Genotoxicity of Aflatoxin B1 and Ochratoxin A after simultaneous application of the in vivo micronucleus and comet assay.

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Abstract (max. 200 words)

Aflatoxin B1 (AFB1) and Ochratoxin A (OTA) are genotoxic mycotoxins that can contaminate a variety of foodstuffs, the liver and the kidney being their target organ, respectively. The micronucleus (MN) assay (bone marrow) and the comet assay (liver and kidney) were performed simultaneously in F344 rats, treated with AFB1 (0.25 mg/kg b.w.), OTA (0.5 mg/kg b.w.) or both mycotoxins. After AFB1 treatment, histopathology and biochemistry analysis showed liver necrosis, focal inflammation and an increase in Alanine Aminotransferase and Aspartate Aminotransferase. OTA alone did not cause any alteration. The acute hepatotoxic effects caused by AFB1 were less pronounced in animals treated with both mycotoxins. With regard to the MN assay, after 24h, positive results were obtained for AFB1 and negative results were obtained for OTA, although both toxins caused bone marrow toxicity. In the combined treatment, OTA reduced the toxicity and the number of MN produced by AFB1. In the comet assay, after 3h, positive results were obtained for AFB1 in the liver and for OTA in the

kidney. The combined treatment reduced DNA damage in the liver and had no influence in the kidney. Altogether, these results may be indicative of an antagonistic relationship regarding the genotoxicity of both mycotoxins.

Keywords: Aflatoxin B1, Ochratoxin A, mycotoxins, micronucleus, comet assay, combined exposure.

Introduction

Mycotoxins are fungal toxins that can be found in many agricultural commodities and processed food (Bennett and Klich, 2003). Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are some of the most relevant due to their toxic effects and demonstrated human exposure (EFSA, 2006; EFSA, 2007).

AFB1 is a genotoxic hepatocarcinogenic compound classified as class 1 (human carcinogen) by the IARC (IARC, 1987). (IARC, 1987). It may also cause tumors in other organs, such as colon and kidney (EFSA, 2007). It is bioactivated in liver by cytochrome P450 and its epoxide metabolite attacks DNA forming adducts (McLean and Dutton, 1995). AFB1 is a clastogen that has been tested extensively for genotoxicity in vivo and in vitro, giving consistently positive results (IARC, 1987). It induces chromosomal aberrations, micronuclei, sister chromatid exchanges, unscheduled DNA synthesis and DNA strand breaks in more than 30 published works since the 80's. OTA is a nephrocarcinogenic compound in rodents and has been classified by the IARC in class 2B (possible human carcinogen). OTA mechanisms of action are not clearly determined but the ability to generate reactive oxygen species (ROS) may explain the lipid, protein and DNA damage (Ringot et al., 2006). The test battery for evaluating OTA genotoxicity gave negative results (IARC, 1993), but some positive results are found in some in vitro and in vivo studies such as DNA breaks in mammalian cell lines (Ehrlich et al., 2002; Lebrun and Follmann, 2002; Arbillaga et al., 2007), DNA damage and micronuclei in primary cultures of human and rat kidney cells (Robbiano et al., 2004) and cytogenetic damage and DNA adducts in rats treated with OTA (Mally et al., 2005; Pfohl-Leszkowicz and Manderville, 2007).

Some in vivo studies with AFB1 and OTA mixtures gave contradictory results with respect to general toxicity. In poultry, these mycotoxins increased mortality in a

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synergetic way but on the contrary, OTA inhibited lipid accumulation normally induced by AFB1 (Huff and Doerr, 1981; Huff et al., 1988; Huff et al., 1992). Other authors could not find any interaction between these mycotoxins with regard to mortality (Micco et al., 1988), relative weight of most organs, blood parameters or immunological status (Ringot et al., 2006). In swine, AFB1 and OTA had additive interactions according to liver weight and blood chemistry but they were antagonists with regard to the degree of renal cortical interstitial fibrosis and relative kidney weight (Harvey et al., 1989). In rats, AFB1 and OTA showed no interaction regarding the measurement of mortality, weight gain, or most serum biological parameters but the anaplastic and hyperchromatic nuclei, necrosis and bile duct proliferation observed were more pronounced in the combined toxin group after 4 months (Rati et al., 1981). In rats and rabbits, the combination resulted in less teratogenicity than OTA alone, although some new manifestations appeared (Wangikar et al., 2004; Wangikar et al., 2005). There was no data available regarding the combined genotoxicity *in vivo*, but *in vitro*, the combination showed genotoxic additive effects in Vero cells (green monkey kidney cells) (El Golli-Bennour et al., 2010). In Hep G2 cells (human hepatocarcinoma cells), a decrease in DNA damage, not only in direct breaks and apurinic sites but also in oxidative damage has been described (Corcuera et al., 2011a).

According to the promotion of the 3R's agenda to Replace, Reduce and Refine animal testing in non-clinical safety, animal reduction can be achieved by integrating in vivo genotoxicity testing into toxicity assays, but there is also an opportunity to reduce animal usage by combination of endpoints into a single acute assay design (Bowen et al., 2011). The ICH (International Conference on Harmonisation) S2 (R1) guideline on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, recomends an in vivo assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay, generally a DNA strand breakage assay, in another tissue (ICH, 2012). In the bone marrow micronucleus assay (MN), the target cells are erythroblasts undergoing their last chromosome replication. The micronuclei are cytoplasmatic chromatin masses with the appearance of small nuclei that arise from chromosomal loss (aneuploidy) or broken chromosomal fragments under the action of clastogenic chemicals. The polychromatic erythrocytes (PCE) extrude their main nucleus (while the micronucleus remains inside), and mature towards normochromatic erythrocytes (NCE) prior to release into the

peripheral blood circulation (MacGregor et al., 1987). OECD guideline 474 describes a validated design for performing the in vivo MN assay and indicates that an increase in the frequency of micronucleated PCEs in treated animals is an indication of induced chromosome damage (OECD, 1997). The comet assay (single-cell gel electrophoresis) is a simple method for measuring DNA damage that can be applied both in vitro and in vivo. Apart from detecting DNA strand breaks (single and double strand) at the level of single cells, the inclusion of different digestion enzymes allows the detection of oxidative DNA damage. In this way, endonuclease III (endo III) is used to detect oxidized pyrimidines, and formamidopyrimidine DNA glycosylase (FPG) is used to detect the major purine oxidation product 8-oxoguanine (8-oxoGua), ring-opened purines and formamidopyrimidines (FAPY) (Collins et al., 1997; Collins et al., 2001; Collins et al., 2008). The comet assay showed high sensitivity (ability to detect carcinogens as positive) and specificity (ability to give negative results with non-carcinogens) in the evaluation of carcinogens when the micronucleus test gave negative or equivocal results (Kirkland and Speit, 2008). After a formal validation of the in vivo rodent comet assay that was coordinated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), in conjunction with the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), a draft version of an OECD testing guideline has been prepared and is under revision. These two in vivo assays are compatible in one short acute design with the same dosage range.

