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# Genotoxicity of Fumonisin B<sub>1</sub>, Beauvericin and Ochratoxin A in Porcine Kidney PK15 Cells: Effects of Individual and Combined Treatment

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After 24- and 48-hour treatment with 0.05, 0.5 and 5 µg/mL of fumonisin (FB<sub>1</sub>), beauvericin (BEA) and ochratoxin A (OTA) alone or combinations of two or all three mycotoxins in equal concentrations, Giemsa-stained binucleated PK15 cells were scored for the presence of micronuclei (MN), nuclear buds (NBs) and nucleoplasmic bridges (NPBs). Single mycotoxins induced MN and NPBs in a dose-dependent manner, showing predominantly clastogenic effect. OTA showed stronger genotoxic potential than FB<sub>1</sub> and BEA, since 0.05 and 0.5 µg/mL OTA increased the frequency of MN and NPBs, respectively. Mycotoxin combinations increased the frequency of MN and NPBs, mostly in additive manner. A dose-dependent increase in NBs was observed after 24-hour exposure to single mycotoxins. Prolonged treatment with 5 µg/mL of each toxin alone as well as combined exposure to all mycotoxin concentrations resulted in a decrease in NBs, which could be related to the extrusion of micronuclei and/or saturation of the genotoxic effect. This is the first report on genotoxicity of BEA.

## INTRODUCTION

Fumonisin B<sub>1</sub> (FB<sub>1</sub>), beauvericin (BEA) and ochratoxin A (OTA) are worldwide-distributed mycotoxins, which contaminate food and feed, particularly maize.<sup>1–3</sup> Consumption of FB<sub>1</sub>-contaminated feed can lead to leukoencephalomalacia in horses, pulmonary oedema in pigs, as well as hepatotoxicity, nephrotoxicity, immunosuppression and carcinogenicity in laboratory animals.<sup>4–8</sup> FB<sub>1</sub> has also been associated with human oesophageal carcinoma in southern Africa and China.<sup>9,10</sup> Currently available informations on FB<sub>1</sub> genotoxic properties are limited and con-

troversial. Negative results were obtained by the gene mutation assay with *Salmonella typhimurium* strains TA98, TA100 and TA 102, SOS chromotest with *E. coli* strain PQ37, DNA repair assay with *E. coli* K-12 strains, and micronucleus (MN) test with rat reticulocytes.<sup>11–13</sup> Evidence of FB<sub>1</sub>-genotoxicity were reported in the bioluminescence test with *Vibrio fischeri* and cytogenetic studies (chromosome aberrations, sister chromatid exchange or induction of micronuclei) on primary rat hepatocytes, mice bone marrow cells, rabbit kidney RK13 cells, human derived hepatoma HepG2 cells, human lymphocytes and *Allium cepa*.<sup>11,12,14–17</sup>

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BEA is a cyclic hexadepsipeptide, which possesses antimicrobial, insecticidal, cytotoxic, ionophoric, immunosuppressive and apoptotic properties.<sup>18–22</sup> BEA increases ion permeability in biological membranes by forming a complex with essential cations ( $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ) and/or by forming cation-selective channels in lipid membranes, which may affect ionic homeostasis.<sup>23</sup> Save for the non-mutagenic activity in the Ames mutagenicity assay,<sup>24</sup> BEA has not yet been investigated for inducing MN formation, as an endpoint for genotoxicity.

OTA is nephrotoxic, carcinogenic, neurotoxic, teratogenic and immunosuppressive in laboratory animals.<sup>25</sup> Moreover, it has been associated with endemic nephropathy and urinary bladder tumours in humans.<sup>26,27</sup> OTA has produced contradictory results in tests for sister chromatid exchange and unscheduled DNA synthesis and did not induce chromosome aberrations in CHO cells.<sup>28</sup> However, OTA induced sister chromatid exchange in porcine urinary bladder epithelial cells as well as chromosome aberrations in human lymphocytes.<sup>29,30</sup> Dose-dependent induction of MN was seen in ovine seminal vesicle cells, syrian hamster embryo fibroblasts and HepG2 cells.<sup>31–33</sup> Moreover, clear genotoxic activity of OTA *e.g.* DNA adduct formation, was observed in laboratory rodents.<sup>34–36</sup>

Recently, Fenech and Crott<sup>37</sup> have shown that nucleoplasmic bridges (NPBs) and nuclear buds (NBs) are good biomarkers of genomic instability within the cytokinesis-block micronucleus assay (CBMN). The authors suggest that the CBMN assay may be a useful model for the study of the breakage-fusion-bridge cycle (BFB), which may be one of the mechanisms of rapid development of cancer cells.

Previously we demonstrated that  $\text{FB}_1$ , BEA and OTA induced lipid peroxidation and decreased intracellular glutathione levels in porcine kidney PK15 cells. Combined treatment with two or all of three mycotoxins resulted mostly in additive effects especially after a 24-hour exposure, although synergistic as well as antagonistic interactions could not be excluded depending on toxin concentrations and time of exposure.<sup>38</sup> In this study we wanted to check genotoxic potential of  $\text{FB}_1$ , BEA and OTA given alone by analysing MN, NPBs and NBs formation in porcine kidney PK15 cells, and to explore whether combinations of  $\text{FB}_1$ , BEA and OTA produce additive, synergistic or antagonistic effect.

