Toxicology 268 (2010) 104–110

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

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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ARTICLE INFO

Article history:
Received 10 September 2009
Received in revised form
16 November 2009
Accepted 7 December 2009
Available online 14 December 2009

Keywords:
Mycotoxins
Fumonisins
Aflatoxin B_1
Genotoxicity
Oxidative stress

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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doi:10.1016/j.tox.2009.12.007

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

50 °C. Olive oil (75.4 μl) was added and the solution vigorously shaken. Then, 1000 g of finely ground commercial basal diet was also added and the mixture continuously shaken until homogeneous. Pieces of approximately 20 g each were molded, and after solidification, stored at −20 °C until being used.

2.3.3. Experimental diet with fumonisins

This was prepared as the control diet, but using an FCM extract obtained as described above, in order to obtain a final FB_1 concentration of 100 ppm in the food.

2.3.4. Experimental diet with AFB_1

This was prepared as the control diet, but adding 75.4 μl of AFB_1-containing olive oil, in order to obtain a final AFB_1 concentration of 40 ppb.

2.3.5. Experimental diet with the mixture of fumonisins and AFB_1

This was prepared as the control diet, but adding 75.4 μl of AFB_1-containing olive oil, and FCM extract obtained as described above, in order to obtain final concentrations of 40 ppb and 100 ppm for AFB_1 and FB_1, respectively.

2.4. Experimental model

Four groups of rats were used. Animals were fed either on the control experimental diet (CED) ($n = 6$), on the experimental diet with fumonisins (FED) ($n = 6$), on the experimental diet with AFB_1 (AFB_1ED) ($n = 6$), or on the experimental diet with the mixture of these toxins (MED) ($n = 6$). Animals were housed in pairs in different cages and fed with the different diets for 90 days. The food ration was replaced daily, and the weights of food portions given and uneaten after 24 h were determined. At the end of the feeding period, the rats were killed by cervical dislocation, and then the spleens were aseptically removed. The *in vitro* genotoxicity of the mycotoxins used individually or in a mixture was evaluated in cells from normal rats, and the results of these experiments were correlated with biomarkers of cellular oxidative stress.

2.5. Preparation and culture of spleen mononuclear cell (SMC) suspensions

SMC suspensions were aseptically prepared by the method described by Kizaki et al. (1991). Briefly, spleens were minced and passed through a stainless-steel mesh to obtain single cell suspensions. The cells were washed with RPMI 1640 medium (Sigma) and resuspended in sterile RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco), gentamicin (50 μg/ml), and 2-mercaptoethanol (5×10^{-5} mol/L). SMC suspensions were adjusted to 10^6 cells/ml.

For *in vitro* assays, a pool of SMC from normal animals was prepared as described above, and then cultured for 24 h at 37 °C and 5% CO_2 in the absence of mycotoxins (Control, $n = 3$), or treated with FB_1 (TFB_1, $n = 3$), AFB_1 ($TAFB_1$, $n = 3$) and with the mixture of both toxins (TM, $n = 3$). Analytical standards of AFB_1 (Sigma–Aldrich, USA) in dimethylsulfoxide (1% of the RPMI final volume) and FB_1 (PROMEC, Republic of South Africa) were added individually or as a combination in order to obtain final concentrations of 20 and 10 μg/ml, respectively.

The cell viability of SMC exposed *in vivo* or *in vitro* to the mycotoxins was assessed by means of the trypan blue exclusion test for membrane integrity and metabolic competence. The cell viability was higher than 97% in all the cases.

