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Thermal treatment of bentonite reduces aflatoxin b_1 adsorption and affects stem cell death☆

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ABSTRACT

Bentonites are clays that highly adsorb aflatoxin B_1 (AFB_1) and, therefore, protect human and animal cells from damage. We have recently demonstrated that bentonite protects the neural crest (NC) stem cells from the toxicity of AFB_1. Its protective effects are due to the physico-chemical properties and chemical composition altered by heat treatment. The aim of this study is to prepare and characterize the natural and thermal treatments (125 to 1000 °C) of bentonite from Criciúma, Santa Catarina, Brazil and to investigate their effects in the AFB_1 adsorption and in NC cell viability after challenging with AFB_1. The displacement of water and mineralogical phases transformations were observed after the thermal treatments. Kaolinite disappeared at 500 °C and muscovite and montmorillonite at 1000 °C. Slight changes in morphology, chemical composition, and density of bentonite were observed. The adsorptive capacity of the bentonite particles progressively reduced with the increase in temperature. The observed alterations in the structure of bentonite suggest that the heat treatments influence its interlayer distance and also its adsorptive capacity. Therefore, bentonite, even after the thermal treatment (125 to 1000 °C), is able to increase the viability of NC stem cells previously treated with AFB_1. Our results demonstrate the effectiveness of bentonite in preventing the toxic effects of AFB_1.

1. Introduction

Bentonite particles are efficient at promoting growth (acting as adsorbents of toxins), improving health (reducing the harmful effects of drugs), and promoting well-being (active principles in cosmetics and pharmaceuticals) [1]. Furthermore, this material can protect human and animal cells from several kinds of damage [2–7]. Furthermore, it has been proved to be efficient as a sequestering agent for aflatoxins [3,8].

Aflatoxin B_1 (AFB_1) is the most potent natural carcinogen known and is usually the major aflatoxin produced by toxigenic strains [9,10]. This compound induced cytotoxicity [11] and it is responsible for inflicting damage on DNA, mutation, abortion, birth deformity, suppression of immune system, and phytotoxic reactions [12,13]. It can influence the very early stages of mammalian embryonic development, affecting the survival and proliferation of neural crest (NC) stem cells, and consequently the formation of the peripheral nervous system [8,14]. Recently, we have also reported that AFB_1 cell toxicity can be reduced by bentonite particles [8].

The physic-chemical properties of the bentonite particles may be modified by various treatments, including thermal treatment [15–17]. Changes in the clay by dehydration, dehydroxylation, recrystallization, fracture, and loss of crystal structure can occur as a result of increasing temperature [18–20]. Furthermore, the thermal treatment leads to a delamination of the aluminosilicate layers, which may enhance the available space between the silicate layers [21] or decrease the number of exchange sites [22]. These transformations are dependent on the temperature treatment [21], mineralogy, and crystal structures [23], effects that differ according to the mineralogy composition of the clay.

Thermally treated clays can provide a simple possibility to change the adsorption properties [22], decreasing adsorption in some cases [16,22,24] or increasing in others [15].

The purpose of this study is to prepare and characterize natural and thermal treatment bentonite and to investigate their effects on the adsorption of AFB_1, using NC stem cell cultures submitted in damage caused by AFB_1 as a model to study the biological effects of both treatments. By using an in vitro system of AFB_1 adsorption and cell culture of quail NC, we demonstrated for the first time that calcined bentonite reduces the toxicity of AFB_1 in the culture medium, decreasing the adsorption and protective effect after calcination at 1000 °C. Perhaps

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thermal treatment was able to protect cells from AFB_1 damage. However, this treatment was not able to increase AFB_1 adsorption. These findings contribute to a better understanding of the effectiveness of bentonite in controlling the toxic effects of AFB_1 and offers new perspectives for understanding the relevance of bentonite use in human and animal therapies.

2. Material and methods

2.1. Drugs

Bentonite samples were collected in Criciúma, Santa Catarina State, in the south of Brazil. The samples were washed with distilled water to remove any impurities and were dried in an electric oven at 60 °C for 8 h. Bentonite was thermally treated and the samples were subjected to the calcination process in a muffle (Jung, N1100) furnace at 125, 250, 500, 750 and 1000 °C for 4 h. Then, the bentonite samples were kept in a stock solution of 40 mg/mL diluted in dimethyl sulfoxide (DMSO) at −20 °C, for cell assay, or in water for adsorption study. AFB_1 (Sigma) was kept in a stock solution of 500 μM and, for the tests, it was diluted in DMSO or acetonitrile at 30 μM for cell assay and adsorption study, respectively.

