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ScienceDirect
Food and Chemical Toxicology 46 (2008) 967–977
www.elsevier.com/locate/foodchemtox

Subchronic mycotoxicoses in rats. Histopathological changes and modulation of the sphinganine to sphingosine (Sa/So) ratio imbalance induced by *Fusarium verticillioides* culture material, due to the coexistence of aflatoxin B1 in the diet

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Received 6 March 2007; accepted 19 October 2007

Abstract

Mycotoxicoses are diseases caused by consumption of diets contaminated with mycotoxins, a special class of fungal secondary metabolites. Fumonisin B1 (FB1) and aflatoxin B1 (AFB1), the main toxins synthesized by toxicogenic stocks of *Fusarium* spp. and *Aspergillus* spp., respectively, can coexist in grains and in its by-products. We investigated a probable synergism of a fumonisins-containing *Fusarium verticillioides* culture material and AFB1 in the induction of hepatocyte apoptosis in rats subchronically fed on a mixture of them. Furthermore, the possibility of modifications in the fumonisins-induced Sa/So ratio imbalance in tissues and urine from rats poisoned with this mycotoxin, due to the presence of AFB1 in the diet, was evaluated. The co-exposure to fumonisins and AFB1 produced a higher liver toxicity, with respect to their individual administration, inducing apoptosis and mitotic hepatocytes. There was an inversion of the typical Sa/So ratio in rats fed on the culture material as well as in those subjected to a diet co-contamined with fumonisins and AFB1. Moreover, the later had a synergistic effect in the induction of Sa/So variations in kidneys. Therefore, the mixture of fumonisins and AFB1 induced toxic responses which could not be considered a sum of the effects caused individually by these mycotoxins.

Keywords: Mycotoxins; Fumonisins; Aflatoxin B1; Biomarkers; Apoptosis

Abbreviations: FCM, *Fusarium verticillioides* culture material; AFB1, aflatoxin B1; FB1, fumonisin B1; CED, animals fed with the control experimental diet ($n = 6$); FED, rats poisoned with the experimental diet with fumonisins ($n = 6$); AFB1ED, rats poisoned with the experimental diet with 40 ppb of AFB1 ($n = 6$); MED, experimental diet containing the mixture of AFB1 (40 ppb) and fumonisins (FB1:100 ppm) ($n = 6$); HCC, hepatocellular carcinoma; Sa, sphinganine; So, sphingosine; Sa-1-PO_4, sphinganine 1 phosphate; So-1-PO_4, sphingosine 1 phosphate; POD, peroxidase.

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doi:10.1016/j.fct.2007.10.041

1. Introduction

Mycotoxicoses are toxic responses induced in human beings and in animals exposed to mycotoxins by a natural route (Bennett, 1987). At present, cases of human acute mycotoxicoses through consumption of food contaminated with high levels of mycotoxins are rarely registered, usually only occurring in some developing countries (Peraica et al., 1999). Other types of mycotoxicoses, in which small amounts of toxins (mainly aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone) enter the organism during a few months (subchronic) or over several months or years (chronic), represent the main problems for human and animal health in the world.

Fumonisin B1 (FB1) and aflatoxin B1 (AFB1), the main mycotoxins synthesized by toxicogenic stocks of *Fusarium* spp. (*Fusarium verticillioides* and *Fusarium proliferatum*) and *Aspergillus* spp. (*Aspergillus flavus* and *Aspergillus parasiticus*), respectively, can coexist in foods destined for human and animal consumption (Ono et al., 2001; Vargas et al., 2001). Moreover, the intake of maize co-contaminated with high levels of both toxins was also related with a high incidence of human primary hepatocellular carcinoma (HCC) in some areas of Guangxi, Republic of China (Li et al., 2001). These data, together with similar results provided by other authors, seem to indicate that the natural cereal contamination with an individual mycotoxin could be an exception, rather than a rule (Ono et al., 2001; Vargas et al., 2001).

There exists a correlation between the chronic exposure at high levels of fumonisins and the higher incidence of esophagus cancer in the population of Transkei (South Africa), China and Italy (Sydenham et al., 1990; Chu and Li, 1994; Doko and Visconti, 1994). However, neither a direct relationship between dietary exposure to fumonisins and esophagus cancer, nor the precise mechanisms involved in the neoplasic induction by these toxins were clearly established.

Fumonisins have immunotoxic, hepatotoxic and carcinogenic properties (Gelderblom et al., 1991, 1996; Theumer et al., 2002; Voss et al., 2002). The data available to date about the toxicology of fumonisins suggest an alteration of the cellular lipid metabolism as one of the main mechanisms of the toxic action induced by these toxins (Riley et al., 2001). The ceramide sintetase activity inhibition by the fumonisins produces a rapid increase in the intracellular concentrations of sphinganine (Sa) and, to a lesser extent, of sphingosine (So), inducing an inversion of the normal intracellular Sa/So ratio, which can be detected in peripheral blood and urine (IPCS-WHO, 2000). However, the usefulness of this Sa/So ratio inversion as a biomarker of exposure to these toxins, for animals or for human epidemiology, is currently under evaluation (Qiu and Liu, 2001; Tardieu et al., 2006; Tran et al., 2006).

Fumonisins could produce alterations in the lipid-mediated signals for the cell growth, differentiation and death. Several studies indicate that fumonisins induce changes in enzymatic activities involved in the cell cycle regulation, and also in the cellular differentiation and apoptosis (Pinelli et al., 1999; Mobio et al., 2000). In this way, some of the immunotoxic effects of the fumonisins (Theumer et al., 2002) could be a consequence of changes in the expression, or in the biological activity of some cell surface receptors, important in the immunological signaling mechanisms (Martynova et al., 1995).

Acute human aflatoxicosis cases are not common at present (Peraica et al., 1999). One of the most severe outbreaks of acute aflatoxicosis was reported in Kenya in the year 2004, which was related to the consumption of contaminated maize, affecting more than 300 persons, and with a case fatality rate of 39% (Muture and Ogana, 2005). Nevertheless, the chronic ingestion of low aflatoxins levels has been associated with the high proportion of primary HCC observed in regions of Italy, China and Africa (Pitt, 2000).

