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Effects of the subchronic exposure to an organomodified clay mineral for food packaging applications on Wistar rats

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ABSTRACT

Organomodified clay minerals have many applications, but their successful technological development should be linked to their safety. There are in vitro toxicological studies that have shown adverse effects induced by organomodified clay minerals in human cell lines. However, in vivo toxicity reports are scarce, particularly those using the oral pathway. The aim of this work was to evaluate the toxicity of an organomodified clay mineral, Clay1, developed from montmorillonite (Mt) and using hexadecyltrimethylammonium bromide as modifier. A repeated dose 90-day oral toxicity study was performed in Wistar rats exposed to 40 mg/kg/day Clay1. The morphological study of the main organ tissues by optic and electronic microscopy did not reveal any adverse effect. Also, blood clinical biochemistry parameters, the reduced/oxidized glutathione (GSH/GSSG) ratio, and interleukin-6 leakage in serum did not show any significant alterations. These results suggest that Clay1 does not cause remarkable toxic effects at the conditions tested.

1. Introduction

Natural clay minerals are widely used in catalysis, as adsorbents, in clay polymer nanocomposites (CPN), as antibacterial materials, nuclear waste storage, pesticide carriers, etc. (Liu, 2007). Particularly food packaging industry has mainly focused its attention on layered inorganic solids, such as clay minerals, due to their availability, low cost, significant enhancements and relative simple processability (De Azeredo, 2009; Hatzigrigoriou et al., 2011). As a result, CPN were the first nanocomposites to be developed and introduced to the market as enhanced materials for the food-packaging sector (Hatzigrigoriou et al., 2011.). The most widely studied clay mineral as filler is montmorillonite (Mt) (Weiss et al., 2006).

The main advantage of using clay minerals as fillers is a marked increase in the barrier of the polymer material to gas and water (Silvestre et al., 2011). Other authors also mention the improvements in mechanical, thermal, optical and rheological properties of the CPN (De Azeredo, 2009; Haztigrigoriou et al., 2011), thus increasing the product shelf life.

One limitation of using clay minerals as nanofillers is the incompatibility between the hydrophilic clay mineral and a hydrophobic polymer (Elmore and Andersen, 2003; Zeng et al., 2005). Therefore, chemical modification of clay minerals is an important step to achieve CPN. In this sense by ion exchange with long-chain organic cations, clay minerals become hydrophobic and thereby compatible with polymers. Such modified clay minerals are referred to as organoclays (OC) (Sharma et al., 2010).

Due to the aforementioned wide range of applications OC can have, the human exposure to this kind of compounds is likely going to increase in the near future. This makes necessary a safety evaluation since potential toxic effects cannot be discarded. Toxic effects of clay minerals have been shown to occur mainly after inhalation (Carretero et al., 2006; Sharma et al., 2010), but the oral pathway is one of the most likely routes of exposure to these clay minerals for the general population, since they are present in food contact materials.

Although toxicological information of clay minerals is scarce, different authors have reported toxic effects on in vitro systems, such as reduced viability or reactive oxygen species production (Houtman et al., 2014; Lordan et al., 2011; Maisanaba et al., 2013, accepted for publication-a), have found a different toxic profile of different modified and unmodified clay minerals in Caco-2 and Hep-G2 cells and have suggested that a case by case toxicological assessment is required for clay minerals, as the modifiers employed to improve their technological aspects play an important role in the toxicity observed.

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In vivo toxicity data of clay minerals are even scarcer, and most of the trials performed are not representative of the likely human exposure to these compounds, which corresponds to a repeated and low dose exposure for a long time. Taking all this into account, the aim of this study was to determine the oral subchronic toxicity of an OC on Wistar rats. A morphological study of the main organ tissues by optic and electronic microscopy was performed. Blood clinical biochemistry parameters, interleukin leakage in serum and blood glutathione levels were also determined.

2. Materials and methods

2.1. Supplies and chemicals

General chemicals were provided by Sigma Aldrich (Madrid, Spain) and VWR International Eurolab (Spain).

The organomodified clay mineral encoded as Clay1 was obtained by cation exchange reaction from Cloisite Na^+® (Southern Clay Products, Inc.), adding the quaternary ammonium salt hexadecyltrimethylammonium bromide (HDTA) in 6 fold the cation exchange capacity (CEC) of pristine clay mineral, following the method described by Jordá et al. (2009), Jordá-Beneyto et al. (2008) and Mittal (2007). The modified clay mineral was characterized by Fourier Transform Infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA) and X-ray diffraction (XRD) as shown in Jordá-Beneyto et al. (2014).

