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Modulation of the mutagenic effect of benzo[a]pyrene and bleomycin by isoflavone extracts in a rat hepatoma cell line

Modulação do efeito mutagênico do benzo[a]pireno e bleomicina por extratos de isoflavonas em células de hepatoma de roedor

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Abstract

Epidemiologic studies show that the intake of foods rich in isoflavones (phytoestrogens), such as soybeans, confers protection against various types of cancer, what increases the scientific and popular interest on these compounds. In the present study, phytoestrogens extracts from soybeans were tested for genotoxic potential and modulatory effects on benzo[a]pyrene and bleomycin. Two phytoestrogens were evaluated in vitro, phytoestrogen "A" was supplied by EMBRAPA-Soja, Londrina – PR, and phytoestrogen "B" was purchased in a local drug store. The methods used were the comet assay (genotoxicity and antigenotoxicity) and micronucleus test with cytokinesis block (mutagenicity) in rat hepatoma cells (HTC cell). The isoflavones were tested at three concentrations pre-established by the MTT cytotoxicity assay. Both isoflavone extracts showed no genotoxic effects in the comet assay, but showed induction of micronucleus. In the evaluation of the phytoestrogens for a modulatory effect, both phytoestrogens extracts showed antigenotoxicity in the comet assay.
Keywords: Soy phytoestrogens. Genotoxicity. Mutagenicity. HTC cells.

Resumo

Estudos epidemiológicos mostram que a ingestão de alimentos ricos em isoflavonas (fitoestrógenos), como a soja, confere proteção contra vários tipos de câncer, o que aumenta o interesse científico e popular sobre esses compostos. No presente estudo, os fitoestrógenos de extrato de soja foram testados quanto aos efeitos genotóxicos e modulador de benzo [a] pireno e bleomicina. Dois fitoestrogênios foram avaliados in vitro, o fitoestrógenos "A" foi fornecido pela Embrapa-Soja, Londrina - PR, e o fitoestrógenos "B" foi comprado em uma farmácia de manipulação local. Os métodos utilizados foram o teste do Cometa (genotoxicidade e antigenotoxicidade) e teste do Micronúcleo com Bloqueio Citocinese

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(mutagenicidade) em células de hepatoma de rato (HTC celulares). As isoflavonas foram testadas em três concentrações pré-estabelecidas pelo ensaio de citotoxidade MTT. Ambos os extratos de isoflavonas não mostraram efeitos genotóxicos no ensaio do cometa, mas mostraram indução de micronúcleo. Na avaliação dos fitoestrogênios para um efeito modulador, ambos os extratos fitoestrogênios mostraram efeito antigenotóxico no ensaio do cometa.
Palavras-Chave: Fitoestrógenos da soja, genotoxicidade, mutagenicidade, células HTC.

Introduction

One of the most prevalent chronic diseases is cancer, a disease primarily initiated by DNA damage. One component of cancer prevention may be dietary supplementation with foods rich in bioactive molecules such as phytoestrogens. The public interest of the scientific community in the benefits of phytoestrogens on the human health has increased in the last years; this interest is associated with their biological activities, such as anticarcinogen, cardioprotector and hormonal replacement in menopause (KLEIN; KING, 2007). Isoflavones are the most known phytoestrogens, they are found in abundance in soybean grains (*Glycine max*) in a natural inactive glycosylated form (linked to sugars) (MOON et al., 2006). After ingestion and absorption in gut, resultant aglycons (active form) are metabolized in the liver and undergo entero-hepatic recirculation (MOON; WANG; MORRIS, 2006; SETCHELL et al., 2002; ESTEVES; MONTEIRO, 2001), being able to exert their activities of modulation of cell proliferation and differentiation, induction of apoptosis and inhibition of angiogenesis and metastasis (RAMOS, 2007).

Soybean products are rich sources of isoflavones, and the daily ingestion of isoflavones in Asian countries can surpass 40 mg/day, at least 10 times more than on the American continents (BOERSMA et al., 2001). Epidemiologic studies show that the ingestion of foods rich in isoflavones confers protection against various types of cancer, particularly those that are hormone dependent, such as mammary and prostate cancers (ADLEUCREUTZ et al., 2002). In these cancers, the cellular malignization is facilitated by the increase of cell proliferation stimulated by hormones. Isoflavones are biologically active phenolic compounds of plant origin that structurally mimic the principal mammalian estrogen 17ß-estradiol, being able to stimulate or inhibit hormone dependent cell proliferation by competing for estrogen receptors (ER) binding sites. Thus, isoflavones act as ER agonists or antagonists (SEBASTIAN; THAMPAN, 2007; THOMSEN et al., 2006; STOPPER; SCHIMITT; KOBRAS, 2005).

