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Research Article

Effects of β-glucan polysaccharide revealed by the dominant lethal assay and micronucleus assays, and reproductive performance of male mice exposed to cyclophosphamide

Rodrigo Juliano Oliveira[1,2,3], João Renato Pesarini[1,2], Maria José Sparça Salles[4], Tatiane Yumi Nakamura Kanno[4], Ana Carolina dos Santos Lourenço[4], Véssia da Silva Leite[4], Ariane Fernanda da Silva[4], Hevenilton José Matiazi[5], Lúcia Regina Ribeiro[6] and Mário Sérgio Mantovani[4]

[1]*Centro de Estudos em Célula Tronco, Terapia Celular e Genética Toxicológica, Núcleo de Hospital Universitário, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil.*
[2]*Programa de Pós-Graduação em Saúde em Desenvolvimento na Região Centro-Oeste, Faculdade de Medicina "Dr. Hélio Mandetta", Universidade Federal de Mato Grosso do Sul, Campo Grande,MS, Brazil.*
[3]*Programa de Mestrado em Farmácia, Centro de Ciências Biológicas e da Saúde, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brazil.*
[4]*Departamento de Biologia Geral, Universidade Estadual de Londrina, Londrina, PR, Brazil.*
[5]*Laboratório de Tecnologia em Alimentos e Medicamentos, Universidade Estadual de Londrina, Londrina, PR, Brazil.*
[6]*Programa de Pós-Graduação em Biologia Celular e Molecular, Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, SP, Brazil.*

Abstract

β-glucan is a well-known polysaccharide for its chemopreventive effect. This study aimed to evaluate the chemopreventive ability of β-glucan in somatic and germ cells through the dominant lethal and micronucleus assays, and its influence on the reproductive performance of male mice exposed to cyclophosphamide. The results indicate that β-glucan is capable of preventing changes in DNA in both germ cells and somatic ones. Changes in germ cells were evaluated by the dominant lethal assay and showed damage reduction percentages of 46.46% and 43.79% for the doses of 100 and 150 mg/kg. For the somatic changes, evaluated by micronucleus assay in peripheral blood cells in the first week of treatment, damage reduction percentages from 80.63-116.32% were found. In the fifth and sixth weeks, the percentage ranged from 10.20-52.54% and -0.95-62.35%, respectively. Besides the chemopreventive efficiency it appears that the β-glucan, when combined with cyclophosphamide, is able to improve the reproductive performance of males verified by the significant reduction in rates of post-implantation losses and reabsorption in the mating of nulliparous females with males treated with cyclophosphamide.

Key words: mutation, post-implantation losses, chemoprevention, micronucleus, nulliparous females.

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Introduction

Occupational exposure, exposure to environmental contaminants and therapeutic compounds are known to induce changes related to the development of infertility, cancer and abnormalities of offspring, amongst other factors (Anderson, 2005). For instance, studies over the last 50 years suggest a reduction of sperm count and volume of semen in fertile men due to increased pollution and chemicals used mainly in water treatment (Lipshultz and Fisch, 1992). Besides the prevalence of clinical causes of infertility (Ferrari, 1991), pharmacological agents, physical and chemical substances are also capable of modifying sperm quality (Van Thiel *et al.*, 1979; De Cherney, 1987; Sadi, 1995; Orejuela and Lipshultz, 1998; Thonneau *et al.*, 1998; Vested *et al.*, 2013).

Among the causes reported for infertility, there is a correlation between the decline in fertility, abnormal sperm and the occurrence of changes in the offspring of men and/or experimental animals (Joffe and Soyka, 1984; Friedler, 1996; Oliveira *et al.*, 2005). Generally, teratogenic tests are performed in pregnant females. However, as the

Send correspondence to Rodrigo Juliano Oliveira. Faculdade de Medicina, Universidade Federal do Mato Grosso do Sul, Cidade Universitária S/N, 79070-900 Campo Grande, MS, Brazil. E-mail: rodrigo.oliveira@ufms.br.

male contributes half of the genetic information to the offspring, males can also be examined as partly responsible for teratogenesis and adverse changes in offspring development. Among these changes, smoking in men, for example, is is reported as related to the development of tumors in their children (Sorahan *et al.*, 1997a,b; Ji *et al.*, 1997; Sorahan *et al.*, 2001; Pang *et al.*, 2003; Cordier *et al.*, 2004; Anderson, 2005). In addition, experiments indicate that the abuse of alcohol, administration of cyclophosphamide and sodium arsenite may also relate to changes in the offspring of parents where the males were exposed to the above xenobiotics (Jenkinson *et al.*, 1987; Jenkinson and Anderson, 1990; Oliveira *et al.*, 2005).