2.2. Bentonite characterization

The chemical composition of bentonite was determined by energy dispersive X-ray spectroscopy (EDX) and the surface morphology of bentonite was investigated using a JEOL JSM - 6390 LV scanning electron microscope (SEM). Bentonite's structural composition was analyzed by infrared absorption assay (FTIR) and X-ray diffraction (XRD). FTIR of bentonite was obtained using a Shimadzu Spectrum, IR Prestige-21. FTIR spectra were taken in the range from 4000 to 400 cm^{-1} in the transmission mode in KBr pellets. The XRD analysis of the bentonite samples was made with the accelerating voltage of 40 kV and 30 mA, Cu K_α ($\lambda = 0.154178$ nm) radiation ranging from 0 to 20° and 2θ scan rate of 0.05°/s (PanAnalytical X'Pert PRO Multi Purpose). Then, the interlayer spacing of each sample was calculated using Bragg's law:

$$n\lambda = 2d \sin\theta \quad (1)$$

where n is the path differences between the reflected waves which equal an integral number of wavelengths (λ) and d is the interlayer spacing (nm), θ the angle of diffraction (°), λ the wavelength (nm). The density was measured using Archimedes' principle. The loss in mass of bentonite was determined by muffle drying at different temperatures (125, 250, 500, 750 and 1000 °C) during 4 h. Loss in mass is defined as the ratio between the mass of water and other impurities and the mass of dry solid expressed as a percentage.

2.3. Aflatoxin B_1 adsorption study

Adsorption of AFB_1 molecules was assessed by UV/visible spectrophotometry [8]. To that end, 50 μL of 0.6 mg/mL bentonite was added to 1 mL of AFB_1-solution (30 μM). The samples were stored overnight and then centrifuged (80-2B Centribio) at 2000 rpm for 57 min. The amount of adsorbed AFB_1 was determined in the supernatant with UV/visible spectrophotometry at 365 nm.

For high-performance liquid chromatography (HPLC) assay, different amounts of bentonite samples were added to 200 μL of AFB_1-solution (30 μM) where a 0.2, 0.4 or 0.6 mg/mL concentration was obtained. Samples were stored overnight and then centrifuged (1500 CFN-II Vision) at 4000 rpm for 30 min. The amount of adsorbed AFB_1 was determined in the supernatant with HPLC analysis. An aliquot of the original AFB_1 test solution was used as the HPLC standard. HPLC analyses were performed on a Waters E2795 AllianceBio Separation Module composed of a quaternary pump with a refrigerated autosampler coupled to a Waters 2475 fluorescence detector (λex = 365 nm; λem = 430 nm). The column was a Polaris C18A Metachem, 5 μm particle size, 150 mm × 4.6 mm, guard column was an Alltech 20 × 2 mm. The mobile phase water: acetonitrile (50:50) was pumped at a flow rate of 0.7 mL/min. Chromatograms were obtained and integrated with the Empower® 2 software (Waters Co., Milford, USA). Percent AFB_1 bound by the bentonites was calculated from the difference between the initial and final AFB_1 concentration in the aqueous supernatant after equilibrium.

2.4. Quail NC cell cultures

Quail NC cell cultures were performed and characterized as previously described by Trentin et al. [25] and Nones et al. [14,26]. Briefly, neural tubes obtained from quail embryos (18–25 somite stage) were dissected at the trunk level and plated in plastic culture dishes (Corning). After 24 h, emigrated NC cells were harvested for secondary plating (400 cells per well of a 96-well plate). Cultures were maintained for an additional 4 days in a medium containing: α-minimum essential medium (α-MEM; Gibco) enriched with 10% fetal bovine serum (Cultlab), 2% chicken embryonic extract, penicillin (200 U/mL) and streptomycin (10 μg/mL) (all from Sigma). Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. The medium was changed every 2 days. Each culture was incubated with DMSO (control group) or 0.6 mg/mL of natural or thermal treatment (125 to 1000 °C) bentonite. In order to determine the role of bentonite on cell survival, AFB_1 (30 μM), was added – alone or concomitantly – to cells treated with a specific concentration (0.6 mg/mL) of bentonite.

2.5. Cell viability

The viability of NC cells was determined by trypan blue [27]. NC cells were treated with natural or thermal treatment at 125, 250, 500, 750 and 1000 °C bentonite (0.6 mg/mL), AFB_1 (30 μM) alone or in combination, for 4 days, and subsequently collected by centrifugation. After washing in PBS, cells were stained with 0.4% trypan blue solution at room temperature for 3 min, and cells were then counted using a hemocytometer and a light microscope. At least one thousand cells were observed and the percentages of unstained (viable) and stained (nonviable) cells were determined.

2.6. Cell death assay

Cell death was quantified by assessing the characteristic nuclear changes (e.g., chromatin condensation and nuclear fragmentation) using DAPI nuclear binding dye [14] and fluorescence microscopy. Briefly, cells treated were fixed with 4% paraformaldehyde and washed in PBS. Next, cell nuclei were stained with DAPI and visualized/analyzed under an epifluorescent microscope (Olympus IX71).

2.7. Quantitative and statistical analysis

Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using GraphPad Prism 4.0 software. $P < 0.05$ was considered statistically significant. The experiments were performed in triplicate and each result represents the mean of at least three independent experiments.

3. Results

3.1. Morphology and chemical composition of bentonites

In order to know the elemental composition of the bentonite samples (natural and thermally treated) and understand the effects of calcination, elemental analysis was performed using scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis.