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Combined Genotoxic Effects of Aflatoxin B1, Ochratoxin A and Zearalenone in Rat Bone Marrow and Blood Leukocytes

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Abstract - Mycotoxins such as aflatoxin B1 (AFB1), ochratoxin A (OTA) and zearalenone (ZEA) are widespread contaminants of food and feedstuffs. It is very likely, that humans and animals are always exposed to mixtures of mycotoxins rather than to individual compounds. Therefore, risk assessments should consider mixture toxicity data. In the present study the combination of AFB1, OTA and ZEA was tested for genotoxicity in rat bone marrow and blood leukocytes after 15, 30 and 60 days treatment. The level of DNA damage was determined by the comet assay. The tail intensity and Olive tail moment in leukocytes and bone marrow cells were significantly higher than in controls. At the same time, the level of DNA damage in bone marrow cells was higher than in leukocytes. The data suggests that prolonged exposure to mycotoxins combination through food consumption can induce DNA damage contributing to the harmful effects *in vivo*.

Key words : mycotoxins, combined genotoxicity, comet assay, bone marrow, leukocytes

INTRODUCTION

Mycotoxins are secondary metabolites of mould; they produce a wide range of adverse and toxic effects in animals in addition to being food borne hazards to humans.

Among hundreds of known mycotoxins, aflatoxins, OTA and ZEA stand out as the most common contaminants in a variety of food. AFs are produced as secondary metabolites by *Aspergillus flavus* and *Aspergillus parasiticus* fungi. AFB1 is considered one of the most powerful human carcinogens. AFB1 is activated by hepatic cytochrome P450 enzyme system to produce a highly reactive intermediate, AFB1-8,9-epoxide, which subsequently binds to nucleophilic sites in DNA (Brahmi *et al.* 2011). OTA, produced by fungi *Aspergillus* and *Penicillium* genus, is mutagenic, teratogenic and nephrotoxic. Its oxidative activity is thought to play a central role in OTA-mediated carcinogenesis and may be divided into direct (covalent DNA adduction) and indirect (oxidative DNA damage) mechanisms of action (Pfohl-Leszkowicz *et al.* 2007). ZEA is a highly estrogenic mycotoxin produced by *Fusarium* species. ZEA caused cancer or at least increased its prevalence, although the mechanism of action is unknown. Many papers mentioned that exposure to ZEA results in genotoxicity and DNA damage (Ben Salah-Abbès *et al.* 2009).

Often more than one mycotoxin is found on a contaminated substrate. There are literature data on co-occurance of AFs, OTA and ZEA in foods (Šegvić Klarić 2012). However most studies are limited to the toxicology of a single mycotoxin, data on their combined action is very limited.

The alkaline single-cell gel electrophoresis (comet) assay is a sensitive and powerful method for determining DNA damage *in vitro* and *in vivo* (Tice *et al.* 2000). It was shown that alkaline comet assay is adequate and reliable biomarker of exposure to dietary/environmental compounds, including mycotoxins (Kamp *et al.* 2005; Zeljezić *et al.* 2006).

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The aim of the present study was to characterize combined genotoxic effects of AFB1, OTA and ZEA *in vivo* in rat bone marrow and blood leukocytes using the comet assay.

MATERIALS AND METHODS

1. Reagents

AFB1, OTA and ZEA were purchased from Scientific Research Institute of Nutrition of RAMS (Russia), heparin sodium and all reagents for comet assay were obtained from Sigma Aldrich (St. Louise, MO, USA), NaOH and salts for phosphate buffered saline were purchased from Medisar LLC (Armenia).

2. Animal treatment

Adult *Wistar* rats weighing 200 ± 20 g were kept in standard environmental conditions with a 12-h light/dark cycle and at a constant temperature of 24°C, fed a standard diet and had free access to water. The study was approved by the Ethical Committee of the Institute of Molecular Biology of the NAS RA (IRB IORG0003427). The rats were randomly assigned to four groups of 3 animals each receiving mixture of AFB1 (0.13 mg kg^{-1} bw per day), OTA (0.064 mg kg^{-1} bw per day) and ZEA (0.032 mg kg^{-1} bw per day) or solvent only (control group) for 15, 30 and 60 days. The mycotoxins doses selection was based on the literature data about individual genotoxicity of AFB1 (Brahmi *et al.* 2011), OTA (Kamp *et al.* 2005; Zeljezić *et al.* 2006) and ZEA (Ouanes *et al.* 2005) in rodents. Considering that the most studies have observed additive or synergistic effects of mycotoxins (Šegvić Klarić 2012), for the mixture we have chosen a lower concentration than those that were effective in the individual action. The rats were killed by cervical dislocation; blood and bone marrow were sampled for comet assay.