The dominant lethal assay is an important method for testing mutagenic substances (Shively *et al.*, 1984). In this context, the presence of implantation sites that did not develop properly in females mated with males exposed to xenobiots is used as a criterion for success in insemination, and the fertility test is considered positive. If implants are not found, the result is negative (Sarkar *et al.*, 2000). However, among the implants, the number of post-implantation losses is indicative of genomic damage that changes gene expression leading to embryonic and/or fetal death.

Identifying environmental substances that are capable of causing genomic damage is essential. Therefore, there is a growing interest in identifying natural and chemical compounds that are able to prevent genomic damage or alter reproductive performance. Among these compounds is β-glucan, a polysaccharide extracted mainly from fungi and cereals. This polysaccharide is known to be an important fiber with antimutagenic activity (Chorvatovicová *et al.*, 1996, 1998; Slamenová *et al.*, 2003; Tohamy *et al.*, 2003; Lin *et al.*, 2004; Oliveira *et al.*, 2006, 2007, 2013; Angeli *et al.*, 2006, 2009a,b; Mantovani *et al.*, 2008; Silva *et al.*, 2013), such as inulin (Mauro *et al.*, 2013) and wheat (Pesarini *et al.*, 2013). When extracted from yeast, it consists of D-glucose molecules linked in position β-(1 → 3) containing side chains β-(1 → 6) (Di Luzio *et al.*, 1979) with a great potential for producing drugs using biotechnology methods. Currently, this polysaccharide is described as an important antimutagenic agent, with ability to increase fetal viability and reduce the rates of post-implantation loss and/or resorption, thus demonstrating an improvement in the performance of reproductive females. However, this molecule showed no ability to prevent congenital malformations induced by cyclophosphamide (Oliveira *et al.*, 2009b). Taken this fact into consideration, this study aimed to investigate the antimutagenic activity of β-glucan, extracted from *Saccharomyces cerevisiae* in somatic cells of mice by means of the micronucleus assay in peripheral blood cell. In addition, the potential for preventing damage in germ cells was evaluated through the dominant lethal assay and the influence of β-glucan on the reproductive performance of male mice exposed to cyclophosphamide was investigated.

Material and Methods

DNA damage-inducing agent

The alkylating agent cyclophosphamide (Fosfaseron®) was 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) and administrated intraperitonially (i.p.) at a final concentration of 50 mg/kg of body weight (b.w.).

Extraction and preparation of β-glucan

The β-glucan molecules tested in this study were extracted from *Saccharomyces cerevisiae.* All methods for extraction and preparation of β-glucan were done according to the protocol of Oliveira *et al.* (2013). 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 chosen according to Oliveira *et al.* (2013).

Animals

Male Swiss mice (*Mus musculus*) at reproductive age and with 30 g average weight were obtained from the Central Vivarium of the State University of Londrina. The experiment was performed at the Sectorial Vivarium of the Department of General Biology. The animals were kept isolated in a polypropylene box, and they had a minimum of seven days for adaptation. Light incidence (12 hours of light: 12 hours of darkness) and temperature (22 ± 2 °C) were controlled. Filtered water and commercial food pellets were provided *ad libitum* and 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. All procedures and protocols followed approved guidelines for the ethical treatment of animals, according to the Ethics Committee in Animal Experimentation from the State University of Londrina (Protocol # 30877/04).

Experimental design and analysis methods

The animals were randomly divided into eight experimental groups (n = 6 each) as follows: animals from the control group (Group 01) received sterile Ca^{+2}- and Mg^{+2}-free PBS at a volume of 0.1 mL/10 g (b.w.; i.p.) during three consecutive days. Animals of the cyclophosphamide group (Group 02) received this chemotherapy at the concentration of 50 mg/kg (b.w.; i.p.) on the second day of treatment and PBS during following days. For mutagenicity evaluation, Groups 03-05 received β-glucan (i.p.) for 3 consecutive days, at the doses of 100, 150 and 200 mg/kg (b.w.), respectively. For antimutagenicity evaluation, Groups 06-08 received β-glucan (i.p.) for 3 consecutive

days at the doses mentioned before and one dose of cyclophosphamide on the second day of treatment (Oliveira *et al.*, 2013).