The human population is probably exposed to multiple mycotoxins because human diet is generally varied and because the same food might be contaminated by several mycotoxins (Ibáñez-Vea et al., 2011; Ibáñez-Vea et al., 2012). Multi-exposure may lead to additive, synergistic or antagonistic effects. However, there are few studies regarding possible interactions of mycotoxins. The objective of this work was to study the genotoxicity of AFB1 and OTA alone and combined in a new study design that adapts two endpoints in only one short study. The selection of AFB1 and OTA is based on the fact that both of them are genotoxic, but their mechanism of action is different.

Materials and methods

Safety precautions

Aflatoxin B1 and ochratoxin A are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures carried out and during the manipulation of treated animals or contaminated samples FPP3 masks were used.

Chemicals

For the analytical standards, AFB1 was purchased as a solution of 2 mg/L in acetonitrile (ACN) and OTA was purchased as a solution of 10 mg/L in ACN, both from OEKANAL® Fluka (Schnelldorf, Germany) as certified reference materials. Cyclophasphamide (CP) and Ethyl methanesulfonate (EMS) were purchased from SIGMA. For oral administration, mycotoxins were purchased in powder from Sigma (Steinheim, Germany) and they were dissolved in 0.1 M NaHCO3 (Riedel-de Haën, Seelze, Germany), adjusted to pH 7.4 with HCl and maintained at -20ºC until their use. For the tissue homogenates, sodium phosphate buffer (0.05 M, pH 6.50) was prepared by adding 6.90 g of NaH2PO4.H2O (Merck, Darmstadt, Germany) to 900 mL of type II water. The pH of the dissolution was adjusted to 6.5 with NaOH (Agilent technologies, Waldbronn, Germany) and the volume was adjusted to 1 L. All reagents used for the HPLC analysis were of analytical grade. ACN and methanol HPLC grade and formic acid were obtained from Sigma Aldrich (St. Quentin Fallavier, France). Millipore type I water was obtained daily from a Milli-Q water-purifying system (Millipore, Bedford, MA, USA).

Animals

The in vivo experiments were approved by the Ethics Committee on Animal Experimentation of the University of Navarra.

Nine-week-old male Fisher 344 (F344) rats, purchased from Harlan (Horst, The Netherlands), were used. On the day of arrival, the animals were weighed (weight variation did not exceed ± 20% (OECD, 2009), and then distributed into polycarbonate cages with stainless steel covers. During one week, the animals were allowed to acclimatize to the environmental conditions: 12 h day/night cycle, temperature 22 ± 2ºC, relative humidity 55 ± 10%, standard diet (Harlan Iberica, Spain) and water ad libitum.

Study design and treatment

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The suggested time endpoints for performing the comet assay are 3 h and 24 h and four or five animals per group for regulatory submissions (Smith et al., 2002). OECD guideline 474 suggests at least 24 h for performing the MN test and using groups of at least 5 animals per sex (OECD, 1997). A combination of both protocols was carried out, applied with two time points (3 h and 24 h) and 5 animals per group.

50 animals were randomly distributed into two big groups of 20 animals each according to the ending timepoints of 3 h and 24 h. In each group the animals were divided into four subgroups of 5 animals each according to the treatments: solvent (Negative control, C-), AFB1, OTA and AFB1+OTA. Additionally, a group with 5 animals was treated with EMS and CP (Positive control, C+). A reserve group of 5 non-treated animals was also included.

Negative control animals (C-) received oral administration of 0.1 M HNaCO3 (pH 7.4) with 0.05% DMSO. The groups treated with AFB1 received orally administration of 0.25 mg/kg bw of AFB1, the OTA groups received oral administration of 0.5 mg/kg of OTA; and the AFB1+OTA groups received oral administration of a mixture of 0.25 mg/kg bw of AFB1 and 0.5 mg/kg of bw OTA. Positive control animals (C+) (n = 5) received intraperitoneal administration of 25 mg/kg bw of CP 24 h before being sacrificed and 300 mg/kg bw of EMS, administered orally, 3 h before being sacrificed (OECD, 1997; Hamada et al., 2001; Vasquez, 2010). The volumes of administration were 10 mL/kg bw via oral route, and 5 mL/kg bw via intraperitoneal route.

Sample collection

After 3 h or 24 h, the animals were sacrificed and samples were obtained: bone marrow cells for the micronucleus assay; liver and kidney samples for the comet assay, gene expression analysis, histopathology examination and mycotoxins quantification; blood for biochemical analyses and mycotoxins quantification.

The animals were euthanized by decapitation and blood was collected into heparinized tubes (BD Vacutainer system) for clinical biochemistry analysis and AFB1 as well as OTA determination. Blood samples were centrifuged (1,085 × g for 15 min at 4°C) in order to obtain plasma that was stored at −80°C. The kidneys and the liver were removed. After extensive washing with water and blotting on filter paper, one half of a kidney and a slice of liver were fixed in 4% formaldehyde solution for histopathological

analysis. Another half of kidney and a piece of liver were flash-frozen in liquid N2 and stored at -80°C for mycotoxin determination. The remaining tissues were dipped in cold Merchant's buffer for performing the comet assay. Both femur bones were released from muscles and separated from the hip and the knee. The head of the femur was cut with a bold cutter and the bone marrow was extracted with a Pasteur pipette (0.5 mL). Bone marrow was placed on a microscope slide with a drop of fetal calf serum (Lonza. Verviers, Belgium). Eight extensions per animal were carried out using both femurs.

Clinical biochemistry and histopathology

Biochemical analyses of plasma samples were performed with a Hitachi 911™ (Roche Diagnostics) analyzer using the protocols obtained from Roche for the determination of the standard parameters: total protein (g/dL), albumin (g/dL), glucose (mg/dL), aspartate transaminase (AST) (U/L), alanine transaminase (ALT) (U/L), alkaline phosphatase (U/L) and urea (mg/dL).

In order to analyze possible weight changes in the target organs due to the administration of the mycotoxins, the relative weight (RW) of liver and kidneys were calculated dividing the weight of each organ by the total weight of the animal.

For the histopathological analysis, the organ sections of groups treated during 24 h were mixed in 4% formaldehyde solution, dehydrated and embedded in paraffin. Paraffin sections (3 μm) were cut, mounted onto glass slides, and dewaxed and stained with hematoxylin and eosin (H&E) for the subsequent histopathological examination. In the observation and evaluation of each sample, the systemic anatomopathological protocol was applied, with special attention to: a) normalcy or alteration of the architecture and proportions of the structures, b) presence of circulatory phenomena; c) evaluation and quantification of degenerative or necrotic phenomena; d) existence or absence of inflammatory phenomena, types and intensity; e) abnormal growths: atrophy, hyperplasia, hypertrophy, neoplasia; f) particular or special findings.

Determination of mycotoxins in plasma, liver and kidney

The concentration of mycotoxins in plasma and tissues was determined by UHPLC with fluorescence detection. The extraction procedure and the UHPLC-FLD quantification

method was previously set up and validated for these biological samples, and has been performed as described (Corcuera et al., 2011b).

