Effects of sulfated and non-sulfated β-glucan extracted from Agaricus brasiensis in breast adenocarcinoma cells – MCF-7

Adrivanio Baranoski¹, Marcelo Tempesta Oliveira¹, Simone Cristine Semprebon¹, Andressa Megumi Niwa¹, Lúcia Regina Ribeiro¹, and Márcio Sérgio Mantovani¹

¹Department of General Biology, State University of Londrina, Londrina, Brazil and ²Department of Pathology, São Paulo State University “Júlio de Mesquita Filho”, São Paulo, Brazil

Abstract

The β-glucans (β-G) are polysaccharides produced by various organisms, and sulfation of β-G renders them more soluble. With the objective to assess the effects of sulfated and non-sulfated β-G extracted from Agaricus brasiensis in MCF-7 cells, assays were used to evaluate cytotoxicity, genotoxicity, cell proliferation and mRNA expression. The sulfated and non-sulfated β-G showed dose-dependent cytotoxicity at concentrations of 5 and 10 μg/mL by the MTT assay. However, only cytotoxicity was observed after 24 h by the Red Neutral test for sulfated β-G, with no genotoxicity for either β-G in comet assay. Proliferation was decreased only at 72 h at a concentration of 100 μg/mL of sulfated β-G. Treatment with 5 μg/mL of sulfated β-G for 6 h reduced the expression of pro-apoptotic genes and stress signaling genes, cell cycle arrest, damage and cell migration. The 5 μg/mL of non-sulfated β-G for 6 h reduced the expression of the stress response gene and signaling damage. These results indicate that the cytotoxicity in the MTT is not cell death, and that, in general, sulfated β-G have greater cytotoxicity compared to non-sulfated β-G.

Introduction

The Agaricus brasiensis, a mushroom native from Brazil, has presented important properties and is primarily consumed in tea form in folk medicine. Studies on the extracts have revealed properties, such as protection against DNA damage and activation of the immune system, among others (Bellini et al., 2006; Gameiro et al., 2013; Guterres et al., 2005; Padilha et al., 2009), which are mainly attributed to the presence of polysaccharides, such as β-glucans. The β-glucans are naturally occurring polysaccharide cell wall constituents of some bacteria and fungi that are produced by various organisms, such as oats, barley and seaweed. The β-glucan of A. brasiensis have a linear backbone composed of D-glucose molecules linked in positions β-(1 → 3) having one or more side ports β-(1 → 6) D-glucose (Angeli et al., 2006). β-glucans have attracted the attention of researchers by stimulating the immune humoral and cellular system, with positive effects on several sources of bacterial, viral, fungal and parasitic infections (Magnani & Gómez, 2008), activation of cytotoxic T cells, natural killer cells, phagocytic cells (Hong et al., 2004) and stimulation of the production of monocytes and neutrophils (Sonck et al., 2010).

Furthermore, β-glucans have been shown to exhibit a protective effect against damage caused by benzo[a]pyrene (Silva et al., 2013). However, a major obstacle in the clinical use of β-glucans is their relative lack of solubility in aqueous media (Han et al., 2008).

To increase their solubility, some biochemical changes were made in β-glucans including sulfation due to biological activities that promote the sulfate groups, causing the molecules to become more soluble. When sulfated, the β-glucans of microorganisms Poria cocos and Pleurotus tuber-regium have shown antiviral and antitumor effects, and sulfated β-glucans extracted from Parmotrema mantiqueirensense mushroom displayed antithrombotic and anticoagulant effects, demonstrating new pharmacological potential (Mantovani et al., 2008). These findings are consistent with studies that suggest that sulfated β-glucans have different effects than non-sulfated β-glucans (Ménard, 2004). These activities can be explained by the higher solubility of sulfated β-glucans due to its higher and increased incorporation of small fragments of β-glucans, resulting in pharmaceutical and dietary benefits (Chang et al., 2006; Mantovani et al., 2008). However, the potency of the biological activities of β-glucans, soluble or insoluble in water, remains debatable (Batbayar et al., 2012).