## EXPERIMENTAL

### Cell Culture and Treatment

Porcine kidney epithelial cells PK15 (American Type Culture Collection, Rockville, Md USA) were grown in 75  $\text{cm}^2$  flasks in DMEM supplemented with 10 % heat-inactivated FBS, penicillin (100 IU/mL; 1 U  $\approx$  67.7  $\mu\text{g/mL}$ ), streptomycin (100  $\mu\text{g/mL}$ ) and amphotericin B (2.5  $\mu\text{g/mL}$ ) at 37 °C in a 5 %  $\text{CO}_2$ . For analysis of MN, NPBs and NBs forma-

tion, cells were seeded on sterile coverslips in 6-well plates. Plating density ranged from  $10^5$  to  $10^6$  cells/mL. Mycotoxin stock solutions (1  $\text{mg/mL}$ ) were prepared by dissolving  $\text{FB}_1$  in sterile water, and BEA and OTA in ethanol (100 %). The final concentrations of mycotoxins were obtained by dilution with the culture medium. The final volume fraction of ethanol in the medium was up to 0.3 %, which was shown not to alter cell viability.

### Micronucleus, Nucleoplasmic Bridges and Nuclear Buds Assay

In order to evaluate the frequency of MN, NBs and NPBs in binucleated cells, the cytokinesis-block micronucleus assay was used. PK15 cells were treated with  $\text{FB}_1$ , BEA and OTA (0.05, 0.5 and 5  $\mu\text{g/mL}$ ) for 24 and 48 h, and with combinations of the two ((0.05 + 0.05), (0.5 + 0.5), and (5 + 5)  $\mu\text{g/mL}$ , respectively) or three mycotoxins ((0.05 + 0.05 + 0.05), (0.5 + 0.5 + 0.5), and (5 + 5 + 5)  $\mu\text{g/mL}$ , respectively). Ethanol was added to control cells in the final volume fraction of 0.3 %. Cytochalasin B (3  $\mu\text{g/mL}$ ), which inhibits cytokinesis producing two daughter nuclei in the cytoplasm, was added for 24 h. Mytomycin C (0.03  $\mu\text{mol dm}^{-3}$ ) as standard clastogen and colchicine (1.2  $\mu\text{mol dm}^{-3}$ ) as aneugen were used as positive control. Cell slides were prepared according to Varga *et al.*<sup>39</sup> with minor modifications. After the treatment coverslips with cells were washed in phosphate buffer solution (pH = 7.4) and treated with 0.9 % NaCl for 5 min at room temperature. Cells on the coverslips were fixed with methanol-acetic acid (vol. ratio 5:1) mixed with an equal volume of 0.9 % NaCl. Afterwards, fixation with undiluted methanol-acetic acid (5:1) was performed three times. Air-dried slides were stained with 7 % (vol. fraction) Giemsa in phosphate buffer (pH = 6.8) for visual counting. 1000 binucleated cells were scored for the presence of MNs, NBs and NPBs under an oil immersion microscope according to Fenech and Crott.<sup>37</sup>

### Statistical Analysis

For each experimental point four cultures were treated in parallel and 1000 cells were evaluated from each culture for induction of MN, NBs and NPBs. Results represented as mean  $\pm$  SEM were statistically analysed by one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Tukey test). The level of  $P < 0.05$  was considered statistically significant. To compare the expected with measured frequency ( $F$ ), the expected value was calculated by adding the mean of frequency after exposure to one mycotoxin alone (or mixture of two mycotoxines) to the mean of frequency obtained after exposure to the second or third substance.<sup>38</sup> Expected frequency ( $F_{\text{expt}}$ ) was calculated as follows:

$$F_{\text{expt}}(\text{FB}_1 + \text{BEA}) = F_{\text{FB}_1} + F_{\text{BEA}} - F_{\text{control}}$$

$$F_{\text{expt}}(\text{FB}_1 + \text{BEA} + \text{OTA}) = F_{(\text{FB}_1 + \text{BEA})} + F_{\text{OTA}} - F_{\text{control}}$$