Micronucleus assay

The slides were maintained in absolute methanol overnight prior to use. They were air dried and used to perform the bone marrow extensions. The extensions were fixed in absolute methanol for 5 min, air dried and stained with 10% Giemsa (Giemsa´s azur eosin methylated blue solution, MERCK. Darmstadt, Germany) for 10 min.

After staining, 1,000 erythrocytes per sample were scored and the ratio of polychromatic erythrocytes (PCEs) / normochromatic erythrocytes (NCEs) was calculated. The PCE/NCE index should be 1 or close to 1, ratios which are lower than 1 are indicative of bone marrow cytotoxicity. The incidence of MN was studied in 2,000 PCEs per sample, and the % of MN/PCE was calculated.

Comet assay

Nuclei were isolated based on the method of Brunborg et al. (1988) for isolating nuclei from tissues such as lung, testis, liver, kidney and brain, with several modifications. The Merchant's buffer (MB) (0.14 M NaCl, 1.47 nM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 10 mM Na_2EDTA, pH 7.4) maintained at 4ºC on ice, was used during the whole process of nuclei isolation. This has to be done as quickly as possible in order to preserve the integrity of the nuclei. First, a small piece of tissue (0.5 cm diameter) was minced with scissors a few times. Afterwards, the pieces were transferred to a stainless steel cylindrical tube (15 mm diameter) with a stainless steel screen of 0.4 mm fitted inside, that was used to squeeze the tissue and obtain the nuclei. A plastic plunger that fits into the cylindrical tube was used to force the tissue through the screen, similar to using a syringe. This was repeated as many times as necessary to ensure that all tissue had been transferred, and the cylinder was rinsed with cold MB to reduce the loss of nuclei. Then, the suspension of released nuclei, cells and tissue fragments was filtered through a nylon filter (100 µm) and centrifuged at 214 x g for 5 min at 4ºC. Finally, the pellet was resuspended in MB (5 mL), filtered and centrifuged once more.

The pellet contained the crude nuclei and was resuspended in 5 mL of MB. Thirty microliters of this suspension were mixed with 140 µL of 1% low melting point agarose; two drops of 70 µL of this mixture were placed on a microscope slide. A cover

slip was put on top of each drop and the gels were allowed to set for 5 min at 4ºC. Once the gels solidified, the cover slip was removed and the slides were dipped in lysis solution at 4ºC (2.5 M NaCl, 0.1 M Na4- EDTA x 2H2O, 0.01 M Trizma-BASE, pH 10.5, TRITON X-100 1%). Four different slides, numbered from 1 to 4, were prepared for each condition. Number 1, for observing DNA breaks and AP sites, and numbers 2, 3 and 4 for obtaining information regarding the presence of oxidized DNA bases using endo III and FPG post-treatments.

Samples were maintained in the lysis solution at 4ºC, during at least 1 h. The positive control slide was dipped into H2O2 (100 µM in PBS) solution for 5 min at 4ºC, then washed with cold PBS and introduced into a lysis solution in a separate jar for at least 1 h.

After lysis, slides 2, 3 and 4 were washed 3 times (5 min each time) with the enzyme reaction buffer (0.04 M HEPES, 0.1 M KCl, 0.0005 M Na4-EDTA x 2 H2O, 0.2 mg/mL BSA, pH 8). Then gels were digested with enzyme reaction buffer (slide 2), endo III (slide 3) or FPG (slide 4), by adding 50 µL of the corresponding solution to each gel and placing a cover slip on top, for 30 min at 37ºC in a humid chamber. After that, all nucleoids were denatured in a high-pH buffer (0.3 M NaOH, 0.001 M Na4-EDTA x 2 H2O) for 40 min at 4ºC. Finally, electrophoresis was carried out at approximately 1 V/cm (Brunborg, 2008) for 30 min and the DNA was then gently re-neutralized in PBS during 10 min, washed in H2O for another 10 min and de-hydrated in ethanol 95% for 5 min.

Nucleoids were stained by adding a drop of 20 µL of DAPI (1 µg/mL) (Sigma-Aldrich) on each gel and comets were analyzed in a fluorescence microscopy (Eclipse 50 i NIKON). A total of 100 comets (50 per gel) were scored using the Comet Assay IV software (Perceptives, UK). The percentage of DNA in tail from slide 1 represents the DNA strand breaks and apurinic (AP) sites. Net endo III and FPG-sensitive sites were calculated by subtracting the % of DNA in tail of slide 2 from the DNA % in tail of the enzymes-treated slides (3 or 4 respectively).

Statistical analysis

Data is presented by descriptive analysis as the mean ± standard deviation (SD) of five animals. The distribution of the data was checked for normality using the Shapiro-Wilk

test. The homogeneity of the variance was verified by the Levene test. The comparisons were performed using the Kruskal-Wallis test followed by DMS test. P-values equal to or below 0.05 were accepted as the level of significance, and p-values equal to or lower than 0.01 were considered very significant.

Results

Clinical biochemistry and histopathology

No clinical signs of toxicity were observed during the study in any of the treatment groups. All liver and kidney samples studied presented normal morphology. The relative weight (RW) of liver and kidney were normal except in AFB1 treated animals after 24 h, which showed a small, but significant, increase in RW. The most evident alterations were observed exclusively in liver (figure 1). The livers from group AFB1 have shown intense diffuse necrosis of hepatocytes, characterized by pyknosis and destruction of the nuclei with shrunken cytoplasms that are strongly acidophilics (Fig. 1 C and D). This necrosis is accompanied by inflammatory infiltrates associated with lysated hepatocytes and the general inflammation of the parenchyma and portal spaces is increased. The kidneys from the OTA group were normal (Fig. 1 B). The livers from the AFB1+OTA group presented necrosis and associated inflammatory infiltration (Fig. 1 E and F). However, the degree of the lesion has been estimated as inferior to that observed in group AFB1 (see table 2).

With regard to plasma biochemical parameters, after 3 h treatment with AFB1, OTA or AFB1 + OTA, the only variation detected was a significant ALT increase in animals treated with OTA, but this was considered not relevant because it is within the normal historical range (Table 1). On the contrary, after 24 h, in AFB1 treated animals, the AST was approximately two times higher than the control and the ALT three times higher (table 1). In OTA treated animals, the enzyme activities were similar to the control animals. In the group treated with both AFB1 and OTA, the ALT level was significantly higher than the control but significantly lower than in group treated with AFB1; moreover, AST was also significantly lower than in the group treated with AFB1, reaching levels similar to those of the control.

Plasma and tissue AFB1 and OTA concentrations

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Mycotoxin concentrations have been determined in plasma, liver and kidney of rats after 3 h and 24 h of administration (table 3). Regarding AFB1, it was not detected after 24h in plasma or tissues and very low concentrations have been found after 3h; the highest concentration was found in liver, in plasma the value was below the limit of quantification (LOQ), and in kidney it was almost undetectable. On the contrary, OTA concentrations were very high in plasma and also in tissues (table 3). The co-administration apparently decreased AFB1 concentration and increased OTA concentration in plasma and tissues. Low levels of OTA were detected in the plasma of control and AFB1 animals. This minimal exposure is justified by natural trace contamination evidence in good quality commercial rat diet (Mantle, 2008; Vettorazzi et al., 2008; Arbillaga et al., 2008).