Much is known about the beneficial effects of β-glucans from different sources and some modified β-glucans (Batbayar et al., 2012; Chorvatovicová et al., 1998; Mantovani et al., 2008; Slameňová et al., 2003).
However, their mechanisms of action have not been fully elucidated, and little is known about sulfated β-glucans. In this context, the study of molecular mechanisms involved in the mode of action of these compounds in experimental models of tumors may help to better understand the mechanisms of action of these molecules. Thus, the present study evaluated the effects of sulfated and non-sulfated β-glucans extracted from *A. brasiliensis* on cytotoxicity, cell proliferation and genotoxicity kinetics correlated with the expression of genes related to apoptosis, cell cycle, metabolism, oxidative stress and repair systems in mammary cell adenocarcinoma (Michigan Cancer Foundation-7 – MCF-7).

### Materials and methods

#### Cell lineage and culture conditions

The breast adenocarcinoma cell line MCF-7 was obtained from the Bank of cells of Rio de Janeiro (UFRJ). The cells were grown in 25 cm² culture flasks with 10 mL of Dulbecco’s Modified Eagle Medium (DMEM) (Gibco®, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco®) and maintained in an atmosphere of 5% CO₂ at 37 °C.

#### Chemical agents and inductors damage

The β-glucans derived from *A. brasiliensis* were used. The β-glucans were extracted by Professor Dr. Sandra de Aguiar Soares of the Federal University of Ceará according to the protocol described in the work of Angeli et al. (2006) and sulfated. Doxorubicin (DXR) (CAS 25316-40-9; Sigma Aldrich, St. Louis, MO) based on the protocol described in the work of Angeli et al. (2006) and Angeli et al. (2008) on six-well plates with approximately 10⁵ cells per well in 1 mL of culture medium with FBS. After 24 h of incubation, an additional 1 mL of fresh medium containing sulfated or non-sulfated β-glucans at final concentrations of 5, 50 and 100 μg/mL, control, and control DXR 1 μM was added. The anticipated treatment time was 3 h, and each treatment was performed in triplicate. After the treatment period, cells were released from the flask bottom with 300 μL of trypsin (0.1% trypsin-EDTA, 37 °C), centrifuged (5 min, 1000 rpm) and resuspended in 500 μL of DMEM medium. Subsequently, 20 μL of cell suspension was added in 120 μL Low Melting Point agarose (0.5%), and distributed in pre-gelatinized slides with normal agarose (1.5%) and overlaid with coverslips. After solidification of the agarose gel, the coverslips were removed. Slides were incubated in lysis buffer [89% (2.5 M sodium chloride, 100 mM EDTA Titriplex, 10 mM tris (hydroxymethyl) aminomethane (Tris), pH 10), 1% Triton X – 100 and 10% DMSO] for 1 h at 4 °C, and then submitted to alkaline conditions (EDTA-Titriplex 200 mM, 10 N sodium hydroxide and pH >13) for 20 min for DNA denaturation. Subsequently, the slides were brought to electrophoresis (pH 13, 25 V, 300 mA) for 20 min, followed by neutralization (0.4 M Tris and pH 7.5), and were then fixed for 15 min in absolute alcohol, dried, and kept in the refrigerator for subsequent staining. For analysis, the slides were stained with 100 μL of ethidium bromide 2 μg/mL, and analyzed visually by fluorescence microscopy (420-490 nm of the excitation filter, and barrier filter 520 nm, 400×). The determination of damage was conducted visually and classified into four classes based on the tail length. Class 0: no tail; Class 1: tail with small size up to the diameter of the head of the nucleoid; Class 2: grand average size between one and two times the diameter of the nucleoid; Class 3: long tail, longer than twice the diameter of the nucleoid. In total, 100 nucleoids per slide were analyzed. The score was obtained by: Score = (Number nucleoid class 0 × 0) + (Number nucleoid...
class $1 \times 1) + (Number\ nucleoid\ class\ 2 \times 2) + (Number\ nucleoid\ class\ 3 \times 3)$. Three experiments were performed, with two replications.