The main toxic effects of AFB1 take place when its metabolite AFB1-*exo*-8,9-epoxide forms aducts with the DNA guanines (Guengerich et al., 1998), causing disruption of transcription and abnormal cell proliferation, and thus increasing the probability of neoplasic cellular transformation. Under normal conditions, the affected guanine is replaced by a new molecule of guanine by means of a depurinating reaction. If the DNA damage persists, the cells undergo cell death (apoptosis) unless there is a selective clonal advantage.

Apoptosis could be considered an effective process for the elimination of transformed cells. However, its inhibition may also be a sort of natural selection for those cells that acquire resistance to the normal death mechanisms, assuring tumor progression. Free sphingoid bases, and their 1-phosphates, cause strong pro- (So, Sa) or anti-apoptotic (So-1-PO_4) activities, and their relative levels have been proposed to be a rheostat that determines the cell's fate (Riley et al., 2001).

Fumonisins–aflatoxins interactions started to be evaluated in recent years (Carlson et al., 2001; Gelderblom et al., 2002; McKean et al., 2006), and the participation of both group of mycotoxins to induce the pathologic responses was demonstrated in most of the cases.

The aim of this work was to evaluate a possible combined action of a fumonisins-containing *F. verticillioides* culture material and AFB1, in the induction of apoptosis in hepatocytes from rats subchronically fed on a mixture of them. The likely modifications in the fumonisins-induced Sa/So ratio imbalance in tissues and urine, due to the presence of AFB1 in the diet, were also studied.

2. Materials and methods

2.1. Animals

Male Wistar inbred rats (6–8 weeks old, weight 316 ± 7 g, $n = 6$) were housed in pairs in stainless-steel cages, and kept in environmentally-controlled rooms with 12-h light/dark cycles. Animals were housed and cared for in the animal resource facilities of the Department of Clinical Bio-

chemistry, Faculty of Chemical Sciences, National University of Córdoba, in accordance with institutional guidelines.

2.2. Mycotoxins

2.2.1. Preparation of Fusarium verticillioides culture material (FCM) extracts

prepared as previously described by Casado et al. (2001). Briefly, maize (300 g) was placed in 1000-ml Erlenmeyer flasks at 35% humidity and sterilized on two consecutive days in autocleave 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 an fumonisins-producer fungal strain. After inoculation of the fungus, the maize was incubated for 28 days in the dark at 25 °C, with manual stirring performed during the first 5 days. An aqueous extract of FCM was prepared following a procedure previously described (Voss et al., 1990). Briefly, the maize was dried in a stove 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 before being centrifuged at 3500 rpm for 10 min, and the supernatant recovered. Then, the supernatants were homogenised and stored at −20 °C until use.

2.2.2. Fumonisins quantification

Samples (100 μl) obtained from the FCM extracts were diluted with acetonitrile (100 μl). Before the quantification essays, the 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 (Shephard et al., 1990). Briefly, an aliquot (50 μl) of the diluted extract was derivatised 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 FB1 (CAS 116355-83-0), FB2 (CAS 116355-84-1) and FB3 (CAS 136379-59-4) were detected and quantified with a Hewlett Packard HPLC equipped with a fluorescence detector. The wavelengths used were 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 used. 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 to those corresponding to analytical standards of FB1, FB2 and FB3 (purity > 95%), provided by PROMEC, Republic of South Africa.

Fumonisins were detected in the FCM extract in a 4.46:1.00:2.03 ratio for FB1, FB2 and FB3, respectively.

2.2.3. Preparation of AFB1 extracts

AFB1 crystalline (CAS 1162-65-8; Sigma, purity > 98%) in benzene–acetonytrile (98:2 v/v) was checked for purity and then spectrophotometrically quantified. An aliquot of this solution was dried in a rotatory evaporator. Then AFB1 was dissolved in olive oil at a final concentration of 1 mg/ml, and kept at −20 °C until diet preparation.

2.3. Diets

2.3.1. Commercial basal diet

Commercial basal diet (mice–rats, Cargill S.A.C.I., Saladillo, Buenos Aires, Argentina), free from fumonisins and aflatoxins, contained total protein > 24%, ether extract > 6%, raw fibre > 7%, calcium > 1%, phosphorus > 0.5%, moisture < 13%, total minerals < 8% and energetic value > 2780 kcal/kg.

2.3.2. Control experimental diet

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 moulded, and after solidification, they were stored at −20 °C until being used.

2.3.3. Experimental diet with fumonisins

Prepared as the control diet, using FCM extract obtained as described above, in order to obtain a final FB1 concentration of 100 ppm in the food.

2.3.4. Experimental diet with AFB1

Prepared as the control diet, adding 75.4 μl of AFB1-containing olive oil, in order to obtain a final AFB1 concentration of 40 ppb.

2.3.5. Experimental diet with the mixture of fumonisins and AFB1

Prepared as the control diet, adding 75.4 μl of AFB1-containing olive oil, and FCM extract obtained as described above, in order to obtain final concentrations of 40 ppb and 100 ppm for AFB1 and FB1, respectively.

2.4. Experimental model

Four groups of rats were used. Animals were fed on the control experimental diet (CED) ($n = 6$), on the experimental diet with fumonisins (FED) ($n = 6$), on the experimental diet with AFB1 (AFB1ED) ($n = 6$), and on the experimental diet with the mixture of these toxins (MED) ($n = 6$). The rats were housed in pairs in different cages and fed on the different diets for 90 days. The food ration was replaced daily. Animals were weighed at the beginning and at the end of the experimental scheme. At the 90th day of being fed, the rats were individually housed in metabolic cages in order to obtain 24-h urine samples. After this period, blood from each animal was obtained by intracardiac puncture. The rats were killed by cervical dislocation, and then organs specimens were collected for tissue microscopic examination and quantification of biomarkers of exposure to FB1.