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 24 hours after the last administration of cyclophosphamide. This experimental design is complementary to the one proposed by Oliveira *et al.* (2013).

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

2.5. 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 with a coverslip. Analyses were performed with a fluorescence microscope (Bioval ®) at 40X magnification, with a 420-490 nm excitation and a 520 nm barrier filter. A total of 2,000 cells were analyzed per animal.

Dominant lethal assay

Animals of different groups were treated for five weeks and then paired with two nulliparous and untreated females. The animals remained together to mate for a period of five days to ensure that the females reached the estrous cycle in the presence of treated male mice. Pregnancy was determined by the detection of a vaginal plug, and this was considered day zero of gestation. Females were submitted to laparotomy after 18 days of pregnancy to evaluate the fertility rate (number of pregnant females x 100 / number of females), number of implants, post-implantation loss rate (number of implants - number of live fetuses x 100 / number of implants), rate of reabsorption or frequency of lethal dominant (number of resorptions x 100 / number of implants) and collection of placentas and fetuses. Placentas were weighed and fetuses were weighed and measured. With these data, the placenta index was calculated (placental weight / fetal weight). The fetuses were classified as fetuses of adequate weight for gestational age (AWGA - weighing between the average weight of fetuses in the control group ± standard deviation); fetuses of low weight for gestational age (LWGA - b.w. below the standard deviation's lower limit of the control fetuses' mean weight) and high weight's fetuses for gestational age (HWGA - b.w. above the standard deviation's upper limit of the control fetuses' mean weight), according to Oliveira *et al.* (2009).

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).

Statistical analysis

The comparison of quantitative results was carried out using parametric and non-parametric tests (ANOVA/Tukey, Kruskal-Wallis/Dunn, unpaired Student's t-test and Chi-square), depending on the nature of the data distribution. In all cases, differences with $p < 0.05$ were considered statistically significant (Oliveira *et al.*, 2009a, 2013).

Results

Table 1 reports parameters related to fertility and fetal development. The statistical analysis showed that there were no significant differences between experimental groups for the fertility parameter. Similarly, no differences were found for the number of implants and fetal viability. Upon analyzing the rate of reabsorption, a higher incidence was found in the group treated with cyclophosphamide and in the association group of cyclophosphamide and β-glucan in highest concentration. Fetal analysis showed that the treatments did not affect the intra-uterine development, as there were no changes in fetal length, fetal weight, placental weight, placental index. Furthermore, the weight to gestational age relationship was adequate and no external malformations were seen.

Tables 2 to 4 shows the frequency, average, standard deviation and DR% for the micronucleus assay in peripheral blood cells. In the first week of treatment, at T0, all animals had the same frequency of micronuclei. The analysis of mutagenicity, assessed at T2, showed that β-glucan presented no mutagenic activity. Antimutagenic activity could be seen at T2, as a prevention of mutagenic damage in somatic cells could be denoted for the three different doses of β-glucan tested. The DR%s were 95.08, 116.32 and 80.63% for the doses of 100, 150 and 200 mg/kg, respectively.

During the fifth week of treatment all groups had the same baseline frequency of micronuclei. At T2, β-glucan did not show mutagenic activity and only the higher dose was chemopreventive ($p < 0.05$). The DR%s were 10.72, 10.20 and 52.54% for the doses of 100, 150 and 200 mg/kg, respectively.

Table 1 - Parameters related to fertility and fetal development.