2.5.1. Alkaline comet assay

The alkaline comet assay was performed as described by Sasaki *et al.* (1997). SMC exposed *in vivo* and *in vitro* to the mycotoxins were homogenized in a chilled homogenization buffer (pH 7.5) containing 75 mM NaCl and 24 mM Na_2EDTA to obtain a 10% tissue solution. Ten microliters of SMC were suspended in 0.5% low-melting agarose sandwiched between a layer of 0.6% normal-melting agarose and a top layer of 0.5% low-melting agarose on fully frosted slides. These slides were kept on ice during the polymerization of each gel-layer. After the solidification of the 0.6% agarose layer, the slides were immersed in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA; 10 mM Tris–HCl, 1% Triton X-100 and DMSO 10%) at 4 °C. After 1 h, slides were placed into an electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) for 10 min at 0 °C to allow the DNA to unwind. Electrophoresis was performed for 10 min at 300 mA and 1 V cm^{-1}. Slides were then neutralized with a Tris–HCl buffer (pH 7.5) and stained with ethidium bromide (20 μg/ml) for 10 min, with each slide being analyzed using a Nikon eclipse TE 2000-U optical microscope equipped with a NIKON digital sight DS-U1 camera. One hundred nuclei were visually analyzed, and the relative tail-fluorescence intensities were used to evaluate the extent of DNA damage. The comets were scored using a 0 (no tail)–4 (100% of DNA in the tail) scale (Collins, 2004).

2.5.2. Micronucleus assay

This was performed in SMC exposed *in vivo* or *in vitro* to the mycotoxins, according to the methodology described by Fenech (2000). The SMC exposed *in vivo* and *in vitro* to the toxins were incubated for 44 h in the presence of Concanavalin A (ConA, 10 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. Then cytochalasin-B (Cyt-B, 6 μg/ml) was added and the cells were incubated for further 28 h. The mitogen-stimulated cells were harvested and fixed for 10 min in absolute methanol prior to be stained with Giemsa dye. One thousand binucleated cells were analyzed using an optical microscope (1000× magnification), and thereafter, the MN frequencies were calculated.

2.6. Markers of oxidative status

2.6.1. Quantification of malondialdehyde (MDA) levels

The MDA levels were estimated in SMC exposed *in vivo* and *in vitro* to the mycotoxins, as an indicator of the lipidic peroxidation extent, by means of the thiobarbituric acid-reactive substances (TBARS) method (Bird and Draper, 1984). The cells were washed and resuspended in 1 ml of KCl 0.15 M and adjusted to 10^6 cells/ml. Then, samples (1 ml) were sonicated, and 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid were added to each tube and well mixed. The aliquots of each vial were then centrifuged at 3000 rpm for 10 min, and the supernatant from each tube was decanted into separate tubes and placed in a bath of boiling water for 10 min. These test tubes were then removed and cooled at room temperature. The absorbance of each aliquot was measured at 532 nm. The rate of lipid peroxidation was expressed as nanomoles of MDA formed per milligram of protein, using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. The protein content of each sample was determined using the Bradford method (Bradford, 1976).

2.6.2. Measurements of catalase (CAT) activity

Ten million cells were lysed by sonication (two 10-s pulses with a 10-s interval) in 0.5 ml PBS (pH 7.4). The resultant sonicate was centrifuged at 14,000 × *g* for 10 min at 4 °C. Then, the catalase (CAT; EC.1.11.1.6) activity was measured in the supernatant by the method of Aebi (1984), with 50 μl of the supernatant being mixed with 600 μl of 15 mM H_2O_2 in a cuvette. The kinetics of the decrease in light absorbance at 240 nm (H_2O_2 decomposition) was determined for 3 min in a Shimadzu UV 1601PC spectrophotometer (Shimadzu Corporation, Japan). A cuvette containing only PBS served as a blank. A cuvette without a sample was used to ensure that H_2O_2 did not decompose spontaneously under our experimental conditions. The amount of H_2O_2 decomposed was calculated using an extinction coefficient of 43.6 mM^{-1} cm^{-1}. Then, the enzymatic activities were expressed as U/mg of protein in the lysates, determined by the Bradford method (1976) (1 U = 1 μmol of substrate converted × min^{-1}).