Micronuclei induction

With regard to bone marrow cytotoxicity, as can be seen in Fig. 2A, a pronounced cytotoxic effect was detected after 3h treatment with AFB1, which was diminished in the combined treatment. OTA treatment also produced a cytotoxic effect after 3 h. After 24 h, no signs of cytotoxicity were observed in any of the treated groups.

As expected, no significant induction of micronuclei was observed after 3 h in any of the treatments. After 24 h, AFB1 increased the amount of MN very significantly, up to 90 times the control levels, while OTA had no effect. The combined treatment was also positive in the MN induction (69 times the control levels) but the amount of MN was lower than in the AFB1 single administration. The difference in the percentage of MN between both groups was not statistically significant (figure 2B).

Comet assay

The comet assay was performed in order to study the genotoxicity potential of the treatments in liver and kidney. No significant induction of DNA strand breaks was observed in any of the groups with the exception of the positive control (figure 3). In liver, a significant increase of net FPG- sensitive sites (3 times the control levels) was observed in isolated AFB1 treatments (3 h and 24 h); the greatest effect was observed after 3 h. In the combined treatmenta significant increase of net FPG-sensitive sites was observed after 24h (figure 3 A, B). In kidney, a significant increase of net FPG-sensitive sites was observed in the OTA and AFB1+OTA groups (3 h and 24 h).

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Discussion

The combined genotoxic effect of two mycotoxins has been studied by the application of a strategy that combines in one single in vivo study, two assays measuring different endpoints: the micronucleus assay in bone marrow cells and the comet assay in liver and kidney cells. In this study liver has been selected because of its central role in the xenobiotics metabolism and also because it is the AFB1 target organ for its general toxicity and carcinogenicity; the second organ has been the kidney for its role in xenobiotic detoxification and because it is the OTA target organ for general toxicity and carcinogenicity. The OECD guideline for the testing of chemicals No. 474 recommends to use 5 animals per group and both sexes (OECD, 1997), but this study was performed only with male rats. Madle et al. observed that AFB1 induced more MN in male rats and mice than in females (Madle et al., 2005) and it is very well known that OTA is more carcinogenic in male rodents than in females (Castegnaro et al., 1998; EFSA, 2006). Therefore, in order to simplify the design of the study which was technically complicated, it was decided to perform the study only in male rats.

A wide range of doses have been used to study AFB1 in rats depending on repeated or single oral doses or long and short experiments. The range of dosage includes from 0.2 up to 12.5 mg/kg bw (Rati et al., 1981; Wong and Hsieh, 1980; Bannasch et al., 1985; Coulombe and Sharma, 1985; Raj et al., 1998; Ellinger-Ziegelbauer et al., 2006; Theumer et al., 2010). Previous studies performed in our laboratory showed that a single oral dose of AFB1+OTA (0.25 mg/kg bw and 0.5 mg/kg bw, respectively) caused general liver deterioration and a marked increase of transaminases and liver paleness after 72 h. All these signs suggested acute toxicity due to AFB1. Although Bowen et al. (2011) suggested a design to perform both comet and MN assays giving three repeated administrations (0, 24, 45h), in this study, we tried to avoid excessive acute toxicity due to the fact that cytotoxicity can confound the ability to determine genotoxicity in the comet assay (Vasquez, 2010). As the aim of the study was to study the combined effect of both toxins and not to obtain a dose-response information, only one dose of each mycotoxin has been tested. The single dose of AFB1 (0.25 mg/kg bw) was similar to the one used to characterize expression profiles of genotoxic carcinogens (0.24 mg/kg bw/day) (Ellinger-Ziegelbauer et al., 2006). The single OTA dose selected (0.5 mg/kg

bw) was approximately the carcinogenic dose described by the NTP studies during 24 months (0.7 mg/kg bw) (NTP, 1989) and has been previously used in other studies (Vettorazzi et al., 2009; Vettorazzi et al., 2011).

No clinical signs of toxicity were observed during the experiment in any of the treated groups, but the biochemical and histopathological findings revealed mild acute liver damage after 24 h in the groups treated with AFB1. These alterations were more pronounced when AFB1 was administered alone than when it was co-administered with OTA. In this study it could be demonstrated that both toxins were absorbed and distributed throughout the organism, but the levels of OTA and AFB1 reflect the different kinetic behavior and metabolism rate of both toxins. After 3h, AFB1 could be detected in plasma and kidney but only quantified in liver, due to its high metabolization rate. On the contrary, the OTA levels remained very high in plasma and also in tissues after 24h, being very similar to those found by Vettorazzi et al. (2009, 2010 and 2011). Apparently, the co-administration decreased the levels of AFB1 and increased the levels of OTA found in plasma and tissues. An influence in the kinetic behavior and metabolism of AFB1 by OTA might be the cause, thus having an impact in toxicity. Nevertheless, a study focused on toxicokinetics and measuring the metabolites would be needed to draw conclusions.

In this study, bone marrow samples were taken after 3 h and 24 h. No positive results in the MN test were expected in the short treatment, because there is not enough time for cells to divide, but sampling time at 3h was included in the experimental design to get tissue samples for comet assay. After 3 h, significant bone marrow cytotoxicity appeared after AFB1, OTA and AFB1+OTA administration, and this is a clear indication of tissue exposure to the mycotoxins. Moreover, these results meet the requirement of the OECD guideline 474 that recommends to reach a dose producing some indication of bone marrow toxicity (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood) (OECD, 1997).

AFB1 treatment produced a significant increase in the percentage of micronuclei of bone marrow PCE and a significant increase in FPG sensitive sites of liver cells, confirming the genotoxic potential of this mycotoxin. The oxidative DNA damage detected in liver was lower after 24 h than after 3 h. OTA did not induce micronuclei in

bone marrow PCE but it induced a significant increase in FPG sensitive sites in kidney cells. The oxidative DNA damage detected in kidney was very similar at 3h and 24 h, Therefore, each mycotoxin induced oxidative DNA damage in their respective target organ. Regarding OTA genotoxicity, the results of this study are in agreement with previous *in vivo* studies in adult Wistar rats after repeated doses (Domijan et al., 2006), and support the idea that the oxidative stress is likely to be responsible for the DNA damage induced by OTA. Moreover, Mally et al. (2004, 2005) after repeated OTA dosing in F344 rats also observed an increase in FPG sensitive sites and the absence of DNA adducts pointing to a non-direct reactive DNA mechanism of toxicity. OTA kinetic parameters described in similar conditions by Vettorazzi et al. (2009) showed that OTA has a long half live in plasma and high apparent volume of distribution. These characteristics would explain the high concentration of OTA in plasma and tissues after 24 h or more, and a continuous exposure of cells to the oxidative stress produced by OTA. AFB1 did not produce any significant genotoxic effect in kidney and did not modify the OTA response in this organ. The fact that AFB1 metabolites could not be analysed, makes impossible to determine if there is no exposure to them in kidney.