2.5. Food consumption, body weight and mycotoxins intake

The daily food consumption was calculated from the difference between the weight of the portions given and uneaten. The body weight was determined on an Ohaus scale, Florham Park, NJ, USA with a precision of 0.05 g. The total AFB1 and FB1 consumption were calculated by taking the food consumption and the toxins concentrations in the food into account. The results are expressed in relation to body weight.

2.6. Tissue examination

2.6.1. Haematoxylin and eosin staining

Specimens of lung, liver, kidney, and small intestine were obtained on the 90th day of feeding, and then were fixed in 10% neutral buffered formalin (pH 7.2). Haematoxylin and eosin stained sections (4 μm thickness) were examined by light microscopy. Photomicrographs were taken with a Zeiss Axiophot instrument using Kodak Plus-X pan (PX 135 to 24) film.

2.6.2. Tunel test

For identification of apoptotic cells in fumonisins and AFB1 target organs, the Tunel test was performed on paraffin sections of tissues prepared as described above. A commercial kit (In Situ Cell Death Detection Kit, POD; Cat. #1 684 817, Roche Diagnostics) was used for this purpose; following to the manufacturer's specifications. Briefly, the paraffin embedded specimens were hydrated and incubated, firstly with dUTP and the terminal desoxinucleotidil transferase (TdT) enzyme, and then with an anti-dUTP-peroxidase (POD) antibody. The nuclei with fragmented DNA were stained by a diaminobencidine immunoprecipitation reaction, while

the cells without DNA fragmentation were colored with a methyl green counterstain. Samples were analyzed by optical microscopy, and photomicrographs were taken with a Zeiss Axiophot instrument using Kodak Plus-X pan (PX 135 to 24) film.

Mitotic (liver) and apoptotic (lung, liver and kidney outer medulla) cells were counted in ten random microscopic fields (magnification: 400×). Then, media values and standard error were calculated for each tissue.

2.7. Quantification of biomarkers of exposure to fumonisins

2.7.1. Preparation of urine, liver and kidney samples for the quantification of sphinganine (Sa) and sphingosine (So)

The samples were analyzed according to procedures previously described (Solfrizzo et al., 1997, 2001).

Briefly, tissues (90 mg) were homogenized with 3 ml of phosphate buffer (8 g NaCl; 1.2 g Na_2HPO_4; 0.2 g KH_2PO_4 and 0.2 g KCl in approximately 990 ml of bidistilled water; the pH was adjusted to 7 with concentrated HCl, and then it was completed to 1 l with bidistilled water). After homogenate centrifugation, the supernatants were collected for the sphingoid base quantification.

The urine samples (2 ml), and the tissue homogenate supernatants, were diluted with 2 ml of methanol, alcalinized with 1.2 ml of 0.35 M NH_4OH, and then extracted with 4 ml of $CHCl_3$. After centrifuging and discarding the aqueous phase, the chloroformic extracts were washed twice with 4 ml of alkaline water (0.6 ml of 0.35 M NH_4OH in 250 ml of bidistilled water), thus recovering the organic phase. The chloroformic extracts (2 ml) were then transferred to silica gel minicolumns for the interferent elimination.

2.7.2. Preparation of serum samples for the Sa and So quantification

This was done according to a methodology previously described (Shephard and van der Westhuizen, 1998). Briefly, 500 μl of serum was deproteinized with 2 ml of CH_3OH, and then centrifuged for 10 min at 1200*g* and 10 °C. An aliquot of the supernatant (1.9 ml) was mixed with water (1.9 ml) and NH_4OH 0.35 M (1.2 ml), and was then extracted with 4 ml of $CHCl_3$. After mixing energetically, the phases were separated by centrifugation at 1200*g* and 10 °C for 10 min. An aliquot of the chloroformic phase (3 ml) was transferred to a silica gel minicolumn to eliminate the interferents.

2.7.3. Interferent elimination with silica gel minicolumns

The chloroformic extracts from urine (2 ml), tissues (2 ml) and sera (3 ml), were cleaned by passing them through polypropylene columns containing 5 g of anhydrous Na_2SO_4 crystals packed on top of 0.2 g of silica gel 60 (15–40 μm). The minicolumns were preconditioned with 3 ml of $CHCl_3$ (maintaining a flow rate < 2 ml/min), the chloroformic extracts were eluted, and then the columns were washed with 1 ml of $CHCl_3$, discarding the eluate. The sphingoid bases (Sa and So) were eluted with 4 ml of a $CHCl_3$:CH_3OH:NH_4OH (50:50:2) solution, thus evaporating the solvent from this eluate.

For the serum samples, the residues obtained in the previous step were dissolved with 1 ml of a 0.125 M KOH solution in CH_3OH:$CHCl_3$ (4:1, v:v), and were incubated at 37 °C for 90 min. Then, 1 ml of $CHCl_3$ was added to each sample, and the organic phase was washed with alkaline water (0.6 ml of NH_4OH 0.35 M in 250 ml of bidistilled water). The chloroformic phases were recovered by centrifugation for 10 min at 1200*g* and 10 °C; before being dried.

The dried residues obtained from urine, tissues and sera were dissolved with 250 μl of CH_3OH:H_2O (9:1). They were then derivatized with 50 μl of an *o*-pthaldialdehide (OPA) reagent, and were analyzed by reverse-phase HPLC. The OPA reagent was prepared by adding 10 ml of boric acid 3% (pH adjusted to 10.5 with KOH); to a solution prepared with 5 mg of OPA dissolved in 100 μl of CH_3OH and 5 μl of 2-mercaptoethanol.

