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Research Article

In vivo evaluation of the antimutagenic and antigenotoxic effects of β-glucan extracted from *Saccharomyces cerevisiae* in acute treatment with multiple doses

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Abstract

Ample evidence suggests that cancer is triggered by mutagenic damage and diets or supplements capable of reducing such incidences can be related to the prevention of neoplasy development or to an improvement in life quality of patients who undergo chemotherapy. This research aimed to evaluate the antimutagenic and antigenotoxic activity of β-glucan. We set up 8 experimental groups: control (Group 1), cyclophosphamide (Group 2), Groups 3-5 to assess the effect of β-glucan administration, and Groups 6-8 to evaluate the association between cyclophosphamide and β-glucan. The intraperitonial concentrations of β-glucan used were 100, 150 and 200 mg/kg. Micronucleus and comet assays showed that within the first week of treatment β-glucan presented a damage reduction rate between 100-62.04% and 94.34-59.52% for mutagenic and genotoxic damages, respectively. This activity decreased as the treatment was extended. During the sixth week of treatment antimutagenicity rates were reduced to 59.51-39.83% and antigenotoxicity was not effective. This leads to the conclusion that the efficacy of β-glucan in preventing DNA damage is limited when treatment is extended, and that its use as a chemotherapeutic adjuvant need to be better clarified.

Keywords: β-glucan, cyclophosphamide, antimutagenicity, antigenotoxicity, mice.

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Introduction

Experimental studies on the interaction between diet, nutrition and cancer can be developed in animal models and/or cell culture, and most of the epidemiological and trial studies on animal models and cell culture focus on the observation, control and analysis of cancer development steps. Thus, trial studies can provide support to epidemiological findings, as testing is done under controlled conditions. Furthermore, they can provide strong evidence on the relationship of diet, nutrition and cancer development and/or treatment (Torrinhas *et al.*, 2006). Correlations between diet, cancer prevention and β-glucan are described in two major reviews (Mantovani *et al.*, 2008; Kumar *et al.*, 2012) which indicate important health implications for humans.

Basically, in studies involving nutrition and cancer, the induction of tumor development in animals can be done by: (I) using different carcinogenic agents: mainly chemical ones or, incertain cases, by irradiation exposure or par-

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ticular strains of viruses; and (II) through tumor cell transplants which are able to grow and turn into tumors when implanted into the host (Torrinhas *et al.*, 2006).

In general, carcinogenicity induced by chemical agents, radiation and viruses is based on the concept of carcinogenicity via multiple steps. This concept implies that cancer development is a process that occurs in sequential steps, named: initiation, promotion, progression and manifestation. Carcinogenesis is an experimentally well-categorized process (Pitot and Dragan, 1991; Pitot, 1993), and it also probable occurs in human beings (Pinkerton and Dubé, 1991; Fearon and Jones, 1992; Sugimura, 1992).

Initiation is associated with a DNA alteration, *e.g.* induced by exposure to a carcinogenic agent. This alteration is considered a genotoxic activity (Camargo *et al.*, 1994). Consequently, diets or dietetic compounds that can diminish the risk of genotoxic or mutagenic effects can be associated indirectly to cancer prevention by decreasing the cancer initiation risk of cells.

β-glucan, a sugar polymer, is currently widely studied for the prevention of DNA damage related to cancer development. This sugar can be extracted from the cell wall of certain cereals, fungi and yeast (Cisneros *et al.*, 1996; Zimmerman *et al.*, 1998; Turnbull *et al.*, 1999; Masihi, 2000). β-glucan of yeast shows is composed of D-glucose molecules linked in the β-(1 → 3) position and containing lateral chains β-(1 → 6) (Di Luzio *et al.*, 1979) with great potential for the biotechnological production of drugs.

The chemopreventive efficacy of this polysaccharide during *in vitro* trials on the prevention of mutagenic damage to the cell lineages V79, CHO-k1, CHO-xrs5, HTC and to human lymphocytes was assessed in several studies (Slamenová *et al.*, 2003; Angeli *et al.*, 2006, 2009a,b; Oliveira *et al.*, 2006, 2007). Other reports are on the prevention against damage induced by doxorubicin, cisplatin and cyclophosphamide *in vivo* (Chorvatovicová *et al.*, 1996, 1998; Tohamy *et al.*, 2003; Lin *et al.*, 2004; Oliveira *et al.*, 2009a).

Notwithstanding such reports concerning chemopreventive effects of this molecule in acute or subchronic trials, there is still the need of studies to show the usefulness of β-glucan in chronic and acute treatments with multiple doses. Thus, the aim of this study was to investigate the antimutagenic and antigenotoxic activity of β-glucan extracted from *Saccharomyces cerevisiae* in damages induced by cyclophosphamide in acute treatments with multiple doses.

Material and Methods

DNA damage-inducing agent

For inducing DNA damage we used the alkylating agent cyclophosphamide (Fosfaseron®), diluted in Ca^{+2}- and Mg^{+2}-free, phosphate-buffered saline (PBS) (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 3.9 mM; KH_2PO_4 1.8 mM, pH 7.4) at a final concentration of 50 mg/kg of body weight (b.w.). This was administrated intraperitonially (i.p.).

Extraction and preparation of β-glucan

The β-glucan molecules tested in this study were extracted from *Saccharomyces cerevisiae.* These β-glucans have their main chains composed of D-glucose molecules linked by the β- type (1 → 3) with lateral branches in which there are links of β- type (1 → 6). They were extracted by autolysis of *Saccharomyces cerevisiae*. The cell wall was separated by means of centrifugation at 6500 *g* for 8 min followed by heat treatment (70 °C for 5 h) in NaOH (10%), washed and centrifuged three times and finally dried in an incubator at 40 °C. Nuclear magnetic resonance (NMR) analysis showed the presence of (1.3 and 1.6) β-D-glucan with purity of 85%. Subsequently, the glucan was solubilized using DMSO (dimethyl sulfoxide) and urea 8 M in the proportion of 100 mL (DMSO): 60 g (Urea). In a warm water bath, 100 mL of DMSO with 10 mL of concentrated sulfuric acid was added, and the mixture was stirred for 4 h at 100 °C. Dialysis was then performed with approximately 100 L of ultrapure water (Milli-Q) and then concentrated in a rotary evaporator at 40 °C followed by lyophilization.