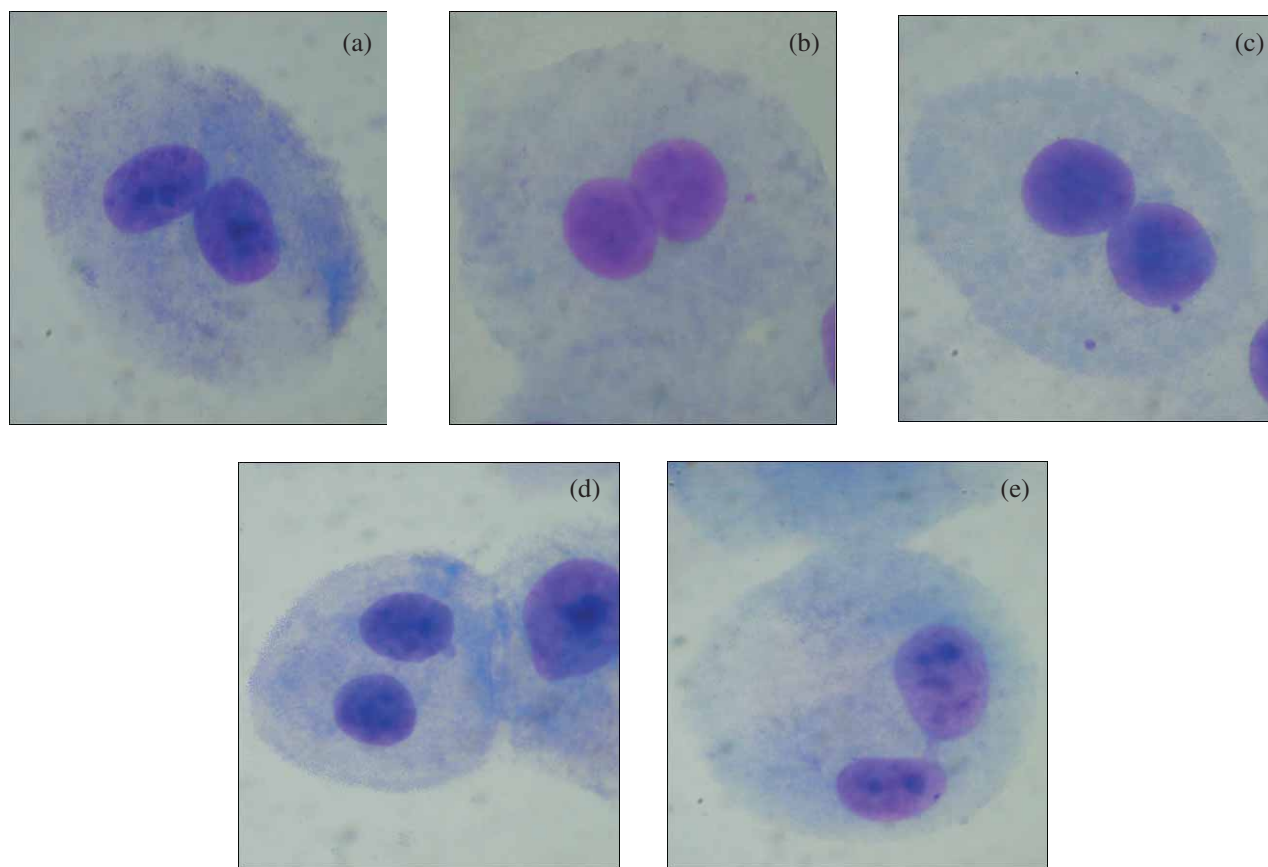


Figure 1. Normal binucleated (BN) PK15 cell (a); BN PK15 cells with 1 micronuclei (MN), treated with 5 µg/mL FB<sub>1</sub> for 24 h (b); BN PK15 cells with 2 MN, treated with combination FB<sub>1</sub> + BEA, 0.5 µg/mL of each toxin, for 24 h (c); BN PK15 cells with 1 nuclear bud treated with 0.05 µg/mL BEA for 24 h (d); and BN PK15 cells with 1 nucleoplasmic bridge treated with combination BEA + OTA, 0.5 µg/mL of each toxin, for 24 h (e).

Expected SEM ( $SEM_{\text{expt}}$ ) was calculated as follows:

$$SEM_{\text{expt}}(\text{FB}_1 + \text{BEA}) = (SEM_{\text{FB}_1}^2 + SEM_{\text{BEA}}^2)^{1/2}$$

$$SEM_{\text{expt}}(\text{FB}_1 + \text{BEA} + \text{OTA}) = (SEM_{(\text{FB}_1 + \text{BEA})}^2 + SEM_{\text{OTA}}^2)^{1/2}$$

The significance of difference between the expected and measured values was calculated using an unpaired *t*-test. The level of  $P < 0.05$  was considered statistically significant. The results were interpreted as follows: an additive effect was recorded if the measured values were not significantly above or below the expected values; a synergistic effect was recorded if the measured values were significantly above the expected values; an antagonistic effect was recorded if the measured values were significantly below the expected values.

## RESULTS AND DISCUSSION

In our experiment we scored MN, NBs and NPBs in binucleated PK15 following the 24- and 48-hour exposure to FB<sub>1</sub>, BEA, OTA and their combinations (Table I, II; Figure 1). The results are presented as the frequency of

MN, NBs and NPBs per 1000 binucleated cells ( $\%$ )  $\pm$  SEM. We used mytomycin C and colchicine to distinguish the clastogenic from aneugenic activity of the tested mycotoxins. Mytomycin C and colchicine significantly increased the frequencies of MN (about 20  $\%$ ) and NBs (about 90  $\%$ ) after 24 and 48 h, as compared to 0.3  $\%$  ethanol (about 6 and 60  $\%$ , respectively). Only mytomycin C, as standard clastogen, could induce formation of NPBs (2.8  $\%$  and 6  $\%$ ) in PK15 cells.

