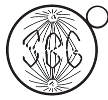

Genetics and Molecular Biology, 30, 1, 105-111 (2007)
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Research Article

Genotoxic and antigenotoxic effects of *Fucus vesiculosus* extract on cultured human lymphocytes using the chromosome aberration and Comet assays

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Abstract

The brown seaweed *Fucus vesiculosus* (Fucales, Fucaceae) was screened for its protective activity using doxorubicin-induced DNA damage in human lymphocytes. In this study, we assessed the genotoxic and antigenotoxic potential of three different concentrations (0.25, 0.5 and 1.0 mg mL^{-1}) of *F. vesiculosus* aqueous extract using the chromosome aberration and Comet assays. Treatment of human lymphocyte cultures with 0.25, 0.5 and 1.0 mg mL^{-1} *F. vesiculosus* aqueous extract had no effect on the chromosome aberration frequency or on the extent of DNA damage detected by the Comet assay. The antigenotoxic effects of the extract were tested in human lymphocyte cultures treated with 15 μg mL^{-1} of doxorubicin, either alone or combined with the different concentrations of the extract, which was added to the cultures before, simultaneously with or after the doxorubicin. Only when lymphocytes were pre-treated with extract there was a reduction in doxorubicin-induced chromosome aberrations and DNA damage as detected by the Comet assay. These results demonstrate that *F. vesiculosus* aqueous extract is not genotoxic in cultured human lymphocytes and indicate that when added to lymphocyte cultures before doxorubicin it has antigenotoxic activity against doxorubicin-induced DNA damage.

Key words: antigenotoxicity, chromosomal aberrations, Comet assay, doxorubicin, *Fucus vesiculosus*.

Received: February 3, 2006; Accepted: May 8, 2006.

Introduction

Doxorubicin is an anthracycline antibiotic used as an antitumor agent against human malignancies such as leukemia, lymphomas and many solid tumors (Young *et al.*, 1981; Booser and Hortobagyi, 1994) but which also has a wide variety of toxic side-effects, including cardiotoxicity, cytotoxicity and the induction of chromosomal aberrations (Singal *et al.*, 2000; Jung and Reszka, 2001). The reduction of oxidative DNA damage by antioxidants has been evaluated as a chemotherapeutic approach for reducing damage caused by chemotherapy agents such as doxorubicin (Quiles *et al.*, 2002). Alcoholic and aqueous extracts of brown seaweeds have been evaluated for antioxidant activity associated to compounds such as polyphenols and polysaccharides (Jiménez-Escrig *et al.*, 2001; Rupérez *et al.*, 2002). Extracts of the brown seaweed *Fucus vesiculosus* (Fucales, Fucaceae) has demonstrated a strong antioxidant activity by the ferric reducing antioxidant power (FRAP) assay (Rupérez *et al.*, 2002).

The alkaline Comet assay is a very useful method for studying genotoxicity in cells exposed *in vitro* or *in vivo* to a variety of physical and chemical agents (Tice *et al.*, 2000). It provides a straightforward visual method for assessing DNA damage quantitatively in single cells and is established as a valuable tool in fundamental DNA damage and repair studies (Visvardis *et al.*, 2000). The chromosome aberration assay is also a powerful classical cytogenetic tool for genotoxicity testing and can be used as a validation test for Comet assay results (Hartmann *et al.*, 2003).

We assessed the genotoxic and antigenotoxic potential of *F. vesiculosus* aqueous extract in cultured human lymphocytes. Genotoxicity was measured by the frequencies of chromosome aberrations and the induction of DNA damage as detected by the Comet assay. The antigenotoxic activity of *F. vesiculosus* aqueous extract was evaluated by determining the protective effect of the extract on the fre-

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quency of chromosome aberrations and Comet assay DNA damage induced by doxorubicin.

Material and Methods

Preparation of *Fucus vesiculosus* aqueous extract

A powdered extract of *Fucus vesiculosus* was kindly donated by Bionatus Botanical Laboratory (São José do Rio Preto, SP, Brazil) and doxorubicin (Eurofarma, São Paulo, Brazil) was kindly provided by the Chemotherapy Center of the University Hospital of Ribeirão Preto-SP, Brazil.

