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Effects of β-glucan from *S. cerevisiae* on the expression of *Casp9*, *Ccna2* and *Sod1* genes in MCF-7 cells

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ABSTRACT. β-Glucans (βG) are polysaccharides widely distributed in nature with chemopreventive properties. The aim of this study was to investigate the effects of βG and the combined treatment with doxorubicin (Dox) on cell viability and mRNA levels of genes involved in cell cycle, apoptosis and antioxidant response. βG was not cytotoxic. The mRNA levels of *CCNA2* of cells exposed to β-glucan was upregulated and the exposure to Dox decreased the expression, while the combination led to an upregulation. Modulation of mRNA levels of *CASP9* suggest that βG could inhibit promotion and progression steps of carcinogenesis, eliminating neoplastic cells. The upregulation of *CCNA2* gene in combined treatment could be occurred due to ability of βG in restoring the cell cycle distribution pattern after treatment with Dox. The upregulation of *SOD1* suggests that βG can enhance the intracellular antioxidant defense, reducing the levels of superoxide dismutase induced by Dox. This response could reduce oxidative damage and attenuate tissue damage during chemotherapeutic treatment. Our data suggest that the drug combination may be less effective in killing tumor cells than the treatment with Dox alone. Thus, future studies should carefully consider this effect on indication of βG during chemotherapy.

Keywords: caspase-9; cyclin A2; superoxide dismutase 1; cell cycle; antioxidant.

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Introduction

Glucans are polysaccharides widely distributed in nature and often studied due to chemopreventive properties. They are constituent of the cell wall of plants (oats and barley), algae, bacteria and fungi. β-glucans (βG) have a common structure comprising a main chain of β-(1,3) and/or β-(1,4) D-glucopyranosyl unit and they differ in length and branching structures. βG of *Saccharomyces cerevisiae* have 1→6 side branches while those of bacteria have 1→4 side branches (Chan, Chan, & Sze, 2009).

βG can prevent DNA damage induced by chemical and physical agents (Ghavami, Goliaei, Taghizadeh, & Nikoofar, 2014). Some authors showed its significant efficacy in preventing mutagenic effects caused by doxorubicin, cyclophosphamide and cisplatin (Tohamy, El-Ghor, El-Nahas, & Noshy, 2003), methyl methanesulfonate (Oliveira et al., 2007) and hydrogen peroxide (Slameňová, 2003). Moreover, some studies have related the antioxidant ability of βG against reactive free radicals formed by endogenous metabolic processes or exogenous chemicals (Tsiapali et al., 2001; Slameňová, 2003; Sener, Ekşioğlu-Demiralp, Cetiner, Ercan, & Yeğen, 2006; Guerra Dore et al., 2007; Kofuji et al., 2012; Lei et al., 2015). Yeast-derived βG have modulating action of humoral and cellular immune responses (Vetvicka et al., 2007). This activity provides protection to the organism against infections and cancer development (Samuelsen, Schrezenmeir, & Knutsen, 2014; Roudbary, Daneshmand, Hajimorad, Roudbarmohammadip, & Hassan, 2015).

Despite postulated modes of action by which β-glucan works are lacking information about the molecular mechanisms involved in the chemopreventive activity of this polysaccharide. In addition, compounds with chemopreventive properties can contribute to reduce side effects and toxicity during the chemotherapeutic treatment. Therefore, the aim of this study was to investigate the effects of βG and the combined treatment with doxorubicin (Dox) on the expression of genes related with apoptosis (*CASP9*), cell cycle control (*CCNA2*) and antioxidant defense (*SOD1*) in human breast cancer MCF-7 cells. Doxorubicin (Dox) was chosen because it is one of the most used chemotherapeutic agent for cancer treatment. The limitation on the use of Dox in cancer treatment is the lack of selectivity against cancer cells and, consequently, its toxicity to patients.

Material and methods

Cell culture

The human breast adenocarcinoma MCF-7 cell line was obtained from the Cell Bank of Rio de Janeiro, Brazil (RJCB). Cells were cultivated in 5.0 mL Dulbecco's Modified Eagle Medium (DMEM, Gibco®), supplemented with 10% fetal bovine serum (FBS, Gibco®) and 1% penicillin/streptomycin (Gibco®). The cell culture was maintained in a humidified incubator containing 5% CO_2 at 37°C. Under these conditions, cell viability remains >80%.