Kinetics of cell proliferation in real time – RTCA

The cell proliferation assay was performed using the apparatus xCELLigence RTCA – Real Time Cell Analyzer – Roche (Mannheim, Germany) according to the manufacturer’s protocol. Microelectrodes positioned at the bottom of the wells detected cell content via the electrical impedance caused by the accumulation of cells in each well, which were then converted to a cell proliferation curve. Cells were seeded at $3.125 \times 10^4$ per well in 96-well plates (E-Plate Roche) containing 100 µL culture medium supplemented with FBS, and kept for 24 h for stabilization. Over 50 µL fresh medium containing β-glucans sulfated or non-sulfated at final concentrations of 5, 50 and 100 µg/mL, control, and control DXR was then added, resulting in 200 µL of culture medium per well. Cell proliferation was monitored in real time for 96 h after treatment. Three experiments containing triplicates were performed.

Gene expression – RT-qPCR

Approximately $10^6$ cells were incubated in 25 cm$^2$ flasks containing 5 mL of DMEM medium and 24 h stabilization awaited. After this period, the medium was replaced with 5 mL of fresh medium containing sulfated or non-sulfated β-glucans at a final concentration of 5 µg/mL and incubated for 6 h of treatment. Treatments were also conducted at concentrations of 10, 25 and 50 µg/mL and incubated for 6 h, as well as treatment with 50 or 100 µg/mL and incubated for 24 h. Total RNA was extracted using Trizol LS reagent (Invitrogen, Life Technology, Grand Island, NY) for cell lysis associated with the RNasea$^9$ Mini Kit Qiagen, Valencia, CA (Cat. No. 74106) according to the manufacturer’s protocol. Part of the total RNA extracted was used for reading in spectrophotometer (NanoDrop 2000, Thermo Scientific, Life Technology, Grand Island, NY) to calculate the concentration, and integrity was evaluated in a 0.8% agarose gel. Three experiments were conducted. The cDNA synthesis was performed in a T100TM Thermal Cycler BIO-RAD, Singapore, where 1000 ng of total RNA were diluted in 8 µL of H$_2$O DEPC, 4 µL of oligo dT (20 pmol/mL) and 4 µL dNTPs (2.5 mM), which was maintained for 15 min at 65°C. They were then placed on ice, and 1.3 µL of DEPC-H$_2$O, 2 µL Buffer 10×, 0.6 µL of MgCl$_2$ (50 mM) 0.05 µL of RNAout (Invitrogen, Life Technologies) 0.05 µL enzyme reverse transcriptase (M-MLV – Invitrogen, Life Technologies) was added to each sample, and the reactions were then maintained at 37°C for 50 min, 15 min at 70°C and then indefinitely maintained at 10°C. For PCR, we used the fluorescent compound (5 µL) Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), 1 µL of each pair of oligonucleotides, and 5 µL of cDNA (50 ng/µL) of each sample. The qPCR reactions were performed under the following conditions: 50°C for 2 min, 95°C for 5 min and 40 cycles [95°C/15 s, 60°C/15 s, 72°C/20 s] in machine CFX96TM Real-Time System BIO-RAD, Singapore. The melting curve analysis was performed at the end of each reaction temperature of 55°C to 95°C every 0.5 degrees for 20 s. The RT reactions were performed in triplicate. The data were normalized to the expression of β-ACTIN values gene which was amplified in each set of experiments qPCRs. The efficiency of the primers was calculated by LinRegPCR, version 2013.1, December 2013, Amsterdam, The Netherlands (Ruijter et al., 2009, 2013).