An aqueous suspension of *F. vesiculosus* was prepared by adding 10 mL of 75 °C deionized distilled water to 0.5 g of *F. vesiculosus* powder and agitating the suspension for 1 h before filtering first through filter paper and then through a 0.22 μm Millipore® bacteriological membrane (Millipore®, São Paulo, Brazil). The *F. vesiculosus* aqueous extract was used immediately after preparation by adding it to the cell culture medium (see below) to produce final concentrations of 0.25, 0.5 and 1 mg mL^{-1}, with respect to the original *F. vesiculosus* powder, these concentrations being chosen on the basis of preliminary screening trials (data not published).

Lymphocyte cells and culture conditions

This study was approved by the Research and Ethics Committee of the University Hospital of Ribeirão Preto, São Paulo State (Process HCRP n. 3254/2003) and by the National Comission of Ethics in Research (CONEP; Process n° 2500.084027/2003-29). Peripheral blood samples were collected by venipuncture from 12 healthy, non-smoking, non-alcoholic female volunteers aged 18 to 40 years who had not taken any medicine recently.

Lymphocytes isolated from the blood samples were incubated at 37 °C in 5 mL of complete medium consisting of 78% (v/v) RPMI culture medium (Sigma, St. Louis, MO) 20% (v/v) fetal calf serum (Cultilab, Campinas, Brazil) and 2% (v/v) phytohemagglutinin (Gibco, Carlsbad, CA) supplemented with 0.001 mg mL^{-1} streptomycin and 0.005 mg mL^{-1} penicillin.

To determine the antigenotoxicity of *F. vesiculosus* aqueous extract at 0.25, 0.5 or 1 mg mL^{-1} of culture medium the lymphocyte cultures were submitted to the following treatments: pre-treatment, this consisted of adding the extract 22 h after starting the incubation of the cultures and 2 h before addition of doxorubicin; simultaneous-treatment, in which the extract and doxorubicin were added 24 h after the start of incubation; and post-treatment, in which the extract was added 26 h after the start of incubation and 2 h after the addition of doxorubicin, which was added 24 h after the start of incubation. In all cases the final doxorubicin concentration was 0.15 μg mL^{-1} and all concentrations of extract were tested. Negative control cultures received neither *F. vesiculosus* aqueous extract nor doxorubicin while the positive control cultures received 0.15 μg $mL^{-1}$of doxorubicin only. The genotoxicity of *F. vesiculosus* aqueous extract was also investigated by adding the extracts at final concentrations of 0.25, 0.5 or 1 mg mL^{-1} to the lymphocyte cultures 24 h after the start of incubation.

The Comet assay was performed 48 h after the start of incubation. To detect chromosomal aberrations cytological preparations were made 50 h after the start of incubation because treatment of mammalian cells *in vitro* with doxorubicin arrests the G2/M phase and delays the cell cycle (O'Loughlin *et al.*, 2000) with 0.016% w/v colchicine (Sigma 12.5 μL in 5 mL) being added to the cultures 90 min before harvesting. The cytological preparations were examined by bright-field optical microscopy to asses the mitotic index (MI) and structural or numerical aberrations. Cell viability was determined by the Trypan blue exclusion technique of Pool Zobel *et al.* (1994) and ranged from 92 to 96%.

Chromosomal aberration assay

This assay was performed in agreement with current OECD (Organization for Economic Co-operation and Development) and ICH (International Conference on Harmonization) guidelines. After 50 h incubation each culture was centrifuged at 200 x g for 5 min, after which the supernatant was carefully removed and the pelleted cells resuspended in 5 mL of hypotonic 0.075 M aqueous KCl at 37 °C and then re-pelleted by centrifugeation for a further 5 min at 200 x g, the supernatant being subsequently removed and the pelleted cells fixed by the addition of 5 mL of freshly-prepared 3:1 (v/v) methanol:glacial acetic acid. The fixed cells were resuspended and centrifuged for at least three times until the supernatant was clear, after which the cells were re-pelleted and then resuspended in a minimal amount of fresh fixative to obtain an homogeneous suspension. The cell suspension was dropped onto microscope slides and left to air-dry and then stained with 3% (w/v) Giemsa in a phosphate buffer.