Chemicals

Doxorubicin (Dox) (CAS 25316-40-9; Adriblastina® - Pharmacia) was prepared in phosphate buffered saline (PBS) and added to the culture. Different Dox concentrations were tested: 0.1; 2.5 and 5 μg mL^{-1}. Dox was diluted in PBS free of Ca^{+2} and Mg^{+2}, pH 7.4, sterile.

βG was extracted from *S. cerevisiae* donated by PhD Marciane Magnoni of the Laboratory of Food Technology and Medicine, Center of Food Sciences, State University of Londrina. β-glucan with main chains containing β-(1→3) bonds and lateral chains with β-(1→6) bonds were extracted by autolysis of *S. cerevisiae*. The cell wall was separated by centrifugation at 6.500 g 8 $min.^{-1}$ and heat treated (70°C for 5 hours) in alkaline medium with NaOH (10%), washed and centrifuged three times and oven dried at 40°C. Analysis was performed on NMR (nuclear magnetic resonance chromatography) to prove the presence of (1,3 and 1,6) β-D-glucan with 85% purity, and then glucan was solubilized using DMSO (dimethyl sulphoxide) plus 8 M urea, at a ratio of 100 mL: 60g. In water bath, 100 mL DMSO was added with 10 mL concentrated sulfuric acid, and stirred at 100°C for 4 hours. Dialysis was run with approximately 100 L ultra-pure water (Milli-Q). The solution was then concentrated in a rotary evaporator at 40°C for subsequent lyophilization. β-glucan was prepared in PBS solution, free of Ca^{+2} and Mg^{+2}, pH 7.4, and dissolved in DMSO.

Cytotoxicity assay (MTT)

The cytotoxic effect of βG and Dox on the MCF-7 cell line was determined by the 3 - [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide assay (MTT), according to the protocol described by Mosmann (1983) with modifications. Briefly, cells were seeded on 96-well plates at a concentration of $2.5x\ 10^4$ cells/well in 100 μl DMEM medium (Gibco), and pre-incubated at 37°C and 5% CO_2 for 24 hours. Then, supernatant was removed and replaced with fresh medium without FBS containing βG (25, 50 and 100 μg mL^{-1}) or Dox (0.1; 2.5; and 5μg mL^{-1}). After 24 hours of exposure, 150 μl MTT solution (0.5 mg mL^{-1}) were added to each well and kept at 37°C for 4 hours. The MTT solution was carefully discarded and formazan crystals were dissolved with 200 μL DMSO. Absorbance was measured at 550 nm using a spectrophotometer (Uniscience). Data were evaluated as relative cell survival rate (%) = (Absorbance Test/Absorbance control) ×100. MTT assay was performed in three experiments.

RT-qPCR

MCF-7 cells (1.0×10^6/flasks) were pre-incubated for 24 hours and treated for 12 hours, being distributed as follows: Control; T1 - βG-treated cells (50μg mL^{-1}); T2 - Dox-treated cells (0.1μg mL^{-1}); T3 – Dox (0.1μg mL^{-1}) + βG (50μg mL^{-1}) treated cells. Total RNA was extracted using the TRIZOL® Reagent (Invitrogen) according to the manufacturer's protocol. RNA samples were incubated with DNase (1U) (CAS 18068-015, Invitrogen) at 37°C for 15 min., and inactivated at 65°C for 5 min. The amount and purity of RNA were determined by spectrophotometry (BioPhotometer - Eppendorf) and the integrity was analyzed by 1% agarose gel electrophoresis. Total RNA (2 μg), 10 pmol μL^{-1} oligo dT primer (1μL, 10 mM dNTPs (2 μL), RNAse OUT and M-MLV Reverse Transcriptase (Invitrogen) were used to synthetize cDNA.

