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Subchronic mycotoxicoses in Wistar rats: Assessment of the *in vivo* and *in vitro* genotoxicity induced by fumonisins and aflatoxin B_1, and oxidative stress biomarkers status

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ABSTRACT

Some evidence suggests that fumonisin B_1 (FB_1), a worldwide toxic contaminant of grains produced by *Fusarium verticillioides*, exhibits an oxidative stress mediated genotoxicity. We studied the DNA damage (by the alkaline comet and the micronucleus tests) and biomarkers of cellular oxidative stress (malondialdehyde, MDA; catalase, CAT; and superoxide dismutase, SOD) in spleen mononuclear cells of male Wistar rats subchronically (90 days) fed on a control experimental diet (CED) or poisoned with experimental diets contaminated with a culture material containing 100 ppm of FB_1 (FED), with 40 ppb of aflatoxin B_1 (a common toxic co-contaminant in cereals, AFB_1ED), and with a mixture of both toxins (MED). The DNA damage was found in 13.7%, 81.7%, 98.0% and 99.3% (comet assay) and in 2.8%, 7.0%, 10.8% and 8.8% (micronucleus technique) in groups CED, FED, AFB_1ED and MED, respectively. The MDA levels as well as the CAT and SOD activities were increased in all the poisoned animals. A similar behavior was observed in cells exposed *in vitro* to the toxins. These data support the hypothesis of an oxidative stress mediated genotoxicity induced by FB_1. Furthermore, the extent of DNA damage assessed by the comet assay suggests a possible protective effect of the fumonisins–AFB_1 mixtures *in vitro* against the genotoxicity induced individually by the toxins.

1. Introduction

Mycotoxins are fungal secondary metabolites commonly present in food. Human exposure occurs mainly by the ingestion of mycotoxin-contaminated products and can lead to serious health problems, including immunosuppresion and even carcinogenesis (Bondy and Pestka, 2000; Richard, 2007). Certain fungal strains belonging to the *Fusarium* and *Aspergillus* families, which are found worldwide, produce fumonisin and aflatoxin mycotoxins, respectively (Pitt, 2000). Fumonisin B_1 (FB_1) and aflatoxin B_1 (AFB_1) are the most important toxins of each group, due to their prevalence as cereal contaminants and their toxicological potency. They are present in several commodities, with humans and animals being constantly exposed to low levels of these mycotoxins, either individually or in combination.

Aflatoxins are hepatotoxic, immunosuppressive, carcinogenic, teratogenic and mutagenic (IPCS-WHO, 1998). Several diseases are associated with the human consumption of these toxins, including toxic hepatitis and even primary hepatocellular carcinomas (Pitt, 2000). Furthermore, a wide spectrum of toxic responses is related to the exposure of animals to aflatoxins, with most of them causing economical losses resulting from decreased production (Peraica et al., 1999).

Fumonisin B_1 was first isolated from cultures of *F. verticillioides* MRC 826 by Gelderblom et al. (1988) at PROMEC, Republic of South Africa, and its chemical structure was then elucidated in collaboration with the Council for Scientific and Industrial Research in

Abbreviations: AFB_1, aflatoxin B_1; AFB_1ED, rats poisoned with an experimental diet containing 40 ppb of AFB_1 ($n=6$); CAT, catalase; CED, animals fed with the control experimental diet ($n=6$); FB_1, fumonisin B_1; FCM, *Fusarium verticillioides* culture material; FED, rats poisoned with the experimental diet containing fumonisins ($n=6$); MDA, malondialdehyde; MED, experimental diet containing a mixture of AFB_1 (40 ppb) and fumonisins (FB_1: 100 ppm) ($n=6$); MN, micronucleus; ROS, reactive oxygen species; SMC, spleen mononuclear cells; SOD, superoxide dismutase; $TAFB_1$, treatment with AFB_1 (20 μg/ml, $n=3$); TBARS, thiobarbituric acid-reactive species; TFB_1, treatment with FB_1 (10 μg/ml, $n=3$); TM, treatment with a mixture of AFB_1 (20 μg/ml) and FB_1 (10 μg/ml) ($n=3$).

