Original Article
DOI: 10.4274/tjps.galenos.2023.76128

Fusariotoxin-Induced Toxicity in Mesenchymal Stem Cells and Fibroblasts: A Comparison Between Differentiated and Undifferentiated Cells

Short Title in English: Fusariotoxin-Induced Toxicity in MSCs and L929

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19.01.2023
30.03.2023
02.04.2023

This study was supported by the Scientific Research Project Center (BAP) of Istanbul University (Project number: 34442).

ABSTRACT
Introduction: Humans are unknowingly exposed to mycotoxins through the consumption of plant-derived foods and processed products contaminated with these toxic compounds. In addition to agricultural losses, *Fusarium* toxins have been shown to pose a threat to human health as well. However, the effects of fusariotoxins on the viability and proliferation of stem cells have not been fully explored. We aimed to investigate the cytotoxic effects of deoxynivalenol and B-trichothecene mix on mesenchymal stem cells and L929 fibroblast cell line.
Methods: Mesenchymal stem cells were isolated from dental pulp tissue. Doubling time and viability of dental pulp stem cells (DPSCs) and L929 cells were determined by MTT assay. The following doses of B-trichothecenes (0.25-16 µg/mL; 24 h and 48 h) were used for evaluating cytotoxicity. Also, changes in the confluency-dependent response of DPSCs to deoxynivalenol toxicity were determined. Moreover, we investigated the effect of deoxynivalenol on cell death via AO/EB double staining.
Results: Deoxynivalenol and B-trichothecene mix, showed a dose- and time-dependent inhibitory effect on the proliferation of both cells. DPSCs exposed to DON for 48 h (IC_{50} =0.5 µg/mL) were found to be 16 folds more sensitive than the L929 cells (IC_{50} = 8 µg/mL). When compared to a culture with 80% confluency, DPSCs from a 50% confluent culture were more sensitive to varying doses of DON (0.25-4 µg/mL, 24-48 h). Moreover, AO/EB staining showed that treatment of DPSCs with DON led to a significant increase in cell death (17% for 2.4 µg/mL; 50% for 4.8 µg/mL).
Discussion and Conclusion: This study reveals that undifferentiated mesenchymal stem cells are significantly more sensitive to DON in comparison to differentiated somatic cells (L929). Given the fact that humans are frequently exposed to these mycotoxins, our findings imply that prolonged exposure to them may also have harmful effects on cellular differentiation and embryonic development.
Keywords: mesenchymal stem cells, mycotoxins, deoxynivalenol, fibroblasts, dental pulp stem cells

Introduction

Mycotoxins are secondary metabolites that support defensive functions for fungi in their ecological niche, however, they can contaminate a wide variety of food sources and animal feed. Therefore, mycotoxins are held as toxicants for both animals and humans. The Food and Agriculture Organization (FAO) proposed that one-fourth of the global food crop is contaminated by mycotoxins.[1] Moreover, the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) called for measures to be taken against widespread mycotoxin contamination.[2] Type B-trichothecenes, produced mostly by *Fusarium*, are prevalent contaminants, and the most important of them are deoxynivalenol (DON) and its derivatives. Essentially, over 80% of agricultural goods from Europe and Asia contain at least one type of mycotoxin and among them, DON is reported to be the most widespread.[3] DON, 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and nivalenol (NIV) can maintain their stability even during storage/milling and processing/cooking of food.[4] Exposure assessments in European countries concluded that consumers and even young children are exposed to DON at levels close to or precisely higher than the tolerable daily intake.[5] Therefore, high doses or prolonged exposure to DON can also pose a threat to human health.