Solutions of β-glucan were prepared in sterile Ca^{+2}- and Mg^{+2}-free PBS (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 3.9 mM; KH_2PO_4 1.8 mM, pH 7.4) at concentrations of 100, 150 and 200 mg/kg (b.w.). The doses were determined in pilot trials according to indications by Tohamy *et al.* (2003) and Oliveira *et al.* (2009a) (data not shown).

Animals

Male Swiss mice were used (*Mus musculus*) (n = 48) at reproductive age, with 30 g of average weight, from the Central Vivarium, State University of Londrina. The experiment was performed at the Sectorial Vivarium, Department of General Biology. The animals were kept in a polypropylene box, isolated and they had a minimum seven days for adaptation. Light incidence (12 hours of light: 12 hours of darkness) and temperature (22 ± 2 °C) were controlled. Feeding constituted of filtered water and commercial ration was provided *ad libitum.* In this phase, the animals were inspected daily. At the end of the experiment, the animals were euthanized by cervical dislocation as the use of any anesthetic could interfere with the effects of the chemical compounds under investigation. The euthanasia procedure was selected to minimize pain and stress, in agreement with the Ethical Principles on Animal Experimentation, elaborated by the Brazilian College of Animal Experimentation (COBEA, 2004), and with the approval of the Ethics Committee on Animal Experimentation, State University of Londrina (registration number 30877/04).

Experimental design and analysis methods

The animals were randomly divided into eight experimental groups (n = 6) as follows: animals from the control group (Group 01) received sterile Ca^{+2}- and Mg^{+2}-free PBS (NaCl 137 mM; KCl 2.7 mM; Na_2HPO_4 3.9 mM; KH_2PO_4 1.8 mM, pH 7.4) at a volume of 0.1 mL/10 g (b.w.; i.p.) during 3 consecutive days. Animals belonging to the cyclophosphamide group (Group 02) received this chemotherapy agent at a concentration of 50 mg/kg (b.w.; i.p.) on the second day of the treatment and PBS during the next couple of days. For the mutagenicity evaluation, Groups 03-05 received β-glucan (i.p.) for three consecutive days, at the doses of 100, 150 and 200 mg/kg (b.w.), respectively. For the antimutagenicity evaluation, Groups 06-08 received β-glucan (i.p.) for three consecutive days at the doses mentioned before and one dose of cyclophosphamide on the second day of treatment (Figure 1).

Peripheral blood was sampled from the experimental groups by puncturing the tail vein at three different time points to evaluate the mutagenic and/or antimutagenic potential by means of micronucleus testing in peripheral blood. Blood sample designated as time points T0 and T1 were always taken before the administration of treatment, within intervals of 24 hours. Time point T2 corresponds to a blood sample taken at 48 hours after the last administration of cyclophosphamide. At this time point, an amount of 30 μL was also collected to evaluate the genotoxicity and antigenotoxicity using the comet test.

Animals were treated during three consecutive days per week, for six consecutive weeks. Blood samples were taken in the first, fifth and sixth week.

Micronucleus assay in peripheral blood

The micronucleus assay was originally described by Hayashi *et al.* (1990), with certain changes proposed by Oliveira *et al.* (2009a). The slides were warmed to 70 °C and covered with a layer of 20 μL of Acridine Orange in an aqueous solution (1.0 mg/mL). After the preparation of the slides, a drop of peripheral blood was deposited on the slide and covered by a coverslip. Analyses were performed with a fluorescence microscope (Bioval®) at 40X magnification, with a 420-490 nm excitation and a 520 nm barrier fil-

