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Evaluation of the mutagenicity and antimutagenicity of soy phytoestrogens using micronucleus and comet assays of the peripheral blood of mice

A.M. Niwa[1], R.J. Oliveira[2] and M.S. Mantovani[1]

[1]Departamento de Biologia Geral, Universidade Estadual de Londrina, Londrina, PR, Brasil
[2]Programa de Pós-Graduação em Saúde e Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina "Dr. Hélio Mandetta",
Programa de Mestrado em Farmácia, Centro de Ciências Biológicas e da Saúde, Coordenadoria de Educação Aberta e a Distância,
Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brasil

Corresponding author: A.M. Niwa
E-mail: andressamn@yahoo.com.br

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ABSTRACT. Studies show that soy imparts many favorable properties in the human body, including the prevention of chronic diseases such as osteoporosis, heart disease, cancer, and diabetes. Soy is rich in isoflavones, and it is a candidate for the chemoprevention of diseases owing to its low toxicity. In this study, a soy phytoestrogen (with high levels of the isoflavones genistin and daidzein) was tested in mice to investigate its mutagenicity and genotoxicity using micronucleus and comet assays of mouse peripheral blood. Phytoestrogen (0.083, 0.83 and 8.3 mg/kg body weight) was evaluated with and without the chemotherapeutic agent cyclophosphamide. For the micronucleus assay, blood was collected

before treatment and after 24 and 48 h. For the comet assay, blood was collected only after 24 h. Phytoestrogen was not mutagenic and reduced cyclophosphamide-induced DNA damage. The results from the comet assay revealed a reduction of DNA damage; however, phytoestrogen did induce genotoxic damage during the 24-h treatment. This genotoxic damage could have been repaired and was therefore not identified in the micronucleus assay, which detects mutations. The results suggested that the reduction of DNA damage observed in associated treatments could also reduce the side effects of chemotherapy. Moreover, they suggested that phytoestrogen might be a candidate of interest for the chemoprevention of cancer because it protects against DNA damage.

Key words: Phytoestrogen; Mutagenicity; Antimutagenicity; *In vivo*

INTRODUCTION

In the last decade, a growing number of studies have indicated that cancers can be prevented not only by avoiding exposure to carcinogens but also by ingesting protective factors that modulate host defense mechanisms. This strategy is referred to as chemoprevention and can be achieved through pharmacological agents, including items included in the diet (Pool-Zobel et al., 2005). In this context, soy is of great interest. Its physiological properties have attracted attention owing to their beneficial effects, especially with respect to the prevention of chronic diseases such as osteoporosis, heart disease, cancer, and diabetes (Esteves and Monteiro, 2001). In addition, soy is the only common food that contains 1 to 3 mg/g isoflavones, has no side effects, and exhibits low toxicity, making it an excellent candidate as a chemopreventive agent.

Isoflavones, also referred to as isoflavonoids, are phenolic compounds of the flavonoid family that are widely distributed in the plant kingdom. Their concentrations are relatively higher in legumes, particularly in soybeans (*Glycine max*) (Bedani and Rossi, 2005). Soybeans contain 3 types of isoflavones that are typically present in 4 forms: glycosylated (daidzin, genistin and glycitin), acetylglycosylated (acetyldaidzin, acetylgenistin and acetylglycitin), malonylglycosylated (malonyldaidzin, malonylgenistin and malonylglycitin), and unconjugated structural forms (daidzein, genistein and glycitein) (Esteves and Monteiro, 2001). After ingestion, the conjugated forms of isoflavones are hydrolyzed by bacterial β-glucosidases in the intestine and release aglycones, daidzein and genistein (Setchell, 2000). Although the conjugated forms are most abundant in soybeans, studies on the bioavailability of isoflavones have shown that glycosidic forms of isoflavones exhibit a low intestinal absorption rate compared to that of the aglycone form. Aglycone forms are absorbed faster and in greater quantities, whereas isoflavone glucosides require more time to reach their maximum plasma concentrations (Izumi et al., 2000).

Isoflavones are non-nutritional bioactive compounds that have important biological properties and a chemical structure similar to that of estradiol, which explains their capability of behaving as estrogens in biological systems (Ferrari and Demiate, 2001). Other properties of isoflavones, such as antioxidant, antimutagenic, and antican-

cer activities, have also been reported. The antimutagenic and anticarcinogenic effects of these compounds have been attributed to several mechanisms, including modulation of enzyme activity that, in turn, decreases the toxic effects of various xenobiotics (Moon et al., 2006).