This study investigated the expression of genes CCNA2 (Cyclin A2), CCNB1 (Cyclin B1), CCNE1 (Cyclin E1), CCND1 (cyclin D2), P53 (Tumor Protein 53), CHECK1 (checkpoint kinase 1), CHECK2 (checkpoint kinase 2), GADD45A (growth arrest and DNA-damage-inducible, alpha), GADD153 (DNA-damage-inducible transcript 3), XPA (xeroderma pigmentosum, complementation group A), PCNA (proliferating cell nuclear antigen), ERCC5 (excision repair Complementing cross-rodent repair deficiency, complementation group 5), NFE2L2 (Nuclear factor erythroid 2-like 2), BAK (BCL2-antagonist/killer 1), BAX (BCL2-associated X protein), BCL-2 (B-cell CLL/lymphoma 2), BCL-XL (BCL2-associated agonist of cell death) CASP-9 (Caspase 9), MAP2K6 (mitogen-activated protein kinase kinase 6), ABL1 (c-abl oncogene 1, non-receptor tyrosine kinase), ABL2 (c-abl oncogene 2, non-receptor tyrosine kinase) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [Supplementary Table 1].

Statistical analysis

The data obtained from the cytotoxicity assays (MTT assay and Neutral Red), genotoxicity, and kinetic cell proliferation real-time assay were compared by ANOVA followed by Tukey’s test ($p < 0.05$), performed using the GraphPad Prism 5 software (San Diego, CA). In RT-qPCR analyses of the relative values, gene expression and normalization for β-ACTIN were performed as described by Pfaffl et al. (2002) using the Pairwise Method Fixed Reallocation Randomization Test inserted into the Rest 2009 program (M. Pfaffl, Munich, Germany and QIAGEN, Hilden, Germany), with a statistical significance set at $p < 0.05$. Significant differences in expression were considered when above two times change was observed.

Results

Cytotoxicity assays

MTT assay

The cytotoxicity of both forms of β-glucans in concentrations of 5, 10, 15, 25, 50, 75 and 100 µg/mL were evaluated and the results obtained in the three replicates are shown as the percentage of cell viability (Figure 1a–c). All sulfated β-glucan concentrations decreased cell viability in a dose-dependent manner, except for the 5 µg/mL concentration, which did not show a reduction of viability after 72 h of treatment. As for the non-sulfated β-glucans, viability was decreased at concentrations of 10, 15, 25, 50, 75 and 100 µg/mL in a dose-dependent manner at all the tested timepoints. For all times tested, the concentration of 5 µg/mL showed no decrease in viability. Sulfated β-glucans were observed to have higher cytotoxicity when compared to non-sulfated β-glucans.
Neutral Red assay

The cytotoxicity results obtained at the three timepoints for both forms of β-glucans are presented in percentage of cell viability (Figure 1d–f). The sulfated β-glucans showed a significant reduction in cell viability at concentrations of 50 and 100 μg/mL, and the concentration of 5 μg/mL showed a significant increase in viability after 24 h of treatment. However, at 48 and 72 h, there was no difference in cell viability compared to the control. The non-sulfated β-glucans showed no interference in cell viability in the studied conditions.

Genotoxicity test – comet assay

All concentrations tested (5, 50 and 100 μg/mL) of both forms of β-glucans showed no significant DNA damage after 3 h of treatment, even with increased damage to the highest concentration of β-glucans sulfated, which was not significant. Figure 2 shows a graphical representation of the score values for both forms of β-glucans in all concentrations for this assay, as well as the values obtained in control and positive control [Supplementary Table 2].

Kinetics of cell proliferation in real time – RTCA

When treated with sulfated β-glucans, the MCF-7 cells showed a significant reduction in proliferation only when treated for approximately 72 h with a concentration of 100 μg/mL. Concentrations of 5 and 50 μg/mL, as well as all concentrations of non-sulfated β-glucans, did not show significant interference with cell proliferation during the treatment time (Figure 3).
Gene expression – RT-qPCR

