Ultrastructural & Analytical Methods in Life Sciences

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Beta-glucan, from mushroom *Pleurotus ostreatus*, induces apoptosis and increases B16F10 cell adhesion and spreading

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The most aggressive form of skin cancer among several cancer types is the malignant melanoma [1]. Its occurrence is one of the most common in the world population [2]. Natural bioactive products discovery is of particular interest to cancer treatment, specially polysaccharides and compounds obtained from plants [3, 4, 5, 6]. *In vitro* assays showed yeast polysaccharides action on B16F10. Cell adhesion was over 75 to 80%, of cell invasion 89-99%, and there was an 80%reduction in metastases, in a dose-dependent manner [5]. Others have demonstrated that treatment with polysaccharides induced cytotoxicity [7], reduced solid tumor in murine models when treated *in vivo* [8]. The treatment with polysaccharide of *Pleurotus ostreatus* mushroom in Sarcoma 180 reduced tumor cells in 90% [9]. Literature suggests that these compounds antitumor mechanism of action involves cellular apoptosis [8].

The aim of this study was to assess the biological potential of -glucan (P2) polysaccharide isolated [10] from *Pleurotus ostreatus var. Florida* mushroom. “In vitro” assays with B16F10 cell line (BCRJ) (murine melanoma) were treated with different concentrations (1, 10, 50, 100, 250 g/mL) and times (24, 48 and 72 h).

MTT method (Fig. 1A), which evaluates cellular cytotoxicity, showed that 1 and 10 g/mL of P2 after 72 hours was able to induce cell cytotoxicity. Neutral red method (NR) (Fig. 1B), which evaluates cell viability, demonstrated that this polysaccharide decreased cell viability, only with 250 g/mL after 24 h of exposure. . After 48 h of exposure, a significant increase in viability was noted, with 1, 50 and 100 g/mL of P2. Also, we have demonstrated that P2 induced cell death mechanisms, using Anexin V/ 7AAD. There was an increase in apoptosis, after 48 hours of treatment with the polysaccharide and an even more significant increase after 72 h (Fig. 1C). Significant difference in necrosis was seen after 48 hours of treatment (Fig. 1D) with decreased cell viability (Fig.1E). Adhesion assay showed that B16F10 cell line tends to adhere more to Fibronectin (FN) when compared to other examined substrates (Fig. 2A, B, C, D). We have also observed that this polysaccharide induced adhesion to Laminin (LN) compared to its control, after 48h of exposure. However, adhesion to Fibronectin, was reduced only after 72 h of treatment, compared to its control (Fig. 2E, F, G, H, I, J). Cells were more sprawled over Vitronectin (VIT), showing increased cell body after 24, 48 and 72 h (Fig. 1F), significantly. No ultra-structural changes were observed in cells treated with polysaccharide (P2) by Transmission Electron Microscopy (TEM) (Fig 2K, L, M, N) and Scanning Electron Microscopy (SEM) (Fig. 2O, P, Q, R). The cells remained without contact inhibition, stacking on one another, which is a typical characteristic of tumorigenic cells, and with similar morphology.

In summary, -glucan treatment on B16F10 cells: was not cytotoxic and did not induce loss of viability, only in a few specific concentrations and times; induced cell death mechanisms, both apoptosis and necrosis; increased cell adhesion and spreading over specific substrates; did not induce ultrastructural cell changes in the concentrations and times analyzed. Interestingly, the increased cell adhesion and spreading, with a possible negative modulation of cell migration and invasion dynamics, may suggest that these cells may become more stationary. Thus, "in vitro" and "in vivo" assays showing efficacy in tumorigenic models, especially lung colonization model and solid tumor growth, employing B16F10 lineage in C57BL/6 Black mice should be performed next.