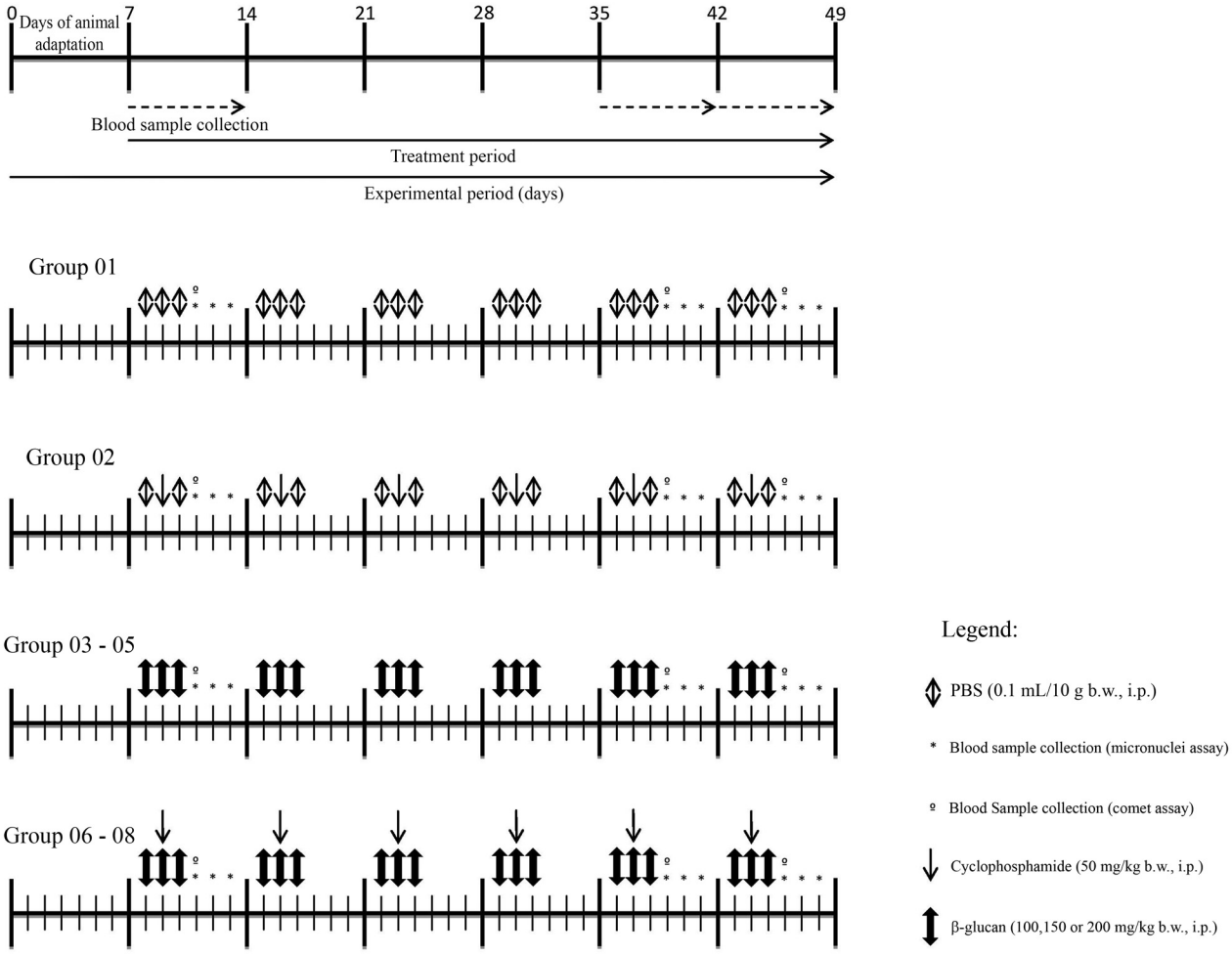

Figure 1 - Diagram of the experimental design and techniques of analysis.

ter. A total of 2,000 cells were analyzed per animal, and the statistical analysis was performed using an unpaired *Student's* t-test ($p < 0.05$).

Comet assay

The alkaline Comet assay proposed by Singh *et al.* (1988) was performed with modifications and under indirect light. Briefly, 20 μL of a blood cell suspension was embedded into 120 μL of 0.5% low melting point agarose and layered onto a pre-coated slide with a thin layer of normal melting point agarose. The slide was covered with a glass coverslip and cooled to 4 °C for 20 min and immersed in lysis solution for 1 h. Next, the slides were transferred to an electrophoresis chamber containing a pH > 13.0 buffer (300 mM NaOH and 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) at 4 °C for 20 min to denature DNA. Electrophoresis was run at 25 V and 300 mA (1.25 V/cm). Subsequently, the slides were neutralized with pH 7.5 buffer (0.4 M Tris-HCl) with three changes of 5 min, air-dried, fixed in absolute ethanol for 10 min and stored for later scoring.

For coloration, the slides were stained with 100 μL of ethidium bromide (20 μg/mL) and evaluated using a fluorescence microscope (Bioval®) at 40X, using a 420-490 nm excitation and a 520 nm barrier filter.

Three independent repetitions were done, and 100 cells were scored per treatment, classifying the comets as follows: (class 0) cells without a comet tail; (class 1) cells with a tail smaller than the diameter of the nucleus; (class 2) cells with a tail 1 to 2 times the diameter of the nucleus; (class 3) cells with a tail greater than 2 times the diameter of the nucleus. Apoptotic cells that showed a completely fragmented nucleus were not counted (Kobayashi *et al.*, 1995).

The total score was calculated by adding the resulting values after the multiplication of the total cells observed in each class of lesion by the number of the class. Statistical analysis was performed using an unpaired *Student's* t-test ($p < 0.05$).

Calculation of the damage reduction percentage

The cyclophosphamide damage reduction percentage (DR%) by β-glucan administration was calculated as the mean of Group 2 minus the mean of an associated group (Groups 6-8) divided by the mean of Group 2 minus the mean of Group l. The result was multiplied by 100 and expressed as DR% (Manoharan and Banerjee, 1985; Waters *et al.*, 1990).

Results

Table 1 shows the frequency, average, standard deviation and DR% related to the comet assay in peripheral blood on the first week of the trial. The baseline micronuclei frequency for all animals did not show a statistically significant difference at the beginning of the experiment.

During mutagenicity assessment, no such activity was confirmed for β-glucan. The animals of Group 3, which received only β-glucan in lower concentrations, showed a statistically significant reduction in the baseline micronuclei frequency at time T2.

Table 1 - Frequency, mean, standard deviation and damage reduction percentage related to the micronucleus test in peripheral blood of mice during the first week of treatment.

Treatment	Micronuclei frequency			mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	49	33	75	8.17 ± 2.71	5.50 ± 2.81	12.5 ± 3.89	-
Group 02	33	36	320	5.50 ± 2.07[a]	6.00 ± 2.28 [a]	53.33 ± 6.83 [a]*	-
Mutagenicity							
Group 03	42	40	37	7.00 ± 2.97[a]	6.67 ± 1.63[a]	6.17 ± 2.48[a]*	-
Group 04	35	53	62	5.83 ± 3.66[a]	8.83 ± 4.02[a]	10.33 ± 5.12[a]	-
Group 05	41	36	42	6.83 ± 2.64[a]	6.00 ± 3.74[a]	7.00 ± 6.42[a]	-
Antimutagenicity							
Group 06	37	20	75	6.17 ± 2.48[b]	3.33 ± 1.75[b]*	12.5 ± 4.41[b]*	100.00
Group 07	30	26	107	5.00 ± 2.37[b]	4.33 ± 2.2 [b]	17.83 ± 5.67[b]*	86.90
Group 08	32	35	168	5.33 ± 2.42[b]	5.83 ± 3.71[b]	28.00 ± 7.77[b]*	62.04

Group 01 - control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
Moments T0, T1 and T2: blood samples were taken within an interval of 24 hours, except for T2, which corresponds to 48 hours.
DR% - Damage reduction percentage (unpaired Student's t-test, $p < 0.05$).
[a]Statistically compared to the control (Group 01); [b]statistically compared to the damage-inducing agent (Group 02); *statistically significant difference.

