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Original article

CYTOTOXICITY OF THE *FUSARIUM* MYCOTOXIN DEOXYNIVALENOL ON MAMMALIAN AND AVIAN CELL LINES

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Summary

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Trichothecenes are mycotoxins that occur in grains and can lead to acute and chronic poisoning in animals and humans. Deoxynivalenol (DON) is a type B trichothecene affecting protein synthesis, immune system, leading to brain, blood and kidney disorders. The aim of this work was to evaluate *in vitro* the cytotoxicity and the pathological effects of DON in short-term experiments on cells from non-tumour and tumour permanent cell lines and to compare their sensitivity. Cell cultivation of BALB/c 3T3, DEC 99, MDA-MB-231, MCF-7 and Hela cells was performed. Quantitative and qualitative methods evaluating cytotoxicity on the base of statistical and morphological analyses for determining the impact on the viability and proliferative activity were used: Neutral Red Uptake (NRU) cytotoxicity test, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and fluorescence microscopy. The cytotoxic effect of DON was assessed after an exposure period of 24 h. DON treatment induced significant alterations in the growth and morphology of the cells, involving early and late apoptosis and necrosis signs. Statistically significant decrease of the viability of all cell lines was established at concentrations of DON starting from 1.9 µg/mL to 3.7 µg/mL, the mean IC_{50} concentrations were calculated. According to the IC_{50} values the hierarchical order of cell lines' sensitivity was determined.

Key words: cytotoxicity, deoxynivalenol, permanent cell lines

INTRODUCTION

Trichothecenes are a widespread group of mycotoxins discovered around 1940 in cereals (Freeman & Morrison, 1948). Apart from *Fusarium,* they are also produced by other genera of moulds: *Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium* and *Stachybotrys* (Ueno, 1984). About 200 compounds were isolated, some of them being found in food and feeds: trichodermol, trichodermin, 4,15-diacetoxyscirpenol (DAS), neosolaniol, T-2 toxin, isotrichodermol,

calonectrin, 7,8-dihydroxy calonectrin, harzianum, nivalenol (NIV), deoxynivalenol (DON), fusarenon-X, trichothecin, trichothecinol A, crotocin, satratoxin H, roridin A, baccharin, verrucarin A etc. These toxins consist of a trichothecenic ring with double bonds at C_9 and C_{10} and epoxide at C_{12}. Their molecular weight is low – about 250–500 g/mol. They are insoluble in water and soluble in organic solvents, resistant to high temperatures and are not decomposed by heat treatment. Trichothecenes are absorbed and distributed rapidly in the body after oral administration to accumulate in the muscles and the liver (Corley *et al.,* 1986). Residues from T-2 and DON for example, could be found in eggs and milk (Prelusky *et al.,* 1987a, b). Trichothecenes inhibit protein, DNA, RNA synthesis, alter membranes and mitochondria, activate apoptosis and cytokines (Rocha *et al.,* 2005).

Deoxynivalenol ($C_{15}H_{20}O_6$) was discovered in 1972 in Japan (Morooka *et al.,* 1972). It is a type B trichothecene, epoxysesquiterpenoid with a molecular weight of 296.32 g/mol, produced by *F. graminearum* and *F. culmorum*. The mechanism of action of DON is associated with suppression of protein synthesis and increased consumption of tryptophan with subsequent accumulation of serotonin excess in the brain. DON affects peripheral and central serotonin receptors (Eriksen & Alexander, 1998), competitively displaces serotonin and increases its levels in CNS. Poisoning with DON causes vomiting, acute stomach pain, diarrhoea, anorexia, haemorrhagic syndrome, dyspnoea, hypothermia, hypotension, tachycardia, tremor, seizures, coma or nerve disorders, nephropathy, embryotoxic effects and abortion and death (Khera *et al.,* 1984; Pestka *et al*., 1987; 1989; Perkowski *et al*., 1990; Jia & Pestka, 2005; Pestka, 2010). DON and T-2 are trichothecenes believed to have been used in the "yellow rain" chemical weapon in the wars in Afghanistan and Cambodia (Desjardins, 2009). The evaluation and elucidation of the pathological effects of mycotoxins in laboratory conditions is an achievable and adequate approach to the study of these natural contaminates of products used for food and feed to supplement the existing information on human health risk. The search for appropriate *in vitro* models in research in mycotoxicology is warranted.