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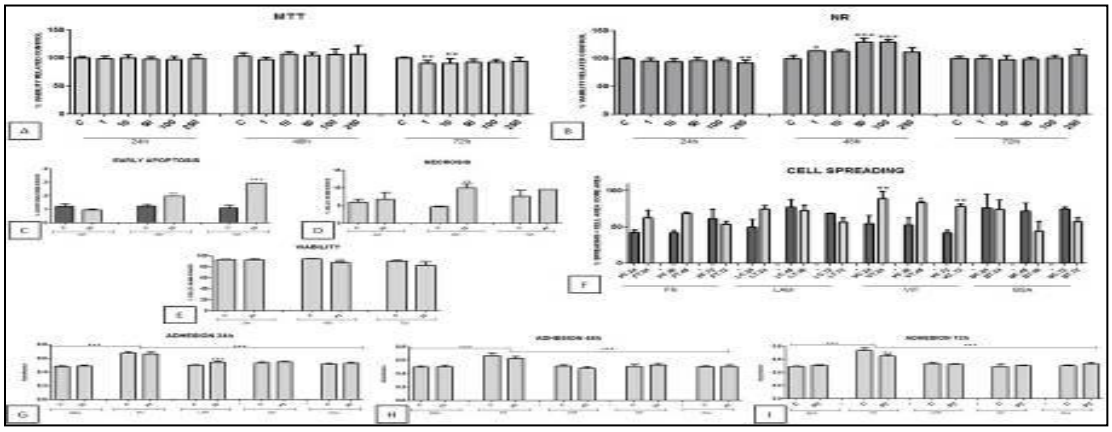

Figure 1. Statistical Results. [A] Cytotoxicity (MTT) and [B] viability (NR) assays B16F10 cells were exposed to P2 polysaccharide, in different concentrations (1, 10, 50, 100, 250 □g/mL), for a period of 24, 48 and 72h. These assays were performed in sextuplicata, in three independent experiments. [C] Early apoptosis; [D] Necrosis; [E] Viability; B16F10 cells were exposed to 100 □g/mL of polysaccharide P2, , for a period of 24, 48 and 72 h. Ten thousand events were analyzed by flow cytometry. These assays were performed in triplicate, in three independent experiments. [F] Spreading Assay. Evaluation of cell spreading degree by analyzing light microscopy images. B16F10 cells were subjected to adhesion assay, and images were acquired by the end (10 images for each replicate). ImageJ software was used to calculate cell area relative to nucleus area. [G] Adhesion Assay 24h; [H] Adhesion Assay 48h; [I] Adhesion Assay 72h. B16F10 cells were exposed to 100 □g/mL of P2, for a period of 24, 48 and 72 h. Subsequently, cells were subjected to adhesion on the following substrates: bovine albumin (BSA), Fibronectin (FN), Laminin (LAM), Vitronectin (VIT) and the plastic plate (PLA); for 2 hours. These assays were performed in triplicate, three times independents. Statistical analysis was assessed by one-way ANOVA and TUKEY post-test (* p<0,05, ** p<0,01 e *** p<0,001). C=control; T=treated.

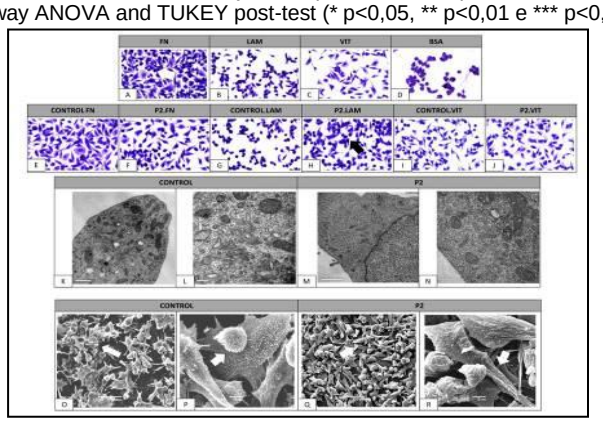

Figure 2. Images of Results. [A, B, C, D] Adhesion Assay Images between groups. The images in the first row represent the difference between groups (the same for all treatment times and concentrations). [E, F, G, H, I, J] Adhesion Assay Images between control and treatment. The second row shows the control with their respective treatment corroborating with statistic data viewed in adhesion assay above. ( ) Cells prefer the FN substrate; ( ) Increase adhesion on LN substrate. [K, L, M, N] Transmission Electron Microscopy (TEM). B16F10 cells were exposed to 1, 10 and 100 □g/mL of polysaccharide (P2), for a period of 24, 48 and 72 h. *The results are the same for all. The images A and C, show 1 □m, magnification 20.000X, and B, D show 0,5 □m, magnification 80.000X. [O, P, Q, R]* Scanning Electron Microscopy (SEM). B16F10 cells is the same morphology ( ) were exposed to 1, 10 and 100 □g/mL of polysaccharide (P2), for a period of 24, 48 and 72 h. *The results are the same for all. The images A and C, show magnification 500X, and B, D magnification 3.000X.*