In the study of antimutagenicity at time T2 there was a chemopreventive activity for the three doses tested. The DR%s were 100, 86.9 and 62.04% for the doses of 100, 150 and 200 mg/kg (b.w.), respectively. Again, there was a reduction compared to the baseline micronuclei frequency. However, this occurred in Group 6, which was the group with the lowest supplemented dose of β-glucan, at time T1, prior to the association of polysaccharide and cyclophosphamide.

The comet assay results are shown in Table 2 indicating that β-glucan has no genotoxic activity. When combined with cyclophosphamide it showed a statistically significant prevention of genotoxic damage caused by the alkylating agent used in the study. The DR%s were 59.52%, 84.05% and 94.34% for the doses of 100, 150 and 200 mg/kg (b.w.), respectively. After taking blood samples, the same animals were kept in the protocol of testing already described and reevaluated in the fifth week of treatment.

The data shown in Table 3 correspond to the fifth week. These show that all groups in the time points T0 and T1 had no statistically significant differences with respect to the frequencies of micronuclei, except for Group 7 at T0. The animals in this group, which received β-glucan in the concentration of 150 mg/kg associated with cyclophosphamide, showed a statistically significant reduction relative to its control group. It is necessary to understand that the statistically significant reduction observed does not correlate to the treatment of the fifth week, but to the treatment that had been done before this week, since the administration of cyclophosphamide in the fifth week was done after the analysis. Thus, data on mutagenicity and/or antimutagenicity at this initial time points (T0 and T1) from the fifth week of analysis correlate to events accumulated from the past weeks of treatment. However, in Group 2 it appears that, upon assessing the mutagenicity, no damage was found as the averages did not differ statistically, suggesting that this prevention correlates with the baseline micronuclei frequency. In the protocol of antimutagenicity, at time point T2, there was a chemoprotective activity for all doses tested and the DR% ranged from 59.77% to 71.63%.

Table 4 shows the data related to the comet assay during the fifth week of treatment. Here we found that the administration of β-glucan lead to a statistically significant increase in the genetic damage frequency. However, the variation found was lower, as the frequency in Group 1 (control group) was 1.83 ± 0.98, while the highest frequency was found in Group 3 (which corresponds to the lower dose of β-glucan), 4.83 ± 1.47. Allied to this increase in the frequency of injured cells in the lower dose of β-glucan it was possible to see that this same dose showed no chemopreventive activity, as its DR% was only 2.39%. Yet, the other two doses tested showed DR%s of 43.79 and 77.0% for the doses of 150 and 200 mg/kg (b.w.), respectively.

During the sixth week of treatment it was possible to see that Group 1 and Groups 3-5, when in T0, T1 and T2, had no statistically significant frequencies of micronuclei.

Table 2 - Total and mean frequency of damaged cells, average distribution between the classes of damage, average scoring and damage reduction percentage related to tests for genotoxicity and antigenotoxicity in peripheral blood of mice during the first week of treatment.

Treatment	Total freq.[1]	mean ± SD					Score	DR%
		mean. freq.[2]	Comet classes					
			0	1	2	3		
Group 01	16	2.67 ± 2.66	97.33 ± 2.66	2.50 ± 2.43	0.17 ± 0.41	0.00 ± 0.00	2.83 ± 2.93	-
Group 02	600	99.83 ± 0.41[a]*	0.17 ± 0.41	84.83 ± 4.75	15.00 ± 4.69	0.17 ± 0.41	115.17 ± 4.49	-
Genotoxicity								
Group 03	41	6.83 ± 4.79[a]	93.17 ± 4.79	6.83 ± 4.79	0.00 ± 0.00	0.00 ± 0.00	6.83 ± 4.79	-
Group 04	20	3.33 ± 1.97[a]	96.67 ± 1.97	3.33 ± 1.97	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 1.97	-
Group 05	25	4.17 ± 3.87[a]	95.83 ± 3.87	4.17 ± 3.87	0.00 ± 0.00	0.00 ± 0.00	4.17 ± 3.87	-
Antigenotoxicity								
Group 06	252	42.00 ± 7.15[b]*	58.00 ± 7.15	42.00 ± 7.15	0.00 ± 0.00	0.00 ± 0.00	42.00 ± 7.15	59.52
Group 07	109	18.17 ± 8.38[b]*	81.83 ± 8.38	18.17 ± 8.38	0.00 ± 0.00	0.00 ± 0.00	18.17 ± 8.38	84.05
Group 08	49	8.17 ± 3.06[b]*	91.83 ± 3.06	8.17 ± 3.06	0.00 ± 0.00	0.00 ± 0.00	8.17 ± 3.06	94.34

Group 01 - control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
[1]Total number of damaged cells by treatment; [2]Average number of damaged cells by treatment; DR% -Damage reduction percentage.
[a]Statistically compared to the control (Group 01); [b]statistically compared to the damage-inducing agent (Group 02); *statistically significant difference (unpaired Student's t-test, $p < 0.05$).

Table 3 - Frequency, mean, standard deviation and damage reduction percentage related to the micronucleus test in peripheral blood of mice during the fifth week of treatment.

Treatment	Micronuclei frequency			mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	55	52	64	9.17 ± 3.82	8.67 ± 6.19	10.67 ± 7.28	-
Group 02	70	55	497	11.7 ± 2.25[a]	9.17 ± 2.93[a]	82.33 ± 16.24[a]*	-
Mutagenicity							
Group 03	36	52	56	6.00 ± 3.10[a]	8.67 ± 5.28[a]	9.33 ± 3.44[a]	-
Group 04	55	42	62	9.17 ± 5.23[a]	7.00 ± 3.46[a]	10.33 ± 2.42[a]	-
Group 05	36	91	46	6.00 ± 3.63[a]	15.17 ± 8.95[a]	7.66 ± 5.28[a]	-
Antimutagenicity							
Group 06	56	46	237	9.33 ± 5.64[b]	7.67 ± 2.80[b]	39.50 ± 6.10[b]*	59.77
Group 07	39	40	186	6.50 ± 5.64[b]*	6.67 ± 4.46[b]	31.00 ± 9.08[b]*	71.63
Group 08	55	52	218	9.17 ± 4.35[b]	8.67 ± 4.13[b]	36.33 ± 13.91[b]*	64.19

Group 01 - control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
Moments T0, T1 and T2: blood samples were taken within an interval of 24 hours, except for T2, which corresponds to 48 hours. DR% - Damage reduction percentage (unpaired Student's t-test, $p < 0.05$).
[a]Statistically compared to the control (Group 01); [b]statistically compared to the damage-inducing agent (Group 02); *statistically significant difference.