The combined AFB1+OTA treatment decreased the number of micronuclei in the bone marrow and the net-FPG sensitive sites in liver with respect to the AFB1 treatment. On the contrary, co-administration of AFB1 did not have any influence on the net-FPG sensitive sites produced by OTA in kidney. In HepG2 cells, co-exposure to OTA significantly decreased DNA damage induced by AFB1, not only in breaks and apurinic sites but also in FPG-sensitive sites (Corcuera et al 2011a). AFB1 is bioactivated in liver by cytochrome P450 and its epoxide metabolite attacks DNA forming adducts (McLean and Dutton, 1995). The primary lesion evolves to secondary injuries such as apurinic sites (AP) that would be detected as direct strand breaks by the comet assay; or imidazole AFB1 formamidopyrimidine opened rings (AFB1-FAPY) (Bedard and Massey, 2006), that could be detected by incubating the DNA with a formamidopyrimidine glycosylase enzyme such as FPG. Moreover, FPG enzyme also detects 8-hydroxydeoxyguanosine (8-oxodG) (Collins and Dusinska, 2002). A time- and dose-dependent increase in 8-oxodG has been described in rat hepatic DNA after a single intraperitoneal injection of AFB1 (Shen et al., 1995). The toxic metabolite of AFB1, the AFB1-epoxide, is converted into AFB1-GSH catalyzed by the cellular

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glutathione-S-transferase and the level of this enzyme is critical to modulate AFB1 metabolism. Glutathion depletors enhanced AFB1-induced micronuclei in bone marrow cells, while inductors of glutathion-S-transferase possess inhibitory activities against AFB1-DNA binding (Raj et al., 1998). The importance of the antioxidant/detoxification enzymes and the regulation via Nrf2/Keap 1/ARE response in AFB1 has been the subject of many studies aimed to demonstrate that activators of this mechanism may modulate mutagenesis and carcinogenesis both in laboratory rats and in humans (Kensler et al, 2005, 2007; Eaton and Schaupp, 2014). Recently, Johnson et al., 2014, pretreating F344 rats with a synthetic oleanane triterpenoid, a powerful activator of Keap 1-Nrf2 signaling pathway, obtained a complete protection against AFB1-induced liver cancer, with a significant reduction in the level of urinary AFB1-N^7-guanine (66%) and an elevation of aflatoxin-N-acetylcysteine (300%). OTA depletes GSH and some enzymes involved in free radical scavenging in vivo (O'Brien and Dietrich, 2005). Besides, in some studies, an inhibition of the pathways regulated by Nrf2 have been described in kidney but not in liver (Marin-Kuan et al, 2006; Cavin et al., 2007). Thus, the protection of OTA against AFB1 genotoxicity would not be explained through this mechanism. Alternatively, this interaction could be explained by a type of competition for the cytochrome enzymes which are involved in the metabolism of both toxins. OTA persists in the organism much more than AFB1, and its maintained presence could somehow avoid or retard AFB1 bioactivation in liver, forcing AFB1 to take other metabolism routes.

In conclusion, in this study, it has been demonstrated that simultaneous exposure to AFB1 and OTA may modify the toxic effects of AFB1, probably due to metabolic factors, although this would need confirmation. From the point of view of risk assessment, these findings provide additional information but do not avoid the application of the precautionary principle of exposure reduction to genotoxic mycotoxins to a level as low as reasonably achievable. On the other hand, the combination of classical genotoxicity tests such as the MN assay and new techniques such as the comet assay, which can be applied to tissues other than bone marrow or blood, gives complementary results. This contributes to the 3Rs strategy by increasing the information that can be obtained from a single animal experiment, and also improves the risk assessment process by producing results that can be interpreted in a more rational way.

Acknowledgements

References

Arbillaga, L., Azqueta, A., Ezpeleta, O. and López de Cerain, A., 2007. Oxidative DNA damage induced by Ochratoxin A in the HK-2 human kidney cell line: evidence of the relationship with cytotoxicity. Mutagenesis 22, 35-42.

Arbillaga, L., Vettorazzi, A., Gil, A.G., van Delft, J.H., García-Jalón, J.A. and López de Cerain, A., 2008. Gene expression changes induced by ochratoxin A in renal and hepatic tissues of male F344 rat after oral repeated administration. Toxicol Appl Pharmacol 230, 197-207.

Bannasch, P., Benner, U., Enzmann, H. and Hacker, H.J., 1985. Tigroid cell foci and neoplastic nodules in the liver of rats treated with a single dose of aflatoxin B1. Carcinogenesis 6, 1641-1648.

Bedard, L.L. and Massey, T.E., 2006. Aflatoxin B1-induced DNA damage and its repair. Cancer Lett 241, 174-183.

Bennett, J.W. and Klich, M., 2003. Mycotoxins. Clin Microbiol Rev 16, 497-516.

Bowen, D.E., Whitwell, J.H., Lillford, L., Henderson, D., Kidd, D., Mc Garry, S., Pearce, G., Beevers, C. and Kirkland, D.J., 2011. Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the Comet assay and the flow-cytometric peripheral blood micronucleus test. Mutat Res-Gen Tox En 722, 7-19.

Brunborg, G., 2008. Spreadsheet to calculate voltages and currents in an electrophoresis tank. http://comics.vitamib.com/electrophoresis-physics.

Brunborg, G., Holme, J.A., Søderlund, E.J., Omichinski, J.G. and Dybing, E., 1988. An automated alkaline elution system: DNA damage induced by 1,2-dibromo-3-chloropropane in vivo and in vitro. Anal Biochem 174, 522-536.

Castegnaro, M., Mohr, U., Pfohl-Leszkowicz, A., Estève, J., Steinmann, J., Tillmann, T., Michelon, J. and Bartsch, H., 1998. Sex and strain specific induction of renal tumors by ochratoxin A in rats correlates with DNA adduction. Int. J Cancer 77, 70-75.

Cavin, C., Delatour, T., Marin-Kuan, M., Holzhäuser, D., Higgins, L, Bezençon, C., Guignard, G., Junod, S., Richoz-Payot, J., Gremaud, E., Hayes, J.D., Nestler, S., Mantle, P. and Schilter, B. 2007. Reduction in antioxidant defenses may contribute to ochratoxin A toxicity and carcinogenicity. Toxicol Sci 96:30-39.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivao, I., Giovannelli, L., Kruszewski, M., Smith, C.C. and Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143-151.

Collins, A.R. and Dusinska, M., 2002. Oxidation of cellular DNA measured with the comet assay. Methods Mol Biol 186, 147-159.

Collins, A.R., Dusinska, M., Horvathova, E., Munro, E., Savio, M. and Stetina, R., 2001. Inter-individual differences in repair of DNA base oxidation, measured in vitro with the comet assay. Mutagenesis 16, 297-301.