The results of the CBMN assay show that FB<sub>1</sub>, BEA and OTA induce MN in PK15 cells in a dose-dependent manner. After 24-hour treatment, the lowest concentration of OTA (0.05 µg/mL) significantly increased MN frequency (13  $\%$ ) as compared to control (5.7  $\%$ ), while the same concentrations of FB<sub>1</sub> and BEA did not significantly affect the formation of MN (7.7  $\%$  and 10.7  $\%$ , respectively). Treatment with 0.5 µg/mL BEA and OTA significantly increased frequency of MN (14  $\%$  and 16.3  $\%$ , respectively), while with 5 µg/mL all of three mycotoxins showed almost equal genotoxic potential (MN was about 17  $\%$  and 18  $\%$ , respectively;  $P < 0.05$ ). Combined treatment with two or all of the three mycotoxins increased MN frequency, mostly in additive manner (Figure 2a,b).

TABLE I. Induction of MN, NBs and NPBs in Giemsa stained binucleated (BN) PK15 cells after 24-hour treatment with FB<sub>1</sub>, BEA and OTA given alone or in combination<sup>(a)</sup>

Treatment	MN ± SEM (%)	NBs ± SEM (%)	NPBs ± SEM (%)
$\gamma$ (FB <sub>1</sub> ) / $\mu\text{g mL}^{-1}$			
0.05	(7.7 ± 1.3) (c)	(58 ± 2.2) (c)	(0.0 ± 0.0) (c)
0.5	(12.6 ± 1.2) (c)	(85.1 ± 3.5) (b)	(0.25 ± 0.25) (c)
5	(17.3 ± 1.2) (b),(c)	(88.3 ± 3.8) (b),(c)	(4.0 ± 1.5) (c)
$\gamma$ (BEA) / $\mu\text{g mL}^{-1}$			
0.05	10.7 ± 1.2	(62.0 ± 3.6) (d)	1.8 ± 0.8
0.5	(14.0 ± 1.5) (b),(d)	(84.3 ± 2.6) (b)	(2.7 ± 0.8) (d)
5	(18.4 ± 2.0) (b),(d)	(87.0 ± 3.6) (b),(d)	(7.6 ± 1.4) (b)
$\gamma$ (OTA) / $\mu\text{g mL}^{-1}$			
0.05	(13.0 ± 1.6) (b)	(60.0 ± 4.0) (e)	1.8 ± 0.8
0.5	(16.3 ± 1.8) (b)	(90.4 ± 4.6) (b)	(7.0 ± 1.0) (b)
5	(17.3 ± 1.2) (b),(e)	(91.0 ± 3.2) (b),(e)	(7.0 ± 1.0) (b)
$\gamma$ (FB <sub>1</sub> +BEA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	(16.3 ± 0.8) (b),(c)	(82.3 ± 2.4) (b),(c),(d)	3.4 ± 1.2
0.5 + 0.5	(16.3 ± 0.8) (b)	(85.7 ± 2.4) (b)	(6.0 ± 1.0) (b)
5 + 5	(18.6 ± 1.2) (b),(f)	(101.8 ± 4.5) (b),(f)	(7.6 ± 2.6) (b)
$\gamma$ (FB <sub>1</sub> +OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	(14.3 ± 1.2) (b),(c)	(88.4 ± 3.0) (b),(c),(e)	2.0 ± 1.0
0.5 + 0.5	(18.4 ± 1.8) (b)	(92.0 ± 2.5) (b)	(8.8 ± 2.4) (b),(c)
5 + 5	(21.6 ± 1.2) (b),(g)	(102.7 ± 2.8) (b),(g)	(10.8 ± 0.8) (b),(c)
$\gamma$ (BEA+OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	(15.3 ± 1.2) (b)	(94.7 ± 3.5) (b),(d),(e)	(5.0 ± 1.8) (b)
0.5 + 0.5	(19.2 ± 1.4) (b)	(97.0 ± 4.0) (b)	(9.0 ± 1.8) (b)
5 + 5	(37.0 ± 3.2) (b),(d),(e)	(51.0 ± 2.6) (d),(e)	(7.8 ± 0.4) (b)
$\gamma$ (FB <sub>1</sub> +BEA+OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05 + 0.05	(15.7 ± 1.8) (b),(c)	(92.3 ± 3.2) (b)-(e)	(4.8 ± 0.4) (b),(c)
0.5 + 0.5 + 0.5	(21.6 ± 2.0) (b),(c),(d)	(94.0 ± 3.0) (b)	(7.7 ± 0.4) (b),(c)
5 + 5 + 5	(40.0 ± 4.3) (b)-(g)	(49.3 ± 3.5) (c)-(g)	(8.3 ± 0.8) (b)
Control <sup>(h)</sup>			
Ethanol	5.7 ± 1.2	59.6 ± 2.3	0.0 ± 0.0
Mitomycin C	(18.2 ± 2.2) (b)	(90.8 ± 4.6) (b)	2.8 ± 1.0
Colchicine	(21.0 ± 2.0) (b)	(92.0 ± 6.2) (b)	0.0 ± 0.0

<sup>(a)</sup> Means of scoring 1000 BN PK15 cells/sample are given.