Table 4 - Total and mean frequency of damaged cells, average distribution between the classes of damage, average scoring and damage reduction percentage related to tests for genotoxicity and antigenotoxicity in peripheral blood of mice during the fifth week of treatment.

Treatment	Total freq.[1]	mean ± SD					Score	DR%
		mean freq.[2]	Comet classes					
			0	1	2	3		
Group 01	11	1.83 ± 0.98	98.17 ± 0.98	1.83 ± 0.98	0.00 ± 0.00	0.00 ± 0.00	1.83 ± 0.98	-
Group 02	598	99.67 ± 0.82[a]*	0.33 ± 0.81	83.33 ± 2.93	15.83 ± 2.79	0.00 ± 0.00	115.50 ± 2.88	-
Genotoxicity								
Group 03	29	4.83 ± 1.47[a]*	95.17 ± 1.47	4.83 ± 1.47	0.00 ± 0.00	0.00 ± 0.00	4.83 ± 1.47	-
Group 04	19	3.17 ± 0.75[a]*	96.83 ± 0.75	3.17 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	3.17 ± 0.75	-
Group 05	27	4.50 ± 2.34[a]*	95.50 ± 2.34	4.50 ± 2.34	0.00 ± 0.00	0.00 ± 0.00	4.50 ± 2.34	-
Antigenotoxicity								
Group 06	584	97.33 ± 2.94[b]	2.67 ± 2.94	96.83 ± 2.86	0.50 ± 0.055	0.00 ± 0.00	97.83 ± 3.12	2.39
Group 07	341	56.83 ± 11.77[b]*	43.00 ± 12.08	56.83 ± 11.77	0.00 ± 0.00	0.00 ± 0.00	56.83 ± 11.77	43.79
Group 08	146	24.33 ± 24.03[b]*	75.67 ± 24.03	24.33 ± 24.03	0.00 ± 0.00	0.00 ± 0.00	24.33 ± 24.03	77.00

Group 01 control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
[1]Total number of damaged cells by treatment; [2]average number of damaged cells by treatment; DR% - Damage reduction percentage.
[a]Statistically compared to the control (Group 01); [b]statistically compared to the damage-inducing agent (Group 02); *statistically significant difference (unpaired Student's t-test, $p < 0.05$).

However, the time point T0 in Groups 2, 6, 7 and 8 had an increased damage frequency in comparison to Group 1. Due to the administration of cyclophosphamide for five consecutive weeks, the damage frequencies were increased by 3.65, 4.35, 4.64 and 4.38 times the control for Groups 2, 6, 7 and 8, respectively. In the protocol of mutagenicity, once again, we found that β-glucan did not have any mutagenic activity, and antimutagenicity harm-reduction percentages ranged from 39.83% to 59.51% (Table 5) at the time point T2. When looking at T1 in this same table, it ap-

pears that the administration of β-glucan caused statistically significant a decrease in the frequency of micronuclei when compared to the control. In Table 6 it is possible to see that only the highest dose of β-glucan showed genotoxic activity. However, unlike the other time points of assessment, none of the associations showed chemopreventive activity, as the DR%s for all were equal to zero. In Figures 2 and 3, the behavior of antimutagenic and antigenotoxic activity is illustrated in terms of the DR%s during the weeks of treatment.

Table 5 - Frequency, mean and standard deviation, and damage reduction percentage related to the micronucleus test in peripheral blood of mice during the sixth week of treatment.

Treatment	Micronuclei frequency			mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	63	55	69	10.50 ± 8.14	9.17 ± 5.91	11.50 ± 6.44	-
Group 02	230	214	511	38.33 ± 8.14[a]*	35.67 ± 2.34[a]*	85.17 ± 21.44[a]*	-
Mutagenicity							
Group 03	60	55	62	8.33 ± 4.50[a]	9.17 ± 4.79[a]	10.33 ± 2.25[a]	-
Group 04	67	44	66	11.17 ± 5.60[a]	7.33 ± 3.14[a]	11.00 ± 2.19[a]	-
Group 05	68	69	50	11.30 ± 4.08[a]	11.50 ± 3.08[a]	8.33 ± 4.97[a]	-
Antimutagenicity							
Group 06	274	130	335	45.67 ± 11.20[b]	21.67 ± 8.62[b]*	55.83 ± 12.86[b]*	39.83
Group 07	292	94	264	48.67 ± 11.20[b]*	15.67 ± 3.72[b]*	44.00 ± 7.32[b]*	55.88
Group 08	276	94	248	46.00 ± 5.51[b]	15.67 ± 2.34[b]*	41.33 ± 7.84[b]*	59.51

Group 01 - control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
Moments T0, T1 and T2: blood samples were taken within an interval of 24 hours, except for T2, which corresponds to 48 hours; DR% - Damage reduction percentage (unpaired Student's t-test, $p < 0.05$).
[a]Statistically compared to the control (Group 01); [b]statistically compared to damage-inducing agent (Group 02); * statistically significant difference.

Table 6 - Total and mean frequency of damaged cells, mean distribution between the classes of damage, mean scoring and damage reduction percentage related to tests for genotoxicity and antigenotoxicity in peripheral blood of mice during the sixth week of treatment.