The effects of an antimutagenic diet are determined in association with numerous pathways involving both extracellular and intracellular mechanisms. Cellular mechanisms comprise antioxidant activities protecting the nucleophilic sites of DNA, induction of detoxification pathways, modulation of DNA metabolism and repair processes, cell cycle control, increased apoptosis, maintenance of genomic stability and modulation of xenobiotic-metabolizing enzymes. The last of these mechanisms has often been associated with isoflavones (Ferguson et al., 2004).

Successive epidemiological studies have shown an inverse association between the consumption of foods rich in these phytochemicals and the incidence of mortality owing to chronic diseases. Diets rich in isoflavones, such as those in Asian countries, provide protection against various forms of cancer, particularly those that are hormone dependent, such as breast and prostate cancers (Adlercreutz et al., 2000).

Owing to the promising therapeutic utility of isoflavones, the aim of this study was to evaluate the safety of soy phytoestrogens through an analysis of mutagenicity and genotoxicity in mice. In addition, the ability of phytoestrogens to protect against cyclophosphamide-induced DNA damage was examined to investigate their chemoprotection capacity.

MATERIAL AND METHODS

Chemicals

The chemotherapeutic drug cyclophosphamide, an indirect inductor of DNA damage, was purchased from Ítaca Laboratórios (Fosfaseron®, REG. M.S. No. 1.2603.0056.002-1; Batch 063020, Brazil) and used in doses of 50.0 mg/kg body weight diluted in phosphate-buffered saline (PBS). Dr. Clara Beatriz Hoffmann Campo of Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) - Soja, Londrina, provided the phytoestrogen, and doses of 0.083, 0.83 and 8.3 mg/kg body weight were used.

Animals

For *in vivo* comet and micronucleus assays, male Swiss mice (*Mus musculus*) weighing 30 g were obtained from the Central Bioterium of the Universidade Estadual de Londrina. The experiments were conducted at the Sectorial Bioterium of Departamento de Biologia Geral. The animals were housed in polypropylene cages and were acclimated for 7 days. The luminosity was controlled on a 12-h light/12-h dark cycle, and temperatures were maintained at approximately 22° ± 2°C. A commercial diet and filtered water were provided *ad libitum*.

The animals were divided into experimental groups consisting of 7 animals for the micronucleus assay and 5 to 7 animals for the comet assay. Three doses of phytoestrogen were tested: 0.083, 0.83 and 8.3 mg/kg body weight. The animal groups were

divided into the following treatment conditions: control (PBS), DNA damage control (cyclophosphamide, 50.0 mg/kg body weight), phytoestrogen, and phytoestrogen + DNA damage-inducing agent (cyclophosphamide). The phytoestrogen concentrations were tested at 3 equivalent *in vitro* concentrations in accordance with Mendes (2008), who evaluated the effects of phytoestrogen in cell culture (Mendes et al., 2012). Phytoestrogen was administered via gavage, and cyclophosphamide was administered intraperitoneally. The control group received treatment with PBS via gavage and intraperitoneally, and the DNA damage control group was treated with PBS via gavage and with cyclophosphamide intraperitoneally.

Peripheral blood for micronucleus assay was obtained by puncturing the tail vein of each animal at the following time points: T0 (before treatment), T24 (24 h after treatment) and T48 (48 h after treatment). For comet assay, the tail veins of the animals were punctured, and 20 μL peripheral blood was collected at T24. All animals were euthanized 48 h after the administration of the treatments.

In vivo single-cell gel electrophoresis

The blood samples were suspended on low-melting point agarose and distributed onto slides pre-gelatinized with common agarose to perform the comet assay according to a method described by Singh et al. (1988) and Ebert et al. (2001). Briefly, the cells were lysed for 60 min at 4°C and subjected to alkaline conditions for 20 min. Electrophoresis was performed at pH 10, 25 V and 300 mA for 20 min. Subsequently, the slides were neutralized and stained with 20 mg/mL ethidium bromide. Comets were classified visually using a fluorescence microscope at 400X magnification with a 420- to 490-nm excitation filter and a 520-nm barrier. The following criteria were used to classify the comets: class 0, nucleoids undamaged and lacking tails; class 1, comets with tails smaller than the nucleoid diameter; class 2, comets with tail sizes ranging from 1 to 2 times the nucleoid diameter, and class 3, comets with tails larger than 2 times the nucleoid diameter. The apoptotic nucleoid pattern was not considered. A total of 100 cells were analyzed per treatment. The total score was obtained by multiplying the number of cells in each class by the damage class.