<sup>(b)</sup>  $P < 0.05$ : as compared to the control value.

<sup>(c),(d),(e)</sup>  $P < 0.05$ : each toxin taken alone as compared to a combination of two and three toxins in equal concentrations.

<sup>(f),(g)</sup>  $P < 0.05$ : two-toxin combination as compared to the combination of three toxins in equal concentrations.

<sup>(h)</sup> 0.3 % ethanol, 0.03  $\mu\text{mol dm}^{-3}$  mitomycin C, 1.2  $\mu\text{mol dm}^{-3}$  colchicine.

A marked increase in MN frequency was observed with combinations BEA + OTA (37 %) and FB<sub>1</sub> + BEA + OTA (40 %), at 5  $\mu\text{g/mL}$  doses for each mycotoxin. Treatment with FB<sub>1</sub> + BEA and FB<sub>1</sub> + OTA, each given at 5  $\mu\text{g/mL}$ , produced a similar genotoxic effect (18.6 and 21.7 %, respectively) as the treatment with the same dose of single toxins. Generally, frequencies of MN after 48-hour exposure to single toxins or their combinations were not

significantly changed comparing to the results of 24-hour treatment. Only the three-toxin treatment resulted with the decrease of MN frequency comparing to the 24-hour exposure, which could be a consequence of saturation of the genotoxic effect, or decreased mitotic index and/or undergoing cell necrosis.

Our findings clearly demonstrate that FB<sub>1</sub>, BEA and OTA are genotoxic to PK15. Induction of MN by FB<sub>1</sub>

TABLE II. Induction of MN, NBs and NPBs in Giemsa stained binucleated (BN) PK15 cells after 48-hour treatment with FB<sub>1</sub>, BEA and OTA given alone or in combination<sup>(a)</sup>

Treatment	MN ± SEM (%)	NBs ± SEM (%)	NPBs ± SEM (%)
$\gamma$ (FB <sub>1</sub> ) / $\mu\text{g mL}^{-1}$			
0.05	(6.2 ± 1.2) <sup>(c)</sup>	63.4 ± 2.0	(0.25 ± 0.25) <sup>(c)</sup>
0.5	(14.0 ± 1.6) <sup>(c)</sup>	(86.0 ± 3.2) <sup>(b),(c)</sup>	(0.25 ± 0.25) <sup>(c)</sup>
5	(19.6 ± 1.4) <sup>(b),(c)</sup>	52.2 ± 2.4	(1.6 ± 1.2) <sup>(c)</sup>
$\gamma$ (BEA) / $\mu\text{g mL}^{-1}$			
0.05	(7.6 ± 1.2) <sup>(d)</sup>	64.0 ± 3.2	2.0 ± 1.0
0.5	(15.2 ± 1.8) <sup>(d)</sup>	(79.6 ± 3.8) <sup>(d)</sup>	(3.6 ± 0.4) <sup>(d)</sup>
5	(18.6 ± 1.8) <sup>(b),(d)</sup>	53.0 ± 5.5	(8.0 ± 1.2) <sup>(b)</sup>
$\gamma$ (OTA) / $\mu\text{g mL}^{-1}$			
0.05	(10.4 ± 1.4) <sup>(e)</sup>	65.2 ± 3.8	2.6 ± 0.6
0.5	(15.2 ± 1.4) <sup>(e)</sup>	(83.6 ± 1.8) <sup>(b),(e)</sup>	(5.6 ± 1.4) <sup>(e)</sup>
5	(17.8 ± 1.8) <sup>(b),(e)</sup>	53.2 ± 2.8	(7.0 ± 1.5) <sup>(b)</sup>
$\gamma$ (FB <sub>1</sub> +BEA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	13.2 ± 1.4	70.0 ± 4.6	3.6 ± 1.4
0.5 + 0.5	(20.2 ± 2.0) <sup>(b)</sup>	(83.0 ± 1.8) <sup>(b),(f)</sup>	(7.2 ± 1.8) <sup>(b),(c)</sup>
5 + 5	(21.8 ± 3.4) <sup>(b)</sup>	(46.2 ± 3.8) <sup>(b)</sup>	(11.0 ± 2.0) <sup>(b),(c)</sup>
$\gamma$ (FB <sub>1</sub> +OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	(12.6 ± 1.2) <sup>(g)</sup>	70.6 ± 1.8	(5.0 ± 1.0) <sup>(b),(c)</sup>
0.5 + 0.5	(18.0 ± 2.0) <sup>(b),(g)</sup>	(60.4 ± 3.8) <sup>(c),(e)</sup>	(8.0 ± 2.0) <sup>(b),(c)</sup>
5 + 5	(24.2 ± 2.8) <sup>(b)</sup>	55.4 ± 2.8	(11.0 ± 2.0) <sup>(b),(c)</sup>
$\gamma$ (BEA+OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05	(16.2 ± 1.8) <sup>(b),(d)</sup>	69.0 ± 5.1	(7.0 ± 1.4) <sup>(b)</sup>
0.5 + 0.5	(29.0 ± 2.5) <sup>(b),(d),(e)</sup>	(59.4 ± 5.2) <sup>(d),(e)</sup>	(10.0 ± 2.0) <sup>(b)</sup>
5 + 5	(36.0 ± 3.0) <sup>(b),(d),(e)</sup>	(43.2 ± 4.0) <sup>(b)</sup>	(9.6 ± 1.8) <sup>(b)</sup>
$\gamma$ (FB <sub>1</sub> +BEA+OTA) / $\mu\text{g mL}^{-1}$			
0.05 + 0.05 + 0.05	(22.0 ± 2.0) <sup>(b)-(g)</sup>	61.0 ± 7.5	(7.0 ± 1.2) <sup>(b),(c)</sup>
0.5 + 0.5 + 0.5	(29.2 ± 3.4) <sup>(b)-(g)</sup>	(64.8 ± 5.2) <sup>(c),(e),(f)</sup>	(12.4 ± 2.4) <sup>(b)-(e)</sup>
5 + 5 + 5	(30.0 ± 2.0) <sup>(b)-(e)</sup>	(40.6 ± 0.6) <sup>(b)</sup>	(8.0 ± 1.2) <sup>(b),(c)</sup>
Control <sup>(h)</sup>			
Ethanol	6.2 ± 1.4	63.4 ± 3.0	0.0 ± 0.0
Mitomycin C	(21.2 ± 3.2) <sup>(b)</sup>	(86.8 ± 6.6) <sup>(b)</sup>	(6.0 ± 2.0) <sup>(b)</sup>
Colchicine	(20.0 ± 2.0) <sup>(b)</sup>	(88.0 ± 4.6) <sup>(b)</sup>	0.0 ± 0.0