However, despite recent attention related to the putative chemoprotective properties of isoflavones, the mechanisms of cancer chemoprevention of isoflavones are not fully understood, especially in relation to antigenotoxic and/or antimutagenic capabilities against common DNA damage agents.

So, the aim of the present study was to determine modulatory effect of soybean phytoestrogens on DNA damage caused by pro-carcinogen benzo[a] pyrene and chemotherapeutic agent bleomycin using the comet assay and micronucleus test with cytokinesis block. The study was made on *Rattus novergicus* hepatoma cells (HTC) *in vitro*. This cell line is worldwide used in drug metabolization studies, especially in biotoxicology ones (FERRO et al., 1993).

Materials and Methods

Phytoestrogens in soybeans (Glycine max)

Two isoflavone extracts from soybeans were evaluated in the powder form, named phytoestrogens

"A" and "B".

Phytoestrogen "A" was supplied by the Brazilian agricultural research corporation, National soybean research center, EMBRAPA-Soja, Londrina – PR. Phytoestrogen "A" was obtained from mixed variety soybean grains and isoflavones chemical characterization was evaluated using HPLC, both procedures were realized according to Piubelli et al. (2003). Under these procedures the phytoestrogen "A" demonstrated high levels of glycosylated forms genistin (94% of total isoflavones) and daidzin (5.8%), and lower levels of other not characterized phytoestrogens (0.2%).

Phytoestrogen "B" was purchased in a local drug store (Vale Varde), as "Isoflavona" commercial name. The quality control report of the manufacturer (DEG®) indicates the presence of the aglycons daidzin (20.7%), genistin (9% of total isoflavones), glycitin (8.9%), and the glycosylated forms daidzein (59.7%), genistein (0.4%), and glycitein (1.3%).

Phytoestrogens "A" and "B" were dissolved at 1% in DMSO (dimethylsulfoxide), diluted in culture medium DMEM/F-12 (Gibco) and sterilized by ultrafiltration (Millex® - Millipore 0.22 μm). For all tests performed the control group cells received treatment of DMSO, not higher than 1% in medium.

DNA Damage-Inducing Agents

In the comet and micronucleus tests, DNA damage was induced by the indirect-acting carcinogen benzo[a]pyrene (Fluka) at a final concentration of 80μM previously diluted in DMSO (0.8%) and by the direct-acting agent bleomycin sulfate (Fluka) at a final concentration of 0.5 μg/mL (approximately 1.25μM), which was diluted in DMEM-/F-12 culture medium.

Cell Culture

The hepatoma cell line of *Rattus novergicus* (HTC) utilized in this study was acquired from the Cell Bank of Rio de Janeiro (UFRJ). These cells were grown in 25-cm2 culture flasks containing DMEM-/F-12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco), and maintained in a BOD incubator at 37°C. Under these conditions, the cell cycle of this line is approximately 24 h (THOMPSON; TOMKINS; CURRAN, 1966).

Experimental Protocols and Procedures in the MTT Cytotoxicity Assay

The MTT cytotoxicity assay, based on the protocol described by Mosmann (1983), was utilized to determine the concentrations of the phytoestrogens to be used in the comet and micronucleus tests.

Six concentrations of each phytoestrogen were tested: 31.25μg/mL, 62.5μg/mL, 125μg/mL, 250μg/mL, 500μg/mL and 1000μg/mL. After 24h of treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the cultures and the cells were incubated at 37ºC, 4h. Afterward, the medium with MTT was completely removed, and 100 μL of DMSO were added to each well to dissolve the formazan crystals formed. Absorbance was determined in a plate reader (Uniscience) at 550 nm.

Experimental Protocols and Procedures in the Comet Assay

The cultures were divided into two experimental groups: (a) evaluation of genotoxicity; (b) evaluation of antigenotoxicity. For both groups 3 concentrations of the phytoestrogens "A" and "B" were tested at 2.5μg/mL, 25μg/mL and 250μg/mL for 24h. These concentrations were previously determined by the MTT assay. For genotoxicity group the cells received only phytoestrogens. After 24h phytoestrogens treatment (step 1), the culture media were removed and fresh media with DMSO were added for 24h (step 2). For antigenotoxicity group the same phytoestrogens concentrations were tested against the damage-inducing agents bleomycin or

benzo[a]pyrene. After 24h phytoestrogens treatment (step 1), the culture media were removed and fresh media were added for 24h damage-inducing treatment (step 2). All the experiments were carried out in triplicate.