2.6.3. Measurement of superoxide dismutase (SOD) activity

The SMC exposed *in vivo* and *in vitro* to the mycotoxins were washed four times with 0.9% NaCl solution and resuspended with cold bidistilled water, before being mixed and left to stand at 4 °C for 15 min. The superoxide dismutase (SOD; EC.1.15.1.1) activity was measured with RANSOD kits (Cat # SD 125; Randox Labs., Crumlin, North Ireland). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity can then be measured by the degree of inhibition of this reaction. Samples were conveniently diluted with saline buffered phosphate solution pH 7.4 so that the percentage of inhibition fell between 30% and 60%. All diluted sample rates were converted into percentages of the sample diluent rate, and subtracted from 100% to give the percentage of inhibition. The activity was measured at 37 °C on a Shimadzu UV 1601PC spectrophotometer (Shimadzu Corporation, Japan), and absorbance was monitored at 505 nm for 3 min, with the unit of activity being defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%.

SOD units were obtained from the standard curve by using the percentage inhibition of the samples (SOD U/ml of SMC suspension, absolute activity) and were converted to SOD units/mg of protein in cell lysates (specific activity), which were quantified by the Bradford method (1976). Standards were prepared by diluting a commercial SOD preparation (Cat # SD 125) in order to obtain a standard curve.

2.7. Statistical evaluation

Data from these studies were analyzed by a two-tailed ANOVA, and the Bonferroni Multiple Comparisons Test was used as a post-test. The mycotoxin intakes were analyzed using the two-tailed Student's *t*-test. Differences were considered statistically significant for p values ≤ 0.05. GraphPad InStat software version 3.01 (La Jolla, CA 92037 USA) was used for the analyses.

3. Results

3.1. Mycotoxin intake

The overall AFB_1 consumption in the AFB_1ED and MED groups was 328.28 ± 13.13 and 298.92 ± 5.74 μg/kg body weight, respectively. No statistically significant differences were observed for this parameter, except that the fumonisin intake (expressed in mg of FB_1/kg body weight) was higher in the FED group (979.92 ± 10.10) than in the MED animals (742.29 ± 14.34).

Table 1
DNA damage induced in SMC from Wistar rats subchronically poisoned with fumonisins, AFB_1, and a mixture of fumonisins and AFB_1, assessed by the alkaline comet assay[1].

Group[2]	Damage				
	0	1	2	3	4
CED	86.3 ± 3.2	10.3 ± 1.5	2.3 ± 1.9	1.0 ± 1.0	ND
FED	18.3 ± 2.0[b,A]	10.3 ± 1.5	12.7 ± 1.5[a]	37.0 ± 0.6[b]	21.7 ± 1.2[b]
AFB_1ED	2.0 ± 0.6[b,A]	10.7 ± 1.2	12.3 ± 1.2[a]	25.3 ± 0.9[b,A]	49.7 ± 2.6[b,A]
MED	0.7 ± 0.3[b,A]	8.7 ± 0.7	12.7 ± 2.0[a]	30.3 ± 0.9[b]	47.7 ± 2.0[b,A]

ND: not detected.
[a] $p < 0.01$ when groups were compared with the CED group.
[b] $p < 0.001$ when groups were compared with the CED group.
[A] $p < 0.001$ when MED was compared with FED.
[1] Data are expressed as percentages of SMC (means ± SE), showing DNA damage scored using a 0 (undamaged)-4 (100% of DNA in the tail) scale.
[2] CED: Control experimental diet; FED: diet containing fumonisins; AFB_1ED: diet containing AFB_1; MED: diet with the fumonisins–AFB_1 mixture.