Parameters	Group 01	Group 02	Group 03	Group 04	Group 05	Group 06	Group 07	Group 08
Number of mated females	12	12	12	12	12	12	12	12
Number of pregnant females	10	10	8	8	10	10	9	8
Fertility rate (%)[1]	83.33	83.33[a]	66.67[a]	66.67[a]	83.33[a]	83.33[b]	75.00[b]	66.67[b]
Number of implants[2]	10.2 ± 5.24[a]	11.1 ± 1.92[a]	11.11 ± 3.10[a]	11.4 ± 3.8[a]	10.6 ± 6.96[a]	9.0 ± 4.42[a]	8.11 ± 4.19[a]	8.11 ± 4.19[a]
Fetal viability (%)[3]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]	98.89 ± 3.51[a]	100 ± 0.00[a]	100 ± 0.00[a]
Rates of post-implantational loss (%)/ lethal dominant frequency (%)[3]	11.97 ± 6.15[a]	41.20 ± 21.05[b]	11.22 ± 17.84[a]	23.82 ± 23.78[a]	23.85 ± 27.79[a]	35.32 ± 22.52[a]	35.13 ± 32.53[a]	45.81 ± 31.56[b]
Reabsorption rates[3]	27.34 ± 18.46[a]	41.20 ± 21.05[b]	11.22 ± 17.84[a]	23.82 ± 23.78[a]	23.85 ± 27.79[a]	34.76 ± 22.34[a]	35.13 ± 32.53[a]	45.81 ± 31.56[b]
DR%[3]	-	-	-	-	-	46.46	43.79	- 33.26
Fetal length (cm)[2]	2.19 ± 0.77[a]	2.38 ± 0.14[a]	2.44 ± 0.27[a]	2.35 ± 0.36[a]	1.92 ± 1.01[a]	2.49 ± 0.06[a]	2.28 ± 0.80[a]	2.20 ± 0.77[a]
Fetal weight (g)[2]	0.98 ± 0.35[a]	1.00 ± 0.11[a]	1.00 ± 0.52[a]	1.08 ± 0.53[a]	0.72 ± 0.69[a]	1.07 ± 0.09[a]	0.94 ± 0.40[a]	0.88 ± 0.51[a]
Placental weight (g)[3]	0.09 ± 0.02[a]	0.09 ± 0.03[a]	0.11 ± 0.05[a]	0.10 ± 0.03[a]	0.15 ± 0.21[a]	0.09 ± 0.03[a]	0.11 ± 0.02[a]	0.10 ± 0.02[a]
Placental index[2]	0.06 ± 0.033[a]	0.08 ± 0.03[a]	0.094 ± 0.07[a]	0.10 ± 0.029[a]	0.14 ± 0.022[a]	0.10 ± 0.04[a]	0.10 ± 0.04[a]	0.07 ± 0.043[a]
Adequacy of weight to gestational age		AWGA	AWGA	AWGA	AWGA	AWGA	AWGA	LWGA
External malformation rate (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

DR%: Damage reduction percentage; AWGA: fetuses of adequate weight for gestational age; LWGA: fetuses of low weight for gestational age.
Values are mean ± standard deviation.
[1]Chi-square - [a]Statistically compared to the control (Group 01); [b]Statistically compared to damage-inducing agent (Group 02); *statistically significant difference.
[2]ANOVA/Tukey - different letters indicate statistically significant differences ($p < 0.05$).
[3]Kruskal-Wallis/Dunn - different letters indicate statistically significant differences ($p < 0.05$).

Table 2 - Frequency, mean ± SD and DR% for the micronucleus assay done on peripheral blood cells during the first week of treatment.

Treatment	Micronuclei frequency			Mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	49	33	56	8.17 ± 2.71	5.50 ± 2.81	9.33 ± 1.97	-
Group 02	33	36	117	5.50 ± 2.07[a]	6.00 ± 2.28 [a]	19.5 ± 4.08 [a*]	-
Mutagenicity							
Group 03	42	40	44	7.00 ± 2.97[a]	6.67 ± 1.63[a]	7.33 ± 4.80[a]	-
Group 04	35	53	54	5.83 ± 3.66[a]	8.83 ± 4.02[a]	9.00 ± 3.74[a]	-
Group 05	41	36	44	6.83 ± 2.64[a]	6.00 ± 3.74[a]	7.33 ± 2.25[a]	-
Antimutagenicity							
Group 06	37	20	59	6.17 ± 2.48[b]	3.33 ± 1.75[b*]	9.83 ± 1.72[b*]	95.08
Group 07	30	26	46	5.00 ± 2.37[b]	4.33 ± 2.25[b]	7.67 ± 4.37[b*]	116.32
Group 08	32	35	68	5.33 ± 2.42[b]	5.83 ± 3.71[b]	11.3 ± 5.01[b*]	80.63

SD: Standard deviation; 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, $p < 0.05$). Time points T0, T1 and T2: samples of blood taken within interval of 24 hours.

Table 3 - Frequency, mean ± SD and DR% for the micronucleus assay done on peripheral blood cells during the fifth week of treatment.