The comet assay was performed in accordance with the method described by Speit and Hartmann (1999). A total of 10^6 HTC cells per culture flask were seeded and incubated for 24h. At the end of the treatments, the cells were trypsinized (0.025% trypsin-EDTA) and a small sample was utilized for the determination of cell viability by the trypan blue exclusion assay. The rest of the material was utilized for the preparation of slides, 20 μL of cell suspension were mixed with 120 μL of low-melting point agarose (0.5% LMP, 37°C), and this mixture was added to pre-gelatinized slides and placed in lysis solution for 1h. After denaturation (20 min) and alkaline electrophoresis (25V, 300mA, 20min, pH > 13), the slides were neutralized and fixed. The slides were then stained with ethidium bromide (0.002 mg/mL) and examined at 400 X magnification using a fluorescence microscope (Nikon) equipped with an excitation filter of 515-560 nm and emission filter of 590 nm. For each culture, 100 cells were examined, making that a total of 300 cells for each treatment. The nucleoids were classified as: type 0: absence of tail; type 1: tail with length up to the diameter of the head of the comet; type 2: tail up to two times the size of the head; type 3: long tail with length greater than two times the diameter of the head (KOBAYASHI et al., 1995). Apoptotic and necrotic cells, with high levels of DNA fragmentation, were excluded from scoring (HARTMAN et al., 2003).

Experimental Protocols and Procedures in the Micronucleus Test With Cytokinesis Block (CBMN assay)

For mutagenicity evaluation, cytochalasin-B (3 μg/mL) was also added in the step 2 of specific treatments for CBMN, and the cells were harvested after 26 h of exposure. All the experiments were carried out in triplicate.

Briefly, the cells were trypsinized (0.025% trypsin-EDTA), hypotonized (1% sodium citrate), and fixed with methanol-acetic acid (3:1). The slides were stained with Giemsa (5%) and examined with a light microscope at 400 X. A total of 3000 cells per treatment were analyzed. To verify the presence of a micronucleus, only binucleated cells with intact cytoplasm were examined. The micronucleus was: size equal to or less than 1/3 the size of the nucleus, had the same staining as the nucleus, were round, and were not linked to the main nucleus (FENECH, 2000).

Statistical Analysis

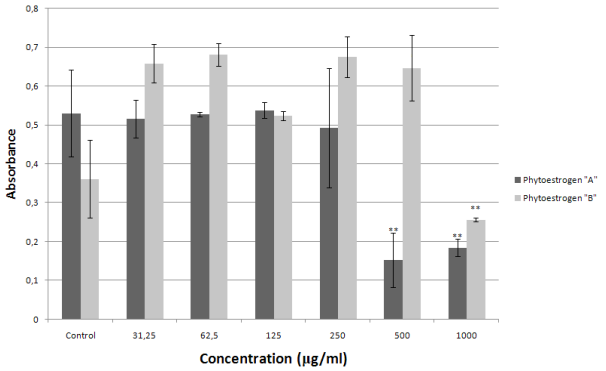
The data were submitted to analysis of variance (ANOVA), combined with Dunnett's test, with the level of significance set at 0.01. The analyses were performed using GraphPad Instat® software, version 3.02.

Results

MTT cytotoxicity assay

The analysis of the MTT data shown in Figure 1 reveals that only concentrations over 500μg/mL of phytoestrogen "A" are cytotoxic to HTC cells. Phytoestrogen "B" appeared to be cytotoxic at concentrations above 1000μg/mL. Thus, concentrations of 2.5μg/mL, 25μg/mL, and 250μg/mL were chosen for the comet and micronucleus tests.

Figure 1 - Determination of cytotoxicity of soybean phytoestrogens ("A" and "B") in HTC cells. A = phytoestrogen from Embrapa; B = commercial phytoestrogen; **statistically significant difference in relation to control, pμ0.01. Concentrations were established in μg/mL.

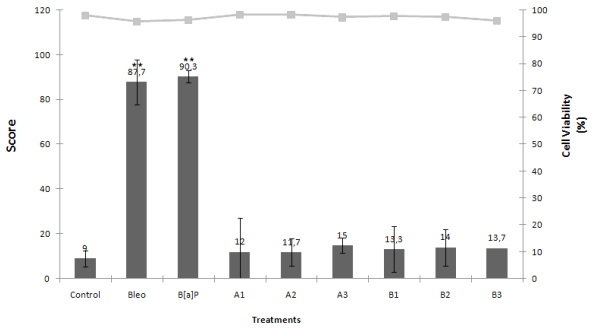


Fonte: Authors.