Quantitative PCR measurement reactions were performed using Platinum® SYBR Green qPCR Supermix-UDG (Invitrogen). The total volume for each primer tested with different cDNAs was 20 μL. Reactions were performed in PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research). Data were normalized with reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), considering an efficiency above 80%, in each cDNA amplified in PCR-RT experiments. The reference gene was selected according to the stability of gene expression among groups. The relative expression was calculated (Pfaffl, Horgan, & Dempfle, 2002). Oligonucleotides used were *SOD-1* (Superoxide dismutase 1), *CCNA2* (Cyclin A2), *CASP9* (Apoptosis-related cysteine peptidase 9) and *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) (Table 1). The expression of target genes was compared to that of the reference gene, *GAPDH*.

The reaction conditions were as follows: 95ºC for 3 min., 40 cycles (95ºC for 30s, 60ºC for 30s, 72ºC for 20s), 95ºC for 10s and 40ºC for 1min., followed by melting curve analysis temperature between 50 and 90ºC (at 0.50°C every 5s). Each experimental protocol was performed in triplicate in two independent experiments. Data are expressed as mean ± standard deviation (SD).

Table 1. Sequences of primers used in RT-qPCR.

Genes	Oligonucleotide	Reference
CCNA2	Forward: 5'AGACCCTGCATTTGGCTGTG3' Reverse: 5'ACAAACTCTGCTACTTCTGG3'	Hsieh, Huang, Lin, & Chung (2006)
CASP9	Forward:5'GCTCTTCCTTTGTTCATCTCC3' Reverse: 5'GTTTTCTAGGGTTGGCTTCG3'	Silva et al. (2013)
SOD1	Forward:5'CTAGCGACTTATGGCGAC3' Reverse: 5'GAATGTTTATTGGGCGATC3	Ding et al. (2004)
GAPDH	Forward: 5'GAAGGTGAAGGTCGGAGTC3' Reverse: 5'AAATCCCATCACCATCTTCC3'	Weglarz Molin, Orchel, Parfiniewicz, and Dzierzewicz (2006) with modifications

Statistical analysis

The MTT assay data were tested by analysis of variance (ANOVA) followed by Tukey's test. Data are presented as mean ± standard deviation. Differences were considered significant for P values less than 0.05. RT-qPCR data were analyzed using REST (Relative Expression Software Tool – 384, REST-384© - version 2) software (Pfaffl et al., 2002). For RT-qPCR, only expression levels above 2-fold with statistically significant differences were considered relevant.

Results

MTT cytotoxicity assay

The evaluation of MCF-7 cell viability by MTT assay showed that all concentrations of Dox were cytotoxic. The analysis also pointed no significant differences in cytotoxic activity of the different evaluated concentrations (Figure 1A). Cell viability evaluation of MCF-7 cells exposed to β-glucan showed that it was not cytotoxic in any tested concentrations (Figure 1B).