3.2. Alkaline comet assay

The results of the comet assay with SMC from animals poisoned for 90 days with the mycotoxins, individually or as a mixture, are summarized in Table 1. All the experimental diets induced a higher frequency of comets, as shown by the smaller percentages of undamaged nuclear DNA in the groups fed on the fungal toxins. Moreover, the overall genetic damage was higher in the AFB_1ED and MED groups ($p < 0.001$ in both cases) than in the FED group. These results demonstrated a greater contribution of damages scored as 3 and 4 in AFB_1ED and MED groups, while the frequency of damages 1 and 2 was similar in the three groups poisoned with the mycotoxins.

For these subchronic mycotoxicoses, the toxins administered individually or as a mixture caused genetic lesions that were mostly classified in the range 2–4 within the scale used, though slight differences were noticed between poisoned rats. Almost half of the nuclei analyzed in the AFB_1ED and MED groups were scored as comets with type-4 damage, which was significantly higher ($p < 0.001$ in both cases) than their homologues in the FED group. The highest percentage recorded in the latter was found in the type-3 damage, which was significantly higher than the observed in the AFB_1ED group.

The alkaline comet assay was also performed with SMC exposed *in vitro* to the mycotoxins, and the results are summarized in Table 2. Similar tendencies to those observed in the poisoned groups were found in *in vitro* exposure to the fungal toxins, though type-4 damage was not observed either in the cells incubated with the toxins or in the controls. The treatments with the mycotoxins induced a higher frequency of comets, but in spite of the higher genetic damage tendencies observed in $TAFB_1$ and TM, no significance was found when compared to the TFB_1 group. Most of the genetic damage observed in cells exposed to the toxins was classified as types 2 and 3. In the SMC treated with the mycotoxins individually, the highest percentage of DNA injury was observed in the type-3 damage. However, the toxin mixture induced mainly type-2 damage, which was significantly higher when compared with its homologue in the TFB_1 group.

Table 2
DNA damage induced in SMC from normal Wistar rats exposed *in vitro* to FB_1, AFB_1 and to a mixture of both toxins, assessed by the alkaline comet assay[1].

Treatment[2]	Damage			
	0	1	2	3
TC	77.3 ± 1.9	14.3 ± 1.4	6.7 ± 1.9	1.7 ± 1.2
TFB_1	12.0 ± 2.3[a]	17.3 ± 1.2	30.3 ± 1.2[a]	40.3 ± 2.3[a]
$TAFB_1$	2.3 ± 1.2[a]	20.7 ± 1.3	33.3 ± 2.4[a]	45.0 ± 2.6[a]
TM	2.7 ± 1.8[a]	21.3 ± 1.4	42.0 ± 1.7[a,A]	34.0 ± 1.7[a,I]

[a] $p < 0.001$ when groups were compared with the TC group.
[A] $p < 0.01$ when TM was compared with TFB_1.
[I] $p < 0.05$ when TM was compared with $TAFB_1$.
[1] Data are expressed as percentages of SMC (means ± SE) showing DNA damage scored using a 0 (undamaged)–4 (100% of DNA in the tail) scale.
[2] Cells were treated for 24 h with FB_1 (TFB_1), AFB_1 ($TAFB_1$), with the mixture of both toxins (TM), and in the absence of mycotoxins (treatment control, TC).

3.3. Micronucleus assay

The ability of mycotoxins to induce MN in SMC was tested, and the results of these experiments are shown in Fig. 1. In the *in vivo* exposure, there was an increase in the percentage of MN in all the animals fed on the diets contaminated with the toxins ($p < 0.001$ for the three cases) (Fig. 1A). The highest frequency of MN was registered in the AFB_1ED group, with this being higher than the ones observed in the FED ($p < 0.001$) and in the MED ($p < 0.05$) groups.