Treatment	Total freq.[1]	mean ± SD					Score	DR%
		mean freq.[2]	Comet classes					
			0	1	2	3		
Group 01	26	4.33 ± 2.58	95.67 ± 2.58	4.33 ± 2.58	0.00 ± 0.00	0.00 ± 0.00	4.33 ± 2.58	-
Grupo 02	599	99.83 ± 0.41[a]*	0.17 ± 0.41	95.50 ± 2.74	4.33 ± 2.73	0.00 ± 0.00	104.17 ± 2.79	-
Genotoxicity								
Group 03	15	2.50 ± 1.05[a]	97.50 ± 1.05	2.50 ± 1.05	0.00 ± 0.00	0.00 ± 0.00	2.50 ± 1.05	-
Group 04	33	5.50 ± 1.97[a]	94.50 ± 1.97	5.50 ± 1.97	0.00 ± 0.00	0.00 ± 0.00	5.50 ± 1.97	-
Group 05	53	8.83 ± 2.48[a]*	91.17 ± 2.48	8.83 ± 2.48	0.00 ± 0.00	0.00 ± 0.00	8.83 ± 2.48	-
Antigenotoxicity								
Group 06	599	99.83 ± 0.41[b]	0.17 ± 0.41	95.83 ± 1.79	4.00 ± 1.79	0.00 ± 0.00	103.83 ± 1.94	0
Group 07	599	99.83 ± 0.41[b]	0.17 ± 0.41	97.50 ± 1.76	2.33 ± 1.75	0.00 ± 0.00	102.17 ± 1.83	0
Group 08	599	99.83 ± 0.41[b]	0.17 ± 0.41	97.83 ± 1.17	2.00 ± 1.26	0.00 ± 0.00	101.83 ± 1.47	0

Group 01 - control (PBS - 0.1 mL/10.0 g), Group 02 - cyclophosphamide (50 mg/kg), Group 03 - β-glucan (100 mg/kg), Group 04 - β-glucan (150 mg/kg), Group 05 - β-glucan (200 mg/kg), Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).
[1]Total number of damaged cells by treatment; [2]Average number of damaged cells by treatment; DR% - Damage reduction percentage.
[a]Statistically compared to the control (Group 01); [b]statistically compared to the damage-inducing agent (Group 02); *statistically significant difference (unpaired Student's t-test, $p < 0.05$).

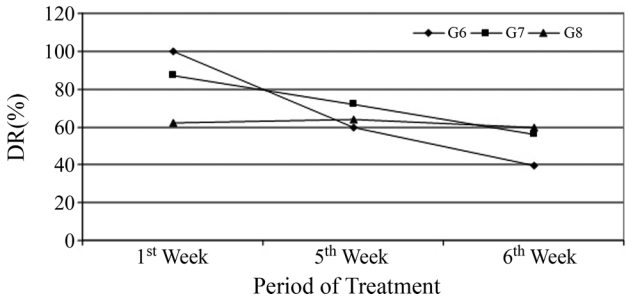

Figure 2 - Antimutagenic behavior of the β-glucan molecule activity measured by DR% in the micronucleus assay in peripheral blood. Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/g).

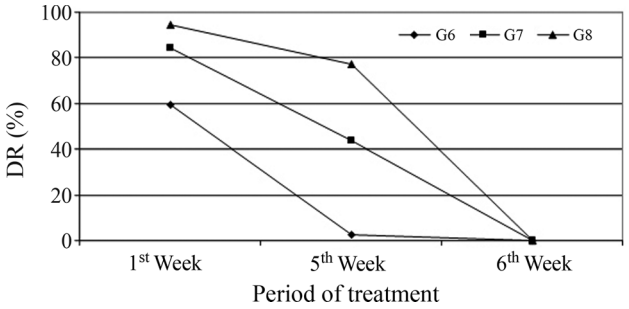

Figure 3 - Antigenotoxic activity of the β-glucan molecule measured by DR% in the comet assay in peripheral blood. Group 06 - β-glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 07 - β-glucan (150 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - β-glucan (200 mg/kg) + cyclophosphamide (50 mg/kg).

The analysis of data concerning the mutagenicity shows that for the doses of 100 and 150 mg/kg (b.w.) this was gradually reduced over the weeks of treatment. There was a decrease of 40.23 and 15.27 percentage points from the first to the fifth week and 19.94 and 15.75 from the fifth to the sixth week, respectively. Yet, the highest dose tested (200 mg/kg b.w.) showed a different behavior from the others, as there was first an increase of 2.15 percentage points followed by a decrease of 4.68 from the first to the fifth week and from the fifth to sixth week, respectively.

For the DR% observed in the comet assay there was a similar behavior for both doses. The data analysis revealed a reduction of 57.13, 40.26 and 17.34 percentage points for the three different doses (100, 150 and 200 mg/kg b.w.) from the first to the fifth week, respectively. From the fifth to the sixth week there was a reduction of 2.39, 43.79 and 77.0 percentage points for the three different doses, respectively. Thus, at the sixth week no antigenotoxic activity was denoted for the β-glucan.

Discussion

In the present study, β-glucan, extracted from *Saccharomyces cerevisiae*, was administered intraperitoneally in mice. The option of using polymer instead of fiber or bran produced from *Saccharomyces cerevisiae* was due to the intention of having a controlled amount administered to the animals, as well as the possibility of finding a more efficient effect of the purified polysaccharide.

In this experimental protocol, an administration of β-glucan was done before cyclophosphamide; one simultaneously and another one 24 h after the onset of chemotherapy. The choice of this substance is due to the mechanism of the antimutagenic β-glucan action, which was previously investigated in our laboratory (Oliveira *et al.*, 2006, 2007). The polysaccharide, regardless of its origin, from cereal or fungus, has its action from both antimutagenesis and bioantimutagenesis. Thus, it is possible to think about a therapy that could combine the protocols for pre-treatment, simultaneous treatment and post-treatment, which would allow greater efficacy of this polysaccharide in the prevention of genetic damage and consequent development of a tumor.