For each treatment and each blood sample (*i.e.* each individual) the frequency of chromosomal abnormalities were estimated in 100-metaphase plates. The mitotic index (MI) was obtained for each treatment and each blood sample (*i.e.* each individual) by counting the number of mitotic cells in 2000 cells. The chromosomal aberration types were classified according to Savage's classification (Savage, 1976).

Comet assay

The Comet assay was performed in its alkaline version following well-established protocols (Singh *et al.*, 1988; Tice *et al.*, 2000). In short, cells from 300 μL of each blood sample culture were pelleted by centrifugation. The pellet was added to 160 μL of 0.5% (w/v) low melting point agarose and the mixture spread onto two microscope slides (Knittel, Germany) pre-coated with 1.5% (w/v) normal

melting point agarose (Gibco, Carlsbad, CA). The slides were covered with coverslips and were refrigerated for 10 min to solidify the agarose. Next, the coverslips were removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 10) plus 10% (v/v) dimethyl-sulfoxide and 1% (v/v) Triton X-100) for at least 60 min at 4 °C. Subsequently, the slides were placed in an alkaline buffer (1 mM EDTA 300 mM NaOH, pH > 13) for 20 min at 4 °C for the DNA to unwind. Following electrophoresis in the same buffer for 20 min at 300 mA and 25 V (0.722 V cm^{-1}), the slides were neutralized in 400 mM Tris-HCl (pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 5 min. The slides were stained with 60 µL of 20 µg mL^{-1} aqueous ethidium bromide and covered with coverslips. One hundred randomly selected cells from each sample were analyzed using an epifluorescence microscope (ZEISS Axiolab HB050; 516-560 nm excitation filter; 590 nm barrier filter). Images were recorded using an intensified video camera (Axiocam: PCO, Germany). All the steps of the Comet assay were conducted under subdued light. In order to quantify the extent of DNA damage, each cell was visually allocated to one of four classes according to tail size (0 = no tail; 1 = short tail with a length smaller than the diameter of the head (nucleus); 2 = tail length between 1 and 2 times the diameter of the head; and 3 = long tail, more than twice the diameter of the head). Comets with no head and those with almost all the DNA in the tail, or with a very wide tail, were excluded from the analysis, since they could represent dead cells (Hartmann and Speit, 1997). A DNA damage-score was obtained for each sample by multiplying the number of cells in each class by the damage class, according to the formula: Total score = (0 x n0) + (1 x n1) + (2 x n2) + (3 x n3), where n = number of cells in each class. Thus, the total score could range from 0 to 300.

Statistical analysis

All results were expressed as mean values ± the standard deviation (SD) and analyzed with SigmaStat software. Analysis of variance (ANOVA) and the Student-Newman-Keuls test were used to assess significant differences ($p < 0.05$) between fractions. Gaps were counted, but not included, in the statistical analysis, since their cytogenetic significance is not well established (Antunes and Takahashi, 1998).

Results

The results of the genotoxicity tests of the *F. vesiculosus* aqueous extracts was assessed in cultured human lymphocytes using the chromosome aberration assay and changes in the mitotic index (MI) are shown in Table 1 and the Comet assay results are shown in Table 2. The fre-

Table 1 - Mitotic index and chromosomal aberrations in human lymphocytes from 12 women treated with *Fucus vesiculosus* aqueous extract (FVAE) and/or 15 µg mL^{-1} of doxorubicin.