Similar findings were registered for the SMC exposed *in vitro* to the fungal toxins (Fig. 1 B), where higher percentages of MN were detected in the TFB_1, $TAFB_1$ ($p < 0.01$ in both cases), and TM ($p < 0.001$) groups. However, no differences were registered when

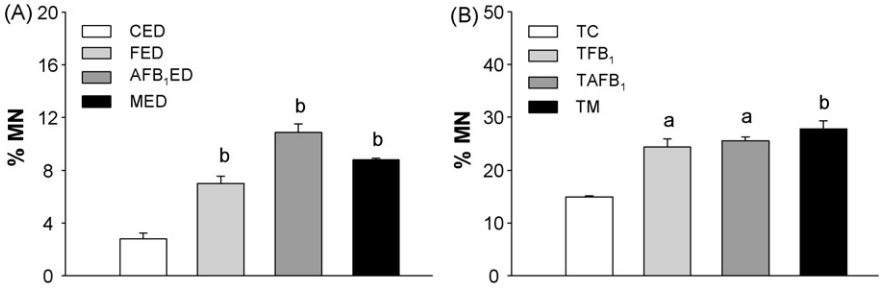

Fig. 1. DNA damage induced in spleen mononuclear cells (SMC) from Wistar rats exposed *in vivo* (A) and *in vitro* (B) to fumonisins, AFB_1, and to a mixture of fumonisins and AFB_1, assessed by the micronucleus technique. Bars represent the percentage of micronuclei (% MN) (means ± SE) found in SMC from: (A) rats fed on the diet without mycotoxins (CED), diets containing fumonisins (FED), AFB_1 (AFB_1ED), and the mixture of both toxins (MED); (B) normal rats cultured in RPMI medium (TC), in the presence of FB_1 (TFB_1), AFB_1 ($TAFB_1$), and a mixture of both toxins (TM). [a] $p < 0.01$; [b] $p < 0.001$ when groups were compared with the CED group.

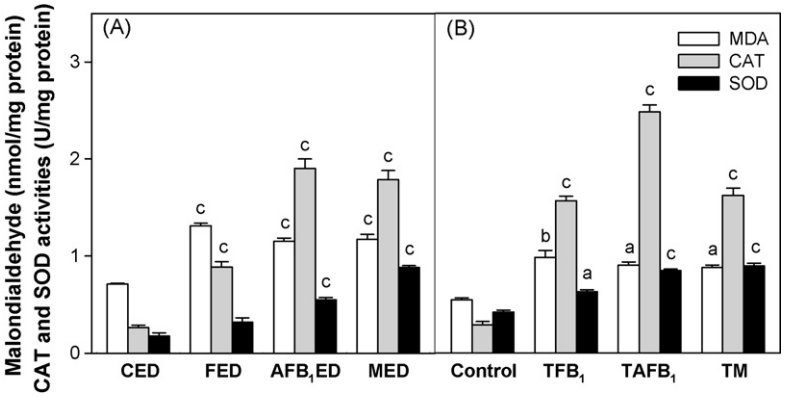

Fig. 2. Measurement of oxidative stress biomarkers in spleen mononuclear cells (SMC) from Wistar rats exposed *in vivo* (A) and *in vitro* (B) to fumonisins, AFB_1, and to a mixture of fumonisins and AFB_1. Bars represent the means ± SE of malondialdehyde levels (MDA, nmol/mg of protein), and the specific catalase (CAT) and superoxide dismutase (SOD) enzymatic activities (Units/mg of protein) found in SMC lysates from: (A) rats fed on the diet without mycotoxins (CED), diets containing fumonisins (FED), AFB_1 (AFB_1ED), and the mixture of both toxins (MED); (B) normal rats cultured in RPMI medium (TC), in the presence of FB_1 (TFB_1), AFB_1 ($TAFB_1$), and a mixture of both toxins (TM). $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ when groups were compared with the CED group.

the different treatments with the mycotoxins were compared with each other.

3.4. Markers of oxidative status

The MDA levels, as well as the CAT and SOD biological activities, were measured in SMC exposed *in vivo* and *in vitro* to the mycotoxins, with the results being shown in Fig. 2.