In vivo micronucleus assay

We used a micronucleus assay technique described by Hayashi et al. (1990) in which a drop of peripheral blood was deposited on a slide previously stained with 1.0 mg/mL acridine orange. A cover slip was placed on each sample, and samples were stored in a freezer (-20°C) for at least 7 days before analysis.

Percentage of damage reduction

The percentage of damage reduction was calculated using the average number of cells showing damage from the DNA damage control group minus the number of cells with damage observed in the antimutagenic treatment group (phytoestrogen + DNA damage-inducing agent) divided by the number of cells with damage observed in the DNA damage control group minus the number of cells with damage in the control group, multiplied by 100.

Statistical analysis

The data were analyzed using analysis of variance followed by the Tukey-Kramer test for the micronucleus assay and the Kruskal-Wallis test followed by the Dunn test for the comet assay. The level of significance for all statistical comparisons was $\alpha < 0.05$.

RESULTS

At T0, no significant difference was observed in the number of micronuclei in the groups, showing that no differences existed among the animals before treatment (Tables 1 and 2). At T24 and T48, based on the micronucleus assay, none of the doses of phytoestrogen evaluated were mutagenic, and they caused no difference in the number of mutations (MNs) compared to that in the control group (see Table 1). The same doses were effective in reducing the damage induced by cyclophosphamide (see Table 2), and a significant reduction in the number of MNs was observed in groups treated with phytoestrogen compared to that in the DNA damage controls.

Table 1. Mutagenic analysis.

Treatments	Times		
	T0	T24	T48
Control	3.14 ± 0.90[a]	4.43 ± 1.72[a]	4.14 ± 2.12[a]
Cyclophosphamide (50 mg/kg)	5.14 ± 2.19[a]	27.3 ± 9.16[b]	34.0 ± 7.79[b]
Phytoestrogen (0.083 mg/kg)	4.00 ± 1.53[a]	4.86 ± 2.41[a]	5.86 ± 2.19[a]
Phytoestrogen (0.83 mg/kg)	4.57 ± 2.44[a]	4.57 ± 2.15[a]	6.57 ± 2.37[a]
Phytoestrogen (8.3 mg/kg)	5.86 ± 1.57[a]	3.17 ± 2.32[a]	9.28 ± 3.40[a]

Data are reported as means ± standard deviation of the number of micronuclei in each group at three treatment times: T0, T24 and T48. Different letters indicate statistically significant differences ($P < 0.05$).

Table 2. Antimutagenicity analysis.

Treatments	Times		
	T0	T24	T48
Control	3.14 ± 0.90[a]	4.43 ± 1.72[a]	4.14 ± 2.12[a]
Cyclophosphamide	5.14 ± 2.19[a]	27.3 ± 9.16[a]	34.0 ± 7.79[a]
Phytoestrogen (0.083 mg/kg) + Cyclo	3.57 ± 0.98[a]	13.1 ± 7.34[b]	24.1 ± 5.43[b,c]
Phytoestrogen (0.83 mg/kg) + Cyclo	2.71 ± 1.60[a]	14.6 ± 5.88[b]	17.3 ± 7.95[b]
Phytoestrogen (8.3 mg/kg) + Cyclo	5.00 ± 1.92[a]	15.3 ± 5.50[b]	17.3 ± 5.16[b]

Data are reported as means ± standard deviation of the number of micronuclei in each group at three treatment times: T0, T24 and T48. Cyclo = DNA damage control (cyclophosphamide, 50 mg/kg). Different letters indicate statistically significant differences ($P < 0.05$).

In the comet assay, the 3 doses of phytoestrogen tested were found to increase DNA damage compared with that in the control group, showing genotoxic effects after 24 h of treatment (Figure 1). The same doses were found to be antigenotoxic, leading to a significant reduction of the scores in relation to those of the DNA damage control (Figure 2).

