010). However, the antioxidant property of this compound (Carsten et al., 2008) should be useful against radiation damage. Damaging effects of ionizing radiation on the deoxyribonucleic acid (DNA) molecule are brought about by both

Abbreviations: Ace, acentric fragments; CA, chromosomal aberrations; CPK, cell proliferation kinetics; Ctb, Chromatid Type Break; DNA, Deoxyribonucleic acid; MI, mitotic index; PI, proliferation index; RMI, relative mitotic index; RPI, relative proliferation index; SCE, sister chromatid exchange; TAR, cigarette-smoke condensate.

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direct and indirect mechanisms. Direct action produces disruption of chemical bonds in the structure of DNA whereas indirect effects result from highly reactive free radicals, mainly produced during the radiolysis of water, and their subsequent interaction with DNA (Okada et al., 1983). Reduction of these free radicals by natural antioxidants will provide a degree of protection against ionizing radiation injury (Hosseinimehr, 2007). It is well known that resveratrol has the ability to scavenge radiation-induced free radicals, therefore is thought that its radioprotective effect results, at least partially, from its ability to scavenge free radicals. Actually, it has been already proved that resveratrol can prevent free radicals production by other agents different from ionizing radiation such as H_2O_2 and cigarette-smoke condensate (TAR) and hence, reduced nuclear DNA fragmentation caused by this factor (Sgambato et al., 2001). Furthermore, prevention of ionizing radiation damage by resveratrol could be assisted by the fact that this polyphenol can induce the action of some endogenous antioxidants such as glutathione and increases enzymes like superoxide dismutase and catalase (Li et al., 2006). In order to quantify the capacity of the *trans*-resveratrol isomer to modulate radiation-induced DNA damage in human cells, scoring chromosomal aberrations (CA) in peripheral blood lymphocytes is considered an important tool due to the fact that DNA of irradiated cells undergoes single- or double-strand breaks and damage to bases and sugars, ultimately leading to chromosomal aberrations (Benkovic et al., 2008).

The aim of this manuscript is to study, *in vitro*, the radioprotective activity, as well as cytotoxic and genotoxic effects, of *trans*-resveratrol, in irradiated and non-irradiated peripheral human blood lymphocytes.

2. Materials and methods

2.1. Reagents and equipment

Standards of *trans*-resveratrol were supplied by Sigma–Aldrich (St. Louis, MO, USA). From the stock solution and using ethanol 95% (Panreac, Barcelona, Spain) as solvent, *trans*-resveratrol dilutions were prepared in order to reach concentrations of 2.19, 21.9 and 219 μM in 12 mL of human peripheral blood samples, adding in all cases a volume of 250 μL of the *trans*-resveratrol dilution. Carnoy's lymphocytes fixative solution was prepared with methanol (Merck, Ramstadt, Germany) and acetic acid (Panreac, Barcelona, Spain) (3:1 v/v). Cytogenetic analyses were carried out by using a conventional microscope (Izasa, Barcelona, Spain) and an image analysis system with the IKAROS-software (MetaSystems). The study was approved by the Ethics Committee of the University of Valencia (Spain) and samples were collected after informed consent.

2.2. Irradiation conditions

Human peripheral blood samples were collected in sterile vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing lithium heparin as anticoagulant. The different concentrations of *trans*-resveratrol were added to blood samples 1 h before irradiation and maintained at 37 °C in a water bath. Samples were irradiated at 2 Gy (dose rate 50 cGy min^{-1}) using a Cobalt Teletherapy Unit located at Hospital la Fe (Valencia). Incubation for 1 h at 37 °C soon after irradiation is required to facilitate the repair mechanisms. Two tubes were irradiated at 2 Gy as controls; one with peripheral blood alone and the other with peripheral blood in the presence of *trans*-resveratrol solvent alone (250 μL of 95% ethanol). International Atomic Energy Agency (2001) recommendations were followed during irradiation to ensure a homogeneous irradiation.

2.3. Culture conditions

For each treatment, separate cultures were set up by mixing 0.75 mL of whole blood with 5 mL of PB-MaxTM Karyotiping medium (Gibco, Barcelona, Spain) and incubated 48 and 72 h at 37 °C. To analyse exclusively first-division metaphases, a final concentration of 12 μg mL^{-1} of bromodeoxyuridine was present since the setting up of the cultures. 150 μL of Colcemid® (Gibco, Barcelona, Spain) from a stock solution of 10 μg mL^{-1} were added 2 h before harvesting to stop the cell culture in metaphase.