The toxic impact of deoxynivalenol on cell proliferation and differentiation was studied and elucidated in numerous short- and long-term experimental studies. The suggested mechanisms of DON toxicity on prokaryotic cells include penetration through the phospholipid bilayer and acting at the subcellular level, interaction with the cellular membranes, free radical mediated phospholipid peroxidation or involvement of more than one of these processes (Rizzo *et al.,* 1992). This multiplicity of the toxic impact of DON has led to investigations on its cell proliferation impact, influence on differentiation and cytokine secretion, the involvement of heat shock protein 90 (Hsp90) and oxidative stress in DON toxicity. Some of its *in vivo* effects have been reviewed in the recent years in *in vitro* models and a variety of cell lines were used: HL60, MOLT-4, A-10, HEp-2, HeLa, Caco-2, T-84, HT-29-D4, HEpG2, 3T3, B lymphocyte REH, human pre-T lymphocyte Jurkat, BHK21, MH-22a, CHO-K1, renal proximal tubule epithelial, normal human lung fibroblast, human dendritic cells, A549, U937, IPEC-J2, MRC-5, K562, human peripheral blood mononuclear cells etc. (Calvert *et al*., 2005; Sobrova *et al*., 2010; Springler *et al*., 2017; Yang *et al.,* 2017; Nagashima, 2018).

This study aimed to obtain additional information and to evaluate the intrinsic cytotoxicity of deoxynivalenol in BALB/c 3T3, DEC, Hela, MDA-MB-231 and MCF-7 cell lines, to characterise its effects on cell viability, proliferation and apoptosis, to compare cell type sensitivity and possible cell type specificity.

MATERIALS AND METHODS

Cell cultivation

Permanent cell lines BALB/c 3T3, DEC 99 (Ivanov *et al*., 2001), MDA-MB-231, MCF-7 and Hela cell were used in the experiments. BALB/c 3T3 (3T3 clone 31) is a standardised fibroblast cell line. The cells are derived from embryos of Swiss albino mice. This cell line has been widely used as an experimental model system in various types of toxicological studies (assessment of cytotoxicity, photo toxicity). DEC99 is a permanent cell line of embryonic cells of a Pekin duck stored in the collection of cell cultures of IEMPAM, BAS. Cells from this line are also non-tumourigenic. The use of avian and mammalian cells will allow clarification of the species-specificity of the cytotoxic effects. Human tumour cell line MDA-MB-231 is an aggressive epithelial human breast cancer cell line from a metastatic poorly differentiated triple-negative mammary adenocarcinoma (Cailleau *et al*., 1978). MCF-7 is a cell line originating from an invasive breast ductal carcinoma, estrogen and progesterone receptors positive. Hela is a cell line derived from an aggressive adenocarcinoma of the cervix. All these lines have been widely used in similar studies. The cell lines BALB/c 3T3, MDA-MB-231, MCF-7 and Hela were obtained from American Type Culture Collection (ATCC).

All cell cultures were grown as monolayers in 75 cm^2 tissue culture flasks in DMEM supplemented with 10% foetal bovine serum and appropriate doses of antibiotics. Cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Test substance

Deoxynivalenol (Sigma-Aldrich) was diluted in ethanol immediately before use and was further diluted in complete culture medium to final concentration of the ethanol below 1% and working concentration of DON ranging from 1.9 to 10 μg/mL.

In vitro tests

After experimental treatment upon different periods of time (from 6 h to 48 h), the duration of the exposure of cells to DON for 24 h was established as a sufficient time for cytotoxicity induction and in dose-response relationship. This period was based on constant observations of the cell morphology and detachment changes from the first hour onwards of the application of DON.

To evaluate cytotoxicity, the quantitative assays neutral red uptake test (NRU) and MTT dye reduction test were used.