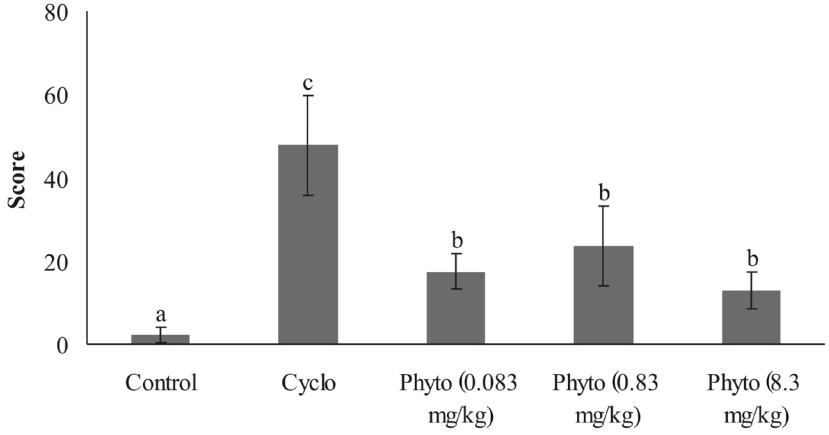

Figure 1. Evaluation of phytoestrogen genotoxicity. Means and standard deviations of scores obtained by the comet assay in peripheral blood from mice after 24 h of treatment with phytoestrogen (Phyto, 0.083, 0.83 and 8.3 mg/kg). Cyclo = DNA damage control (cyclophosphamide, 50 mg/kg). Different letters indicate statistically significant differences ($P < 0.05$).

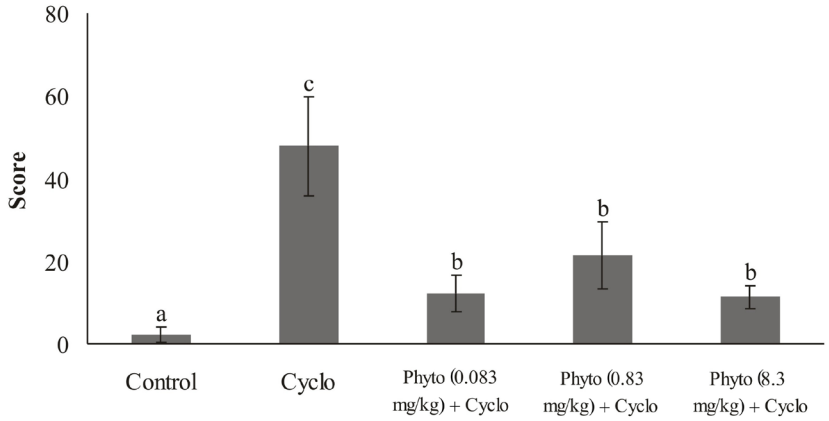

Figure 2. Evaluation of phytoestrogen antigenotoxicity. Means and standard deviations of scores obtained by the comet assay in peripheral blood of mice after 24 h of treatment with phytoestrogen (Phyto, 0.083, 0.83 and 8.3 mg/kg). Cyclo = DNA damage control (cyclophosphamide, 50 mg/kg). Different letters indicate statistically significant differences ($P < 0.05$).

DISCUSSION

Identifying substances that provide protection against mutations is a topic of great interest. Antimutagenic and anticarcinogenic activities have been attributed to various constituents of the diet; thus, the intake of chemopreventive compounds might protect against damage caused by xenobiotics (Ribeiro and Salvadori, 2003; Stopper et al., 2005). Diets rich

in phytoestrogens could aid in controlling many chronic diseases, including cancer (Esteves and Monteiro, 2001). However, inadequate levels of ingestion of these phytochemicals could lead to genotoxicity (Klein and King, 2007). Therefore, in this study, we proposed to evaluate the *in vivo* mutagenic effect of soy phytoestrogen to ascertain its safe use, and we evaluated the *in vivo* antimutagenic effect of soy phytoestrogen in association with cyclophosphamide in view of assessing its protective effect against DNA damage.

Cytotoxicity tests are widely used in toxicology studies to determine the cytotoxic doses of a substance in exposed cells (Fotakis and Timbrell, 2006). The concentrations of phytoestrogens used in the micronucleus and comet assays were determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] cytotoxicity assay performed by Mendes (Mendes J, unpublished results), which excluded toxic doses of phytoestrogens. Only non-cytotoxic doses were evaluated for genotoxicity and mutagenicity. The doses of phytoestrogen (0.083, 0.83 and 8.3 mg/kg body weight) used in this study were not mutagenic, but they were genotoxic, as demonstrated by the increased number of damaged cells in the comet assay. In combination with cyclophosphamide, phytoestrogen was antimutagenic and antigenotoxic, reducing DNA damage in both tests.