Collins, A.R., Dobson, V.L., Dusinska, M., Kennedy, G. and Stetina, R., 1997. The comet assay: what can it really tell us? Mutat Res 375, 183-193.

Corcuera, L.A., Arbillaga, L., Vettorazzi, A., Azqueta, A. and López de Cerain, A., 2011a. Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. Food Chem Toxicol 49, 2883-2889.

Corcuera, L., Ibáñez-Vea, M., Vettorazzi, A., González-Peñas, E. and Cerain, A.L.d., 2011b. Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin a in rat plasma, liver and kidney. J Chromatogr B 879, 2733-2740.

Coulombe Jr, R.A. and Sharma, R.P., 1985. Clearance and excretion of intratracheally and orally administered aflatoxin B1 in the rat. Food Chem Toxicol 23, 827-830.

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Domijan, A., Želježić, D., Žlender, V., Fuchs, R. and Peraica, M., 2006. Ochratoxin A causes DNA damage in rats kidney. Toxicol Lett 164, S291-S292.

Eaton, D.L. and Schaupp, C.M. 2014 Of mice, rats, and men: Could Nrf2 activation protect against Aflatoxin hepatocarcinogenesis in humans? Cancer Prev Res 7 doi: 10.1158/1940-6207.

EFSA, 2006. Opinion of the Scientific Panel on Contaminats in the Food Chain on a request from the commision related to Ochratoxin A in food. EFSA J 365, 1-56.

EFSA, 2007. Opinion of the Scientific Panel on Contaminats in the Food Chain on a request from the Commission related to the potential increase of consumer health risk by a possible increase of the existing maximun levels for Aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA J 446, 1-127.

Ehrlich, V., Darroudi, F., Uhl, M., Steinkellner, H., Gann, M., Majer, B.J., Eisenbauer, M. and Knasmuller, S., 2002. Genotoxic effects of ochratoxin A in human-derived hepatoma (Hep G2) cells. Food Chem Toxicol 40, 1085-1090.

El Golli-Bennour, E., Kouidhi, B., Bouslimi, A., Abid-Essefi, S., Hassen, W. and Bacha, H., 2010. Cytotoxicity and genotoxicity induced by aflatoxin B1, ochratoxin A, and their combination in cultured Vero cells. J Biochem Mol Toxicol 24, 42-50.

Ellinger-Ziegelbauer, H., Ahr, H.-J., Gmuender, H., Pallez, C. and Koenig, J., 2006. Characteristic expression profiles induced by carcinogens in rat liver. Toxicol Lett 164, S293-S294.

Hamada, S., Yamasaki, K., Nakanishi, S., Omori, T., Serikawa, T. and Hayashi, M., 2001. Evaluation of the general suitability of the rat for the micronucleus assay: the effect of cyclophosphamide in 14 strains. Mutat Res-Gen Tox En 495, 127-134.

Harvey, R.B., Huff, W.E., Kubena, L.F. and Phillips, T.D., 1989. Evaluation of diets contaminated with aflatoxin and ochratoxin fed to growing pigs. Am J Vet Res 50, 1400-1405.

Huff, W.E., Kubena, L.F., Harvey, R.B. and Phillips, T.D., 1992. Efficacy of hydrated sodium calcium aluminosilicate to reduce the individual and combined toxicity of aflatoxin and ochratoxin A. Poult Sci 71, 64-69.

Huff, W.E., Kubena, L.F., Harvey, R.B. and Doerr, J.A., 1988. Mycotoxin interactions in poultry and swine. J Anim Sci 66, 2351-2355.

Huff, W.E. and Doerr, J.A., 1981. Synergism between aflatoxin and ochratoxin A in broiler chickens. Poult Sci 60, 550-555.

IARC, 1987. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42. Supplement 7, 440.

IARC, 1993. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. 56, 33.

Ibáñez-Vea, M., Martínez, R., González-Peñas, E., Lizarraga, E. and López de Cerain, A., 2011. Co-occurrence of aflatoxins, ochratoxin A and zearalenone in breakfast cereals from spanish market. Food Control 22, 1949-1955.

Ibáñez-Vea, M., González-Peñas, E., Lizarraga, E. and López de Cerain, A., 2012. Co-occurrence of aflatoxins, ochratoxin A and zearalenone in barley from a northern region of Spain. Food Chem 132, 35-42.

ICH, 2012. Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1). EMA/CHMP/ICH/126642/2008.

Johnson, N.M., Egner, P.A., Baxter, V.K., Sporn, M.B., Wilbe, R.S., Sutter, T.R., Groopman, J.D., Kensler, T.W.and Roebuck, B.D. 2014. Complete protection against Aflatoxin B1-induced liver cancer with a triterpenoid: DNA adduct dosimetry, molecular signature, and genotoxicity threshold. Cancer Prev Res 7: 658-665.

Kensler T.W., Chen, J.G., Egner, P.A., Fahey, J.W., Jacobson, L.P., Stephenson, K.K., et al. 2005. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. Cancer Epidemiol Biomarkers Prev 14: 2605–2613.

Kensler, T.W., Wakabayashi, N., Biswal. S. 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47: 89–116.

Kirkland, D. and Speit, G., 2008. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens: III. Appropriate follow-up testing in vivo. Mutat Res-Gen Tox En 654, 114-132.

Lebrun, S. and Follmann, W., 2002. Detection of ochratoxin A-induced DNA damage in MDCK cells by alkaline single cell gel electrophoresis (comet assay). Arch Toxicol 75, 734-741.

MacGregor, J.T., Heddle, J.A., Hite, M., Margolin, B.H., Ramel, C., Salamone, M.F., Tice, R.R. and Wild, D., 1987. Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutat Res-Gen Tox 189, 103-112.

Madle, E., Korte, A. and Beek, B., 2005. Species differences in mutagenicity testing: I. Micronucleus and SCE tests in rats, mice, and Chinese hamsters with aflatoxin B1. Teratog Carcinog Mutagen 6, 1-13.

Mally, A., Pepe, G., Ravoori, S., Fiore, M., Gupta, R.C., Dekant, W. and Mosesso, P., 2005. Ochratoxin a causes DNA damage and cytogenetic effects but no DNA adducts in rats. Chem Res Toxicol 18, 1253-1261.

Mally, A., Zepnik, H., Wanek, P., Eder, E., Dingley, K., Ihmels, H., Volkel, W. and Dekant, W., 2004. Ochratoxin A: lack of formation of covalent DNA adducts. Chem Res Toxicol 17, 234-242.

Mantle, P.G., 2008. Interpretation of the pharmacokinetics of ochratoxin A in blood plasma of rats, during and after acute or chronic ingestion. Food Chem Toxicol 46, 1808-1816.

Marin-Kuan, M., Nestler, S., Verguet, C., Bezençon, C., Piguet, D., Mansourian, R., Holzwarth, J., Grigorov, M., Delatour, T., Mantle, P., Cavin, C. and Schilter. 2006. A toxicogenomic approach to identify new plausible epigenetic mechanisms of ochratoxin A carcinogenicity in rat. Toxicol Sci 89: 120-134.