Neutral red uptake test (NRU) for cytotoxicity. The NRU test is based on the ability of viable cells to incorporate and bind the neutral red dye in their lysosomes, while lysosomal membrane damage due to toxic effect results in a decrease of the uptake of the neutral red dye. Cytotoxic effect of DON on the non-tumourigenic and tumour cell lines was studied by neutral red uptake test as described previously (Borenfreund & Puerner, 1985). Briefly, cells were suspended in growth medium with 10% FBS and seeded in 96-well plates (1×10^5

cells/mL). After 24-hour incubation period, the cell cultures were treated with DON in growth medium containing 5% FBS. Cells were treated with six different concentrations of DON, namely: 1.9 µg/mL, 2.7 µg/mL, 3.7 µg/mL, 5.2 µg/mL, 7.2 µg/mL, 10 µg/mL in the medium (dilution factor 1.389; 6 wells for each concentration) for 24 h. Untreated (control) cultures were grown at the same conditions in the growth medium with 5% FBS. The morphology of the cell cultures was monitored by inverted microscopy. Control and treated cell cultures were then incubated for 3 hours in a culture medium containing 0.25 µg/mL neutral red. The absorbed intracellular vital dye was extracted by adding a solution containing 50% ethanol, 49% water and 1% acetic acid. The optical density of the control and treated cultures was measured using an ELISA spectrophotometer (TECAN, SunriseTM, Grödig / Salzburg, Austria) at a wavelength of 540 nm. Three independent experiments were performed for each cell line.

MTT dye reduction assay. MTT dye reduction test is a colorimetric assay for assessment of the metabolic activity of cells. Rapidly dividing cells exhibit high rates of reduction of the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble form – formazan in a reaction catalysed by the mitochondrial succinate dehydrogenase. Thus, the MTT method is mitochondrial respiration dependent and can evaluate the cellular energy capacity. The cytotoxic and antiproliferative potential of DON was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, performed on the normal and tumour cells according the standard procedure (Mossman, 1983). Briefly, cells in the exponential phase of growth were trypsinised and seeded in 96-well microtiter plates (100 µL/well) at a density of 1×10^5 cells/mL. The cells were allowed to adhere for 24 h and then were treated with abovementioned six concentrations of DON in the medium (six replicates/concentration). The control and treated cells were incubated for 24 hours in an incubator at 37 °C and 5% CO_2. The treatment medium was replaced with DMEM containing 0.5 mg/mL MTT and the cells were incubated for three hours. The MTT solution was removed from the plates and the formed formazan crystals were dissolved in DMSO: 96% ethanol (1:1 v/v) solution. Optical density of the samples was measured by microplate reader (TECAN, Sunrise TM, Groedig/Salzburg, Austria) at 540 nm. The experiments were performed in triplicate.

Fluorescence microscopy

Qualitative fluorescence methods for determining the effect of the substance on the viability and proliferative activity of cells were used: double fluorochroming with acridine orange and ethidium bromide and DAPI staining. These assays were used for evaluation of the cytomorphological alterations of DON-treated normal and tumour cells – Balb/c3T3 and MDA-MB-231 cells. The cells were cultured on 13-mm in diameter cover glasses in 24-well plates and were treated for 24 hours with DON at a concentration approximating the IC_{50} value established by the MTT test (5 µg/mL). Cells cultivated in the same conditions, without application of DON, were used as a negative control of the experiments. The control and DON-treated cells were washed with PBS and stained by two different methods.

Acridine orange/ ethidium bromide double staining. Acridine orange (AO) and ethidium bromide (EtBr) (live/dead)

intravital staining was performed to assess the apoptosis-inducing ability of DON. Briefly, cell preparations of Balb/c3T3 and MDA-MB-231 were stained with the fluorescent dyes AO (5 µg/mL) and EtBr (5 µg/mL) in PBS. The stained cells were mounted on microscope slides and were immediately examined by a fluorescence microscope [Y3 (BP 535/50- BP 610/ 75)], Leica DM 5000B, Wetzlar, Germany), before the fluorescence started to fade.