Comet Assay

Cell viability based on trypan blue exclusion was carried out concomitantly with the comet assay and showed satisfactory results in all treatments (mean 95% of viability) (Figure 2). Figure 2 shows the results for the different treatments of genotoxicity with phytoestrogens "A" and "B". The data demonstrate that there was no induction of comets by these extracts, and the scores were low and did not differ significantly from control. Thus, the phytoestrogens themselves did not show genotoxic activity in HTC cells at tested concentrations.

Figure 2 - Mean score of comets (barrs) and cell viability (line) observed in HTC cells in genotoxicity evaluation, after treatment with two soybean phytoestrogens ("A" and "B"). Bleo = bleomycin; B[a]P = benzo[a]pyrene; "A" = phytoestrogen from Embrapa; "B" = commercial phytoestrogen; 1 = 2.5μg/mL, 2 = 25μg/mL and 3 = 250μg/mL; **statistically significant difference in relation to control, pμ0.01.



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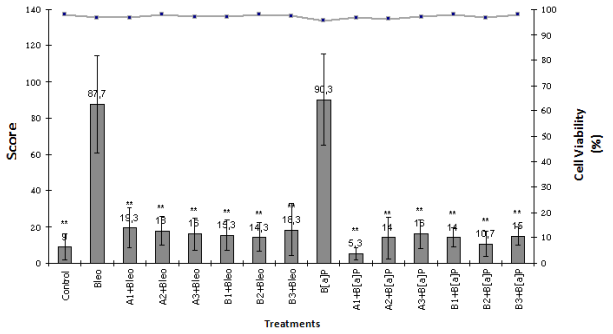
Statistical analysis demonstrated a significant decrease of DNA damage in both phytoestrogens treatments (phytoestrogens "A" and "B"), when compared to both positive controls (bleomycin and benzo[a]pyrene) (Figure 3).

Figure 3 - Mean score of comets (barrs) and cell viability (line) observed in HTC cells in antigenotoxicity evaluation, after treatment with two soybean phytoestrogens ("A" and "B") in association with bleomicin or benzo[a]pyrene. Bleo = bleomycin; B[a]P = benzo[a]pyrene; "A" = phytoestrogen from Embrapa; "B" = commercial phytoestrogen; 1 = 2.5μg/mL, 2 = 25μg/mL and 3 = 250μg/mL; **statistically significant difference in relation to control, pμ0.01.

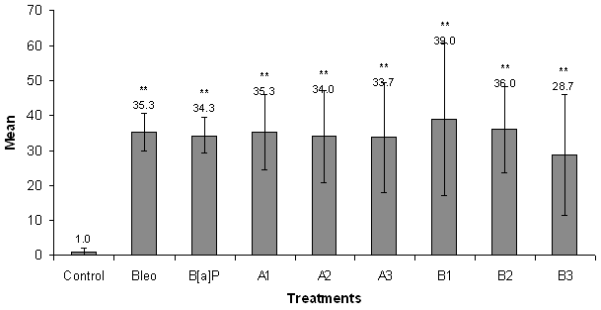


Fonte: Authors.

Micronucleus Test With Cytokinesis Block (CBMN assay)

The data presented in Figure 4 were obtained in evaluating phytoestrogens "A" and "B" for mutagenicity, both phytoestrogens showed mutagenic effect at all tested concentrations, demonstrating an increased frequency of binucleated cells with micronuclei similar to positive controls.

Figure 4 - Mean number of binucleated cells with MN observed in HTC cells in mutagenicity evaluation, after treatment with two soybean phytoestrogens ("A" and "B"). Bleo = bleomycin; B[a]P = benzo[a]pyrene; "A" = phytoestrogen from Embrapa; "B" = commercial phytoestrogen; 1 = 2.5μg/mL, 2 = 25μg/mL and 3 = 250μg/mL; **statistically significant difference in relation to negative control, pμ0.01



Fonte: Authors.

Discussion

This work demonstrates the cytotoxic activity of phytoestrogens extracts in cultured rat hepatoma cells (HTC). Phytoestrogen "A" demonstrated a higher cytotoxicity potential, probably due to a high concentration of genistin, which is converted to genistein.

