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 neoplasia 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 intraperitoneal 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, in certain cases, by irradiation exposure or par-
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; Mashihi, 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 linesages 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 (Fosfason®), diluted in Ca²⁺- and Mg²⁺-free, phosphate-buffered saline (PBS) (NaCl 137 mM; KCl 2.7 mM; Na₂HPO₄ 3.9 mM; KH₂PO₄ 1.8 mM, pH 7.4) at a final concentration of 50 mg/kg of body weight (b.w.). This was administrated intraperitoneally (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 Ca²⁺- and Mg²⁺-free PBS (NaCl 137 mM; KCl 2.7 mM; Na₂HPO₄ 3.9 mM; KH₂PO₄ 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\(_2\)HPO\(_4\) 3.9 mM; KH\(_2\)PO\(_4\) 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-

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**Figure 1** - Diagram of the experimental design and techniques of analysis.
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 and then covered with 120 L of 0.5% low melting point agarose. The slide was covered with a glass coverslip and cooled to 4 °C for 20 min and immersed in 416 Oliveira Lo f et al.

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* | 6.00 ± 2.28* | 53.33 ± 6.83 ²* | - |
| Mutagenicity                       |    |    |    | 7.00 ± 2.97* | 6.67 ± 1.63³ | 6.17 ± 2.48* | - |
| Group 03                           | 42 | 40 | 37 | 5.83 ± 3.66* | 8.83 ± 4.02* | 10.33 ± 5.12* | - |
| Group 04                           | 35 | 53 | 62 | 6.83 ± 2.64* | 6.00 ± 3.74* | 7.00 ± 6.42* | - |
| Group 05                           | 41 | 36 | 42 | 6.17 ± 2.48* | 3.33 ± 1.75* | 12.5 ± 4.41* | 100.00 |
| Antimutagenicity                   |    |    |    | 5.00 ± 2.37* | 4.33 ± 2.2 ² | 17.83 ± 5.67* | 86.90 |
| Group 06                           | 37 | 20 | 75 | 5.33 ± 2.42* | 5.83 ± 3.71* | 28.00 ± 7.77* | 62.04 |
| Group 07                           | 30 | 26 | 107 | 5.00 ± 2.37* | 4.33 ± 2.2 ² | 17.83 ± 5.67* | 86.90 |
| Group 08                           | 32 | 35 | 168 | 5.33 ± 2.42* | 5.83 ± 3.71* | 28.00 ± 7.77* | 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).

*Statistically compared to the control (Group 01); ²statistically 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% 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% 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.

Yet, the other two doses tested showed DR% 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. | mean ± SD | Comet classes | Score | DR% |
|-----------|-------------|-----------|---------------|-------|-----|
|           |             |           | mean freq.    | 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** | 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 | 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 | 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 | 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** | 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** | 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** | 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).

1Total number of damaged cells by treatment; 2Average number of damaged cells by treatment; DR% - Damage reduction percentage.

Statistically compared to the control (Group 01); 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* | 9.17 ± 2.93* | 82.33 ± 16.24** | -    |

Mutagenicity

| Treatment                              | mean freq. | Comet classes | Score | DR%  |
|----------------------------------------|------------|---------------|-------|------|
|                                        | 0          | 1             | 2     | 3    |    |
| Group 03                               | 36         | 52            | 56    | 6.00 ± 3.10* | 8.67 ± 5.28* | 9.33 ± 3.44* | -    |
| Group 04                               | 55         | 42            | 62    | 9.17 ± 5.23* | 7.00 ± 3.46* | 10.33 ± 2.42* | -    |
| Group 05                               | 36         | 91            | 46    | 6.00 ± 3.63* | 15.17 ± 8.95* | 7.66 ± 5.28* | -    |

Antimutagenicity

| Treatment                              | mean freq. | Comet classes | Score | DR%  |
|----------------------------------------|------------|---------------|-------|------|
|                                        | 0          | 1             | 2     | 3    |    |
| Group 06                               | 56         | 46            | 237   | 9.33 ± 5.64b | 7.67 ± 2.80b | 39.50 ± 6.10** | 59.77 |
| Group 07                               | 39         | 40            | 186   | 6.50 ± 5.64** | 6.67 ± 4.46b | 31.00 ± 9.08** | 71.63 |
| Group 08                               | 55         | 52            | 218   | 9.17 ± 4.35b | 8.67 ± 4.13b | 36.33 ± 13.91** | 64.19 |