2.7.4. Quantification of Sa and So levels by HPLC

This was done according to a methodology previously described (Shephard and van der Westhuizen, 1998); using a Hewlett Packard 1100 HPLC equipped with a fluorescence detector. The wavelengths used were 335 and 440 nm for excitation and emission, respectively. The separation of the sample components was made in a C_{18} column (150 mm × 4.6 mm; particle size of 5 μm), connected to a pre-column C_{18} (20 mm × 4.6 mm; particle size of 5 μm). As the mobile phase, a CH_3OH:H_2O (9:1, v:v) solution was used at a flow rate of 1.5 ml/min. The Sa and So levels were calculated by extrapolating the peak areas obtained for the problem samples, in a calibration curve constructed by injection of commercial standards of Sa and So (44.4; 177.7 and 355.3 ng/ml).

2.8. Statistical evaluation

Data from these studies were analyzed by two-tailed ANOVA. Results giving p values $\leqslant 0.05$ were considered significantly different, and further analyzed by the Tukey–Kramer post hoc test. Differences between groups were considered statistically different for p values $\leqslant 0.05$.

3. Results

3.1. Food consumption, body weight, and mycotoxins intake

The Table 1 summarizes the daily food consumption, the total amounts of mycotoxins ingested, and the body weight of the animals at the end of the experimental period.

The rats belonging to the FED group consumed lower amounts of food, and a decrease in their body weights was detected with respect to the controls. No significant differences were registered in the total fumonisins and AFB1 intakes in group MED, with respect to those observed in FED and AFB1ED, respectively.

3.2. Examination of tissues

The incidence and severity of the main histopathologic findings in the rats fed on the different experimental diets are depicted in Table 2.

The main histopathologic alterations in group AFB1ED were observed in the lungs, where thickening of the alveolar walls and lymphocytic infiltrates were found (Fig. 1A). However, these alterations were not observed in the CED group (Fig. 1B).

The liver and the kidney were the main target organs of the fumonisins-AFB1 mixture. The most important alterations were registered in the liver, where mitotic cells (Fig. 1C, arrow head) and hepatocytes with morphologic characteristics compatible with apoptosis (Fig. 1C, arrow) were observed. A comparison with the observations in tissues coming from the CED group can be seen in Fig. 1D. On the other hand, in the kidneys of these animals, tubular cells with morphologic characteristics compatible with those seen in the apoptosis were observed (Fig. 1E, arrows), whereas these changes were not found in the CED group (Fig. 1F). Also, in the MED group, animals were observed with lymphocytic infiltrates (vellocitis) in the small intestine (Fig. 1G, arrow heads), and with thickening of the alveolar walls and lymphocytic infiltrates in the lungs (data not shown) similar to those registered in the AFB1ED. However, these alterations were not found in the rats from the CED group (Fig. 1H and B, respectively).

Table 1
Summary of food consumption, body weight and mycotoxins intake[1]

Group[2]	Daily food consumption (g)	Body weight (g)	Mycotoxin intake (kg bw)	
			AFB1 (μg)	FB1 (mg)
CED	45.57 ± 2.95	415.65 ± 5.32	–	–
FED	36.38 ± 2.33[a]	373.87 ± 3.75[a]	–	901.13 ± 20.21
AFB1ED	45.53 ± 1.21	444.4 ± 3.26	353.9 ± 10.6	–
MED	36.86 ± 2.38	354.2 ± 17.5[b]	349.4 ± 17.5	864.8 ± 42.5

[a] $p < 0.05$; [b] $p < 0.001$ when groups were compared with the control (CED).
[1] Data are expressed as means ± SE.
[2] CED: Control experimental diet; FED: diet containing fumonisins; AFB1ED: diet containing AFB1; MED: diet with the fumonisins–AFB1 mixture.

Table 2
Incidence and severity of the main alterations found in mycotoxins target organs[1]

Organ	Type of lesion	Experimental group[2]			
		CED	FB1ED[3]	AFB1ED	MED
Lung	Alveolar walls thickening and lymphocytic infiltrates	ND	ND	D (6/6)	D (6/6)
	Apoptosis	ND	ND	6.2 ± 1.6[b] (6/6)	6.7 ± 1.6[b] (6/6)
Liver	Mitosis	ND	ND	ND	2.7 ± 0.6[b] (5/6)
	Apoptosis	ND	ND	ND	1.9 ± 0.4[b] (4/6)
Kidney	Tubular apoptotic cells	ND	1.0 ± 0.3[a] (4/6)	ND	3.0 ± 0.1[b,A] (6/6)
Small instestine	Vellocitis	ND	D (6/6)	ND	D (6/6)

ND: not detected; D: detected.
Values between brackets indicate the incidence of the lesion in the rats belonging to each group.
[a] $p < 0.01$; [b] $p < 0.001$ when groups were compared with the control (CED).
[A] $p < 0.001$ when MED was compared with FED.
[1] Light microscopy examination was performed in hematoxylin-and-eosin-stained sections. The score of mitotic (liver) and apoptotic (lung, liver and kidney) cells were computed in the Tunel-stained tissue sections.
[2] CED: Control experimental diet; FED: diet containing fumonisins; AFB1ED: diet containing AFB1; MED: diet with the fumonisins–AFB1 mixture.
[3] Data already published by our research group (Theumer et al., 2002).

3.3. Tunel test

Liver, lung and kidney samples from each animal were analyzed by means of the Tunel test to identify the apoptotic cells. Figs. 2 and 3 show representative images of the findings during microscopic examination of animal tissues from all the experimental groups.

Apoptotic cells were found in the pulmonary alveolar wall thickening in the AFB1ED group (Fig. 2A, arrows), whereas in group CED, apoptosis was not detected in the lung sections (Fig. 2B). Apoptosis was also observed in the lungs of rats belonging to the MED group (data not shown), but the score of apoptotic cells in these animals was similar to that registered in the AFB1ED group (Table 2).

The Tunel test was also performed on liver and kidney samples from FED, in order to confirm previous results reported by our group using this experimental model (Theumer et al., 2002), and the test was also carried out on samples from MED rats.