After the treatment with 5 \( \mu \)g/mL for 6 h, the CCNA2, CCNB1, CCNE1, CCND1, p53, CHECK1 and CHECK2 genes showed no difference in expression compared to the control treatment for both types of \( \beta \)-glucans (Figure 4a). The GADD45A, GADD153, XPA, PCNA, ERCC5 and NFE2L2 genes did not have differential expression compared to the control treatment for both forms of \( \beta \)-glucans (Figure 4b). Regarding BAK, BAX, BCL-2, BCL-XL and CASP-9 genes, we observed a significant decrease in gene expression of BAK (3.44-fold) and BCL-XL (2.46-fold) in cells treated with sulfated \( \beta \)-glucans. The other genes showed no change, and there was no variation of expression for any of these in cells treated with non-sulfated \( \beta \)-glucans (Figure 4c). For the MAP2K6, ABL1, ABL2 and GAPDH genes, it was observed...
that MAP2K6 and ABL1 expression showed a significant reduction (4.4- and 9.8-fold, respectively) in cells treated with sulfated β-glucans, while only the ABL1 gene expression was significantly decreased (2.1-fold) in cells treated with non-sulfated β-glucan (Figure 4d). When the treatment was conducted with 10, 25 and 50 μg/mL for 6 h, and the treatments of 50 and 100 μg/mL for 24 h, all of the reference genes tested (GAPDH, β-ACTIN and RPL13a) showed variation of expression compared to the control, especially for the treatment of sulfated β-glucans. Therefore, it was not possible to use an expression study for these concentrations and times in the present study.

Discussion

Molecules can be chemically modified to improve the effects of interest, the sulfation being one of these modifications. The sulfation of biomolecules plays a key role in physiological functions in plants and animals (Ménard et al., 2004), and can bring new effects to be studied. In the present study, two different assays were used to evaluate the cytotoxicity of β-glucans, one being the MTT assay, which is based on the reduction of MTT in formazan crystals, a reflection of its potential for growth and survival. The second test, Neutral Red, which provides an estimate of the number of viable cells in a culture, is based on the number of cells with intact membranes and viability, based on the ability to incorporate and store the Neutral Red dye in their lysosomes (Repetto et al., 2008).

When both forms of β-glucans were studied using the MTT assay, we observed that viability was reduced in a dose-dependent manner. Furthermore, it was observed that sulfated β-glucans showed higher cytotoxicity when compared with non-sulfated β-glucans in this test. The sulfation enhances the solubility of β-glucan (Han et al., 2008) and our results indicate that the increased solubility also increases with the potential dose-dependent cytotoxic effect. In general, the results obtained in this study by MTT are similar to those reported by Zhang et al. (2006a). The authors investigated the cytotoxicity and cell proliferation by MTT assay in MCF-7 cells treated with β-glucan carboxymethylated, a soluble form of β-glucans, extracted mushroom Pleurotus tuber-regium. The researchers tested six concentrations (12.5, 25, 50, 100, 200 and 400 μg/mL), where it was also dose-dependent cytotoxicity and the IC50 obtained was approximately 200 μg/mL. A second study was conducted in the same year and was led by the same author (Zhang et al., 2006b), which evaluated fungal β-glucans extracted from Poria cocos using the same conditions. The authors obtained similar results, with dose-dependent cytotoxicity, but with an IC50 of 400 μg/mL. These results, compared with that of the present work, demonstrated that β-glucans from different sources may influence cytotoxicity at different levels using the MTT assay.