DAPI staining. The alterations in the nuclear morphology of Balb/c3T3 and MDA-MB-231 cells induced by DON were studied after staining with DNA binding dye 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI). The cells were fixed with methanol, incubated for 15 minutes in 1 µg/mL DAPI in methanol according to the manufacturer's protocol. The cover glasses with the stained cells were mounted with glycerol on microscope slides and were stored in the dark until they were observed under a fluorescence microscope [(I3 (BP 450-490- LP 515)], Leica DM 5000B, Wetzlar, Germany). The main reagents were purchased by Sigma Aldrich-Merck KGaA, Darmstadt Germany.

Statistical analysis

The statistical processing of the data from the NRU and MTT assays was carried out by one-way analysis of variances (ANOVA), followed by Bonferroni *post hoc* test with the GraphPAD Prism software. The IC_{50} values were determined by a nonlinear curve-fit analysis (GraphPAD Prism). The results were presented as mean ± standard deviation; $P \leq 0.05$ was accepted as the lowest level of statistical significance.

RESULTS

The mean IC_{50} concentrations obtained by colorimetric NRU and MTT tests from three consecutive experiments with each cell line are presented in Table 1.

Cytotoxicity of DON was expressed as a concentration-dependent reduction of the uptake of the vital dye neutral red and decreased ability of the cells to metabolise the tetrazolium dye MTT to formazan as explained above. The dose-response graphs of DON-induced cytotoxicity with all cell culture systems were presented. Data showed that MDA cells were sensitive to DON at all concentrations higher than 2.7 µg/mL and that cell viability was significantly decreased ($P \leq 0.001$) (Fig. 1).

MCF-7 cells reacted with a significant decrease ($P \leq 0.001$) of the number of live cells at concentrations of 3.7 µg/mL and 2.7 µg/mL in the NRU and MTT tests, respectively (Fig. 2). Hela cells showed a significantly decreased viability at a DON concentration of 1.9 µg/mL evaluated by both tests ($P \leq 0.001$) (Fig. 3).

Statistically significant decrease of the viability of both non-tumour cell lines BALB/c 3T3 ($P \leq 0.001$) and DEC99 ($P \leq 0.01$) was found out at a DON concentration of 2.7 µg/mL (Fig. 4 and 5).

Table 1. IC_{50} values determined by colorimetric NRU and MTT assays. Values are presented as mean ± SD (n=3).

IC_{50}, µg/mL	MCF-7	MDA-MB 231	Hela	3T3	DEC99
MTT	4.084±0.43	5.465±0.57	3.714±0.75	4.163±0.78	4.120±0.85
NRU	6.510±0.83	4.829±0.92	4.002±0.64	4.269±0.93	4.082±0.69

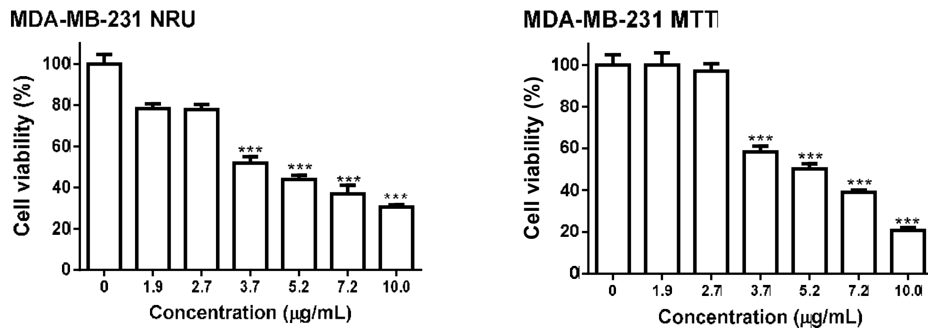


Fig. 1. Antiproliferative and cytotoxic effects of different concentrations of deoxynivalenol on MDA-MB-231 cells evaluated by NRU and MTT tests. Results are presented as mean ±SD (n=6); *** P≤0.001 vs control (0 μg/mL).