<sup>(a)</sup> Means of scoring 1000 BN PK15 cells/sample are given.

<sup>(b)</sup>  $P < 0.05$ : as compared to the control value.

<sup>(c),(d),(e)</sup>  $P < 0.05$ : each toxin taken alone as compared to a combination of two and three toxins in equal concentrations.

<sup>(f),(g)</sup>  $P < 0.05$ : two-toxin combination as compared to the combination of three toxins in equal concentrations.

<sup>(h)</sup> 0.3 % ethanol, 0.03  $\mu\text{mol dm}^{-3}$  mitomycin C, 1.2  $\mu\text{mol dm}^{-3}$  colchicine.

was reported for polychromatic erythrocytes in the bone marrow of mice at the mass ratio of 25 mg/kg, in rabbit kidney RK13 cells at the concentration of 0.07  $\mu\text{g/mL}$ , in human derived hepatoma HepG2 cells at 25  $\mu\text{g/mL}$ , and in human lymphocytes exposed to 5  $\mu\text{g/mL}$ .<sup>12,15-17</sup> The observed dominant clastogenic effect of FB<sub>1</sub> is in agreement with these literature reports. The mechanism by which FB<sub>1</sub> could induce genotoxicity is not fully

understood. *In vitro* and *in vivo* studies indicate that FB<sub>1</sub> can damage DNA indirectly by increasing the oxidative stress.<sup>40</sup> Our previous study demonstrated that FB<sub>1</sub> at the concentration of 0.5  $\mu\text{g/mL}$  significantly decreased the glutathione level in PK15 cells which is probably due to the production of reactive oxygen species.<sup>38</sup> To our knowledge, the induction of MN by BEA has not been reported before. Since BEA acts as an ionophore and in-

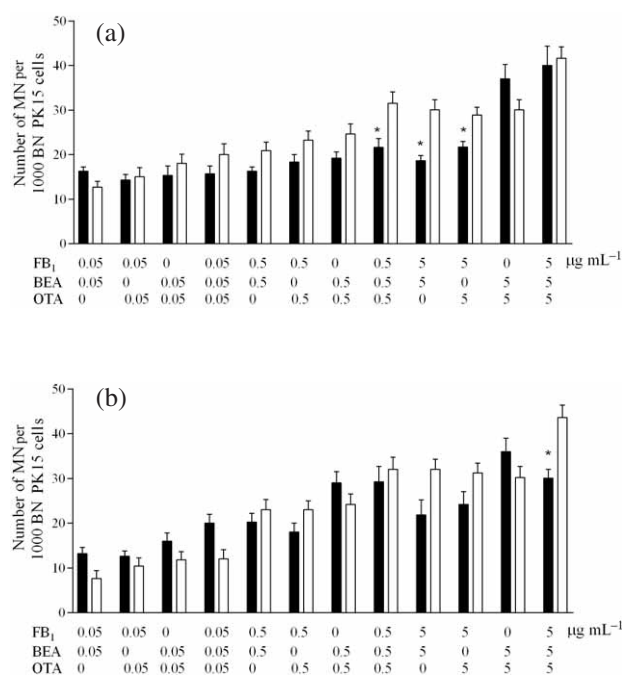


Figure 2. Induction of micronuclei (MN) in binucleated (BN) PK15 cells after 24-hour (a) and 48-hour (b) combined treatment with fumonisin B<sub>1</sub> (FB<sub>1</sub>), beauvericin (BEA) and ochratoxin A (OTA). Dark bars represent the measured frequency and white bars the expected frequency. Asterisk (\*) represent significant antagonistic effect ( $P < 0.05$ ).