At the end of time treatments for the MTT cytotoxicity evaluation, it was noted that cells treated with different concentrations of both forms of β-glucans and at all treatment times were not microscopically different in the respective controls. Therefore, a second cytotoxicity test (Neutral Red) was used to assess cell viability. In this assay, we observed that concentrations of 50 and 100 μg/mL initially had a significant decrease in cell viability upon treatment with β-glucans sulfated for 24 h, but no difference after 48 and 72 h of treatment compared to controls. Kinetic Cell Proliferation Assay performed by the RTCA apparatus, which monitors the changes of number of cells in real time using the change in electrical impedance obtained by microelectrodes (Abassi et al., 2009) confirms the results of Neutral Red. In this assay, only the concentration of 100 μg/mL of sulfated β-glucans showed a slight, but significant, decrease in cell proliferation after approximately 72 h of treatment. This outcome demonstrates that the interference found in the MTT assay, in both forms of β-glucans, does not decrease the cell number. The reduction of tetrazolium dye (MTT) is NADH and NAD(P)H-dependent, and therefore, differs depending on the metabolic activity. Viable cells at rest, but metabolically low, reduce little MTT. Agents that decrease the mitochondrial metabolic activity can therefore reduce the tetrazolium dye to the formazan reduction without necessarily causing cell death (Berridge et al., 2005). It is also known that MTT is reduced in other cellular compartments, such as the cytoplasm and regions of the plasma membrane (Bernas & Dobrucki, 2002).

With respect to genotoxicity evaluated in the comet assay, both forms of β-glucans did not separately induce DNA damage. The lack of genotoxicity observed corroborates the results found by Gutieres et al. (2005) and Angeli et al. (2009). Gutieres et al. (2005) studied the extract of A. blazei in lung cells of Chinese hamsters, in which 3 mg extract residues were diluted in 400 μL of dimethyl sulfoxide and 4600 μL of PBS, and a final concentration 60 μL/mL of this solution was applied to the comet assay. In the present study, the authors did not observe any genotoxic effects, and there was a significant reduction of DNA damage caused by methyl methanesulfonate – MMS. Angeli et al. (2009) demonstrated that β-glucans extracted from A. blazei did not exert genotoxic or mutagenic effects at concentrations of 7, 21 and 63 μg/mL, and that there was also a dose-dependent protective effect against DNA damage caused by benzo[a]pyrene (20 μM) in a human hepatoma cell line (HepG2) by a comet assay. Thus, the authors suggest that, by a binding action with benzo[a]pyrene, the β-glucans captures free radicals produced during its activation and β-glucans that modulate cellular metabolism.

The lack of changes in gene expression in cycle, repair and cellular stress pathways are consistent with the results of the RTCA and Neutral Red assays. There was a reduction in the expression of BAK and BCL-XL genes; however, this expression profile found does not indicate apoptosis. BAK and BCL-XL genes, together with BAX, BCL-2 and others, are members of the Bcl-2 family. BAX and BAK belong to the group encoding pro-apoptotic proteins. BCL-2 and BCL-XL encodes proteins that belong to the group of anti-apoptotic proteins (Someya et al., 2009). The greatest expression reduction of BAK with respect to BCL-XL indicates a deflection of pro and anti-apoptotic proteins in which the anti-apoptotic is protrude. However, as the RTCA showed no difference in cell proliferation and Neutral Red assay showed no cytotoxicity, this demonstrates that there is cell death. These results corroborate those obtained by Silva et al. (2013), where the authors studied the expression of CASP-9 in HepG2 cells treated for 6 h with non-sulfated β-glucans (50 μg/mL).
extracted from *A. blazei*, and also indicated that expression of β-glucans did not influence apoptotic cells. Additionally, some components of the apoptotic pathway have been shown to influence cell cycle or modulate the proliferation in a very general way, or control the specific phases and the metabolism. The *BCL-2* and *BCL-XL* genes function as negative regulators of the cell cycle. *BCL-2*, by inhibiting CDK2, delays the G1/S transition, a similar role as has been assigned to the *BCL- XL*, *BAD*–and *BAX*-encoded proteins can stimulate cell cycle progression. This cycle regulatory role for members of the *BCL-2* family is mediated by a specific domain of the protein does not participate in the regulation of apoptosis. In addition, the BCL-2 family of proteins, which are involved in the regulation of mitochondrial morphology and dynamics, have important implications not only in apoptosis, but also for the bioenergetic functions of mitochondria in various stress situations (Galluzzi et al., 2012). Such evidence reinforces the supposition that the results found using an MTT assay in the present study do not represent cell death, but rather effects on energy metabolism.