2.2. Animals and experimental design

A repeated dose 90-day oral toxicity study was performed in rats. Briefly, twenty male Wistar rats provided by Janvier S.A.S. (France) with a mean mass of 240.2 ± 2.04 g were used. Rats were fed during the acclimation week with a standard laboratory diet (Harlan 2014, Harlan Laboratories, Barcelona, Spain), and water, both ad libitum, in a controlled-temperature room (23 ± 1 °C) with 12 h dark/light cycle, and free from any source of chemical contamination. After this period, animals were randomly divided into two groups, a control group (n = 10) and an exposed group (n = 10). The control group was fed with the standard diet, while the exposed group was orally administered with 40 mg/kg/day of Clay1 in the diet. This dose was selected to reproduce 2-fold the dose that a person would receive daily from a 1.5 L bottle made of the CPN. This is, assuming that all the clay mineral incorporated into the packaging polymer is present in the food. During the treatment period clinical signs, body mass, and food and water consumption were recorded weekly.

All animals received human care in compliance with the guidelines for the protection of animals used for scientific purposes and all the procedures were previously accepted by the Ethic Committee of the University of Seville.

2.3. Organs and blood sampling

At the end of the experimental period, rats were fasted for 18 h before sacrifice. Liver, kidneys, lungs, spleen, brain, testes, gastrointestinal (g.i.) tract and heart were excised, rinsed with cold saline solution and weighted. Blood samples were obtained by cardiac puncture and collected in test tubes with/without lithium heparin depending on the experiment. Serum was separated by low speed centrifugation at 1500 × *g* at 4 °C for 15 min, and stored at −80 °C until analysis of IL-6 leakage and clinical biochemistry parameters.

2.4. Histopathogical analysis

The histopathological examination by optic (Hematoxylin–Eosin HE staining) and electronic microscopy was performed as described by Maisanaba et al. (accepted for publication-b).

2.5. GSH/GSSG levels

The ratio GSH/GSSG was determined in blood samples using a commercial kit (Bioxytech GSH/GSSG-412; Oxis Research, Foster City, CA, USA).

2.6. IL-6 leakage

For this assay the blood sera of experimental animals were used. Manufacturer instructions from the kit (Thermo Scientific Rat Interleukin-6 (IL-6) ELISA) were followed.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation of ten animals per group. Statistical analysis was performed by analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA).

3. Results

No rats died during the experimental period and there were no remarkable clinical signs. Body mass, body mass gain, food and water consumption, organ mass and the somatic index of the different organs (Table 1) did not show statistical differences between the control and the exposed group.

3.1. Histopathogical results

Microscopic examination of the HE-stained tissue sections of the exposed animals did not show remarkable changes in comparison to the control group (Fig. 1). Moreover, the ultrastructural study did not reveal alterations related with the treatment (Fig. 2).

3.2. Clinical biochemistry parameters

Results showed no significant changes in any of them in comparison to the control group (Table 2).

3.3. GSH/GSSG levels

Blood GSH/GSSG ratio experienced a non-significant alteration in rats exposed to Clay1 in comparison to the control group (Fig. 3).

Table 1
Organ mass (g) and somatic index (%) of the different organs of control Wistar rats and rats exposed to 40 mg/kg/day Clay1 for 90 days. Results are expressed as mean ± SD.

	Control group Mean ± SD	40 mg/kg/day Clay1 Mean ± SD
Liver (g)	13.12 ± 2.44	12.84 ± 1.67
Liver somatic index (%)	2.83 ± 0.40	2.53 ± 0.30
Kidneys (g)	3.02 ± 0.63	2.89 ± 0.26
Kidney somatic index (%)	0.65 ± 0.11	0.60 ± 0.05
Lungs (g)	2.75 ± 0.78	2.72 ± 0.66
Lung somatic index (%)	0.60 ± 0.09	0.53 ± 0.12
Spleen (g)	1.02 ± 0.17	1.04 ± 0.13
Splenic somatic index (%)	0.22 ± 0.02	0.20 ± 0.02
Brain (g)	2.06 ± 0.18	2.14 ± 0.22
Brain somatic index (%)	0.45 ± 0.05	0.42 ± 0.05
Testes (g)	3.66 ± 0.75	3.77 ± 0.52
Testicle somatic index (%)	0.79 ± 0.11	0.75 ± 0.14
Intestine (g)	4.99 ± 1.022	5.68 ± 1.86
Intestine somatic index (%)	1.10 ± 0.37	1.00 ± 0.50
Heart (g)	1.82 ± 0.12	1.75 ± 0.19
Heart somatic index (%)	0.40 ± 0.05	0.34 ± 0.04