Comet assay detects DNA damage that can be repaired. The micronucleus assay examines aneugenic or clastogenic chromosomal mutations that are installed and cannot be repaired (Fenech, 2000; Gontijo and Tice, 2003). In this study, phytoestrogen was not mutagenic but was genotoxic. Thus, the genotoxic damage induced by phytoestrogen might have been repaired and was therefore not observed in the micronucleus assay, which detects mutations. Hence, the use of these 2 complementary tests is of great value in assessing mutagenesis because they result in a more accurate definition of the mutagenic potential of a substance.

Adverse effects and genotoxicity of phytoestrogens have been reported in animal studies, *in vitro* experiments and clinical studies in humans (Klein and King, 2007). The data obtained from the micronucleus assay showed that soy phytoestrogen could be safely used because it was not mutagenic. However, even though the damage might not transform into MNs, the data obtained from the comet assay are important because most cancers are caused by genomic mutations that arise through exchanges, deletions, or additions of base pairs, which are often due to inadequate repair systems.

Evidence showing that isoflavones protect the human body against various chronic diseases has stimulated researchers to study the physiological and pharmacological effects of these compounds. Among isoflavones, aglycone forms have been the most studied, and genistein has been reported to inhibit the development of breast (Peterson and Barnes, 1991) and prostate (Adlercreutz et al., 2000) cancer cells.

The genotoxic effects of the isoflavones genistein, daidzein and equol, a metabolite of daidzein, have been evaluated by Di Virgilio et al. (2004) in a hamster lung cell line (V79). Genistein was found to induce micronuclei at concentrations ranging from 5 to 25 μM, which declined at higher doses (Di Virgilio et al., 2004). Daidzein (25 μM) induced a significant increase in the number of micronuclei. Additional staining with antikinetochore (CREST) antibodies displayed CREST (-) under genistein treatment, indicating a clastogenic effect for this isoflavone (Di Virgilio et al., 2004). Equol treatment led to the detection of CREST (+), indicating aneugenic action. Daidzein presented mixed effects. The comet assay showed that genistein induced damage only at high concentrations (Di Virgilio et al., 2004).

Genistein was investigated for its mutagenic and clastogenic potential *in vitro* using

the Ames test in mouse lymphoma cells and in *in vivo* experiments using the micronucleus assay in rats and mice (Michael et al., 2006). The results of the Ames test showed that genistein was not mutagenic, nor was it found to be mutagenic or clastogenic *in vivo* in rats and mice. In mouse lymphoma cells, genistein increased the number of small colonies, indicating clastogenic action (Michael et al., 2006). Kulling et al. (2002) and Stopper et al. (2005) have also reported *in vitro* clastogenic effects of genistein.

In this study, genotoxic effects were observed in the peripheral blood of mice in response to phytoestrogen treatment. However, phytoestrogen also showed antigenotoxic and antimutagenic activities against DNA damage induced by cyclophosphamide. Phytoestrogens act as antimutagens that modulate xenobiotic-metabolizing enzymes and thus, inhibit the activation of promutagens (Ferguson et al., 2004). Such inhibition might have been the explanation for the observed reduction of the mutagenic effects of cyclophosphamide, which is an indirect agent that causes DNA damage only after metabolism.

Dietary supplementation with soy isoflavones has been reported to reduce oxidative damage in human DNA, and this effect might represent the protective mechanism of these compounds in preventing cancer (Djuric et al., 2001). The consumption of soy is important in the Asian diet and is associated with a lower risk of prostate cancer in Asian men. Soybean products are rich sources of isoflavones, and their intake in Asian countries exceeds 40 mg/day, which is at least 10 times greater than that in American countries (Boersma et al., 2001). Thus, the increased consumption of phytoestrogens has contributed significantly to reducing the incidence of cancer (Klein and King, 2007). Furthermore, the antimutagenic effect of soy in relation to the anticancer agent observed in this study could reduce the side effects in patients who are undergoing chemotherapy, improving their life quality.

Data accumulated from epidemiological and experimental studies have allowed a deeper understanding of the effects of phytoestrogens. However, further studies are still necessary to understand the interactions of these molecules in cell biology and their real consequences for human health.

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