Treatment	Micronuclei frequency			Mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	55	52	76	9.17 ± 3.82	8.67 ± 6.19	12.67 ± 4.97	-
Group 02	70	55	272	11.7 ± 2.25[a]	9.17 ± 2.93[a]	45.33 ± 16.03[a*]	-
Mutagenicity							
Group 03	36	52	76	6.00 ± 3.10[a]	8.67 ± 5.28[a]	12.67 ± 8.57[a]	-
Group 04	55	42	52	9.17 ± 5.23[a]	7.00 ± 3.46[a]	8.67 ± 2.80[a]	-
Group 05	36	91	74	6.00 ± 3.63[a]	15.17 ± 8.95[a]	12.33 ± 5.32[a]	-
Antimutagenicity							
Group 06	56	46	251	9.33 ± 5.64[b]	7.67 ± 2.80[b]	41.83 ± 18.60[b*]	10.72
Group 07	39	40	252	6.50 ± 5.64[b*]	6.67 ± 4.46[b]	42.00 ± 12.38[b*]	10.20
Group 08	55	52	169	9.17 ± 4.35[b]	8.67 ± 4.13[b]	28.17 ± 11.14[b*]	52.54

SD: Standard deviation, 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, $p < 0.05$). Time points T0, T1 and T2: samples of blood taken within interval of 24 hours.

Analysis of the sixth week of treatment showed that β-glucan did not exert a mutagenic effect. However, groups 02, 06, 07 and 08 (which received cyclophosphamide during the six weeks of treatment) showed very high frequencies of micronuclei (at least 3.6 times). The mutagenicity remained similar to the control at T1 and T2, indicating once again that β-glucan showed no mutagenic activity. In the assessment of T1 it was noticied that after administration of β-glucan there was a considerable reduction in damage relating to the ones present at T0. The evaluation of T2 indicated chemoprevention only for the two lower doses and the respective DR%s were 62.35, 39.87 and -0.95% for the doses of 100, 150 and 200 mg/kg (Figure 1).

Discussion

Several reports indicate β-glucan chemoprevention activity *in vitro* (Slamenová *et al.*, 2003; Oliveira *et al.*, 2006, 2007; Angeli *et al.*, 2006, 2009a,b; Mantovani *et al.*, 2008; Silva *et al.*, 2013), including molecular level treatments (Da Silva *et al.*, 2013), *in vivo* (Chorvatovicová *et al.*, 1996, 1998; Tohamy *et al.*, 2003; Lin *et al.*, 2004; Mantovani *et al.*, 2008; Oliveira *et al.*, 2009., 2013) and recently also in humans (Magnani *et al.*, 2011). These results suggest that β-glucan is potentially useful in improving short-term survival. However the literature is scarce when it comes to studying the influence of this polysaccharide in

Table 4 - Frequency, mean ± SD and DR% for the micronucleus assay done on peripheral blood cells during the sixth week of treatment.

Treatment	Micronuclei frequency			Mean ± SD			DR%
	T0	T1	T2	T0	T1	T2	T2
Group 01	63	55	78	10.50 ± 8.14	9.17 ± 5.91	13.00 ± 4.56	-
Group 02	230[a]*	214	394[a]*	38.33 ± 8.14[a*]	35.67 ± 2.34[a*]	65.67 ± 13.88[a*]	-
Mutagenicity							
Group 03	60[a]	55 [a]	80[a]	8.33 ± 4.50[a]	9.17 ± 4.79[a]	13.33 ± 7.94[a]	-
Group 04	67[a]	44 [a]	55[a]	11.17 ± 5.60[a]	7.33 ± 3.14[a]	9.17 ± 2.64[a]	-
Group 05	68[a]	69 [a]	67	11.30 ± 4.08[a]	11.50 ± 3.08[a]	11.17 ± 1.8[a]	-
Antimutagenicity							
Group 06	274	130	197	45.67 ± 11.20[b]	21.67 ± 8.62[b*]	32.83 ± 6.24[b*]	62.35
Group 07	292	94	268	48.67 ± 11.20[b*]	15.67 ± 3.72[b*]	44.67 ± 5.71[b*]	39.87
Group 08	276	94	397	46.00 ± 5.51[b*]	15.67 ± 2.34[b*]	66.17 ± 13.30[b*]	-0.95

SD: Standard deviation; 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, $p < 0.05$). Time points T0, T1 and T2: samples of blood taken within interval of 24 hours.