McLean, M. and Dutton, M.F., 1995. Cellular interactions and metabolism of aflatoxin: An update. Pharmac Ther 65, 163-192.

Micco, C., Miraglia, M., Benelli, L., Onori, R., Ioppolo, A. and Mantovani, A., 1988. Long term administration of low doses of mycotoxins in poultry. 2. Residues of ochratoxin A and aflatoxins in broilers and laying hens after combined administration of ochratoxin A and aflatoxin B1. Food Addit Contam 5, 309-314.

NTP, 1989. Toxicology and carcinogenesis studies of ochratoxin A (CAS No 303-47-9) in F344/N rats (gavage studies). Natl Toxicol Program Tech Rep Ser 358, 1-142.

O'Brien, E. and Dietrich, D.R., 2005. Ochratoxin A: The Continuing Enigma. Crit Rev Toxicol 35, 33-60.

OECD, 1984. OECD Guidelines for the Testing of Chemicals, Number 417: Toxicokinetics.

OECD, 1997. Guideline for the testing of chemicals 474: Mammalian Erythrocyte Micronucleus Test. OECD guideline , 1-10.

OECD, 2009. OECD Guidelines for the Testing of Chemicals. Draft proposal for a revised TG417: Toxicokinetics.

Pfohl-Leszkowicz, A. and Manderville, R.A., 2007. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res 51, 61-99.

Raj, H.G., Gupta, K., Rohil, V., Bose, M., Biswas, G., Singh, S.K., Jain, S.C., Parmar, V.S., Olsen, C.E. and Wengel, J., 1998. Aflatoxin B1-induced micronuclei and cell cycle alterations in lung and bone marrow cells and their modulation by Piper argyrophyllum extract. Teratog Carcinog Mutagen 18, 249-261.

Rati, E.R., Basappa, S.C., Sreenivasa Murthy, V., Ramesh, H.P., Ramesh, B.S. and Singh, G.B., 1981. The sinergistic effect of aflatoxin B1 and ochratoxin A in rats. J. Food Scie Tech 18, 176-179.

Ringot, D., Chango, A., Schneider, Y.J. and Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem Biol Interact 159, 18-46.

Robbiano, L., Baroni, D., Carrozzino, R., Mereto, E. and Brambilla, G., 2004. DNA damage and micronuclei induced in rat and human kidney cells by six chemicals carcinogenic to the rat kidney. Toxicology 204, 187-195.

Shen, H.M., Ong, C.N., Lee, B.L. and Shi, C.Y., 1995. Aflatoxin B1-induced 8-hydroxydeoxyguanosine formation in rat hepatic DNA. Carcinogenesis 16, 419-422.

Theumer, M.G., Cánepa, M.C., López, A.G., Mary, V.S., Dambolena, J.S. and Rubinstein, H.R., 2010. Subchronic mycotoxicoses in Wistar rats: Assessment of the in vivo and in vitro genotoxicity induced by fumonisins and aflatoxin B1, and oxidative stress biomarkers status. Toxicology 268, 104-110.

Vasquez, M., 2010. Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. Mutagenesis 25, 187-199.

Vettorazzi, A., Gonzalez-Penas, E., Arbillaga, L., Corcuera, L.A. and Lopez de Cerain, A., 2008. Simple high-performance liquid chromatography-fluorescence detection method for plasma, kidney and liver of rat as a tool for toxicology studies. J Chromatogr A 1215, 100-106.

Vettorazzi, A., Gonzalez-Penas, E., Troconiz, I.F., Arbillaga, L., Corcuera, L.A., Gil, A.G. and de Cerain, A.L., 2009. A different kinetic profile of ochratoxin A in mature male rats. Food Chem Toxicol 47, 1921-1927.

Vettorazzi, A., Tracóniz, I.F., Gonzalez-Peñas, E., Corcuera, L.A., Arbillaga, L., Gil, A.G., Nagy, J.M., Mantle, P.G. and López de Cerain, A., 2010. Effects of fasting and gender on ochratoxin A toxicokinetics in F344 rats. Food Chem Toxicol 48, 3159-3166.

Vettorazzi, A., de Tracóniz, I.F., González-Peñas, E., Arbillaga, L., Corcuera, L., Gil, A.G. and de Cerain, A.L., 2011. Kidney and liver distribution of ochratoxin A in male and female F344 rats. Food Chem Toxicol 49, 1935-1942.

Wangikar, P.B., Dwivedi, P. and Sinha, N., 2004. Effect in rats of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. I. Maternal toxicity and fetal malformations. Birth defects research. Part B, Dev Rep Toxicol 71, 343-351.

Wangikar, P.B., Dwivedi, P., Sinha, N., Sharma, A.K. and Telang, A.G., 2005. Teratogenic effects in rabbits of simultaneous exposure to ochratoxin A and aflatoxin B1 with special reference to microscopic effects. Toxicology 215, 37-47.

Wong, Z.A. and Hsieh, D.P.H., 1980. The comparative metabolism and toxicokinetics of aflatoxin B1 in the monkey, rat, and mouse. Toxicol Appl Pharmacol 55, 115-125.