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Pretoria (Bezuidenhout et al., 1988). Afterwards, a strong correlation between the consumption of maize highly contaminated with fumonisins (up to 155 ppm FB1) and a high-incidence of human esophageal tumors in the population of China was detected (Chu and Li, 1994). Consequently, it was proposed that these toxins had a carcinogenic potential. However, at present, there is inadequate evidence to classify fumonisins as being carcinogenic for humans.

Li et al. (2001) observed co-contamination of maize with AFB_1 (9–2496 ppb) and FB_1 (0.058–1.976 ppm) in a high-incidence area of human primary hepatocellular carcinoma in Guangxi, Republic of China, which led to the probable participation of both toxins in the genesis of this pathology being suggested. Supporting this proposal, the immune system is considered a target of several mycotoxins (Bondy and Pestka, 2000). The physiological immunosurveillance, a key function to prevent tumor development, could be suppressed as consequence of the immunotoxic action of aflatoxins and fumonisins individually or as mixtures, as was previously stated in an experimental mycotoxicoses in Wistar rats (Theumer et al., 2002, 2003).

At the molecular level, the toxicology of AFB_1 involves its metabolic conversion by the cytochrome p450 system to the highly electrophilic AFB_1-*exo*-8,9-epoxide, which in turn binds to the DNA guanines to form adducts (IPCS-WHO, 1998; Wang and Groopman, 1999). In addition to its previously described genotoxic action, some evidence indicates that AFB_1 can produce genetic damage by causing reactive oxygen species (ROS) accumulation (Shen and Ong, 1996).

With regard to the physiopathologic action of FB_1 on cells, the toxic responses appear to be related at least in part, to the inhibition of the ceramide synthetase activity and the subsequent imbalance in the cell lipidic metabolism (IPCS-WHO, 2000). The possible genotoxicity of fumonisins has been evaluated in recent years, and it was proposed that this toxin could produce genetic damage by means of an indirect mechanism involving the cellular oxidative stress. Sahu et al. (1998) observed that FB_1 caused DNA strand breaks in isolated rat liver nuclei, and concluded that such lesions may be caused by increased lipid peroxidation. Although other authors also found that FB_1 increased lipid peroxidation (Abel and Gelderblom, 1998; Klaric et al., 2007; Mobio et al., 2003; Stockmann-Juvala et al., 2004), Galvano et al. (2002a,b) showed that ROS production did not increase with the appearance of DNA lesions in FB_1-exposed cells.

To determine whether or not a chemical is genotoxic or epigenetic, and its related mode of action, it is necessary to collect experimental information coming from several *in vivo* and *in vitro* assays (Dybing et al., 2008). For this purpose, the *in vitro* micronucleus (MN) technique has emerged as one of the preferred laboratory tools for assessing chemically induced chromosomal damage, an important event in carcinogenesis (Fenech, 2000). The alkaline comet assay is also a widely accepted genotoxicity-testing method for novel pharmaceuticals or other chemicals, and is a simple and sensitive procedure for detecting DNA strand breaks that requires a small number of cells and a short time to complete the study (Burlinson et al., 2007; Collins, 2004; Wang et al., 2005). Both methods to assess DNA damage induced *in vitro* or *in vivo* were readily adapted to virtually any cell population feasible of being obtained as a single cell suspension, including lymphocytes isolated either from the spleen or peripheral blood (Burlinson et al., 2007; Collins, 2004; Fenech, 2000).

The aim of the present work was to provide additional information to evaluate the hypothesis that FB_1 is a genotoxicant to mammalian cells, also elucidating whether the induction of genetic damage is involved in the subchronic immunotoxic action of fumonisins, with emphasis on cellular oxidative stress as a mechanism that may be involved in the eventual genotoxicity of this mycotoxin. Furthermore, a possible interaction of FB_1 and AFB_1 was evaluated, related to the probable genotoxic damage that could be produced by a mixture of both toxins in a subchronic mycotoxicoses model in Wistar rats, as well as in *in vitro* tests.

2. Materials and methods

2.1. Animals

Male Wistar inbred rats (6–8 weeks old, body weight 220 ± 5 g, $n = 6$) were housed in stainless-steel cages, and kept in environmentally controlled rooms with a 12-h light/dark cycle. Animals were housed and cared for in the animal resource facilities of the Department of Clinical Biochemistry, Faculty of Chemical Sciences, National University of Córdoba. The Institutional Experimentation Animal Committee (authorization # 15-09-69934) approved animal handling and experimental procedures.