Treatment	Mitotic index (%) ± SD	Chromosomal aberrations						TA/100 cells	AM/100 cells
		G'	G"	B'	B"	Qr	Sf		
Control group	6.11 ± 0.687	35	14	28	12	0	0	3.33	3.33
Doxorubicin group	2.52 ± 0.397	314	288	244	80	16	0	28.33	20.00
FVAE group (mg mL^{-1})									
0.25	5.68 ± 0.449	34	10	33	9	0	2	3.66	3.43*
0.5	5.77 ± 0.496	38	6	33	8	0	2	3.58	3.54*
1.0	5.90 ± 0.461	44	22	33	10	0	0	3.58	3.58*
Pre-treatment with FVAE									
0.25 mg mL^{-1} FVAE + doxorubicin	5.90 ± 0.567	45	15	31	10	0	0	3.41	3.37*
0.5 mg mL^{-1} FVAE + doxorubicin	5.65 ± 0.647	55	11	29	13	0	0	3.50	3.40*
1.0 mg mL^{-1} FVAE + doxorubicin	5.68 ± 0.551	31	13	31	14	0	0	3.75	3.35*
Simultaneous-treatment with FVAE									
0.25 mg mL^{-1} FVAE + doxorubicin	2.54 ± 0.250	335	238	228	45	0	0	22.75	19.33
0.5 mg mL^{-1} FVAE + doxorubicin	2.89 ± 0.687	339	299	245	37	0	0	23.5	19.35
1.0 mg mL^{-1} FVAE + doxorubicin	3.13 ± 0.446	353	280	253	21	0	0	22.83	19.36
Post-treatment with FVAE									
Doxorubicin + 0.25 mg mL^{-1} FVAE	3.07 ± 0.431	359	290	242	40	5	1	24.00	19.50
Doxorubicin + 0.5 mg mL^{-1} FVAE	3.01 ± 0.434	343	280	250	34	7	1	24.33	19.33
Doxorubicin + 1.0 mg mL^{-1} FVAE	2.91 ± 0.434	353	200	262	22	9	1	24.5	19.66

Key: G' = chromatid gap; G" = chromosomal gap; B' = chromatid break; B" = chromosomal break; Qr = quadriradial; Sf = simple fragment; TA = total aberrations; and AM = abnormal metaphases. Gaps were not included in the number of abnormal metaphases.A total of 2000 cells per culture were analyzed for mitotic index and 100 cells per culture (1200 cells per treatment) for chromosomal aberrations.

* Significantly different from the doxorubicin group ($p < 0.05$).

Table 2 - Comet assay scores for lymphocytes of 12 women treated with three different concentrations of *Fucus vesiculosus* aqueous extract (FVAE) and/or 15 µg mL^{-1} of doxorubicin. For each treatment 100 nuclei were scored (n = 1200 cell/treatment).

Treatments	Number of cells with Comet class				Mean score
	0	1	2	3	
Control group	880	227	31	12	27.08
Doxorubicin group	189	694	236	81	117.41
FVAE group (mg mL^{-1})					
0.25	872	291	25	12	31.41*
0.5	884	285	16	15	30.16*
1.0	883	282	21	14	30.5*
Pre-treatment with FVAE					
0.25 mg mL^{-1} FVAE + doxorubicin	880	281	26	13	31*
0.5 mg mL^{-1} FVAE + doxorubicin	875	287	25	13	31.33*
1.0 mg mL^{-1} FVAE + doxorubicin	883	276	27	14	31*
Simultaneous-treatment					
0.25 mg mL^{-1} FVAE + doxorubicin	410	490	250	50	95.0
0.5 mg mL^{-1} FVAE + doxorubicin	359	570	232	39	95.92
1.0 mg mL^{-1} FVAE + doxorubicin	350	574	245	31	96.42
Post-treatment					
Doxorubicin DXR + 0.25 mg mL^{-1} FVAE	334	567	247	52	101.42
Doxorubicin DXR + 0.5 mg mL^{-1} FVAE	298	603	252	47	104
Doxorubicin DXR + 1.0 mg mL^{-1} FVAE	291	607	261	41	104.3

* Significantly different from doxorubicin group ($p < 0.05$).

quency of chromosome aberrations, the mitotic index and the extent of DNA damage in cells treated with different concentrations of the extract were similar to those of the control ($p > 0.05$), with the most frequent type of chromosome aberration being chromatid and chromosomal gaps (Table 1). In the Comet assay, the most frequent classes were 0 and 1 (Table 2).