Zeljezic, D., Domijan, A.M. and Peraica, M., 2006. DNA damage by ochratoxin A in rat kidney assessed by the alkaline comet assay. Braz J Med Biol Res 39, 1563.
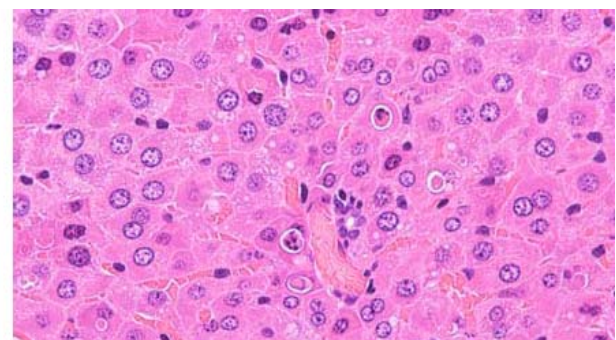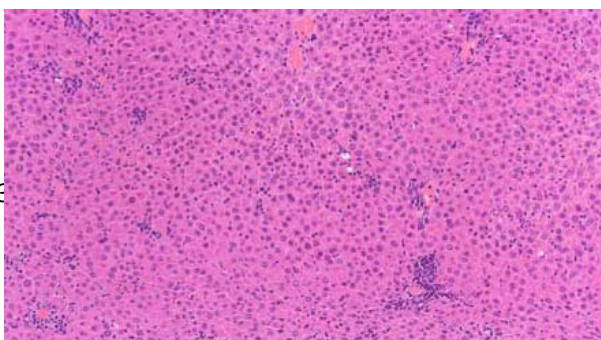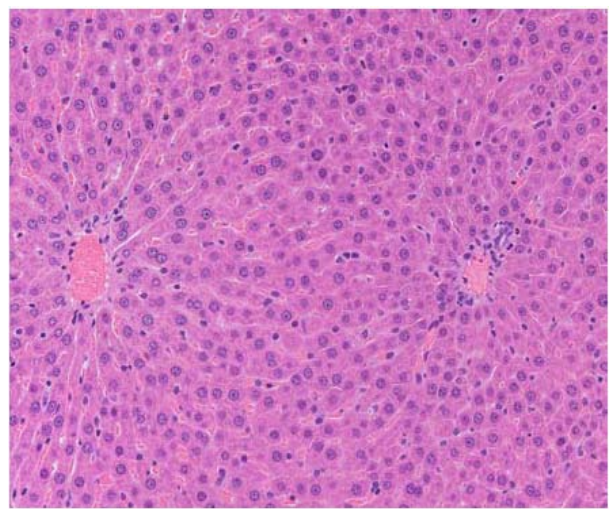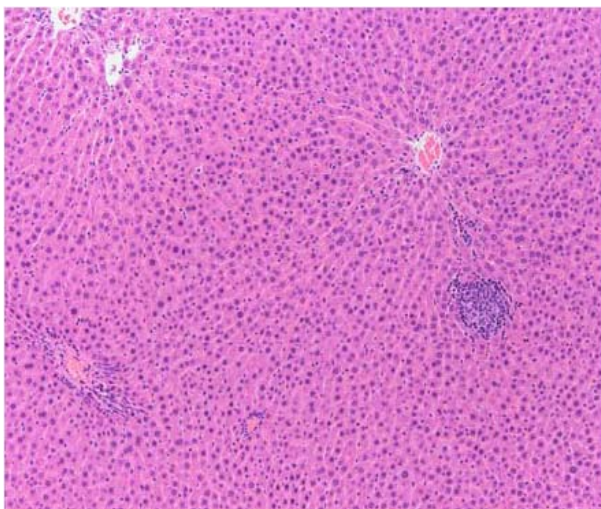
Fig. 1: Histopathology of H&E stained liver sections of (A) a control rat (24h) x 100, (B) a rat treated with OTA (24h) x 200, (C) a rat treated with AFB1 (24h) x 100. Inflammatory infiltrates are indicated, (D) a rat treated with AFB1 (24h) x 400. Intense diffuse necrosis of hepatocytes, characterized by pyknosis and destruction of the nuclei with shrunken cytoplasms that are strongly acidophilics are indicated with arrows, (E) a rat treated with AFB1+OTA x 100 (24h) and (F) a rat treated with AFB1 + OTA (24h) x 400. Necrosis and associated inflammatory infiltration are indicated.
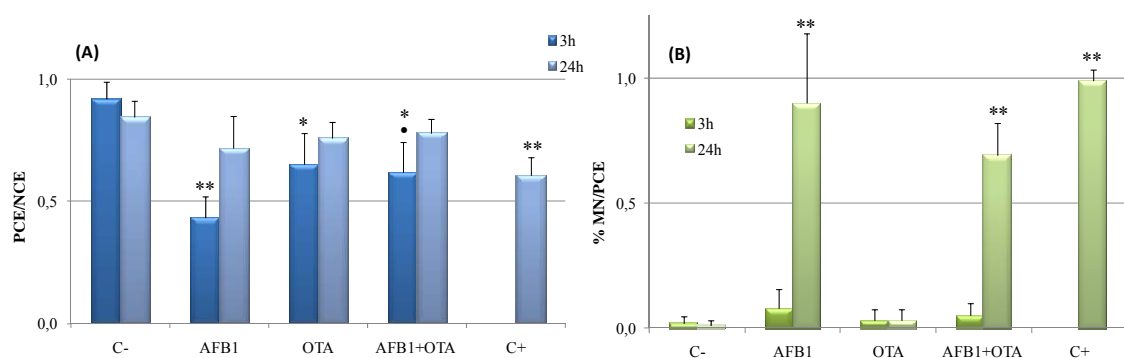
Figure 2: Micronucleus assay results. (A) Bone marrow cytotoxicity expressed as polychromatic erythrocytes / normochromatic erythrocytes ratio (PCE/NCE) after 3h or 24h treatment with the mycotoxins. * Significantly different from C- ($p \leq 0.05$). ** Very significantly different from C- ($p \leq 0.01$). AFB1+OTA treatments have been compared with AFB1 treatment • Significantly different from AFB1 ($p \leq 0.05$). (B) Micronuclei induction of expressed as % of micronuclei in 2000 polychromatic erythrocytes ** Very significantly different from C- ($p \leq 0.01$).
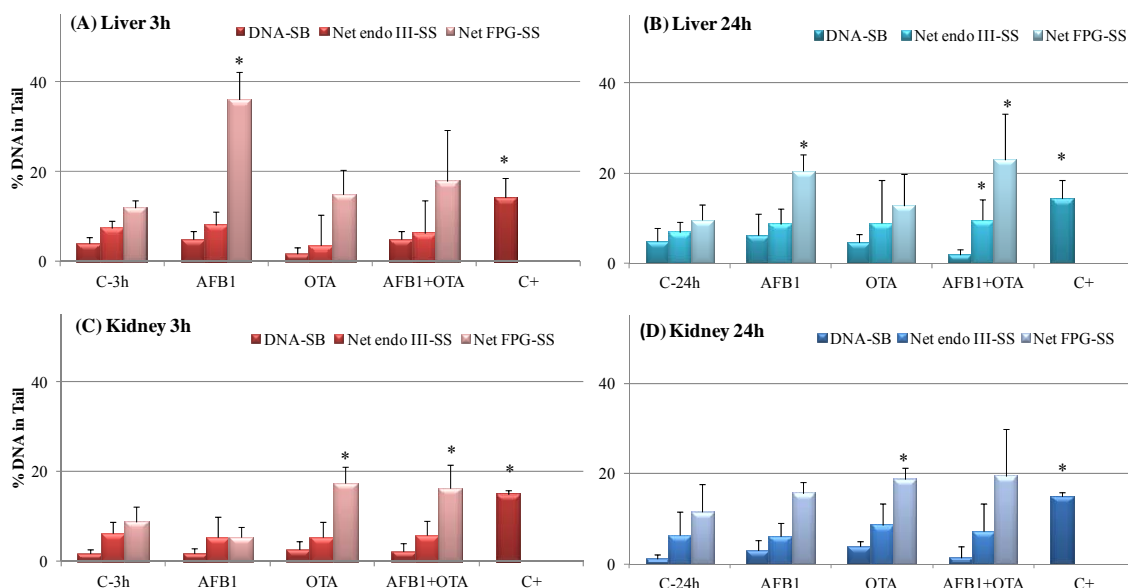

Figure 3: Liver and kindey DNA damage after 3h/24h of treatment expressed as strand breaks (SB: single and double strand breaks and AP sites) and oxidative DNA damage as net endo III sensitive sites (Net endo III-SS: oxidized pyrimidines) and net FPG sensitive sites (Net FPG-SS: 8-oxodGua, FAPY, FAPY-AFB1. * Significantly different from C- ($p \leq 0.05$). The mean and SD have been represented (n=5).