The genes found in the *tri5* gene cluster and regulatory mechanisms have been described for the production of B-trichothecene in *Fusarium* species. Insertion-deletions (in-dels), changes in tandem repeats, and single nucleotide polymorphisms (SNPs) in the gene cluster determine the final product as DON and its acetylated derivatives or NIV.[6,7] However we demonstrated in a research project (unpublished data) that both DON and NIV biosynthesis were carried out at the same time in *F. graminearum* and *F. culmorum* as reported Thus, regardless of the fact that DON is the most prevalent mycotoxin, the cytotoxic effects of NIV also should not be ignored. A number of screening studies of *Fusarium* contaminated food stuff and products showed that two or more mycotoxins frequently co-existed, and also co-contamination of DON and NIV was predominant.[2,3,8] All of these studies demonstrate that DON and NIV contaminations are widespread all over the globe and therefore can also be regarded as an important risk factor for public health. *In vitro* analyses showed that these mycotoxins could both suppress and stimulate immune functions.[9] Also, they inhibit RNA, DNA, and protein synthesis by binding to the 60S subunit of eukaryotic ribosomes and disrupting the activity of peptidyl transferase. It was shown that DON led to changes in mRNA alternative splicing in human cells (HepG2, HEK293 and Caco-2 exposed to relatively low dosages (2 µg/mL).[10] It was revealed that DON suppressed the activity of the Wnt/β-catenin signaling pathway, which affects stem cell fate during development and in adult tissues.[11,12] Also, DON has been shown to have a strong embryotoxic effect by causing cell growth inhibition in embryonic stem cell lines.[13] Moreover, DON induced apoptosis and inflammation in intestinal cells through increasing ROS accumulation, activating the NF-kB and the apoptotic signaling pathways.[14] In human gastric epithelial and intestinal cells, 3ADON was fewer adverse effects than DON, whereas 15ADON appeared to be slightly more effective than DON. It was also revealed that 15ADON was a more potent MAPKs' inducer compared to DON and 3ADON.[15-17] Outcomes of various studies on somatic and cancer cell lines revealed that the co-existence of B-trichothecenes might cause a synergistic effect.[16,18-21] Thus co-occurrence of B-trichothecenes in foods and diets can cause more health problems than predicted.

Stable cell lines undergo morphological and genetic changes in culture during transformation and/or multiple passage cycles. It can be argued that due to these genetic and phenotypic instabilities, they are not ideal model systems for toxicology studies. Mesenchymal stem cells (MSCs) have attracted attention as an alternative and more sensitive screening platform for assessing chemical toxicity.[22,23] Even so, the effects of fusariotoxins on stem cells have received little consideration. Since dental pulp stem cells (DPSC) can easily be obtained from dental waste, they provide a suitable source for MSCs. A growing body of evidence suggests that differentiated cells are more resistant to DON treatment compared to undifferentiated stem cells.[19,24-26] However, a comparative analysis of DON-induced effects on MSCs and stable cell lines has not been reported to date. Findings suggest that human MSCs and fibroblasts share many physiological and molecular properties such as cell surface markers and gene expression patterns.[27,28] Because L929 fibroblasts can serve as an ideal stable cell line model for comparative analysis, L929 cell line was selected to use in the comparative analysis of the cytotoxic effects of B-trichothecenes on MSCs.

In this study, the effects of DON and B-trichothecene mix (MIX), which contains DON, 3ADON, 15ADON, and NIV, were investigated on DPSCs as a model for undifferentiated MSCs and differentiated L929 fibroblast cells.

MATERIALS AND METHODS

Isolation of stem cells

Human DPSCs were harvested from extracted human third molars of adult patients. The teeth were collected under guidelines approved by the Istanbul University Medical Faculty Clinical Research Ethics Committee (No: 2019/455) and informed consent was obtained from the patients. The extracted teeth were transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS; Wisent) solution containing 200 U/mL penicillin and 200 µg/mL streptomycin (Thermo). After the teeth were cleaned, the dental pulp tissue was separated from the pulp chamber and root canal. The dental pulp tissue was then chopped into pieces and digested in a 2 mL solution of 1 mg/mL collagenase type I (Biochrom) for 1 h at 37°C in 5% CO_2 to generate a single-cell

suspension, followed by centrifugation at 1000 rpm for 5 minutes. Afterward, the cells were seeded on 10 cm^2 plates.

Cell culture conditions

The DPSCs were cultured in growth medium: alpha-modified Eagle's medium (α-MEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Gibco), 2.5 mM L-glutamine (Gibco) 50 U/mL penicillin, 50 µg/mL streptomycin (Gibco) and 1.25 µg/mL amphotericin B (Capricorn). Mouse fibroblast L929 cells were a kind gift from Istanbul Yeni Yuzyil University Cell Culture Collections. They were maintained in Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, USA). Cells were incubated in a humidified incubator at 37°C (5% CO_2). The incubation medium was refreshed every 2-3 days.