Apoptotic cells were not found in the FED liver specimens (Fig. 3A), similar to the observation in the CED group (Fig. 3E). On the other hand, apoptosis (Fig. 3C, arrows) and a high proportion of mitotic hepatocytes (Fig. 3C, arrow heads) were identified in MED. The study of the kidneys enabled the identification of apoptotic tubular cells in groups FED (Fig. 3B, arrow) and MED (Fig. 3D, arrow), whereas these cell modifications were not found in the rats belonging to CED (Fig. 3F). A higher incidence of apoptosis was observed in the kidneys of the MED group rats, compared to that registered in FED (Table 2).

3.4. Quantification of Sa and So levels in urine, serum and tissues

Table 3 summarizes the mean Sa and So concentrations, and the Sa/So ratios found in urine, serum, liver and kidney samples.

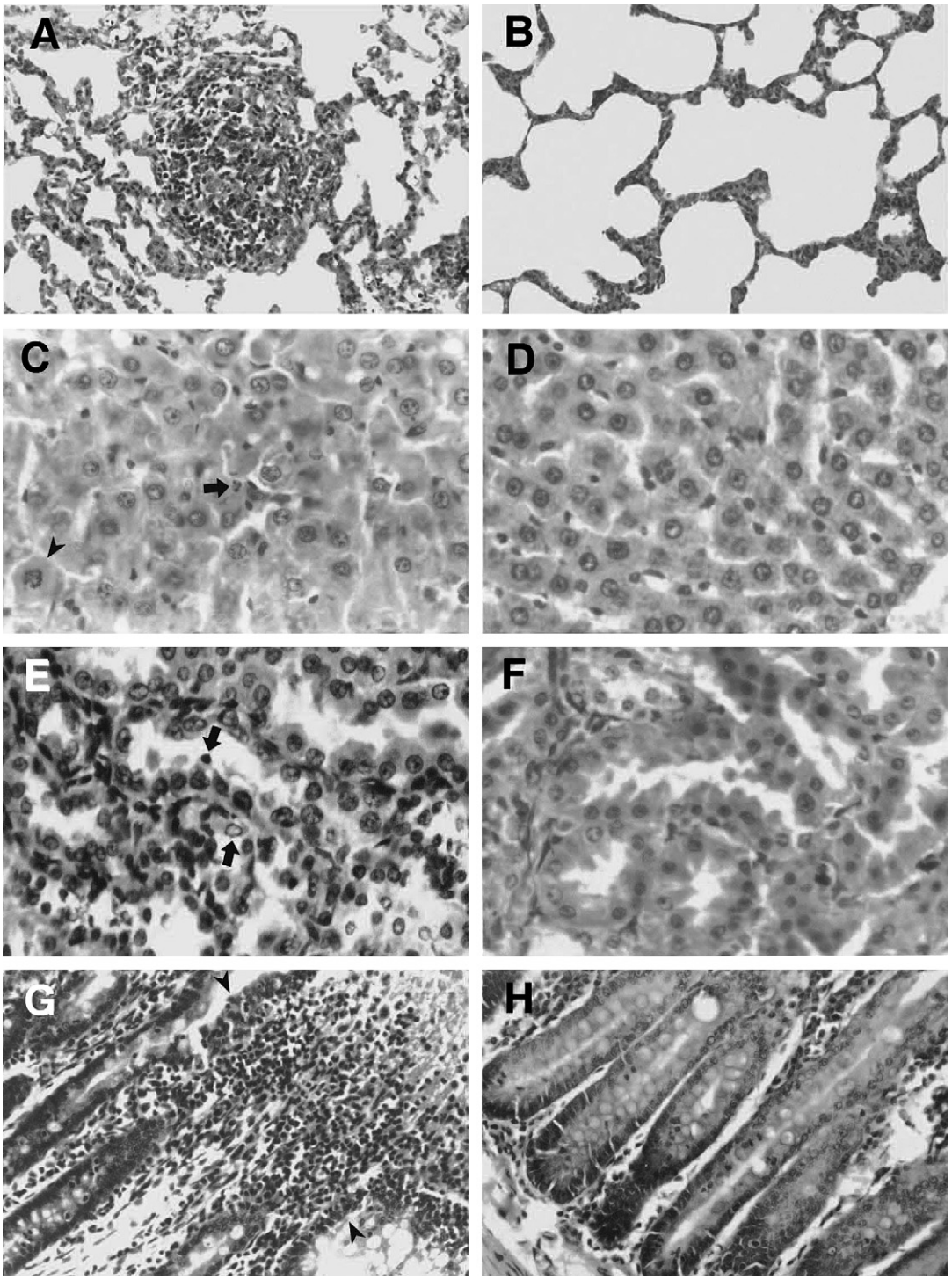

Fig. 1. Haematoxylin-and-eosin-stained sections of rat organs from groups AFB1ED (A), MED (C, E and G) and CED (B, D, F and H). The main alterations in AFB1ED were observed in the lung, where thickening of alveolar walls and lymphocytic infiltrates were found (A), compared to the lungs from animals fed for 90 days with the mycotoxin-free diet (B). Mitotic cells (C, arrow head) and apoptotic hepatocytes (C, arrow) were observed in livers from MED group rats, while these alterations were not found in the CED group (D). Furthermore, histopathologic findings in animals fed on the fumonisins–AFB1 mixture included apoptotic tubular cells in kidneys (E, arrows) and lymphocytic infiltrates in the small intestine (G, arrow heads); whereas these changes were not observed in either the kidney or small intestine in the CED group (F and H, respectively). Magnifications: 400× (C, D), 200× (E, F) and 100× (A, B, G, H).

There was an inversion of the Sa/So ratio in urine from the FED and MED rats (Table 3), with the increase registered in this index being higher in the MED animals.