The idea proposed previously found support in studies published by other authors. According to these, antimutagenic substances are those capable of preventing the action of damage-inducing agents mainly by their adsorption. Therefore these act preferably in the extracellular environment. On the other hand, bioantimutagenic agents are the ones capable of involved in injury prevention or DNA repair, thus acting within the cell (Kada *et al.*, 1982; Kada and Shimoi, 1987, De Flora, 1998).

The experimental results show that β-glucan presented no mutagenic activity, and in the experimental groups and the groups treated with the lowest and highest dose tested it was capable of reducing the basal frequency of micronuclei. This suggests that in the future this sugar polymer can be an important dietary supplement that can help to prevent the development of cancer because of its chemopreventive ability against spontaneous DNA damage.

The study of antimutagenicity showed a high chemopreventive efficacy against the damage caused. When assessing the lowest dose, there was a 100% prevention against these injuries. In this study, there was no correlation between an increased dose of β-glucan and increased chemoprevention. On the contrary, with increasing concentrations there was actually a reduction in chemopreventive capacity. Although there are no reports yet in the literature to support this, the administration of high doses of β-glucan could relate to a blockage of cellular metabolism, and thus does not achieve the desired chemoprotective effect but could rather cause cell toxicity. Another possibility would be an inflammatory reaction, and this in turn would lead to an increased frequency of micronuclei.

A possible explanation for the increased frequency of micronuclei in an inflammatory reaction could be the accumulation of free radicals that can be generated by the metabolism of arginine, as this molecule is the precursor of

nitric oxide. Corroborating this, the study of Oliveira *et al.* (2009b) showed that glutamine can be converted into nitric oxide precursors, and their excess in a body could be related to the increase in DNA damage. A possible explanation for the increased frequency of micronuclei in an inflammatory reaction could be the accumulation of free radicals that can be generated by the metabolism of arginine and/or glutamine, as this molecule is the precursor of nitric oxide, which has many functions in the body, including the stimulation of the immune response mediated by lymphocytes and macrophages.

In certain situations where the production of nitric oxide is increased there may be tissue damage (Dusse *et al.*, 2003; Luiking *et al.*, 2005) and this route is important for this study because it helps in understanding mutagenic data. This would imply a direct proportional relationship between the increase of free radicals and the increase of damage to the DNA.

In acute and/or moderate inflammation, such as the possible reaction caused by high doses of β-glucan administered in the first week of treatment, there could have been a change in arginine metabolism and the use of arginine by the body could be high. To meet this need, there could be a breakdown of muscle protein and the endogenous synthesis of arginine leading to an increase in nitric oxide capable of increasing the level of damage caused by this free radical.

Another subject that needs to be discussed is the potential generation of free oxygen radicals due to the oxidative burst generated by the activated neutrophils and macrophages. The study made by Demir *et al.* (2007) demonstrated that β-glucan extracted from *S. cerevisiae*, when administered orally during 14 days in women with breast cancer in advanced stage, caused the activation of monocytes in peripheral blood, as well as the stimulation of their proliferation. Also, clinical examinations did not show any side effects of oral administration of β-glucan. Added to this fact, Xiao *et al.* (2004) questioned if β-glucan immunomodulatory activity occurs by activating or increasing the host immune response through leukocyte activation and the production of inflammatory cytokines, when treating with anticancer drugs. Thus, to define the exact β-glucan anticancer mechanism, more research is necessary.

Today it is known that cell and/or tissue damage causes the release of a number of cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, that in turn cause neutrophils to become activated and produce a host of cytotoxic substances, including reactive oxygen species, such as superoxide anions, hypochlorite and hydrogen peroxide (Best *et al.*, 1999; Brickson *et al.*, 2001). The cytokines IL-1, IL-6 and TNF-α all stimulate pathways that contribute to the activation of the enzyme NADPH-oxidase, which generates a "respiratory burst". The subsequent release of reactive oxygen species (Butterfield *et al.*, 2006) could then be responsible for the increased frequency of micronuclei.

Thus, even though β-glucan is described as an antioxidant agent, and the mechanism of desmutagenic action is its greatest effectiveness (Patchen *et al.*, 1987; Chorvatovicová, 1991; Slameňová *et al.*, 2003; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007; Magnani *et al.*, 2011; Silva *et al.*, 2012), this could not prevent the damage caused by metabolites of arginine generated in the inflammatory process and the metabolism of chemotherapy. On the other hand, when evaluating the comet assay data, a dose-response curve in chemoprevention of genotoxic damage was seen in the first week of treatment.

Notwithstanding, the dose response curve in seen in chemoprevention of genotoxic damage was not observed for mutagenic damage. One possible explanation for this is that the two tests used assessed different types of damage. The micronucleus test quantifies cytogenetic damage, such as mutation events already set in the cell genome (Salvadori *et al.*, 2003), whereas the comet assay indicates genotoxic damage, which may or may not result in mutations (Oliveira *et al.*, 2007).

When assessing the fifth week of treatment we again noticed that β-glucan showed no mutagenic activity and, again, there was no correlation between an increased dose of β-glucan and increased chemopreventive activity. However, the intermediate dose was more effective in preventing damage caused by mutagenic alkylating agent. When compared to the first and fifth week of treatment, there was a gradual reduction in chemopreventive ability from the polysaccharide in this study. In the first week, the DR%s showed greater variation (37.96 percentage points between the lowest and highest dose tested) compared to the fifth week (11.86 percentage points between the lowest dose and the intermediate dose).

When analyzing the comet assay, the β-glucan proved to be genotoxic in three of the doses tested in the weeks following the first one. A curve dose response was seen in antigenotoxicity, and it is even possible to infer that the lowest dose tested showed no chemopreventive ability, whereas the higher dose was better at preventing damage to DNA.

With respect to the micronucleus test, in T0, statistically significant differences were found when comparing Groups 2, 6, 7 and 8 to control (Group 1) during the sixth week. This indicates that exposure to five weeks of chemotherapy associated with β-glucan promoted a significant increase in the initial ratio of micronuclei. Thus, since the damage was accumulated over the weeks, the time between both applications of cyclophosphamide during the sixth week was not enough for the organism to recover from mutagenic damages caused by the treatment with chemotherapy. Until the fifth week, the interval between treatments was enough for the animal to go through a process of recovery and the level of damage at T0 was always along the lines of control. When evaluating the protocol of mutagenicity, it appears that the animals that received only

β-glucan showed no statistically significant difference over that week and the entire experiment. Thus, once again it can be inferred that the polysaccharide shows no mutagenic activity.