Calculation of doubling time

In order to determine the cell expansion efficiency and to calculate the doubling time of cells, DPSCs (passage 5) and L929 were seeded on a 24-well cell culture plate at a density of 5×10^3 cells/cm^2. The DPSC and L929 cells were incubated for 7 days and 4 days, respectively. Cell viability was measured with the MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) assay on the 1st, 2nd, 3rd, 4th, and 7th days of the culture. The growth curves were created by plotting MTT assay absorbance values. The doubling times for DPSCs and L929 were calculated by using the equations obtained from the growth curves. The region that best represents a straight line within the Log phase was delineated and transformed into a logarithmic scale. The specific growth rate was calculated by applying the following equation: $y=Ae^{Bx}$, [y – absorbance (optical density), B – specific growth rate, and x – time]. Doubling time (td) was determined according to the equation $td=\ln(2)/B$.[28]

Treatments with DON and MIX

Acetonitrile was used as the solvent for the preparation of mycotoxins. Firstly, to eliminate the solvent effect we exposed cells to acetonitrile for 48 h. The final concentration of acetonitrile (0.8 µg/mL), that existed in the solution for the highest DON concentration (8 µg/mL) was added to the cell media. The stock solutions of DON (100 µg/mL) and MIX (100 µg/mL from each mycotoxin) were added to the media at the specified concentrations. The final concentration of acetonitrile solvent in a cell culture containing mycotoxin did not exceed 1%. Two different sets of experiments were designed: (1) When the DPSCs and L929 cultures reached 50-55% confluence, the medium was refreshed and DON or MIX at various concentrations (0.25, 0.5, 1, 2, 4, 8, 16 µg/mL) were added; (2) When the DPSCs cultures reached 50% and 80% density, the medium was replaced with a fresh medium containing DON at 0.25, 0.5, 1, 2, 4 µg/mL concentrations. A regular growth medium was used as the control. After incubation for 24 h and 48 h the cell viability was measured (570 nm) via the MTT assay.

The mean half-maximal inhibitory concentration (IC_{50}), representing the concentration of DON or MIX that inhibits the proliferation of treated cells by 50% in comparison to untreated controls, was calculated using the mean absorbance values obtained from the MTT assay. All experiments were performed in three biological and technical replicates.

Cell viability testing

The cytotoxic effects of DON or MIX on undifferentiated DPSCs or differentiated L929 cells were evaluated by MTT assay. 50 µL of MTT solution (2.5 mg/mL in PBS) was added to 500 µL of culture media. The cells were incubated in a 5% CO_2 incubator at 37 °C for 3 h in the dark. After discarding media, 400 µL DMSO was added into each well and the formazan crystals were dissolved. The optical density (OD) was measured at 570 nm using a spectrophotometer (BioTek Eon™) and the percentage of cell viability was calculated with the following Formula: Cell viability (%) = (OD sample/OD control) × 100. The percentage of cell inhibition was calculated with the Formula: 100 - % cell viability.

Fluorescent imaging of cell viability

Acridine orange (AO) penetrates into both viable and nonviable cells and emits green fluorescence. Ethidium bromide (EB) is taken up only by nonviable cells and emits red fluorescence. After double staining with AO/EB, four types of cells can be detected according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei: (1) Viable, (2) Early apoptotic, (3) Late apoptotic, (4) Necrotic.[29,30] Dye mixture (300 µg/mL AO and 300 µg/mL EB) added to the media of cells treated with DON (0.6-4.8 µg/mL) was incubated for 2-3 min in the dark, and was then immediately (fast uptake) examined by fluorescence microscopy (Carl-Zeiss, Axio Observer 3); 604 nm/Texas Red filter for EB and 520 nm/Green fluorescent protein (GFP) filter for AO.

Statistical Analysis

Cytotoxicity was expressed as the mean percentage change relative to the untreated control. Control values were set as 100% of viable cells. All experiments include three biological and three technical repetitions (n = 3). Statistical analysis was performed by using the Graph Pad Prism 8 program. Two-way ANOVA, followed by post hoc Dunnett's multiple comparison tests with a single pooled variance, was used to test for differences between the control and the treated cells groups. The statistical significance of time-dependent variations was tested by unpaired T-test. The results were presented as Mean±SEM (standard error of the mean) and p values

show statistical significance: statistically significant ($p<0.05$), very statistically significant ($p<0.01$), highly statistically significant ($p<0.001$).

RESULTS

Morphology and growth characteristics of DPSCs and L929 cells

Within the first 5 days of the primary culture obtained from the dental pulp tissue various cell types with different morphological features were observed. By day 5 the majority of cells exhibited an elongated, fibroblast-like (spindle-shaped) morphology under normal culture conditions (Fig. 1-A). Although there were some variations among the cells from different tissue samples, all established cells exhibited similar growth characteristics and morphology. The primary cultures reached confluency in about 10 days. Subcultures from all cells had the inclination to exhibit accelerated growth, thus the cultures reached confluency at a faster rate than primary cultures (in about 5-7 days). The cells maintained their spindle-shaped morphology along passages and during the whole culture period (Fig. 1-B). L929 stable cell lines exhibited a healthy proliferation profile with a typical fibroblastic morphology (Fig. 2).