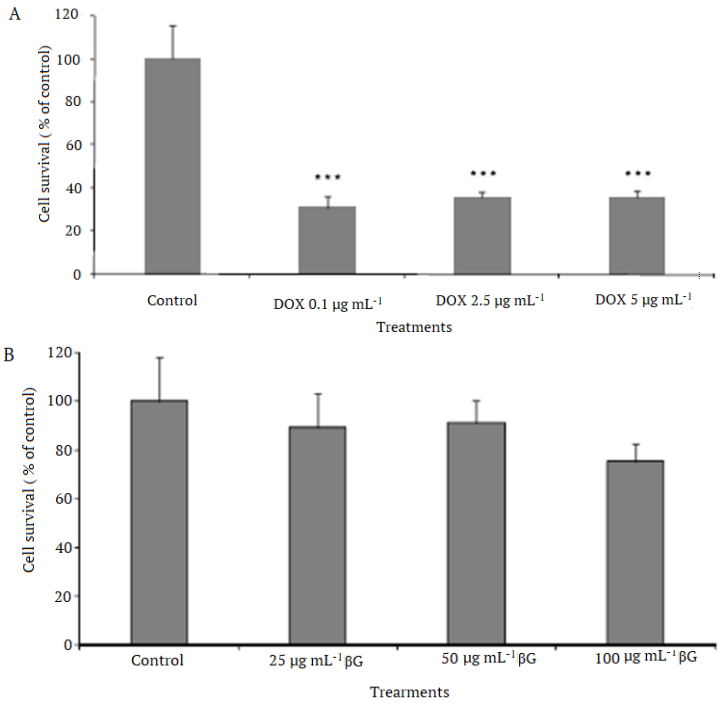

Figure 1. Cell survival (% control) of MCF-7 cells after incubation for 24 hours with (A) doxorubicin (0.1; 2.5 and 5 µg mL^{-1}) and (B) βG (25; 50 and 100 µg mL^{-1}) measured using the MTT assay. Results are presented as mean ± SD of three experiments. * p <0.05, ** p <0.01, *** p <0.001 relative to control using ANOVA followed by Tukey's test.

RT-qPCR analysis

Based on cell viability data, the concentration selected for RT-qPCR experiments was 0.1 μg mL^{-1} for Dox and 50 μg mL^{-1} for βG. To analyze the effects of βG (50 μg mL^{-1}) and of the combination of βG (50 μg mL^{-1}) and Dox (0.1 μg mL^{-1}), cells were exposed to treatments for 12 hours and mRNA levels analysis was performed by RT-qPCR.

mRNA levels of *CCNA2* of cells exposed to βG (T1) was upregulated (4-fold-change). On the other hand, treatment with doxorubicin (T2) decreased *CCNA2* mRNA levels (4.7-fold-change). The combined treatment (T3) led to an upregulation of about 10.4-fold-change (Figure 2A).

mRNA levels of *CASP9* were upregulated after the exposure to βG (T1) (5.2-fold-change), while treatments T2 and T3 had no significant influence on gene expression (Figure 2B).

The treatment combining βG and Dox (T3) resulted in an upregulation of 5.2-fold-change in mRNA levels of *SOD1* gene (Figure 2C). The other groups (T1 and T2) had no significant changes.