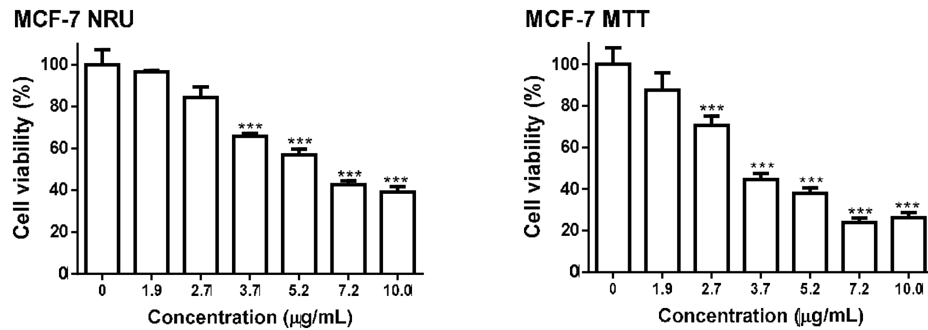


Fig. 2. Antiproliferative and cytotoxic effects of different concentrations of deoxynivalenol on MCF-7 cells evaluated by NRU and MTT tests. Results are presented as mean ±SD (n=6), *** P≤0.001 vs control (0 μg/mL).

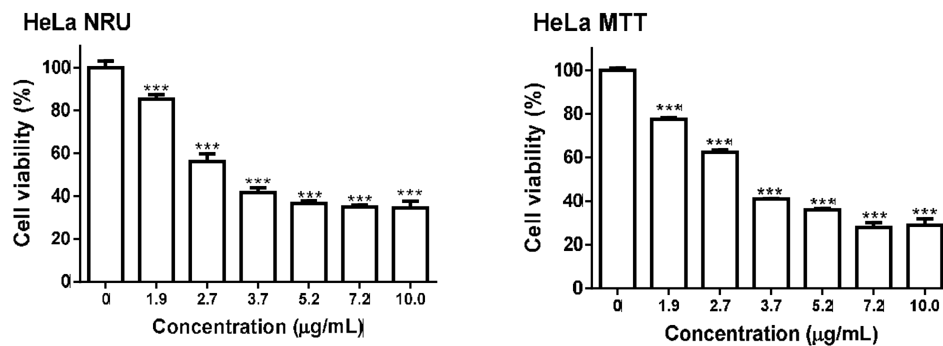
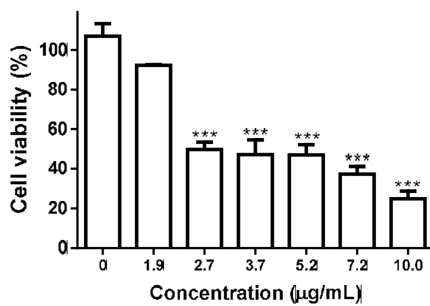


Fig. 3. Antiproliferative and cytotoxic effects of different concentrations of deoxynivalenol on Hela cells evaluated by NRU and MTT tests. Results are presented as mean ±SD (n=6), *** P≤0.001 vs control (0 μg/mL).

The sensitivity of the cell lines on the basis of the IC_{50} values calculated by NRU was estimated to be MCF-7>MDA-MB-231 >3T3>DEC99>Hela cells. According to IC_{50} values obtained by MTT assays, similar results were found out: MDA-MB-231>3T3>DEC99>MCF-7> Hela with only one position changed.

The microscopic study of viable and dead cells of control and DON-treated Balb/c3T3 and MDA-MB-231 cell lines by acridine orange/ethidium bromide staining showed pronounced alteration of the morphology and cytotoxicity of DON, confirming the results of NRU and MTT tests. The control cells showed normal morphology and monolayer growth typical of the corresponding cell type and green fluorescence colour due to the diffusion of acridine orange stain through the cellular membrane (Fig. 6A, 7A).

DON treatment induced significant alterations in cell growth and morphology. Early apoptotic cells with intense yellow-green fluorescence and late apoptotic and necrotic cells with orange and red fluorescence due to the loss of membrane integrity and the passage of ethidium bromide into cell were observed in both cell lines after treatment with DON (Fig. 6B, 7B).