The growth curves of DPSCs and L929 cells were constructed by using absorbance values obtained from MTT assays (Fig. 3). Doubling time of DPSCs was calculated as 32 h and the specific growth rate was determined as 0.0215 h^{-1}. The doubling time and the specific growth rate for L929 were calculated as 19 h and 0.0354 h^{-1}, respectively. Calculations were verified by using an online computation software (https://www.omnicalculator.com).

Effects of DON and MIX on the viability of DPSCs and L929 cell line

The dose-response curves indicated that treatment with DON or MIX induced a concentration-dependent reduction in the viability of both cells (Fig. 4-A). Acetonitrile exposure showed no significant effect compared with untreated control (Fig. 4-B). Treatments with DON (8 µg/mL for 24 h) reduced the cell viability by almost 50% in both DPSC and L929 cell lines compared to the untreated controls. However, at lower concentrations DON treatment for 48 h showed a more pronounced toxicity in DPSCs (49.7% inhibition at 0.5 µg/mL) at lower concentrations compared to L929 (36.7% inhibition at 0.5 µg/mL). Likewise, after 24 h of treatment with the MIX at the concentration of 7 µg/mL the survival rate of DPSCs decreased to 50% and 48 h of exposure decreased cell viability to 50% at the concentration of 0.25 µg/mL. Next, we calculated IC_{50} values (the concentration that reduces cell viability by 50%) for DPSCs and L929 cells (Table 1). Both DON and MIX showed similar inhibitory effects on cell viability after treatment for 24 h. However, at longer exposures (48 h) DON was found to be more toxic to DPSCs. As shown in Table 1, IC_{50} value for DPSCs was 16 folds lower than that of the L929 cells (0.5 µg/mL for DPSCs; 8 µg/mL for L929).

Four different concentrations of DON were tested for toxicity (0.25, 0.5, 1, and 2 µg/mL), and we also tested the toxicity of DON when combined with the B-trichothecene mix (containing 0.25, 0.5, 1, and 2 µg/mL of DON). We found that both DON and MIX dramatically reduced viability of DPSCs and L929 cells (Fig. 5). However, at higher concentrations (1 µg/mL and 2 µg/mL) significant changes were not observed between DON and MIX treatments in DPSCs (Fig. 5-A), suggesting that the highest toxicity in MIX was caused by DON.

Incubation with the DON and MIX for 24 h did not cause significant changes in cellular morphology (Fig. 6). However, the morphology of DPSCs remarkably changed after 48 h of exposure to DON and MIX. It was observed that cells rounded up and detached from the culture dishes, and the number of viable cells was relatively reduced at higher concentrations of the fusariotoxins (>2 µg/mL). DPSCs lost their spindle-shaped morphology and turned into flattened spread-out morphology in contrast to the morphology of untreated control cells. Fusariotoxins induced similar changes in the morphology of L929 cells at concentrations of 4 µg/mL and above.

Relevance between confluency and DON toxicity in DPSC culture

As shown in the previous sections our findings suggested that DON is the most potent mycotoxin in the mixture (Fig. 5) and DPSCs are more sensitive to DON toxicity (Fig. 5 and 6). Therefore, further analyses were conducted using only DON and DSPCs. We investigated the relation between DON toxicity and cell density. DPSCs at 50% confluency were found to be more sensitive to DON. (Fig. 7-B). A 24 h DON exposure (4 µg/mL) caused 40% inhibition in a 50% confluent cell culture. Whereas the same dose of DON, exhibited only 20% inhibition in an 80% confluent culture (Table 2). However, a prolonged DON treatment (48 h) showed a different inhibitory effect on DPSCs. In a 50% confluent culture, 4 µg/mL of DON inhibited the proliferation of cells by 60%. On the other hand, in an 80% confluent culture inhibitory effect of DON was 35% (Table 2).

DON-induced cell death in DSPC culture

Fluorescence microscope images of AO/EB stained samples revealed that treatment with 0.6 µg/mL DON did not cause a change in the number of apoptotic or necrotic cells compared to the control (control 1.7±0.3% and 0.6 µg/mL DON treated group resulted in 2.0±0.3%). 1.2 µg/mL of DON (48h) increased the rate of apoptotic and necrotic cells by 3.7±0.3% (Fig. 8). Higher DON doses (2.4 µg/mL and 4.8 µg/mL) dramatically increased the rate of apoptotic and necrotic cells (up to 50%) (Table 3).