3. Cell preparation

Bone marrow

The left femur was removed and the bone marrow at both ends was exposed with bone cutters. Cells were flushed out with 3 mL PBS (pH 7.4) using a needle and syringe, and the cell suspension was filtered through a three layer bolting cloth.

Blood

Tail vein blood (1 mL) was collected into syringe with heparin (0.3 mL).

4. Comet assay

The level of DNA damage was evaluated by standard alkaline single-cell gel electrophoresis (comet assay) (Tice *et al.* 2000). Slides were examined at 250× magnifcation on a fluorescent microscope (ZEISS, Germany). At least 150 cells were scored per animal (50 cells scored per each of three replicate slides). Images of comets were recorded with a video camera with high sensitivity (Variocam, PCO, Germany) and processed on a computer program Comet Assay IV (Version 4.3). The tail intensity and Olive tail moment are used to evaluate the extent of DNA damage. Statistical analysis of the results was performed using SPSS 19 with application of non-parametric Mann-Whitney test (U test).

RESULTS AND DISCUSSION

In recent years, research efforts have concentrated on the investigation of the individual effects of AFB1 (Brahmi *et al.* 2011), OTA (Kamp *et al.* 2005, Zeljezić *et al.* 2006) and ZEA (Ouanes *et al.* 2005; Pfohl-Leszkowicz *et al.* 2007). However, considering the coincident production of mycotoxins, mixture toxicity data is needed for an improved and more realistic risk assessment. The *in vivo* effects of AFB1, OTA and ZEA combination commonly produced by fungi species has to our knowledge, not yet been investigated.

Combination of AFB1, OTA and ZEA found to be genotoxic to bone marrow and blood cells of rats, treated 15, 30 and 60 days (Figs. 1 and 2). In bone marrow cells the mycotoxins-induced increase of tail intensity was observed after 15, 30 and 60 days of exposure ($p < 0.05$). The increase of Olive tail moment was revealed only after 30 and 60 days of treatment ($p < 0.05$). In leukocytes the significant increase of tail intensity and Olive tail moment was observed after 30 and 60 days ($p < 0.05$). DNA damage, induced

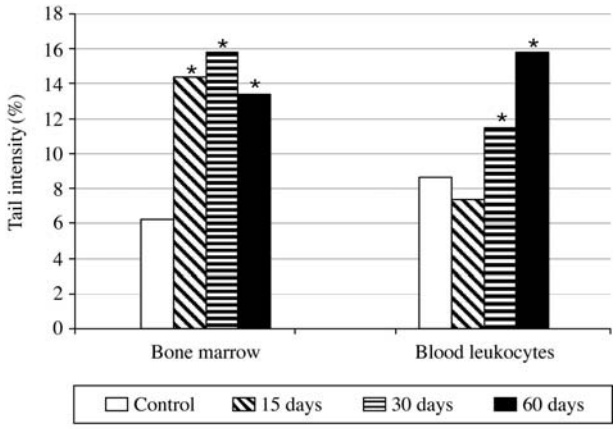

Fig. 1. The levels of tail intensity in the bone marrow cells and blood leukocytes of rats treated with mixture of AFB1, OTA and ZEA (*Significant difference ($p < 0.05$) compared with control).

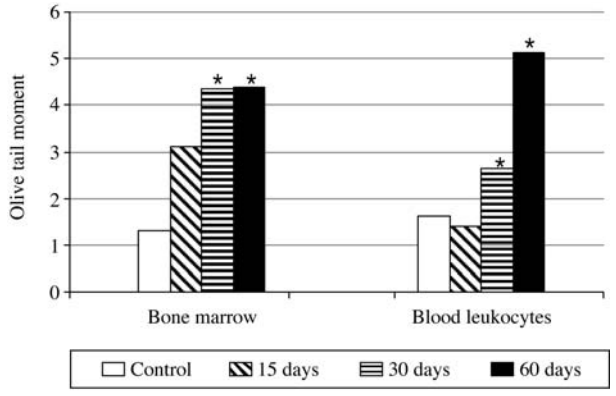

Fig. 2. The levels of Olive tail moment in the bone marrow cells and blood leukocytes of rats treated with mixture of AFB1, OTA and ZEA (*Significant difference ($p < 0.05$) compared with control).

by mycotoxins, was more pronounced in bone marrow cells than in leukocytes. This effect may be due to high metabolic activity of bone marrow cells and requires further elucidation.

Our study of combined AFB1, OTA and ZEA genotoxicity *in vivo* confirmed the genotoxic potential of mycotoxins combination, and show that the severity of DNA lesions correlates with time of treatment and depends on tissue type. The data obtained suggests that prolonged exposure to mycotoxins combination through food consumption can induce DNA damage contributing to the harmful effects *in vivo*.

The application of comet assay confirmed its potential value as a sensitive tissue specific biomarker of mycotoxins genotoxicity.

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