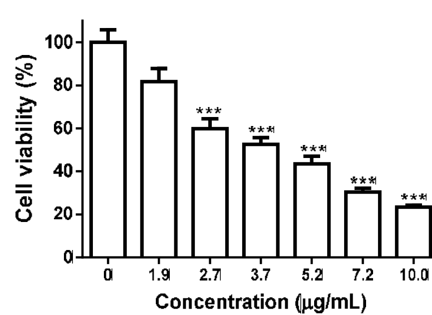
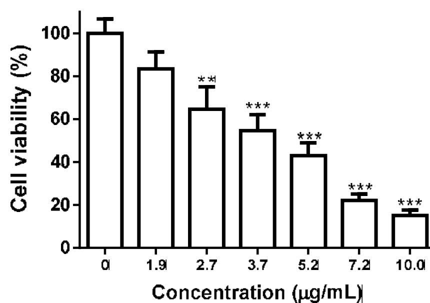


Fig. 4. Antiproliferative and cytotoxic effects of different concentrations of deoxynivalenol on BALB/c 3T3 cells evaluated by NRU and MTT tests. Results are presented as mean ±SD (n=6), *** P≤0.001 vs control (0 µg/mL).



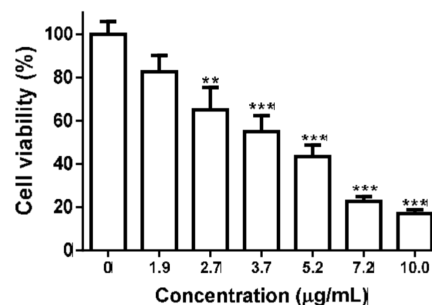


Fig. 5. Antiproliferative and cytotoxic effects of different concentrations of deoxynivalenol on DEC99 cells evaluated by NRU and MTT tests. Results are presented as mean ±SD (n=6), ** P≤0.05; *** P≤0.001 vs control (0 µg/mL).

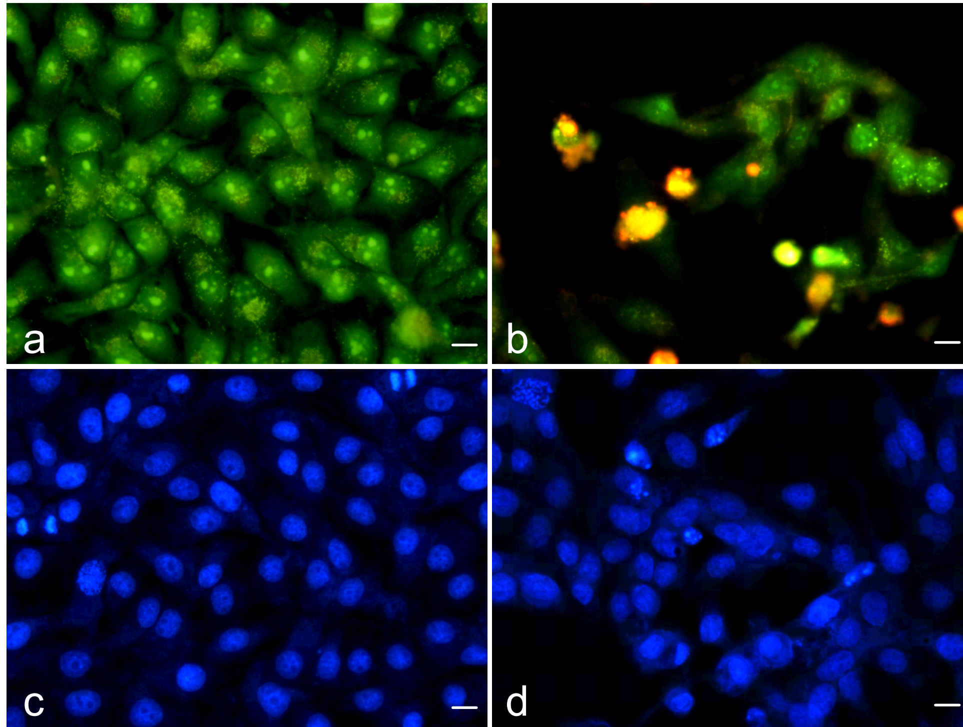


Fig. 6. Fluorescence microscopy images of the morphological alterations induced by DON in Balb/c3T3 cells after 24-hour treatment: **A, C:** Balb/c3T3 control; **B, D:** Balb/c3T3 treated with 5 μg/mL DON. **A, B:** AO/EtBr staining; **C, D:** DAPI staining; bar=50 μm.