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. | mean ± SD | Score | DR%  |
|----------------------------------------|-------------|-----------|-------|------|
|                                        | mean freq. | 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** | 0.33 ± 0.81 | 83.33 ± 2.93 | 15.83 ± 2.79 | 0.00 ± 0.00 | 115.50 ± 2.88 | -    |

Genotoxicity

| Treatment                              | mean freq. | Comet classes | Score | DR%  |
|                                        | 0          | 1          | 2    | 3    |    |
| Group 03                               | 29         | 4.83 ± 1.47** | 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** | 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** | 95.50 ± 2.34 | 4.50 ± 2.34 | 0.00 ± 0.00 | 0.00 ± 0.00 | 4.50 ± 2.34 | -    |

Antigenotoxicity

| Treatment                              | mean freq. | Comet classes | Score | DR%  |
|                                        | 0          | 1          | 2    | 3    |    |
| Group 06                               | 584        | 97.33 ± 2.94b | 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** | 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** | 75.67 ± 24.03 | 24.33 ± 24.03 | 0.00 ± 0.00 | 0.00 ± 0.00 | 24.33 ± 24.03 | 77.00 |

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% 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% 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*** | 35.67 ± 2.34*** | 85.17 ± 21.44*** | - |

**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. | mean ± SD | Score | DR% |
|-----------|------------|-----------|-------|-----|
|           |           | Comet classes | DR% |
|           | mean freq. | 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 | - |
| Grpuo 02  | 599 | 99.83 ± 0.41*** | 0.17 ± 0.41 | 95.50 ± 2.74 | 4.33 ± 2.73 | 0.00 ± 0.00 | 104.17 ± 2.79 | - |

**Mutagenicity**

| Group 03  | 60 | 55 | 62 | 8.33 ± 4.50a | 9.17 ± 4.79a | 10.33 ± 2.25a | - |
| Group 04  | 67 | 44 | 66 | 11.17 ± 5.60a | 7.33 ± 3.14a | 11.00 ± 2.19a | - |
| Group 05  | 68 | 69 | 50 | 11.30 ± 4.08a | 11.50 ± 3.08a | 8.33 ± 4.97a | - |

**Antimutagenicity**

| Group 06  | 274 | 130 | 335 | 45.67 ± 11.20b | 21.67 ± 8.62b*** | 55.83 ± 12.86b*** | 39.83 |
| Group 07  | 292 | 94 | 264 | 48.67 ± 11.20a* | 15.67 ± 3.72a* | 44.00 ± 7.32a* | 55.88 |
| Group 08  | 276 | 94 | 248 | 46.00 ± 5.51b | 15.67 ± 2.34b** | 41.33 ± 7.84b** | 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).

In vivo -glucan chemoprevention -glucan (200 mg/kg), Group 06 - -glucan (150 mg/kg), Group 07 - -glucan (100 mg/kg) + cyclophosphamide (50 mg/kg), Group 08 - -glucan (150 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).

*Statistically compared to the control (Group 01); bstatistically 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. | mean ± SD | Score | DR% |
|-----------|------------|-----------|-------|-----|
|           |           | Comet classes | DR% |
|           | mean freq. | 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 | - |
| Grpuo 02  | 599 | 99.83 ± 0.41*** | 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 ± 1.05a | 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 ± 1.97b | 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** | 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 ± 0.41b | 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 ± 0.41b | 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 ± 0.41b | 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).

1Total number of damaged cells by treatment; 2Average number of damaged cells by treatment; DR% - Damage reduction percentage.

*Statistically compared to the control (Group 01); bstatistically compared to the damage-inducing agent (Group 02); * statistically significant difference (unpaired Student’s t-test, p < 0.05).
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; Chorvatovícová, 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% 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
\( \beta \)-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 \( \beta \)-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 \( \beta \)-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 the first (DR% = 100% – lower dose) and fifth (DR% = 71.63% – intermediate dose) weeks.

In the comet assay, the \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-glucan could have 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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 animals which had radiation exposure, regarding hematopoietic regeneration and the ability of \( \beta \)-glucan in inactivating free radicals. Silva et al. (2012) showed the anticlastogenic effect of \( \beta \)-glucan in cells exposed to ultraviolet radiation (UV) suggesting that \( \beta \)-glucan has more than one mechanism of action, being capable of exerting desmutagenic as well as bioantimutagenic action and, therefore, these results indicate that \( \beta \)-glucan from Saccharomyces cerevisiae can be used in the prevention and/or reduction of DNA damage. Chortavoticová 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. Slava...
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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