increases the intracellular calcium level by increasing the formation of cation-selective channels in lipid membrane,<sup>23</sup> we can speculate that the induction of mitotic disturbances can be related to changes in intracellular calcium concentrations. OTA induction of MN was reported for seminal vesicle OSV cells, Syrian hamster embryo SHE fibroblasts and HepG2 cells at concentrations near 5 μg/mL, as well as for primary cultures of rat and human kidney at the concentration of 0.002 μg/mL and 0.05 μg/mL, respectively.<sup>32–34,41</sup> In kinetochore analysis Dopp *et al.*<sup>33</sup> clearly demonstrated that OTA induces predominantly clastogenic effect, which is in agreement with our findings. OTA also causes an intracellular calcium rise by enhancing the permeability of the cellular membrane to calcium, as well as free radical production, which is closely related to the DNA damage.<sup>33,42</sup> These mechanisms could be the reason for marked additive genotoxic effects, which were observed after combined treatment of PK15 cells with BEA + OTA. Our previous study showed that FB<sub>1</sub>, BEA and OTA decreased glutathione level in PK15 cells in concentrations so low (0.05 and 0.5 μg/mL) that they did not affect cell viability and lipid peroxidation. Combined treatment also resulted mostly in additive effects,<sup>38</sup> which correlates with the genotoxic effects of single and combined toxins. We can speculate that oxidative stress might be related to genotoxicity of FB<sub>1</sub>, BEA and OTA.

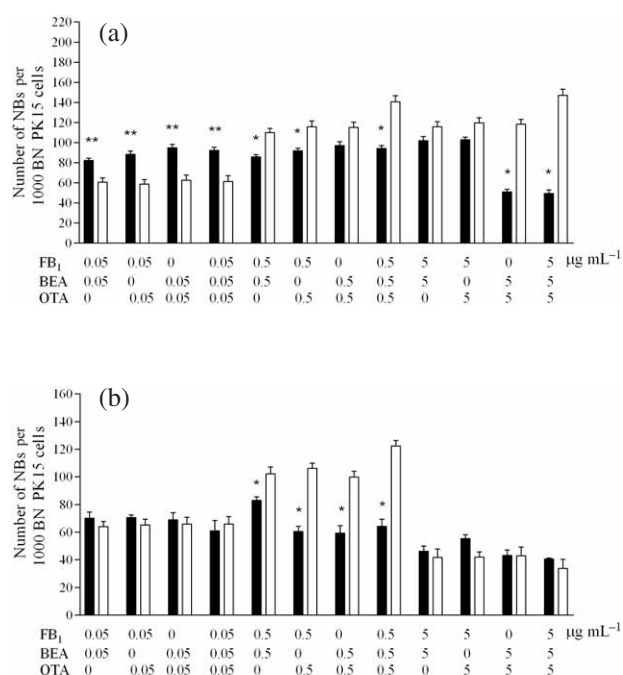


Figure 3. Induction of nuclear buds (NBs) in binucleated (BN) PK15 cells after 24-hour (a) and 48-hour (b) combined treatment with fumonisin B<sub>1</sub> (FB<sub>1</sub>), beauvericin (BEA) and ochratoxin A (OTA). Dark bars represent the measured frequency and white bars the expected frequency. Asterisk (\*) represent significant antagonistic effect ( $P < 0.05$ ). Two asterisks (\*\*) represent significant synergistic effect.

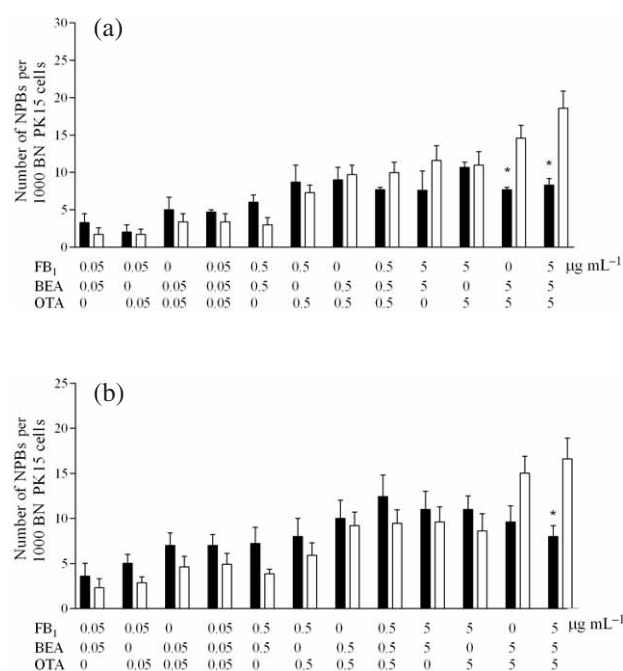


Figure 4. Induction of nucleoplasmic bridges (NPBs) in binucleated (BN) PK15 cells after 24-hour (a) and 48-hour (b) combined treatment with fumonisin B<sub>1</sub> (FB<sub>1</sub>), beauvericin (BEA) and ochratoxin A (OTA). Dark bars represent the measured frequency and white bars the expected frequency. Asterisk (\*) represent significant antagonistic effect ( $P < 0.05$ ).