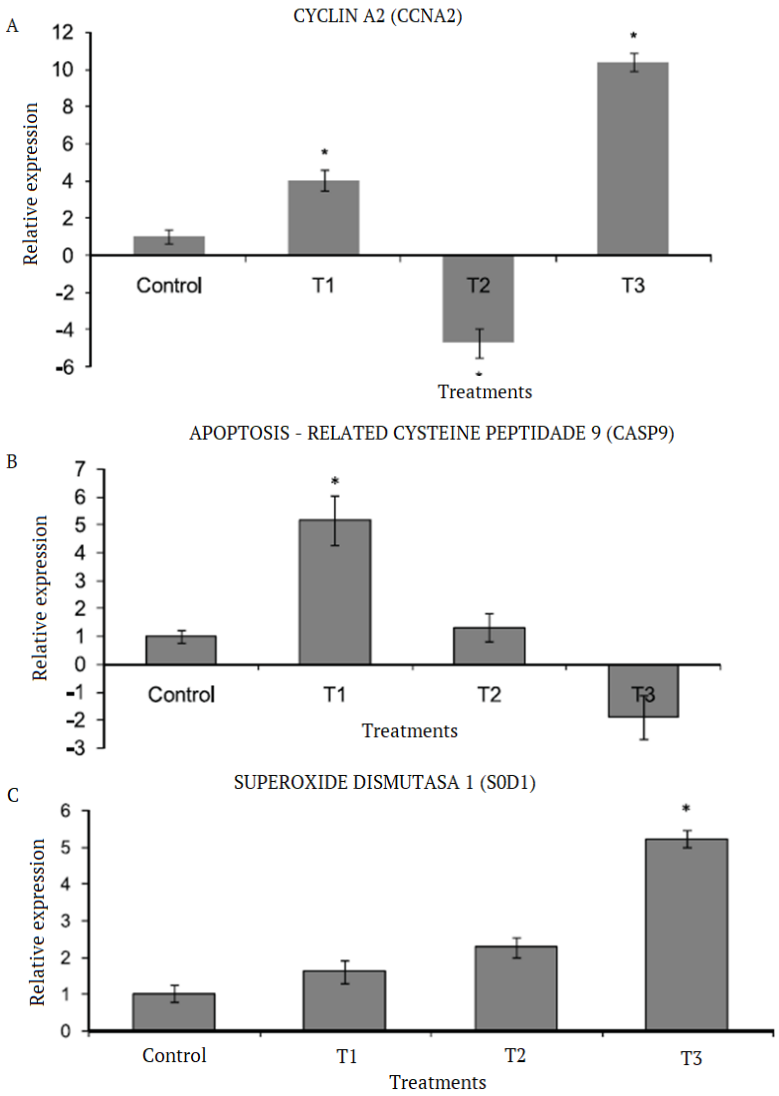

Figure 2. RT-qPCR analysis of gene expression. A) *CCNA2*; B) *CASP9;* C) *SOD1*. Graphs show the relative gene expression (fold-change) in MCF-7 cells after 12 hours of exposure to the treatments: Control; T1 - βG-treated cells; T2 - DOX-treated cells; T3 - DOX + βG treated cells. The relative gene expression was normalized with reference gene GAPDH. Data are expressed as fold changes relative to control group. Statistical differences compared to control are indicated by * (Relative Expression Software Tool – 384, REST-384© - version 2).

Discussion

Some reports show that glucans can prevent DNA damage induced by chemical and physical agents (Tohamy et al., 2003; Pillai, Maurya, Salvi, Janardhanan, & Nair, 2014). However, the mechanisms involved in the chemopreventive activity of βG are poorly known. In this study, we investigated the effect of βG from

S. cerevisiae on the cytotoxicity and gene expression of *CCNA2* (Cyclin A2), *CASP9* (Caspase-9, Apoptosis-related cysteine peptidase 9) and *SOD-1* (Superoxide dismutase 1) in human breast adenocarcinoma MCF-7 cells. The RT-qPCR analysis was performed after treatment with βG alone or combined with chemotherapeutic agent doxorubicin.

Chemopreventive agents are compounds with ability to prevent, slow, suppress or reverse the carcinogenic process (Steward & Brown, 2013; Ko & Moon, 2015). Moreover, these compounds can contribute to reduce side effects and toxicity of cancer cells during chemotherapeutic treatment (Ko & Moon, 2015). The MTT assay showed that β-glucan was not cytotoxic in any tested concentrations. Previous studies of our group have shown that β-glucan at concentrations near to 50 μg mL^{-1} have chemopreventive activity (Oliveira et al., 2007; Silva et al., 2013). Therefore, this concentration was selected for gene expression studies.

The chemotherapeutic agent doxorubicin showed cytotoxic activity against MCF-7 cells at all tested concentrations. This drug is one of the most widely used chemotherapeutic agent for treatment of cancer, since it has a great efficacy in inducing death of cells in solid tumors (tumors of breast, prostate, stomach, thyroid, uterus, lung, etc.) and hematological malignancies (Tacar, Sriamornsak, & Dass, 2013). The intercalation with DNA and generation of free radicals are the two main mechanisms of action suggested for this chemotherapeutic agent (Thorn et al., 2011).

The machinery of the cell cycle must enable the progression of the cell cycle ensuring genomic fidelity. However, tumor cells are characterized by deregulation of cell cycle checkpoints, leading to uncontrolled cell proliferation. Cyclin A is essential for continuation of S-phase and for entry into mitosis (Hochegger, Takeda, & Hunt, 2008). In the present study, data showed that treatment with doxorubicin (T2) significantly decreased the mRNA levels of CCNA2. The intercalation of doxorubicin with DNA leads to destabilization of topoisomerase II, which consequently results in DNA damage. As consequence of DNA damage, cell cycle arrest may occur or cells may be driven to apoptosis (Gewirtz, 1999). Downregulation of *CCNA2* may be related to cell cycle arrest after treatment of MCF-7 cells with Dox. Federico et al. (2010) also showed that doxorubicin at concentrations below 750 nM caused downregulation in mRNA levels of *CCNA2* in human lung adenocarcinoma A549 cells as a consequence of the arrest of S phase cell cycle. On the other hand, the treatment of MCF-7 cells with β-glucan alone (T1) or combined with doxorubicin (T3) caused an increase in mRNA levels of *CCNA2*. The upregulation of *CCNA2* gene in combined treatment may have occurred due to ability of βG in restoring the cell cycle distribution pattern after treatment with Dox.