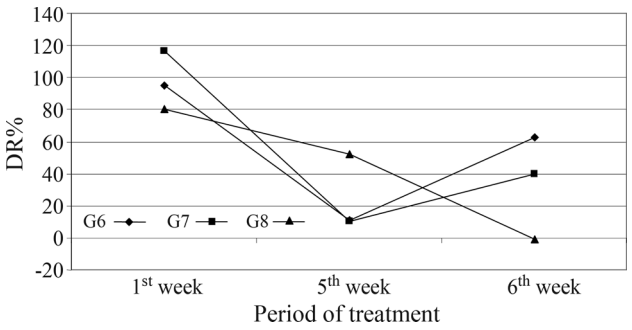

Figure 1 - Antimutagenic behavior of the β-glucan molecule measured by DR% in the micronucleus assay.

maintaining the reproductive performance of males and females and fertility of experimental animals exposed to gonadotoxic agents such as cyclophosphamide. Another fact that draws attention is that there are no studies to assess the effectiveness of β-glucan over several weeks in acute multiple dose protocols associated with male reproductive performance. In this sense, the current study represents pioneering research.

We did not observe any changes in fertility rate, number of implants and fetal viability. However, the rate of reabsorption was higher in the group receiving cyclophosphamide in association to β-glucan at the highest concentration. Analysis of the rate of post-implantation losses is also known as the dominant lethal assay (Degraeve *et al.*, 1985; Morrissey *et al.*, 1990).

The presented results show that the dose of cyclophosphamide used did not reduce the fertility of animals and also did not affect the number of implants and fetal viability. However, cyclophosphamide caused changes in the development of implanted embryos, frequently leading to death. Analysis of the rates of post-implantation loss and resorption are complementary data to this conclusion. Thus, it appears that β-glucan was able to reduce the rates of post-implantation losses and reabsorption when used in the two lower doses, therefore suggesting that it is efficient in preventing genetic damage in germ cells. Moreover, embryos without dominant lethal mutations had an appropriate intrauterine growth and corroborate to this the parameters of fetal length, fetal weight, adequacy of weight to gestational age, placental weight and placental index.

When it comes to the validation of the dominant lethal assay, there is a need to consider that the embryonic or fetal deaths must be understood as resulting from a dominant lethal mutation that occurred and was transmitted to the offspring by DNA via the sperm. Thus, the basis for the use of dominant lethals is that zygotic embryonic or fetal deaths occur due to changes or chromosomal rearrangements. Evidence for this comes from cytogenetic analysis performed on embryo samples (Maxwell and Newell, 1973).

Overall, the conclusion is that the doses of β-glucan administered did not cause any changes in the evaluated reproductive parameters. Furhtermore, cyclophosphamide at the administered dose resulted in an increase in rates of post-implantation loss and reabsorption, but when associated with β-glucan, a decrease in the reabsorption rate and an improvement in reproductive performance was observed. Herein, the two lower doses tested had very similar efficiency while the highest dose did not contribute to the prevention of such damage.

According to Tohamy *et al.* (2003) the protocol of pre-treatment with β-glucan 100 mg/kg (b.w.; i.p.) tested in mice that received 2.5 mg/kg (b.w.) of cyclophosphamide, 12 mg/kg (b.w.) of adriamycin, and 5mg/kg (b.w.) of

cisplatin (i.p.) showed a DR% of 47.11, 28.5 and 69.15% of chromosomal aberrations in bone marrow cells for cyclophosphamide, adriamycin and cisplatin respectively. In spermatogonia, these were 49.44, 67.33 and 69.20% for cyclophosphamide, adriamycin and cisplatin respectively. Thus, this data indicates that the polysaccharide has chemopreventive capacity in both somatic and germ cells. Notwithstanding, despite using different tests, our study supports the data from Tohamy *et al.* (2003), since both indicate DR%s in germ cells, except for the higher dose of β-glucan.

The micronucleus assay is often used to evaluate the organisms exposure to chemical or physical agents (Tucker and Preston, 1996), being an important procedure in genetic toxicology and when evaluating the carcinogenic potential of compounds (Krishna and Hayashi 2000). According to Kishi *et al.* (1992), comparative studies between the conventional technique in bone marrow stained by Giemsa and the technique that uses pre-staining with Acridine Orange show a good correlation. Thus, we opted for the Acridine Orange pre-staining. With peripheral blood cells, this technique has an advantage as each animal can provide multiple samples without the need of euthanasia (CSGMT, 1992).