In the serum samples, there was also an increase in the Sa concentrations in the FED group. However, no significant changes in the absolute levels of So were found when

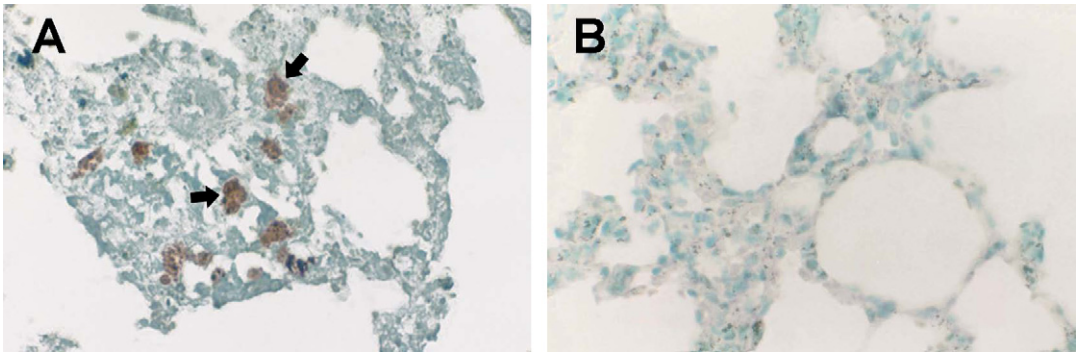

Fig. 2. Tunel-stained sections for identification and score of apoptotic cells in lung from groups AFB1ED (A), and CED (B). Apoptotic cells were identified in the pulmonary alveolar wall thickenings registered in the AFB1ED group (A, arrows), while apoptosis was not found in lungs from animals fed 90 days on the mycotoxin-free diet (B). Magnifications: 400× (A, B).

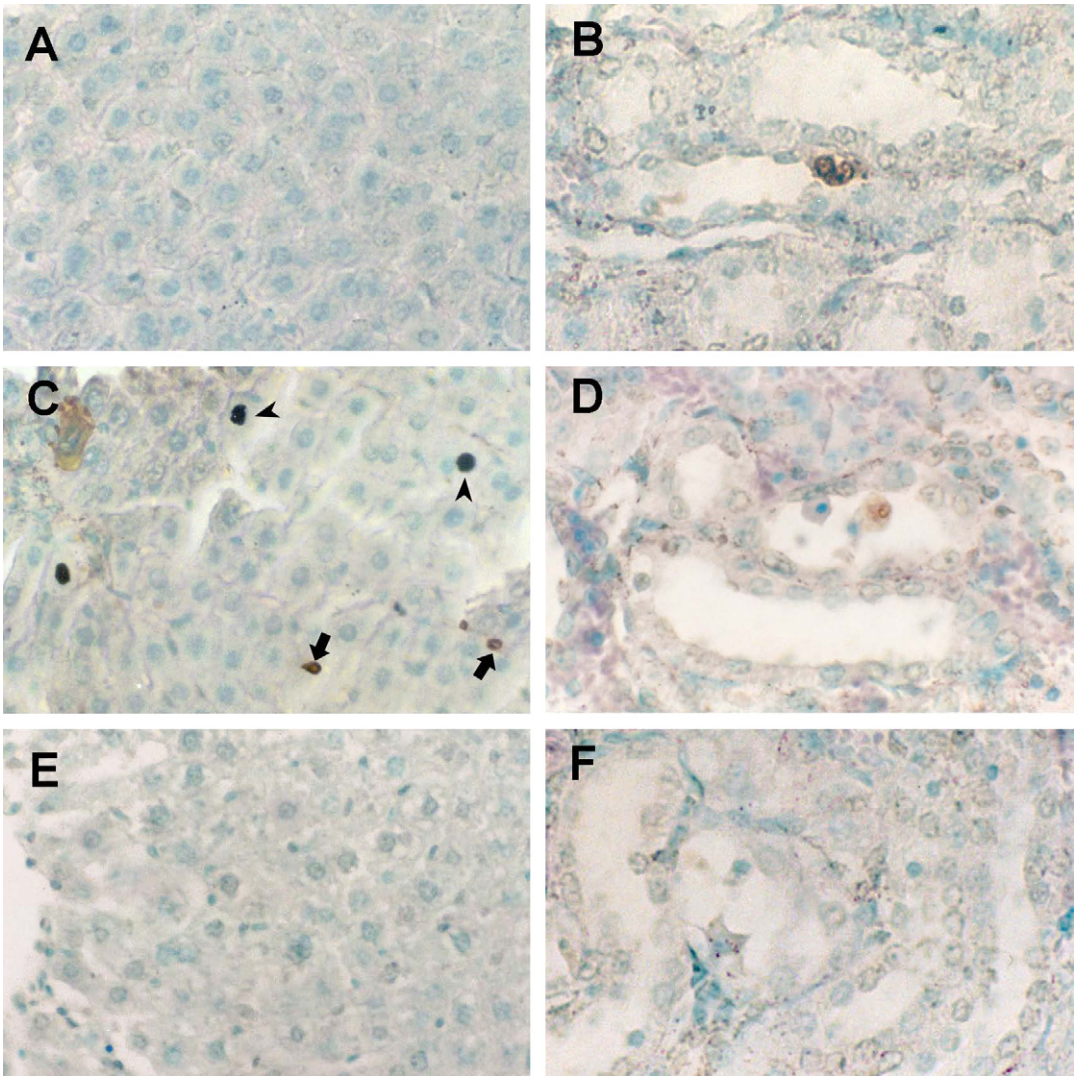

Fig. 3. Tunel-stained sections for identification of apoptotic cells in of liver and kidney from rats belonging to the groups FED (A, B), MED (C, D), and CED (E, F). Apoptotic cells were not found either in the livers from groups FED (A) or CED (E). However, apoptosis (C, arrows) and a higher proportion of mitotic hepatocytes (C, arrow heads) were identified in the livers of the MED group, compared to the observations in CED (E). Microscopic examination of Tunel-stained sections of kidneys revealed apoptotic tubular cells in groups FED (B, arrow) as well as in MED (D, arrow); whereas these changes were not found in the rats from the CED group (F). Magnifications: 200×.