The MAP2K6 gene encodes a member of the protein family of dual-specificity kinase, the MAP kinase kinase 6 (MAPKK6). MAPKKK 6, also known as extracellular signal regulated kinases, acts as an integration point used for various biochemical signals. This protein activates p38 MAP kinase by phosphorylation in response to environmental stress or inflammatory cytokines. Thus, the MAP2K6 gene is involved in many cellular processes, such as cell cycle arrest induced by stress, transcriptional activation and apoptosis (Kaur et al., 2010). The reduction of expression found in the present work by treatment with sulfated β-glucans indicates that under these treatment conditions, the cells did not need to stop the cell cycle, as observed in the test RTCA proliferation. Furthermore, the cells were not under stress as observed in the expression of the gene *NFE2 L2*, and there was no need to enter apoptosis. There was a decrease in the expression of the ABL1 gene for both treatments. The product of this gene is located in various cell sites, making it involved in many cellular processes, such as growth regulation, cell survival, oxidative stress, cell damage responses and migration (Szczylik et al., 1991). The low expression of this gene suggests that cells, when treated with β-glucans (primarily the sulfated version), modulate to reduce damage and stress.

For gene expression studies in different biological conditions, it is necessary to use reference genes to address any changes in the amount of mRNA, and to minimize deviations due to differences in efficiency in reverse transcription, being that expressions of reference genes should not be influenced by experimental conditions. However, eventually, the levels of the reference gene expression commonly used can be affected by biological conditions of the experiments (Dupasquier et al., 2014). In the present study, there was no difference in the expression of the *GAPDH* gene when treatments were performed with both forms of β-glucans at a concentration of 5 μg/mL. However, when used at concentrations of 10 to 100 μg/mL for 6h of treatment or longer, the β-glucans induced a reduction of expression of this gene (data not shown). The *GAPDH* gene is considered to be a constitutive gene, and its product is typically identified as an enzyme involved in glycolysis processes. However, the product has been linked to other important cellular functions, such as membrane transport between the endoplasmic reticulum and Golgi complex and the regulation of mRNA stability, among others, which are due to the ability of GAPDH to bind macromolecules in different cells (Colell et al., 2009). The mechanisms used by β-glucans to interfere in the expression of this gene have not been identified, but it has been reported that β-glucans can interfere with cellular metabolism (Jurczynska et al., 2012). Moreover, in relation to treatments with higher concentrations, β-actin and RPL13a genes also showed reduced expression after treatments with both forms of β-glucans. The *β-ACTIN* gene is used to normalize RT-qPCR reactions; the product of this gene is involved in many cellular functions, such as cell division, migration and transcriptional regulation, among others (Perrin & Ervasti, 2010). The *RPL13a* gene is also considered to be a normalizing gene and has been validated for RT-qPCR mesenchymal stromal cells. *RPL13a* encodes a protein family member ribosomal L13P, which is a component of the 60 S subunit of the ribosome (Curtis et al., 2010). Thus, the reduction of expression of these genes for treatment with concentrations of 10 to 100 μg/mL, both forms of β-glucans, prevented its use for normalizing and these concentrations to study expression. In conclusion, the results indicate that sulfated β-glucans have a greater power of action relative to non-sulfated β-glucans, providing preliminary insight for the potentiation effects of different forms of glucans.

Declaration of interest

The authors report no declarations of interest.

References

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.

Angeli JP, Ribeiro LR, Gonzaga ML, et al. (2006). Protective effects of beta-glucan extracted from *Agaricus brasiliensis* against chemically induced DNA damage in human lymphocytes. Cell Biol Toxicol 22:285–91.

Babayar S, Lee DH, Kim HW. (2012). Immunomodulation of fungal β-Glucan in host defense signaling by Dectin-1. Biomol Ther 20:433–45.