The analysis of T1 in the sixth week in the protocol of antimutagenicity indicates that the administration of β-glucan prior to the new treatment with cyclophosphamide showed a tendency to reduce the incidence of micronuclei observed at the time T0. However, when evaluating T2, 48 hours after the administration of cyclophosphamide combined with β-glucan, we observed a chemopreventive activity of the latter. But this capacity was low compared to the other weeks, with a variation of 19.68 percentage points. Another fact that draws attention is that the increased chemopreventive activity was denoted at the highest dose. But this was still far below the DR% observed in in the first (DR% = 100% - lower dose) and fifth (DR% = 71.63% - intermediate dose) weeks.

In the comet assay, the β-glucan again presented itself as genotoxic, but only for the highest dose. However, before the real genotoxic activity of the polysaccharide can be assessed, further studies are needed to support this finding, since the average values of damage shown in the fifth and sixth weeks were low and close to each other. Therefore, the actual biological significance of these values must be better understood to infer whether or not there is a toxic effect of β-glucan.

Contrary to what has been observed so far, the comet assay in the sixth week of treatment indicates a total chemopreventive inefficacy against the genotoxic damage assessed. The behavior analysis of the mutagenic and/or genotoxic DR% during the 3 weeks of evaluation in general showed that acute treatment with multiple doses induced a loss in the chemopreventive efficacy of β-glucan, particularly in its antigenotoxic activity, which was canceled during the last week of the study.

The values found for antimutagenicity and antigenotoxicity in the acute treatment are very important and encourage the use of this polysaccharide in the prevention of cancer and/or genetic damage that can lead to the development of cancer. Another important fact already mentioned by some authors is the possibility of using β-glucan as an adjunct to chemotherapy, as it is able to prevent some unwanted side effects (Kaneno *et al.*, 1989; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007), which means that the supplementation of this polysaccharide could help to decrease mutagenic effects in non-tumor cells.

Facing all these facts, it is still an open question whether and when β-glucan could has effects that are related to improving the quality of life, both in humans and experimental animal models. Clearly, further work will be necessary to clarify this issue. Nonetheless, some data are of interest. As it is known that the cells are exposed to oxidant and antioxidant sources. The multiple chemical reactions involving oxygen are the most effective mechanisms of energy production and can generate intermediate compounds or reactive oxygen species commonly named free radicals. Free radicals are highly reactive compounds with one or more unpaired electrons that are not evenly neutralized by enzymatic and non-enzymatic systems (McCord, 1993). Nitric oxide is one of these free radicals and plays an important role in carcinogenesis and tumor progression. Thus, the cellular exposure to high levels of nitric oxide induced by nitric oxide synthase during the inflammatory process may induce carcinogenesis due to the mutagenic properties of this compound (Dragsted *et al.*, 1993).

Drawing a parallel with the data obtained herein it is feasible that the exposure to chemotherapy for six consecutive weeks, associated with the administration of β-glucan, might result in releasing free oxygen radicals due to an oxidative burst by activating neutrophils and macrophages. These free oxygen radicals could promote a loss in the antigenotoxic activity, mainly during the sixth week, when β-glucan loses all its antigenotoxic capacity and has its antimutagenic capacity reduced. These effects may occur due to the saturation of repair mechanisms and/or altered cell cycle kinetics. The level of DNA damage that is sustained by the cell might be important in this context. At low levels of damage, DNA-repair factors, which are highly specific for damage, could recognize and repair damage before its detection by the checkpoint proteins. However, if damage reaches a higher threshold, checkpoint proteins such as ATR could also find DNA lesions. This repair system might have been saturated, and it either needs time to work or cannot restore genomic integrity. If ATR signals lead to cell-cycle arrest, ATR might either dissociate from DNA to allow repair enzymes access to the lesion or participate in as-yet-undiscovered interactions with excision repair factors to target the damage for repair (Cline and Hanawalt, 2003).

Based on all these reports, it is suggested that β-glucan is a strong candidate for cancer prevention and control of genetic damage due to its antioxidant activity. Corroborating this, the work of Patchen *et al.* (1987) indicates improvement in the quality of life of animal which had radiation exposure, regarding hematopoietic regeneration and the ability of β-glucan in inactivating free radicals. Silva *et al.* (2012) showed the anticlastogenic effect of β-glucan in cells exposed to ultraviolet radiation (UV) suggesting that β-glucan has more than one mechanism of action, being capable of exerting desmutagenic as well as bioantimutagenic action and, therefore, these results indicate that β-glucan from *Saccharomyces cerevisiae* can be used in the prevention and/or reduction of DNA damage. Chorvatovicová *et al.* (1991, 1996, 1998) reported the prevention of genetic damage caused by cobalt and cyclophosphamide. Oliveira *et al.* (2006, 2007) suggested the antioxidant function in cell cultures of strains CHO-K1, CHO-xrs5 and HTC. Sla-

menová *et al.* (2003) and Lazarová *et al.* (2006) described the sequestrant ability of free radicals in V79 cells and in mice, respectively, when damage is caused by hydrogen peroxide. Adding to these facts, Magnani *et al.* (2011) have recently shown a protective effect of Carboxymethyl-Glucan against DNA damage in patients with advanced prostate cancer, which was shown for the first time in humans. These results suggest that β-glucan is potentially useful in improving the short-term survival.

However, for chronic therapies, there is still doubt about the true activity of β-glucan and its form of administration. But the opportunity to use this polymer concurrently with chemotherapy cannot be dismissed. Certainly, further work is necessary to reproduce other acute models that use multiple doses, as well as protocols where the administrations of chemotherapeutics are made in at longer intervals, such as chemotherapy protocols in humans.

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