Table 3
Sphinganine (Sa) and Sphingosine (So) concentrations, and Sa/So ratios in samples of urine, serum, liver and kidney from animals subchronically fed on the different experimental diets

Biological material	Group[2]	Sphingoid bases[1]		
		Sphinganine (Sa)	Sphingosine (So)	Sa/So ratio
Urine	CED	4.91 ± 0.22	15.15 ± 3.36	0.37 ± 0.06
	FED	214.47 ± 39.85^{a}	133.67 ± 24.24	1.72 ± 0.23^{a}
	AFB1ED	5.51 ± 0.97	13.25 ± 1.49	0.45 ± 0.08
	MED	$402.63 \pm 81.77^{c,A}$	207.51 ± 72.64^{b}	2.47 ± 0.60^{c}
Serum	CED	29.39 ± 6.64	67.10 ± 1.82	0.43 ± 0.09
	FED	113.81 ± 9.33^{c}	77.86 ± 5.82	1.48 ± 0.14^{c}
	AFB1ED	36.70 ± 0.10	88.36 ± 4.79	0.42 ± 0.01
	MED	$159.22 \pm 18.65^{c,A}$	87.90 ± 11.00	1.91 ± 0.22^{c}
Liver	CED	0.61 ± 0.01	1.75 ± 0.26	0.39 ± 0.07
	FED	13.78 ± 1.34^{c}	3.81 ± 0.57^{b}	3.97 ± 0.64^{c}
	AFB1ED	0.58 ± 0.01	1.37 ± 0.25	0.46 ± 0.07
	MED	14.46 ± 1.29^{c}	3.27 ± 0.43	4.50 ± 0.30^{c}
Kidney	CED	1.32 ± 0.10	2.85 ± 0.28	0.48 ± 0.05
	FED	19.07 ± 1.77^{b}	4.04 ± 0.35	4.81 ± 0.42^{b}
	AFB1ED	1.33 ± 0.07	2.80 ± 0.42	0.51 ± 0.12
	MED	$46.26 \pm 6.24^{c,B}$	3.33 ± 0.51	$14.54 \pm 1.35^{c,B}$

The Sa and So levels (ng/ml for urine and serum samples and μg/g for liver and kidney homogenates), were evaluated in duplicate in the samples coming from each animal.
$^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$ when groups were compared with the control (CED).
$^{A}p < 0.05$; $^{B}p < 0.001$ when groups FB1ED and MED were compared.
[1] Data are expressed as means ± SE, $n = 6$ per group.
[2] CED: Control experimental diet; FED: diet containing fumonisins; AFB1ED: diet containing AFB1; MED: diet with the fumonisins–AFB1 mixture.

comparing them with those observed in CED. The same tendency was registered in sera coming from the MED group rats, although a greater relative increase in Sa was found in these animals with respect to those found in the FED group.

The absolute and relative Sa levels observed in tissue homogenates from groups FED and MED were significantly greater than the ones registered in group CED. In the same samples, slight modifications in the So concentrations were identified, which were statistically significant only in the liver homogenates from the FED group. The changes in the Sa levels, and the absence of modifications in the So concentrations, caused increases in the Sa/So ratio in liver and kidney from FED and MED rats. Moreover, the Sa/So ratios in kidneys from the MED group were significantly greater than those observed in FED.

No significant differences were observed either in the Sa and So concentrations or in the Sa/So ratios in all the biological samples coming from rats belonging to the AFB1ED, compared to those found in the samples coming from CED.

4. Discussion

During recent years, interest in the behavior of aflatoxins and fumonisins mixtures in *in vitro* and *in vivo* systems has increased, with the poisonous effects of these mixtures on animal cells in culture being studied, and the first schemes of experimental poisoning in animals being carried out (Carlson et al., 2001; Gelderblom et al., 2002). Nevertheless, the administration route of the mycotoxins, a critical factor when studying the toxicity of these compounds, has not reproduced in all the cases what normally happens in nature.

Previous studies performed by our laboratory have led us to identify some general poisonous effects and immunobiological alterations, induced in an experimental subchronic mycotoxicosis model in Wistar rats fed on diets contaminated with AFB1, fumonisins, and on a mixture of them (Theumer et al., 2002, 2003). In the present work, the toxicity of a fumonisins–AFB1 mixture was further characterized, with special emphasis being laid on in the possible combined action that these fungal toxins could have from a toxicological point of view, compared to their individual effects.

The main histopathological changes in the AFB1ED group were found in the lungs, where pulmonary alveolar walls thickening and apoptotic cells were observed (Figs. 1A and 2A, respectively). Related to this, autoradiographies of [^{3}H]aflatoxin B1 in monkeys have enabled other authors to identify the nasal olfactory and respiratory mucosa, and also the mucosa of the nasopharyngeal duct, the pharynx, the larynx, the trachea and the oesophagus, as extra-hepatic binding sites for AFB1. The results from the present study may indicate that AFB1, or some of their metabolites, could also alter the normal structure of lung tissue in rats; and even participate in the induction of the lung parenchimal cell apoptosis observed in our experimental model (Fig. 2A, arrows).

Histopathological changes found in kidneys (apoptotic tubular epithelial cells, Figs. 1E and 3D) and in the small

intestine (lymphocytic infiltrates, Fig. 1G) in rats belonging to the MED group, were similar to those already published by our laboratory in a mycotoxicoses in rats induced by the subchronic consumption of a diet containing 100 ppm of FB1 (Theumer et al., 2002). However, the score of apoptotic cells was higher in the MED group, with respect to the observed in FED (Table 2). Also, the lung alterations registered in the MED group (data not shown) were similar to those found in animals belonging to AFB1ED (Figs. 1A and 2A). Taken together, these results seem to indicate that in this experimental model the fumonisins and the AFB1 have no additive effects on the induction of the intestinal and pulmonary histopathology found in the MED group. However, in the MED group the AFB1 increased by means of an unknown mechanism, the kidney injury caused by the ingestion of FED.