In apoptosis, an execution machinery is activated and culminates in cell death in an orderly and controlled manner (Jin & El-Deiry, 2005). The induction of apoptosis is an important mechanism of chemoprevention, because apoptotic events can eliminate damaged cells in preneoplastic or neoplastic stage (Tanaka, 2013). In this study, RT-qPCR showed an increase in mRNA levels of *CASP9*, the gene encoding the initiator caspase of the intrinsic pathway, in cells exposed to βG (T1), while treatments T2 and T3 had no significant influence. This finding suggests that βG can induce apoptosis in MCF-7 cells by activating the intrinsic pathway. In line with our study, other authors have reported the potencial of β-glucan to induce apoptosis. Kim et al. (2009) showed the ability of β-glucan from bacteria to activate the intrinsic pathway of apoptosis in human colon cancer cells (SNU-C4), decreasing mRNA levels of the anti-apoptotic gene *BCL-2* and increasing the expression of pro-apoptotic gene *BAX* and the executor Caspase-*3*. Kobayashi et al. (2005) also reported that β-glucan from *Agaricus blazei* induces Bax translocation, cytochrome c release, and caspase-9 activation in human ovarian cancer HRA cells.

Another outstanding aspect in the search for chemoprevention mechanisms of βG include studies related to its antioxidant ability. The Cu/Zn superoxide dismutase or SOD1 enzyme belongs to the SOD family and is found in the cytosol, nucleus and intermembrane space of mitochondria. This enzyme catalyzes the dismutation of superoxide into H_2O_2 and oxygen. Juarez et al. (2008) showed that inhibition of SOD1 induces prooxidant effects due to excess superoxide in the cell. The generation of free radicals is one of the mechanisms by which doxorubicin acts in cancer cells, causing lipid peroxidation, membrane damage, DNA damage, oxidative stress, etc. Some of the enzymes with ability to inactivate free radicals are glutathione peroxidase, catalase and superoxide dismutase (Thorn et al., 2011). The SOD1 enzyme is an important cellular defense molecule against damage caused by superoxide radicals. Our results indicated that β-glucan combined with doxorubicin significantly increased mRNA levels of the *SOD1* gene. This suggests that upregulation of *SOD1* in the combined treatment with βG can enhance intracellular antioxidant defenses by reducing the levels of superoxide induced by doxorubicin. Other studies have already demonstrated that βG

have antioxidant properties (Tsiapali et al. 2001; Guerra Dore et al. 2007; Kofuji et al., 2012). Şener et al. (2006) showed that βG from *S. cerevisiae* was able to reduce oxidative damage induced by the chemotherapeutic agent methotrexate restoring GSH levels, inhibiting the increase of malondialdehyde (MDA), and attenuating tissue damage induced by methotrexate (Sener et al., 2006). Lei et al. (2015) showed that sulfated glucans from *S. cerevisiae* displayed scavenging effects on DPPH, superoxide anion and hydroxyl radicals *in vitro*. Further, the antioxidant activity of βG may contribute to its DNA protecting property, preventing mutagenic effects induced by hydrogen peroxide (Slameňová, 2003).

Conclusion

In summary, β-glucan from *S. cerevisiae* showed ability to markedly increase levels of the *CASP9* gene; upregulate the *SOD1* gene when combined with chemotherapeutic agent Dox; and cause upregulation of *CCNA2* alone and when combined with Dox. Modulation of mRNA levels of *CASP9* suggests that βG could be able to inhibit the promotion and progression of carcinogenesis, eliminating neoplastic cells. On the other hand, upregulation of the *CCNA2* gene in combined treatment could be occurred due to ability of βG in restoring the cell cycle distribution pattern after treatment with Dox. In addition, upregulation of *SOD1* suggests that βG can enhance the intracellular antioxidant defense, reducing the levels of superoxide dismutase induced by Dox. This response could reduce oxidative damage and attenuate tissue damage during chemotherapeutic treatment. Taken together, these data suggest that the drug combination (βG and Dox) may be less effective in killing tumor cells than the treatment with Dox alone. Thus, future studies should carefully consider this effect on indication of βG during chemotherapeutic treatment.

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