The results for the antigenotoxicity assays conducted using doxorubicin in combination with different concentrations of *F. vesiculosus* aqueous extract are also presented in Table 1 for chromosome aberrations and mitotic index and Table 2 for the Comet assay as a measure of DNA damage. No significant difference was found in the chromosome aberration frequency, mitotic index or damage score (*i.e.* Comet assay results) of cultures submitted to simultaneous and post-treatment with the extract when compared to the doxorubicin group (Table 1 and 2). However, for all the cultures pre-treated with extract the mean mitotic index, chromosome aberration frequency and DNA damage score were all significantly lower than the corresponding values for the doxorubicin group ($p < 0.05$) (Tables 1 and 2).

The most common chromosome aberrations observed in all cultures treated with doxorubicin were chromatid and chromosomal gaps, followed by chromatid and chromosomal breaks, although quadriradial figures and acentric fragments were also found (Table 1 and Figure 1). In addition, Comet assay cells with class 1 (low-level) damage were the most frequent (Table 2).

Discussion

Genotoxicity studies have frequently been conducted on mammalian systems to evaluate the mutagenic potential associated with acute or chronic exposure to chemical agents. Recently, particular attention has been devoted to the Comet assay in order to identify substances with genotoxic activity. This test allows the detection of DNA damage such as single and double-strand breaks and alkali labile lesions in individual cells after acute and/or chronic exposure to a genotoxic agent (Tice *et al.*, 2000). According to Hartmann *et al.* (2003) the chromosome aberration assay can be used to validate the results of the Comet assay.

Our results show that *F. vesiculosus* aqueous extract had no genotoxic effects on human lymphocyte cultures, suggesting that substances present in the extract are not clastogenic nor do they promote DNA damage. Few studies have investigated the toxic effects of this brown seaweed, but according to Oliveira *et al.*(2003) *F. vesiculosus* extract could generate reactive oxygen species (ROS), which can damage DNA (Gewirtz, 1999). In contrast, other authors have associated the compounds in this seaweed to antioxidant activity (Jiménez-Escrig, 2001; Rupérez *et al.*, 2002).

Figure 1 - Chromosomal aberrations observed in cultured human lymphocytes treated with doxorubicin only or doxorubicin plus *Fucus vesiculosus* aqueous extract using different experimental protocols (pre-treatment, simultaneous-treatment and post-treatment). Arrows indicate chromatid gap (**A**), chromatid break (**B**) and quadrirradial figure (**C**). Arrowheads indicate chromosomal gap (**A**) and chromosomal break (**B**).

Genotoxic chemotherapy treatments include: alkylating agent that modify DNA bases and thus interfere with DNA replication and transcription; intercalating agents that wedge into the spaces between the nucleotides in the DNA double helix and interfere with transcription and replication; and enzyme inhibitors which induce DNA damage by inhibiting key enzymes such as the topoisomerases which are involved in DNA replication (Jung and Reszka, 2001). According to Gewirtz (1999) doxorubicin is believed to be involved in induction of DNA damage through the inhibition of topoisomerase II and free radical generation by redox reactions.

We found that doxorubicin induced a significant increase ($p < 0.05$) in DNA damage score and the frequency of chromosome abnormalities, these results being consistent with those reported by other authors (Anderson *et al.*, 1998). In addition, we also found that doxorubicin induced a significant decrease ($p < 0.05$) in mitotic index, which can be associated with apoptosis. In fact, according to Ling *et al.* (1996) doxorubicin could induce apoptosis by promoting cyclin B accumulation.

Natural antioxidants have been used to prevent chromosome damage induced by antitumor-agents as a strategy to attenuate the toxicity of chemotherapy with agents such as doxorubicin (Antunes and Takahashi, 1998; Quiles *et al.*, 2002). Algal extracts have demonstrated antioxidative and antitumoral activities on mammals (Funahashi *et al.*, 1999; Rupérez *et al.*, 2002) and for edible brown algae, antioxidant and antimutagenic properties having been identified in alcoholic extracts (Le Tutour *et al.*, 1998) of *Laminaria digitata, Himanthalia elongata, Fucus serratus* and hot-water-soluble extracts of *Laminaria japonica* (Okai *et al.*, 1993) and *Hijikia fusiforme* (Okai and Higashi-Okai, 1994).