Typical signs of apoptosis, such as cell shrinking, cell wrinkling, nucleus fragmentation and a formation of apoptotic bodies were frequently found and more pronounced in Balb/c3T3 cells as compared to MDA-MB-231 cells. The nuclear morphology of control and DON-treated Balb/c3T3 and MDA-MB-231 were examined by DAPI staining. The nuclei of the control cells showed normal morphology with smooth edges and homogenous blue staining. A number of mitotic figures were observed in the untreated Balb/c3T3 and MDA-MB-231 cell cultures (Fig. 6C, 7C). Some of DON-treated cells were with smaller and more brightly stained nuclei and irregular shape. Cells in the phase of mitosis were not found in cell cultures exposed to DON. Chromatin condensation intensifying the DAPI staining as well as nuclear fragmentation and formation of apoptotic bodies were observed in both cell lines. The established apoptotic changes were more clearly expressed in DON-treated Balb/c3T3 cells (Fig. 6D, 7D).

DISCUSSION

Because of their sensitivity and repeatability, the cell culture systems are usually used for preliminary toxicity screening of mycotoxins, or further investigations on the structure-activity relationship and elucidation of toxicological mechanisms at a

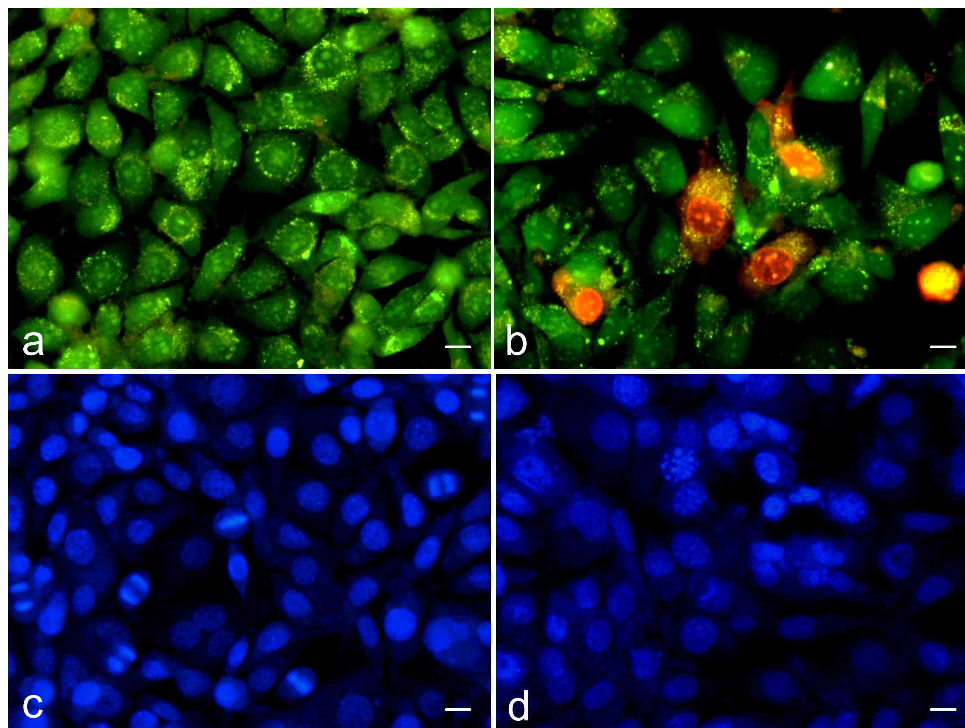


Fig. 7. Fluorescence microscopy images of the morphological alterations induced by DON in MDA-MB-231 cells after 24-hour treatment: **A, C:** MDA-MB-231 control; **B, D:** MDA-MB-231 treated with 5 μg/mL DON. **A, B:** AO/EtBr staining; **C, D:** DAPI staining; bar=50 μm.

