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Organophilic treatments of bentonite increase the adsorption of aflatoxin B_1 and protect stem cells against cellular damage

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ABSTRACT

Bentonite clays exhibit high adsorptive capacity for contaminants, including aflatoxin B_1 (AFB_1), a mycotoxin responsible for causing severe toxicity in several species including pigs, poultry and man. Organophilic treatments is known to increase the adsorption capacity of bentonites, and the primary aim of this study was to evaluate the ability of Brazilian bentonite and two organic salts - benzalkonium chloride (BAC) and cetyltrimethylammonium bromide (CTAB) to adsorb AFB_1. For this end, 2^2 factorial designs were used in order to analyze if BAC or CTAB was able to increase AFB_1 adsorption when submitted in different temperature and concentration. Both BAC and CTAB treatment (at 30 °C and 2% of salt concentration) were found to increase the adsorption of AFB_1 significantly compared with untreated bentonite. After organophilic bentonite treatments with BAC or CTAB, a vibration of C—H stretch (2850 and 2920 cm^{-1}) were detected. A frequency of the Si—O stretch (1020 and 1090 cm^{-1}) was changed by intercalation of organic cation. Furthermore, the interlayer spacing of bentonite increases to 1.23 nm (d_{001} reflection at $2\theta = 7.16$) and 1.22 (d_{001} reflection at $2\theta = 7.22$) after the addition of BAC and CTAB, respectively. Another aim of the study was to observe the effects of these two bentonite salts in neural crest stem cell cultures. The two materials that were created by organophilic treatments were not found to be toxic to stem cells. Furthermore the results indicate that the two materials tested may protect the neural crest stem cells against damage caused by AFB_1.

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1. Introduction

Bentonites are argillaceous materials in clay, which contain a high rate montmorillonite (2:1 type aluminosilicate), that can be effectively employed as adsorbents for many pollutants and contaminants [1,2]. Previously researches have demonstrated the effectiveness of this material against toxicity caused by mycotoxins [3–5], due to the ability of bentonites to bind these toxic substances - *e.g.* aflatoxin B_1 (AFB_1) [4,6–9].

AFB_1, which is produced by different fungal species [10,11] is highly toxic to humans [7,12,13] as well as to several animal species such as pigs [14] and poultry [15]. AFB_1 has been reported to cause serious biochemical and structural alterations in different organs, including liver, lungs, kidneys and heart [16–18], as well as it is carcinogenic [19]. In the early stages of mammalian embryonic development, AFB_1 may disturb nerve cell functions and cause damage in the central nervous system [4,20–23].

Bentonite given as a feed additive reduces the impact of mycotoxins by adsorbing mycotoxins in the gastrointestinal tract and thus preventing absorption of the toxin [9,24–26]. The efficacy of bentonite to adsorb aflatoxins and other organic molecules depends on the crystal structure, chemical properties of the molecules and the surface properties of the clay [8,27,28]. The adsorption capacity of bentonite can be increased by intercalation of organic molecules into their interlayer surface, including quaternary ammonium compounds [29–32].

The quaternary ammonium compounds (QAC) are cationic surfactants, with the alkyl chain of 16–20 carbon atoms, and the most used organic source to prepare organoclays [31,33,34]. The structure of QAC contains at least one hydrophobic hydrocarbon chain linked to a positive charged nitrogen atom [R_4N^+], and other alkyl groups which are mostly short-chained substituents such as methyl or benzyl groups [35].

Some studies have reported that bentonite modified by QACs, such as benzalkonium chloride (BAC) and cetyltrimethylammo-

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nium bromide (CTAB) show an increase adsorption capacity for textile dye [28,36], aromatic compounds [37] and petroleum-derived fuels [38]. This increased capacity for adsorption of organic compounds results from the conversion of hydrophilic to hydrophobic characteristics of clay by incorporation of surfactants [2,39–42].