Our results show a protective effect of *F. vesiculosus* aqueous extract (FVAE) on pre-treated lymphocyte cultures, although simultaneous or post-treatment of lymphocyte cultures with extract in combination with doxorubicin appeared to result in no protection against the doxorubicin genotoxicity.

Other extracts of brown algae such as *Ascophyllum nodosum* and *Sargassum micracanthum* have been shown to produce *in vitro* protective effects (Le Tutour *et al.*, 1998; Mori *et al.*, 2003) and anti-tumor activity has been observed in mice pre-treated with *Undaria pinnatifida* extract (Ohigashi *et al.*, 1992).

Antioxidants can be classified into two groups, primary, or chain-breaking, antioxidants and secondary, or preventive, antioxidants (Frei, 1994). In our study, pretreatment of lymphocyte cultures with *F. vesiculosus* aqueous extracts resulted in strong protection against doxorubicin-induced DNA damage, while the extracts themselves produced no significant increase in chromosome aberrations or mitotic index. This suggests that *F.*

vesiculosus aqueous extracts act as a preventive antioxidant only.

Although many studies highlight the protective activity of seaweeds, there are controversies about the compounds responsible for this activity (Matsukawa *et al.*, 1997; Funahashi *et al.*, 1999; Lim *et al.*, 2002; Ponce *et al.*, 2003). According to Rupérez *et al.* (2002) the antioxidant activity of the *F. vesiculosus* aqueous extract is due to the sulfated-polysaccharide fucoidan. In brown seaweeds, soluble dietary fiber polysaccharides are mainly represented by alginates, fucans and laminarans and the insoluble fibers are essentially made of cellulose (Bobin-Dubigeon *et al.*, 1997). Sulfated polysaccharides (especially fucans and alginic acid derivatives) of soluble fibers from marine algae are atoxic for humans and, moreover, are known for exhibiting various biological properties, such as anticoagulant, anti-inflammatory, antiviral or antitumoral activities. However, the antioxidant activity of aqueous *Fucus* extracts has also been associated to its high polyphenolic content (Jiménez-Escrig *et al.*, 2001) and it has been suggested that dietary phytochemical polyphenols prevent oxidative damage in important biological membranes (Decker, 1995). Many algal species also contain polyphloroglucinol phenolics (phlorotannins) (Nakamura *et al.*, 1996) and in several cases, the antioxidant activity of algae could be due to these compounds. Other authors have associated the protective effect of seaweeds to their iodine content. For example, Funahashi *et al.* (1999) have suggested that the iodine contained in edible seaweed have suppressive effects on mammary tumor cells. According Moro and Basile (2000) iodine is the most important active principle in *F. vesiculosus*, although its constituents also include polysaccharides, sterols and other minerals. Iodine is known to play an important role in the treatment of obesity and thyroid disorders and iodine deficiency has also been associated with the development of mammary pathology and cancer (Venturi, 2001).

Our results demonstrate that *Fucus vesiculosus* aqueous extracts have no genotoxic effect on lymphocyte cultures and can provide effective protection to lymphocytes against doxorubicin-induced DNA damage, but only when administered before doxorubicin. Our findings indicate that further investigations are necessary to evaluate the *in vivo* benefits of *Fucus vesiculosus* aqueous extract. Moreover, the evaluation of other seaweed extracts using the model described in this paper may also contribute to the discovery of new marine resources able to protect DNA against the undesirable effects of antitumor agents.

Acknowledgments

The authors thank the women who spontaneously participated in this study and Sílvio A. dos Santos, Raquel A. dos Santos, Ana Paula L. Montaldi and Luiz A. da Costa Junior for technical assistance. This research was supported by grants from the Brazilian agencies Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Apoio ao Ensino, Pesquisa e Assistência (FAEPA).

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Associate Editor: Carlos F.M. Menck