2.2. Mycotoxins

2.2.1. Preparation of *F. verticillioides* culture material (FCM) extracts

The extracts were prepared as previously described by Theumer et al. (2008, 2003). Briefly, maize (300 g) was placed in 1000-ml Erlenmeyer flasks at 35% humidity and sterilised for two consecutive days in autoclave at 121 °C for 15 min. *F. verticillioides* MRC 826, kindly provided by PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, Republic of South Africa), was used as a fumonisine-producing fungal strain. After inoculation of the fungus, the maize was incubated for 28 days in the dark at 25 °C, with manual stirring performed for the first 5 days. An aqueous extract of FCM was prepared following a procedure previously described by Voss et al. (1990). Briefly, the maize was dried in an oven with circulating air at 60 °C for 24 h. Some of this maize (300 g) was ground and placed in 500 ml of distilled water in an orbit agitator at room temperature for 1 h. The solution was centrifuged at 3500 rpm for 10 min and the supernatant recovered. Then, the supernatants were homogenized and stored at −20 °C until use.

2.2.2. Fumonisin quantifications

Samples (100 μl) obtained from the FCM extracts were diluted with acetonitrile (100 μl). Then, before the quantification assays, these samples were diluted with acetonitrile/water (1:1, v/v). The quantification of the diluted extracts was performed by means of a method previously described by Shephard et al. (1990). Briefly, an aliquot (50 μl) of the diluted extract was derivatized with 200 μl of an o-phthaldialdehyde solution, obtained by adding 5 ml of 0.1 M sodium tetraborate and 50 μl of 2-mercaptoethanol to 1 ml of methanol containing 40 mg of o-phthaldialdehyde. The mycotoxins FB_1 (CAS 116355-83-0), FB_2 (CAS 116355-84-1) and FB_3 (CAS 136379-59-4) were detected and quantified with a Hewlett Packard 1100 HPLC equipped with a fluorescence detector, using wavelengths of 335 and 440 nm for excitation and emission of fluorescence, respectively. An analytical reverse-phase C_{18} column (150 mm by 4.6 mm (internal diameter); 5 μm particle size), connected to a C_{18} pre-column (20 mm by 4.6 mm; 5 μm particle size), was utilized. The mobile phase was methanol: 0.1 M NaH_2PO_4 at a 75:25 ratio (v/v), the pH was set at 3.35 ± 0.20 with orthophosphoric acid, and a flow rate of 1.5 ml/min was used. The quantification of fumonisins was carried out by comparing the peak areas obtained for the FCM extracts with those corresponding to analytical standards of FB_1, FB_2 and FB_3 (purity > 95%), provided by PROMEC, Republic of South Africa.

Fumonisins were detected in the FCM extract at 4.46:1.00:2.03 ratios for FB_1, FB_2 and FB_3, respectively.

2.2.3. Preparation and quantification of AFB_1 solutions

AFB_1 crystalline (CAS 1162-65-8; Sigma, purity > 98%) in benzene–acetonitrile (98:2 v/v) was checked for purity and then spectrophotometrically quantified. An aliquot of this solution was dried in a rotatory evaporator. Then AFB_1 was dissolved in olive oil to a final concentration of 1 mg/ml, and kept at −20 °C until diet preparation.

2.3. Diets

2.3.1. Commercial basal diet

The commercial basal diet (mice–rats, Cargill S.A.C.I., Saladillo, Buenos Aires, Argentina) was certified by the supplier as free from fumonisins and aflatoxins, and contained total protein > 24%, ether extract > 6%, raw fiber > 7%, calcium > 1%, phosphorus > 0.5%, moisture < 13% and total minerals < 8%, with an energetic value > 2780 kcal/kg. The commercial basal diet was finely ground and then used to prepare the different experimental diets, as previously described by Theumer et al. (2008).

2.3.2. Control experimental diet

This was prepared by adding 435 ml of maize aqueous extract without inoculation of *F. verticillioides* to an agar solution (Difco) in 435 ml of distilled water. This mixture was warmed until the agar dilution was completed and then cooled to