Nuclear budding is a unique mechanism of MN formation where amplified DNA is localised to a specific site at the periphery of the nucleus during the S phase of the cell cycle.<sup>43</sup> The NBs have the same morphology as MN with the exception that they are linked to the nucleus by a wide or narrow stalk of nucleoplasmic material, which depends on the stage of budding process. The duration of budding and final formation of MN is not known. However, it has been shown that NBs and MN are formed simultaneously after exposure to genotoxins.<sup>37</sup> In this study, the frequency of NBs significantly increased after 24-hour treatment with 0.5 µg/mL of FB<sub>1</sub> (85.1 %), BEA (84.3 %) and OTA (90.3 %), as compared to the control (59.6 %). Combined treatment with 0.05 µg/mL of each toxin resulted in synergistic increase of NBs, while after exposure to toxin combinations, each given at 0.5 or 5 µg/mL, additive or antagonistic effects were recorded (Figure 3a). Exposure of 48 h to mycotoxins given alone or to their combinations resulted in decrease of NBs comparing to the 24-hour treatment (Figure 3b). Decrease of NBs frequency is probably connected with the extrusion and formation of micronuclei after 24 h and/or saturation of genotoxic effect after prolonged exposure to mycotoxin combinations.

NPBs between nuclei in BN cells have been proposed for scoring in the CBMN assay because they were observed after exposure to clastogens and are thought to originate from rearranged chromosomes with more than one centromere.<sup>44</sup> Our results show that OTA (0.5 µg/mL) and BEA (5 µg/mL) significantly increased the frequency of NPB (about 7 %) after the 24-hour treatment in respect to the control (0 %), while FB<sub>1</sub> did not affect significantly the formation of NPB (0–4 %) at any of concentrations considered. Combined mycotoxin treatment showed mostly additive genotoxic effect (Figure 4). The results of scoring NPBs following the 48-hour exposure to mycotoxins did not differ significantly from the results obtained for the 24-hour treatment. All three mycotoxins were predominantly clastogenic as compared to the positive controls as well as considering the formation of NPBs. To our knowledge, this study is the first to report induction of NBs and NPBs by FB<sub>1</sub>, BEA and OTA.

## CONCLUSIONS

FB<sub>1</sub>, BEA and OTA are genotoxic to PK15, showing predominant clastogenic effects. All of three mycotoxins induce MN and NPBs in PK15 cells in a dose-dependent manner. Combined mycotoxin treatment showed mostly additive effects in the formation of MN and NPBs. A dose-dependent increase in NB frequency was observed following the 24-hour mycotoxin treatment. Prolonged exposure to each toxin alone (at the concentration 5 µg/mL) as well as combined treatment with all mycotoxin concentrations resulted in a decrease in NBs, which could

be related to the extrusion of micronuclei and/or saturation of the genotoxic effect. In conclusion, this study provides additional evidence for the hypothesis that OTA and FB<sub>1</sub> are carcinogens with genotoxic properties. Since these mycotoxins were effective in relatively low concentrations, these effects might also occur in animals and humans following the consumption of mycotoxin-contaminated feed and food. This study is the first to report clastogenic effects of BEA in cell cultures.

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## SAŽETAK

### Genotoksičnost fumonizina B<sub>1</sub>, bovericina i okratoksina A u stanicama bubrežnog epitela svinje (PK15): djelovanje pojedinačnih i kombiniranih mikotoksina

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Stanice bubrežnog epitela svinje (PK15) tretirane su s 0,05, 0,5 i 5 µg/mL fumonizina (FB<sub>1</sub>), bovericina (BEA) i okratoksina A (OTA) te kombinacijama dva ili tri mikotoksina u istim koncentracijama tijekom 24 i 48 h. Binuklearne PK15 stanice (Giemsa bojenje) pretražene su na prisutnost mikronukleusa (MN), nuklearnih pupova (NP) i nukleoplazmatskih mostova (NPM). Pojedinačni toksini inducirali su tvorbu MN i NPM ovisno o dozi, pokazujući dominantno klastogeno djelovanje. OTA ima jači genotoksični potencijal od FB<sub>1</sub> i BEA, s obzirom na to da 0,05 i 0,5 µg/mL OTA značajno povećava učestalost MN i NPM. Mikotoksini aplicirani u kombinaciji uglavnom pokazuju aditivno djelovanje. Učestalost NP povećava se ovisno o dozi nakon 24-satnog tretmana s pojedinačnim toksinima. Dulje izlaganje pojedinačnim mikotoksinima (5 µg/mL) te kombinacijama toksina u svim koncentracijama dovodi do smanjenja učestalosti NP, što se može pripisati odvajanju MN i/ili zasićenosti genotoksičnog djelovanja. Ovo je prvo izvješće o genotoksičnom djelovanju BEA.