The poisonings with the three experimental diets induced increases in the MDA levels and in the CAT activity (Fig. 2A). A similar behavior was also observed in the SOD activity. However, the increase of this biochemical parameter was significant only in the AFB_1ED and MED groups.

While no differences were noticed in the MDA levels between FED, AFB_1ED and MED, the CAT and SOD activities were higher in the last two groups, with respect to FED ($p < 0.001$ in all the cases but for the SOD activity in AFB_1ED, $p < 0.01$). Also, the SOD activity was higher in the MED ($p < 0.001$) than in the AFB_1ED group.

The *in vitro* exposure of normal SMC to the mycotoxins induced a similar behavior in the cell oxidative status markers (Fig. 2B). Higher levels of MDA, together with increases in the CAT and SOD biological activities were found in cells exposed to the toxins, either individually or as a mixture. No differences were detected in the MDA levels found in the TFB_1, $TAFB_1$ or TM groups. Furthermore, the CAT activity was higher in $TAFB_1$ compared to TFB_1 and TM ($p < 0.001$ in both cases), with the SOD activities being higher in the $TAFB_1$ ($p < 0.05$) and TM ($p < 0.01$) groups when compared with TFB_1.

4. Discussion

Since a link between the high-incidence of human esophageal cancer and the increased consumption of foodstuffs contaminated with fumonisins was established in the region of Transkei, South Africa (Sydenham et al., 1990), many studies have been performed worldwide to try to elucidate whether these toxins are carcinogens for humans and animals. However, contradictory results have been reported when FB_1 was evaluated in bacterial genotoxicity tests. Moreover, Knasmuller et al. (1997), and then Aranda et al. (2000), observed a lack of mutagenic action when the fungal toxin was tested in gene mutation assays with *Salmonella typhimurium*. On the other hand, Sun and Stahr (1993) found a positive result when the FB_1 was tested in a commercial bioluminescent bacterial (*Vibrio fischeri*) mutagenicity assay, even in the absence of an exogenous metabolic system from rat liver, thus showing that this mycotoxin had a direct genotoxic-like behavior. A similar tendency was also observed when the ability of FB_1 to induce *in vitro* genetic damage was tested. Related to this, Norred et al. (1992) reported that FB_1 could not be considered to be a genotoxic mycotoxin, when evaluated by its ability to induce unscheduled DNA synthesis (UDS) in primary rat hepatocytes. However, genetic damage was observed by other authors when the toxin was tested by its ability to induce MN in primary hepatocytes (Knasmuller et al., 1997), rabbit kidney RK13 cells (Rumora et al., 2002), human lymphocytes and *Allium cepa* (onion) (Lerda et al., 2005). Moreover, the FB_1 was also found to be genotoxic *in vitro* when it was evaluated by means of comet assay in rat astrocytes (Galvano et al., 2002a) and in human fibroblasts (Galvano et al., 2002b).

Experimental mycotoxicoses in rats were developed in order to characterize the genetic alterations induced by subchronic consumption of diets containing known levels of FB_1 and AFB_1, individually or as a mixture, which could mimic those found in nature. Biological and pharmacokinetic data were also taken into account for the choice of the doses used in the *in vitro* exposures, since p. o. bioavailability of FB_1 was informed to 3.5% in Wistar rats (Martinez-Larranaga et al., 1999).

In the present work, when the SMC coming from normal rats were incubated for 24 with FB_1 (TFB_1) and then subjected to further analysis by the comet assay (Table 2) as well as by the MN technique (Fig. 1B), the genotoxic action of FB_1 was observed by both methods. The overall damage registered by both the comet assay (Table 2) and the MN test (Fig. 1B) was similar in the TFB_1, $TAFB_1$ and TM cells. Curiously, the extent of the genetic lesions shown by the comet assay in the latter group, mainly depicted by the percentage of cells showing damage scored as 3, was lower than that found in SMC exposed *in vitro* to the toxins individually, suggesting a possible protective action of the mycotoxin mixture against genetic damage (Table 2). However, further research needs to be carried out in order to elucidate the exact meaning of this result.