2.4. Stain technique

Two- to three-days-old slides were stained with Fluorescence plus Giemsa stain technique. Briefly, old slides were treated 20 min in Hoechst 33258 at room temperature. The slides were washed with distillated water. The slides were covered with 2× SSC and treated with UV light (300 W) for 2 min. Once washed the slides with water and dried 30 min, the slides were stained with Leishman (Merck, Ramstadt, Germany) for 5 min being useful in the identification of first-, second- and third-division metaphases.

2.5. Cytogenetic analysis

2.5.1. Chromosomal aberrations in irradiated cultures with and without trans-resveratrol

Chromosomal analysis was carried out exclusively on first-division metaphases containing 46 centromeres in a total of one hundred cells. Chromosomal aberrations were classified as follows: dicentric chromosomes and rings, that were only considered when an acentric fragment was present. Acentric fragments, not associated with dicentric and ring chromosomes, were classified as extra acentric fragments (ace). Other chromosome aberrations, like chromatid breaks (ctb), gaps, translocations and inversions, were also recorded were only recorded when the morphology of the derivative chromosome was clearly indicative of this kind. Frequency of dicentrics chromosomes were recorded for blood cultures with any treatment, 250 μL of ethanol 95% and different concentrations of *trans*-resveratrol.

2.5.2. Sister chromatid exchange (SCE)

The incidence of SCE was determined from the analysis of 50 s division metaphases for each treatment. The evaluation of SCE scores in lymphocytes included scoring total exchanges in total number of analyzed cells for each treatment to establish its frequency (Y_{SCE}). Moreover, we scored the number of chromosomes with one ($Y_{1\ SCE}$), two ($Y_{2\ SCE}$) or three ($Y_{3\ SCE}$) chromatid exchanges in the total number of analyzed cells for each treatment.

2.5.3. Mitotic index (MI)

For each concentration, the mitotic index was calculated exclusively on first-division metaphases as the proportion of metaphases in 500 cells according to Rojas et al. (1993). The relative mitotic index (RMI) was evaluated as RMI = [MI treated/MI control] × 100. The inhibition of mitotic index was evaluated as 100 − [MI treated × 100/MI control].

2.5.4. Cell proliferation kinetics (CPK)

For each concentration, in order to evaluate de CPK, the proportion of cells in first (M1), second (M2) and third (M3) division was obtained from 100 consecutive metaphases. The proliferation index (PI) was calculated according to the formula [PI = (M1 + 2M2 + 3M3)/100] and the relative proliferation index (RPI) was evaluated as RPI = [PI treated/PI control] × 100 (Rojas et al., 1993).

2.6. Statistical analysis

For statistical analysis, Student *t* test was used and *p*-values < 0.05 were considered significant. Correlation was assessed using Spearman's rank correlation coefficient. All statistical analyses were carried out using SPSS (Statistical Package for Social Sciences) version 10.0 for Windows. The Poisson distribution was checked by the test quantity *U* of the dispersion index (variance/mean), a value of $U > 1.96$ indicates over dispersion at the 5% level of significance.

3. Results and discussion

3.1. Cytogenetic results in trans-resveratrol pre-treated cultures exposed at 2 Gy of γ-Ray

The cytogenetic results obtained after the analysis of human lymphocytes exposed at 2 Gy of γ-ray in the presence of different concentrations of *trans*-resveratrol are shown in Table 1. After 2 Gy irradiation, the frequency of dicentrics in samples pre-treated with *trans*-resveratrol were reduced compared to samples irradiated without this compound. The differences in the frequencies were statistically significant ($p < 0.05$) for 2.19 μM of *trans*-resveratrol. The maximum level of radioprotection achieved when lymphocytes were exposed to 2 Gy of gamma rays was around of 47% corresponding to the concentration of 2.19 μM. This value was obtained for each concentration of *trans*-resveratrol from the formula $Y = 100 - ((\text{Dicentrics frequency with } trans\text{-resveratrol} \times 100)/\text{Dicentrics frequency in control blood sample})$.