The liver structure in the MED animals was clearly altered (Figs. 1C and 3C) compared to the findings in the CED (Figs. 1D and 3E), and AFB1ED (data not shown) groups, or in rats subchronically poisoned with an experimental diet containing 100 ppm of FB1 (Theumer et al., 2002). Similar results were found in a short-term carcinogenesis model in rat liver, where the induction of GSTP(+) foci was significantly increased when both mycotoxins were administered in a sequential manner, in contrast with the individual AFB1 and FB1-treatments where no such increase was encountered (Gelderblom et al., 2002). A few apoptotic bodies in the central vein regions of the rats poisoned with FB1 were also observed in the former study.

The dose–response relationship for FB1 and organ-specific toxicity was studied in different laboratory animals (Voss et al., 1995). Hepatotoxicity signs were not found either in male or female Fischer 344 rats fed diets containing up to 81 ppm FB1 for 90 days. Moreover, significant interspecies differences in the no observed effect levels and organ-specific responses were found in these animals with respect to male and female B6C3F1 mice. These results could be closely related with our findings, where a subchronic poisoning with a diet containing *F. verticillioides* culture material extract with 100 ppm of FB1 did not induce hepatocyte apoptosis in male Wistar rats (Theumer et al., 2002).

In our experimental model, co-exposure to fumonisins and AFB1 produced greater toxic effects in liver from the MED group, inducing apoptosis and mitotic hepatocytes (Figs. 1C and 3C), while these alterations were found neither in the AFB1ED group (data not shown) nor in rats fed on a diet containing FB1 (Theumer et al., 2002). The lack of apoptotic hepatocytes in the former could be related to several factors including time, dose and type of exposure to fumonisins, and also to the susceptibility of the animals used in the present work to the toxic action of this mycotoxin.

Increases registered in the Sa and So levels, and particularly in the Sa/So ratio, which were detected before the appearance of other indicators of the cellular insult caused by fumonisins, have led in recent years to evaluate the possibility of using the Sa/So ratio in different biological samples as a biomarker of exposure to foods contaminated with these mycotoxins (Turner et al., 1999). In spite of this, it is still unknown whether the aflatoxins, which could be co-contaminant agents in the fumonisin-containing foods, can somehow modify the cellular Sa and So imbalance caused by exposure to this last group of toxins. In this work, a study was made of the probable modulation of AFB1 on the inversion of the Sa/So ratio caused by the subchronic ingestion of food contaminated with fumonisins.

Of all the biological samples studied, the liver and kidney homogenates presented the greatest increases in Sa, and the Sa/So ratio (Table 3). In our experimental model, poisoning with a fumonisins–AFB1 mixture produced significant increases in the Sa levels and in the Sa/So ratio in kidney, with respect to those observed in FED. Nevertheless, there were no significant differences when comparing these parameters in the livers, between animals belonging to the MED and FED groups. Although other fungal toxins, structurally related to the fumonisins, can induce imbalances in the Sa and So levels (van der Westhuizen et al., 1998); the studies made to date suggest that AFB1 does not produce modifications in the sphingolipid metabolism in liver (Norred et al., 1997). This hypothesis is somehow supported by our findings in the AFB1ED group, where no significant differences were observed neither in the Sa and So concentrations nor in the Sa/So ratios in all the biological samples studied, compared to those found in controls.

The variable susceptibility of different cellular types to the sphingolipid imbalance induced by fumonisins is related, at least partly, to the alternative metabolic pathways existing in cells to compensate for these changes. In some cellular types, including renal cells, a portion of the accumulated Sa can undergo additional metabolic reactions, and thus be redirected to other lipid biosynthesis routes (Riley and Voss, 2006).

The results obtained in this work appear to indicate that AFB1 could be interfering directly or indirectly, by some unknown mechanism, in the lipid metabolism of renal cells, leading to synergism with FB1 in the induction of the cellular sphingolipid imbalance. Consequently, the modulation of the renal sphingolipid metabolism by AFB1, and therefore the effects of this mycotoxin on the Sa/So ratio modifications induced in animals exposed to fumonisins in the diet, remain unclear. Further studies should be focused on clarifying these points.

The liver Sa/So ratio imbalance was observed in both the FED and MED groups. However, apoptosis was confirmed by the Tunel test only in the former. Since the sphingoid bases Sa and So, as well as some of their metabolites such as sphingosine-1-phosphate (So-1-P) and sphinganine-1-phosphate (Sa-1-P) are strong cell cycle regulators, proliferation or cell death by necrosis or apoptosis can result (Merrill et al., 1997; Watterson et al., 2003; van der Westhuizen et al., 2004). The Sa/So ratio was

increased in rat livers from the MED as well as from the FED groups (Table 3). However, hepatocyte apoptosis was observed only when AFB1 and fumonisins were administered as a mixture (Table 2). Several mechanisms could induce or prevent the hepatocyte apoptosis in our experimental model. In this sense, AFB1 could act as an enhancer of the fumonisins apoptotic activity to the hepatic cells, or vice versa. In the MED group, AFB1 could be blocking some pathway that prevents the hepatocytes undergoing apoptosis in rats fed on a diet containing fumonisins. On the other hand, the sphingolipid imbalance observed in the MED group livers (with respect to CED) could impair the depurination mechanisms that repair the DNA damage induced by the AFB1-*exo*-epoxide, the genotoxic derivative from AFB1, and thereby cause hepatocyte apoptosis.

Genotoxic and epigenetic xenobiotics may be involved in cancer development through different mechanisms, leading to imbalances in cell growth and death (Schulte-Hermann et al., 1999). Moreover, the rates of cell replication and death may vary during the initiation and promotion of cancer (Kountouras et al., 2003).

Future studies should reveal the action modes of AFB1–fumonisins mixtures in inducing hepatocyte proliferation and apoptosis, as well as clarifying the significance of these cell cycle alterations in different stages of liver cancer caused by subchronic and chronic consumption of diets co-contaminated with fumonisins and AFB1.

Acknowledgements

This work was supported by Agencia Nacional de Ciencia y Tecnología grant PICT 2005 No. 15-32256 and Agencia Córdoba Ciencia grant No. 0279-005429/2006.

M.G. Theumer is recipient of post-doctoral fellowship from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). M.P. Aoki is Member of the Research Career from CONICET, Argentina.

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