The culture materials containing known levels of fumonisins have been widely used to characterize the toxicity of these compounds in laboratory and farm animals (Asrani et al., 2006; Deshmukh et al., 2007; Theumer et al., 2008, 2003). In spite of this, other factors than the test ones may be present in culture materials and thus to interfere either negatively or positively in the changes induced by the toxin of interest. Scarce information is available on the eventual *in vivo* genetic damage induction by fumonisins, and by FB_1 in particular, with this being the first report regarding the possible genotoxic action of fumonisins intake through a subchronically induced mycotoxicoses in rats, where a possible

fumonisins-AFB_1 interaction to produce DNA lesions was also evaluated. Such experimental designs could have some advantages with regards to acute exposures, since integrated information on the possible genotoxicity of a substance as well as the adaptive response of the subchronically poisoned animals would be evidenced by assessing any eventual genetic injury.

In this experimental model, the oral poisoning of rats with fumonisins caused DNA damage, which was shown by means of the alkaline comet (Table 1) and the MN (Fig. 1A) tests. These results agree with those reported for intraperitoneal administrations of FB_1 by Domijan et al. (2007) and Aranda et al. (2000), who noted increases in the tail length and tail intensity when the comet assay was performed in rat renal cells, and a rise in the frequency of MN in mice bone marrow polychromatic erythrocytes, respectively. Under the experimental conditions used in this study, the extent of the genetic lesions was lower in the FED group, than in the AFB_1ED or MED groups. Furthermore, while almost half of the comets analyzed in the latter two groups was scored as type-4 damage, for comets in FED type-3 damage was the most common (Table 1), suggesting that the DNA lesions observed in MED had been produced by a major contribution of the AFB_1 toxicity. Moreover, the fraction of undamaged cells in MED and AFB_1ED (2%) groups was almost zero compared to FED (18.3%), probably due to saturation effects that could be present in SMC from animals poisoned with both diets containing AFB_1. However, this was not the case in the findings of the MN technique, where the extent of the genetic lesions, determined by the percentage of micronucleated cells, was lower in MED than in the AFB_1ED group (Fig. 1A). These results mimic the ones obtained in the comet assay of SMC exposed *in vitro* to the toxins. However, spleen hematopoesis in response to a higher toxicity could be influencing the outcome of MN induced by the *in vivo* exposure to the mycotoxins.

Oxidative stress arises when the generation of ROS, by-products of the oxidative metabolism primarily produced in the mitochondria, exceeds the cellular ability to eliminate them and to repair cellular damage, thus leading to oxidation of biomolecules including DNA, lipids and proteins (Hwang and Kim, 2007). Oxidative genetic damage is a general definition referring to all types of changes (structural or functional) in the DNA due to its interaction with ROS, and may be an initial step in the genotoxicity induced by xenobiotics.

In this work, we observed that fumonisins increased the TBARS levels, which is suggestive of increased lipid peroxidation. This finding is similar to that of Abel and Gelderblom (1998) in a 21-day poisoning of rats, and is in agreement with the increases reported in the TBARS concentrations detected in several cell lines exposed *in vitro* to FB_1 (Klaric et al., 2007; Mobio et al., 2003; Stockmann-Juvala et al., 2004). However, in contrast with the observations of Galvano et al. (2002a,b) in rat astrocytes and in human fibroblasts, we found a direct correlation between genetic damage and biomarkers of oxidative stress in SMC exposed *in vivo* and *in vitro* to the fumonisins, supporting the hypothesis of an oxidative stress mediated indirect genotoxicity of FB_1.