In human body inactive glycosilated forms of isoflavones are previous metabolized by gut flora and converted to aglycon active, some mammalian cells also express cytosolic β-glucosidases able to hydrolyse various phenolic glucosides, glucosidases were reported in cells of human small intestine and liver (DAY et al., 1998). There is no report on β-glucosidases in HTC cells, the literature of this cell is poor, but the result of cytotoxicity with phytoestrogen "A" suggests that it is probably occurring.

According to many reports, genistein inhibits the growth of several cancer cells through the modulation of genes that are intimately related to the regulation of apoptosis, but also to other processes such as cell growth or signal transduction pathways, since this isoflavone is an inhibitor of protein tyrosine kinases (LIAN et al., 1999).

Phytoestrogen "B" also demonstrated cytotoxicity effect, however only above 1000μg/mL. This phytoestrogen extract has low concentrations of genistin and genistein, and a high concentration of daidzein. Daidzein demonstrated to arrest G1 and G2/M cell cycle phases in cancer cells, but the mechanism is still not understood (CHOI; KIM, 2008).

Cytotoxicity tests are also widely used in *in vitro* toxicology studies to determine the cytotoxic concentrations avoiding the use of these concentrations in genotoxic and mutagenic evaluations (FOTAKIS; TIMBRELL, 2006).

In this study, soybean phytoestrogens were tested for genotoxicity and antigenotoxicity using the comet assay, and mutagenicity using CBMN assay. The phytoestrogen extracts "A" and "B" were not genotoxic in comet assay, but both showed mutagenic response in CBMN assay.

Adverse effects and the genotoxicity of phytoestrogens have been reported in *in vitro, in vivo*, and clinical studies in humans (KLEIN; KING, 2007). According to Di Virgilio et al. (2004), the micronucleus test appears to be more sensitive than the comet assay in detecting DNA damage induced by isoflavones. Genistein induced comets in V79 Chinese hamster cells (not proficient in

metabolization) only at concentrations of 250μM or higher, while in the micronucleus test, genistein induced an increase in the number of micronuclei at much lower concentrations (5 to 25 μM) by clastogenic effect. Tsutsui et al. (2003) showed that genistein induced chromosomal aberrations, aneuploidies, DNA adducts, and transformation of Syrian hamster embryonal cells. Genistein was reported to cause DNA strand breaks due to its inhibition of topoisomerase II (MARKOVITS et al., 1989).

Di Virgilio et al. (2004) also reported that the genotoxicity of genistein seems to be higher than daidzein and equol (last daidzein metabolite). We did not observed this result in our work, since phytoestrogen "A" (with higher concentrations of genistin) showed similar genotoxic effect of phytoestrogen "B" (lower genistin and genistein concentrations). For phytoestrogen "A", genistein (after genistin metabolization) is responsible for the genotoxicity. For phytoestrogen "B", it is known that reductive and oxidative metabolites of daidzein exhibit genotoxic potential *in vitro* (SCHMITT et al., 2003) and these metabolites can be produced by metabolic proficient HTC cells. Similar effects observed between the phytoestrogens extracts, perhaps, can be due to an influence of the convertion of genistin to genistein by HTC cells.

In association with a DNA damage-inducing agent, bleomycin (direct-acting) and benzo[a] pyrene (indirect-acting), both phytoestrogens extracts showed antigenotoxicity in comet assay. Phytoestrogens can act as antigenotoxic, modulating enzymes that metabolize xenobiotics, inhibiting the activation of promutagens (FERGUSON et al., 2004), what explains the protective effect observed against benzo[a]pyrene. This inhibition is correlated with the capacity of genistin, daidzin, and their respective aglycon forms (genistein and daidzein) to prevent benzo[a]pyrene activation by CYP1A1, avoiding covalent binding of its metabolites to DNA (SHERTZER et al., 1999).

For bleomycin other mechanisms can be involved, such as the reduction of oxidative lesions (DJURIC et al., 2001; RASCHKE et al., 2006) that is associated to prevention of cancer. Genistein has been extensively investigated for its chemopreventive ability, especially against breast and prostate cancer. Its effect involves antioxidant properties, modulation of enzymes, inhibition of cell cycle (CHOI et al., 1998), and induction of apoptosis in transformed cells (KUMI-KIAKA; SANDERSON; HALL, 2000; SARKAR; LI, 2004). Daidzein is just as effective as genistein in protecting cells against oxidative damage especially with respect to DNA (FOTI et al., 2005).

In conclusion, our study sustains the fact that phytoestrogens extracts has a chemoprotective effects. However, the findings of a positive response in micronucleus test demonstrate the concern for the utilization of these phytoestrogens by the human population. Further studies are still needed to understand the involvement of these molecules in cell biology and their consequences for human health.

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