biochemical level (Buckle & Senders, 1990; Rodraiguez & Haun, 1990). A correlation between the *in vitro* cytotoxicity of fungal extracts on 3T3 fibroblasts and GM3349 fibroblasts and effects of weight loss and feed refusal were observed in rat feeding studies (Abbas *et al*., 1984), which rationalise the *in vitro* studies of the effects prior to animal tests. Our investigation aimed to observe the *in vitro* effects of DON on permanent lines of non-tumour (BALB/c 3T3 and DEC 99) and tumour (MDA-MB-231, MCF-7, Hela) cells of avian and mammalian origin. This comparative study in different cell systems was performed to obtain supplementary information, concerning predictive toxicity screening of the mycotoxin deoxynivalenol – a potential food-borne hazard. Particularly, the dose-dependency of the cytotoxic effect, the influence on apoptosis and proliferative activity were determined to analyse and summarise the toxic impact of DON on MDA-MB-231, 3T3, DEC99, MCF-7 and Hela cells. The results showed that the MDA tumour cells were more resistant to mitochondrial-lysosomal damage than non-tumour BALB/c 3T3 and DEC99 cells. The MCF-7 cell line did not show a consistent reaction, but yet was the most resistant in the NRU test. Studies similar to ours revealed decline of cell viability and increased cytotoxicity after DON exposure of CHO-K1, HepG2, Caco-2, C5-O, V79 and Hela with impact on proliferation and increased

indicators of oxidative stress status (Calvert *et al*., 2005; Cetin & Bullerman, 2005; Juan-García *et al*., 2019). In some similar studies evaluating the toxicity of *Fusarium* mycotoxins by MTT assay (Cetin & Bullerman, 2005), DON was found to be the most cytotoxic among the tested *Fusarium* toxins. Measured IC_{50} values were from 0.27 to 8.36 µg/mL for the mammalian cell lines used and the IC_{50} values of DON in BALB/c, 3T3, DEC 99, MDA, MCF-7 and Hela cells obtained in the present study were also within this concentration range. Dose- and time-dependent cytotoxic effect on SK, MDCK, and Hela cell lines was reported in another comparable study by MTT with tested concentrations of DON ranging between 0.01 to 100 µg/mL where SK cells exhibited higher sensitivity than the other two cell lines (Hanelt *et al.,* 1994). Also, by MTT test, it was found that 24-hour exposure to DON at concentrations of 10–100 µg/mL induced a marked suppressive effect on the activity of SK, MDCK, Vero and BEL (Reubel *et al*., 1989). These results were obtained with DON levels exceeding the cytotoxic concentrations in our experiments (between 1.9 and 10 µg/mL). From the three tumour cell lines of epithelial origin, the Hela cells showed the strongest intolerance and the viability decreased at a lower DON concentration (1.9 µg/mL, $P \leq 0.001$). Hela cells in this study were the most sensitive to DON cytotoxicity in both tests, which could be due to the inherited HPV genome. According to Calvert (2005) the survival is dependent on the concentration and exposure period to toxins but a cell type-specific mycotoxin sensitivity exists, too. The HeLa cell line in their study showed cytotoxic response to all the toxins, while the other lines where more resistant.

The differences in the cytotoxic effects of fungal mycotoxins depend on different biochemical properties, structural conformation, ability to bind to particular cellular receptors, or penetrate through the cell membranes, and to interfere with metabolic pathways, as shown in different studies and assays. Cell cultures showed different sensitivity and varying responses because of their specific metabolic activity and different enzyme potential to react against mycotoxins too. In addition, genomic specificity of cell lines plays an important role in their resistance to xenobiotics. All these data justify future research studie on the cell type- and species-specific action of mycotoxins and elucidation of the molecular cytotoxicity mechanisms.

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