As mentioned previously in the present study, the genotoxic action of AFB_1 is already acknowledged, and this toxin has been classified as carcinogenic for humans, Group 1 (IPCS-WHO, 1998). Also, it is widely recognized that AFB_1 is capable of inducing oxidative damage in cells, with most of the evidence generated by means of studies with hepatocytes or subcellular fractions of them. In this context, it is difficult to establish the degree of the contribution of the oxidative stress to the observed genotoxicity of AFB_1, due to most of the genetic damage in hepatocytes expected to be a consequence of the metabolic conversion of the toxin into its *exo*-epoxide in cells, which, on the other hand, are highly specialized in detoxifying xenobiotics.

Toxicokinetic considerations must be taken into account when the correct interpretation of laboratory data regarding the toxicology of oral exposures to AFB_1 is attempted. Similarly, the intrinsic and any eventual exogenously improved metabolic capacity of cells should also be considered in order to obtain a better understanding of data generated by *in vitro* exposure of metabolically competent or incompetent cells to the toxin.

In this work, the subchronic exposure of rats to a diet contaminated with AFB_1 produced genetic damage and increased the levels of oxidative stress biomarkers in SMC, which was in agreement with the studies of Shen et al. (1994), where a single dose of AFB_1 stimulated lipid peroxidation and oxidative damage Shen et al. (1995). On the other hand, the *in vitro* exposure of normal SMC to AFB_1-induced genetic damage, which was observed by the MN technique and the alkaline comet assay, and increases in the levels of oxidative stress biomarkers. However, the precise contribution of the ROS to the genotoxicity observed in the poorly metabolically specialized SMC remains unclear.

The interaction of fumonisins and AFB_1 in the induction of DNA damage and its correlation with biomarkers of cellular oxidative status was also evaluated in this work. Similarly to the findings in the histopathological alterations as well as in the sphingoid bases imbalance induced in this experimental mycotoxicoses (Theumer et al., 2008), a clear synergism of the tested fungal toxins to induce genetic damage was not found. The *in vitro* exposure to the mycotoxin mixture had a protective behavior against the genotoxicity induced individually by the toxins, when the genetic damage was assessed by the alkaline comet assay. Such unexpected results are of special interest, and future research should be focused on further characterization of these effects.

As clearly stated by Blumberg (2004), measuring the oxidative stress can be difficult due to the complexity of the endogenous systems involved in correction and repair (e.g., as may occur when a brief increase in oxidative stress rapidly induces various antioxidant defenses, such as the antioxidant enzymes SOD, CAT, and glutathione peroxidase), which quickly reduce the stress and limit the ability of testing methods to detect a change. It was previously reported the absence of change in the basal levels of H_2O_2 released by adherent peritoneal cells, when exposed *in vivo* and *in vitro* to fumonisins (Theumer et al., 2002), AFB_1, or to fumonisins-AFB_1 mixtures (Theumer et al., 2003). However, the results from the present work do not contradict those in our previous reports, because the absence of modifications in the H_2O_2 release could be a consequence of higher ROS scavenging by antioxidant enzymes, induced by a sustained aggression of the mycotoxins.

Summing up, the data presented in this paper support the theory of an indirect genotoxic action of fumonisins on mammalian cells that could be related somehow with the previously reported immunotoxic effects induced by aflatoxins and fumonisins, individually or as mixtures. Such genetic lesions could be mediated by the oxidative stress induced by the toxins and the subsequent widespread oxidation of biomolecules including the DNA. Furthermore, in the comet assay the mycotoxins mixture seemed to protect the cells against the genetic damage induced by the *in vitro* exposure to the toxins individually. However, the mechanisms underlying these effects are still unclear.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was partially supported by grants form Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SeCyT-UNC), Agencia Nacional de Ciencia y Tecnología grants PICT

2005. No. 15-32256 and PICTO-UNC 2005. No. 35857, and Agencia Córdoba Ciencia, grant No. 0279-005429/2006. Cánepa MC and Dambolena JS are recipients of fellowships from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Theumer MG and López AG are career investigators of the later institution.

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