It has been reported that organo-modification of bentonite clay can increase the adsorption capacity for mycotoxins [6,43]. There is however no information available regarding the effects of this treatment on Brazilian bentonites and its ability to increase the binding capacity for AFB_1.

The knowledge on the effects of bentonites or surfactants on cells or cell cultures is scarce [4,44,45]. Moreover, there are to our knowledge no data reported from *in vitro* studies of the effects of organo-modified bentonites on stem cells.

This paper is the first report on the adsorption capacity for AFB_1 of Brazilian bentonite treated with two organic salts (BAC and CTAB). Furthermore it reports the result from toxicity studies of organic bentonites (BAC e CTAB) alone and in combination with AFB_1 using neural crest stem cell culture as a study model.

2. Material and methods

2.1. Materials

Bentonite samples, characterized by Nones et al. [4,8], were collected in the city of Criciúma, located in the State of Santa Catarina, 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, as described by Nones et al. [4]. 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 methanol 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 water at 30 μM for cell assay and adsorption study, respectively.

2.2. Preparation of organobentonites

The organobentonites were synthesized according to the following process: 5 g of bentonite was first added to 50 mL of distilled water at 30 or 60 °C and stirred until they were thoroughly dispersed. Desired amounts of benzalkonium chloride (BAC) and cetyltrimethylammonium bromide (CTAB) were mixed in 50 mL distilled water at 30 or 60 °C for 30 min. Then the modifying agents were added to the bentonite suspension under vigorous stirring. The mixed suspensions were stirred at 30 or 60 °C for 4 h and then stored at room temperature (around 25 °C) overnight. After that, the resulting products were washed with distilled water and dried at 80 °C. The added amounts of BAC and CTAB were 2 or 6% of the bentonite's weight.

2.3. Factorial design

In this study, 2^2 factorial design was used in order to analyze if BAC or CTAB was able to increase AFB_1 adsorption when submitted in different temperature and concentration. The adsorption of AFB_1 was determined as average of three parallel experiments analyzed. The details about factorial design with the low (−1) and high (+1) levels are presented in Table 1. The results were evaluated using the program Statistica (version 8.0), when the effects (temperature and concentration) and interactions between them were evaluated.

2.4. Bentonite characterization

The surface morphology of bentonite was investigated using a JEOLJSM-6390LV scanningelectron 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 Agilent Technologies Spectrum – Cary 600 Series. 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 (λ = 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).

Table 1
Experiment design for organic salts intercalation in natural bentonite.

Experiment	Sample	Reaction temperature (°C)	Concentration of salt (%)
Benzalkonium chloride (BAC)			
1	BAC302	30 (−1)	2 (−1)
2	BAC306	30 (−1)	6 (+1)
3	BAC602	60 (+1)	2 (−1)
4	BAC606	60 (+1)	6 (+1)
Cetyltrimethylammonium bromide (CTAB)			
1	CTAB302	30 (−1)	2 (−1)
2	CTAB306	30 (−1)	6 (+1)
3	CTAB602	60 (+1)	2 (−1)
4	CTAB606	60 (+1)	6 (+1)

2.5. Aflatoxin B_1 adsorption

Adsorption of AFB_1 molecules was assessed by high-performance liquid chromatography (HPLC) using a modified method described by Nones et al. [8]. To that end, bentonite samples were added to 200 μL of AFB_1-solution (30 μM) where a 0.6 mg/mL concentration was obtained. Samples were stored overnight at 25 °C, without light control, 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 auto sampler coupled to a Waters 2475 fluorescence detector (λex = 365 nm; λem = 430 nm). The column was a Synergi Hydro-RP, 4 μm particle size, 150 mm × 2.0 mm, protected by a security guard column (both Phenomenex, Torrance, USA). The mobile phase water: acetonitrile (50:50) was pumped at a flow rate of 0.2 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.

2.6. Quail NC cell cultures

Quail NC cell cultures were performed and characterized as previously described by Trentin et al. [46] and Nones et al. [20,47]. 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, peni-

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