We observed that in the first week of treatment, β-glucan did not cause changes in micronucleus frequency and that all β-glucan doses were chemopreventive, as there was a decrease in the frequency of micronuclei in the treatment group. In the fifth week, β-glucan alone also did not result in an increased micronucleus frequency, but when associated with cyclophosphamide there was a drastic reduction in the chemopreventive capacity of the β-glucan. It is worthy of note that the highest dose of β-glucan in the first week had the lowest DR%. However, during the fifth week, this same dose was the only one that showed a statistically significant difference in DR%.

For the sixth week of treatment, at T0 all animals that had received cyclophosphamide in the weeks before showed 3.6 times higher micronucleus frequency than the control. Compared to the groups where β-glucan was associated with the damage-inducing agent, the group which received the lowest dose, group 6, started out with a similar number of micronuclei ($p > 0.05$). However, groups 7 and 8 which received the middle and highest doses of β-glucan had significantly higher frequencies ($p < 0.05$). After administration of β-glucan there was considerable decrease in the frequency of micronuclei for the two lower doses. For the highest dose there was no damage reduction.

When assessing the behavior of the three doses used during the experimental weeks, we found that in the first week of treatment all doses had a good chemopreventive activity, and the middle dose gave the best results, followed by the lower and higher dose, respectively. Yet, in the fifth week, the situation had changed because only the higher dose presented chemopreventive capacity. For the sixth week, we noted again that only the lower and middle doses presented chemopreventive capacity. Thus, in this study a dose-response relationship was not established. These results are similar to what we observed in previous *in vitro* and *in vivo* studies (Oliveira *et al.*, 2006, 2007, 2009) and also are in agreement with the findings of a multiple doses protocol (Oliveira *et al.*, 2013) showing that in the first week of treatment β-glucan presented DR%s between 62.04-100.00% and 59.52-94.34% 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 39.83-59.51% and no antigenotoxicity effect was seen.

For several years now the polysaccharide β-glucan has being tested for its chemopreventive ability. Patchen *et al.* (1987) showed that it improves survival of animals treated with radiation, and that this improvement is due not only to hematopoietic regeneration, but the authors also assume that β-glucan is able to inactivate free radicals that could cause damage to the body Chorvatovicová (1991) also suggested that the polysaccharide acts as an antioxidant. However, only in 2006 the antimutagenic mode of action of β-glucan extracted from barley was described (Oliveira *et al.*, 2006), reporting that β-glucan has desmutagenenic and bioantimutagenic activity. Thus, there is evidence that β-glucan can act by intercepting agents that cause damage to the DNA while they are still in the extracellular environment, or act as an antioxidant capable of modulating the DNA repair system in the intracellular environment. A study with β-glucan extracted from *Saccharomyces cerevisiae* confirmed that β-glucan has desmutagenic and bioantimutagenic activity (Oliveira *et al.*, 2007). The protocols established in the current study were based upon the β-glucan modes of action described previously. Thus, an administration of β-glucan prior to that of cyclophosphamide, one concomitantly and another thereafter were used to obtain a maximal capacity of β-glucan chemopreventive ability, and the data suggest chemoprevention to both somatic and germ cells. Also, corroborate findings on the decrease in the rates of reabsorption and post-implantation losses. This study is also in accordance with data from Morrissey *et al.* (1990) showing a correlation with mutagenic events in somatic cells.

An important aspect in the analysis of reproductive performance is sperm morphology, and current results show an increase in exchanges between sister chromatids leading to the increase in the frequency of dominant lethal mutations and decreased sperm quality. McGregor (1984) also strengthens the relationship between changes in sperm head morphology and the increasing in frequency in occurrence of dominant lethal mutations.

It is thus feasible that in the near future β-glucan may be used as an effective chemopreventive agent being able to

modulate changes that affect both the individual and its offspring. Some studies already suggest the consumption of β-glucan for injury prevention in genomic somatic and germ cells. The current study underlines the effectiveness of this chemopreventive polysaccharide, but this chemoprevention is not consistent in multiple doses treatment. Further studies are thus still clearly needed to firmly establish the mode of action of β-glucan as well its indication or contra-indication in chemoprevention and/or for preventing alterations in reproductive performance, embryo-fetal development and genomic integrity of germ cells.

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