Bellini MF, Angeli JPF, Matuo R, et al. (2006). Antigenotoxicity of *Agaricus blazei* mushroom organic and aqueous extracts in chromosomal aberration and cytokinesis block micronucleus assays in CHO-k1 and HTC cells. Toxicol in Vitro 20:355–60.

Bemis T, Dobrecki J. (2002). Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 47:236–42.

Berridge MV, Herst PM, Tan AS. (2005). Tetrazolium dyes as tools in study expression. In conclusion, the results indicate that sulfated β-glucans have a greater power of action relative to non-sulfated β-glucans, providing preliminary insight for the potentiation effects of different forms of glucans.

Declaration of interest

The authors report no declarations of interest.

References

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.

Angeli JP, Ribeiro LR, Gonzaga ML, et al. (2006). Protective effects of beta-glucan extracted from *Agaricus brasiliensis* against chemically induced DNA damage in human lymphocytes. Cell Biol Toxicol 22:285–91.

Babayar S, Lee DH, Kim HW. (2012). Immunomodulation of fungal β-Glucan in host defense signaling by Dectin-1. Biomol Ther 20:433–45.

Bellini MF, Angeli JPF, Matuo R, et al. (2006). Antigenotoxicity of *Agaricus blazei* mushroom organic and aqueous extracts in chromosomal aberration and cytokinesis block micronucleus assays in CHO-k1 and HTC cells. Toxicol in Vitro 20:355–60.

Bemis T, Dobrecki J. (2002). Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 47:236–42.

Berridge MV, Herst PM, Tan AS. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev 11:127–52.

Chang YJ, Lee S, Yoo MA, Lee HG. (2006). Structural and biological characterization of sulfated-derivatized oat β-glucan. J Agric Food Chem 54:3815–18.

Chorvatovicˇova´ D, Machova´ E, Sˇandula J. (1998). Ultrasonication: the characterization of sulfated-derivatized oat β-glucan. J Agric Food Chem 54:3815–18.

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.

References

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.

References

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.

References

Abassi YA, Xi B, Zhang W, et al. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. Chem Biol 16:712–23.

Angeli JPF, Ribeiro LR, Bellini MF, Mantovani MS. (2009). β-Glucan extracted from the medicinal mushroom *Agaricus blazei* prevents the genotoxic effects of benzo[a]pyrene in the human hepatoma cell line HepG2. Arch Toxicol 83:81–6.
Curtis KM, Gomez LA, Rios C, et al. (2010). EF1α and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells. BMC Mol Biol 11:61.

Dupasquier S, Delmarcelle AS, Marbaix E, et al. (2014). Validation of housekeeping gene and impact on normalized gene expression in clear cell renal cell carcinoma: critical reassessment of YBX3/ZONAB/CSDA expression. BMC Mol Biol 15:9.

Galluzzi L, Kepp O, Trojl-Hansen C, Kroemer G. (2012). Non-apoptotic functions of apoptosis-regulatory proteins. EMBO Rep 13:322–30.

Gameiro PH, Nascimento JS, Rocha BH, et al. (2013). Antimutagenic and antigenotoxic effects of organic extracts of mushroom Agaricus blazei Murrill on V79 cells. Genet Mol Biol 28:458–63.

Han MD, Han YS, Hyun SH, Shin HW. (2008). Solubilization of water-insoluble β-glucan isolated from Ganoderma lucidum. J Environ Biol 29:237–42.

Hong F, Yan J, Baran JT, Allendorf DJ, et al. (2004). Mechanism by which orally administered β-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. J Immunol 173:797–806.

Jurczyn´ska E, Saczko J, Kulbacka J, et al. (2012). β-glucan as a housekeeping gene and impact on normalized gene expression in clear cell renal cell carcinoma: critical reassessment of YBX3/ZONAB/CSDA expression. BMC Mol Biol 15:9.

Perrin BJ, Ervasti JM. (2010). The actin gene family: function follows isoform. Cytoskeleton (Hoboken) 67:630–4.

Supplementary material available online
Supplementary Tables 1 and 2

β-Glucans sulfated and non-sulfated 679