DISCUSSION

Analysis of recent data revealed that the most prevalent mycotoxins in the contaminated food samples are DON, its acetylated forms (3ADON, 15ADON), and NIV. Plants infected by *Fusarium* spp. can produce conjugated forms of these mycotoxins named masked forms, which are less toxic than the main forms (DON-3-glucoside, NIV-glucoside). DON conjugations can also occur during food processing.[31] Although conjugated forms of mycotoxins are considered to be less toxic, their hydrolysis in the digestive tract may cause the conversion to the main form and thereby exposure to higher toxicity.[32] Climate change and the increasing world population may have an impact on the augmentation of mycotoxin contaminations and distributions on a large scale in the upcoming years.[33]

Although a number of researchers have investigated the toxic effects of *Fusarium* mycotoxins on various cells using transformed stable cell lines, limited information is available on the effects of these mycotoxins on stem cells.[11,34,35] Nowadays stem cells, particularly MSCs, have taken a tremendous interest as an alternative high-efficiency screening platform for appraising the toxicity of several chemicals such as drugs, and nanoparticles.[36] In contrast to the traditional *in vitro* systems based on stable cell lines, MSCs provide a sensitive platform for toxicological studies. One of the readily available sources for MSCs is the dental pulp that can be obtained from dental waste.

Various studies have demonstrated that MSCs and fibroblasts in humans share many characteristics. Both cell types can be isolated from almost all human tissues, in addition to morphological features, they share similar gene expression patterns and cell surface markers.[27,28] Therefore, the L929 fibroblast cell line was chosen as the differentiated stable cell line model for comparison with undifferentiated MSCs.

Stem cells from dental pulp tissue showed a high proliferation rate and there were no obvious alterations in the cell morphology and growth patterns in subsequent passages. Also, the doubling times of cells were in accordance with the data reported by Rajendran[37] for DPSCs, and for L929 by Kubat[38]. The inhibitory effect of *Fusarium* toxins on proliferation of both cells (24 h and 48 h) was evaluated via MTT assay. Results showed that fusariotoxins affected both cell types in a dose-dependent and time-dependent manner (Fig. 4-A). Similar to our findings, Lee and colleagues[36] demonstrated that exposure to chemicals was more toxic to stem cells than terminally differentiated fibroblasts. Identically, several groups reported that differentiated cells were less sensitive to the mycotoxins (DON, 3ADON, 15ADON, DON3G) than their proliferative counterparts.[19,24] We determined concentrations of DON and MIX that lead to the inhibition of cell viability by 50% (Table 1). It was suggested that DON inhibited the proliferation of DPSCs and L929 cells in a time-dependent manner. Indeed, we observed that DON-induced toxicity at 24 h (7 µg/mL) was much lower than at 48 h (0.5 µg/mL) (Table 1). However, it can be argued that the significant reduction in proliferation rate can also be attributed to the long doubling time (32 h) of DPSCs compared to fibroblasts (doubling time:19 h).

Compared to DON (alone), MIX reduced the viability of DPSCs and L929 cells significantly at both 24 h and 48 h of exposure (0.25-2 µg/mL) (Fig. 5). Intriguingly, at higher concentrations (1 µg/mL and 2 µg/mL) significant changes were not observed between DON and MIX treatments in DPSCs (Fig. 5-A), suggesting that the highest toxicity in MIX was caused by DON. These observations may be interpreted as a result of the synergistic effects of mycotoxins in MIX on stem cells. We also observed dramatic changes in cellular morphologies and viability rates of DPSCs and L929 at concentrations of 2 µg/mL and above, especially after 48 h of exposure (Fig. 6).

Cell confluency enormously affected the response of the cells to the tested toxins. Several groups reported that subconfluent cultures responded vigorously to the toxic effects of a similar amount of chemicals or drugs, in comparison to confluent cultures of cells.[39,40] Data in the literature suggests that the main differences between cell confluencies originate from proliferative capacity, motility, and intercellular cell−cell adhesion contacts. Additionally, confluent cells excrete extracellular matrix components that inhibit cell proliferation, make cells quiescent, and induce growth arrest.[41] In this context, we evaluated the relation between the confluency of DPSCs and DON toxicity. DON treatment (4 µg/mL) inhibited the proliferation of DPSCs by 20% at 80% confluency, whereas 4 µg/mL DON inhibited the proliferation of DPSCs by 40% at 50% confluency. So far, this is the only finding that has been reported for DON toxicity in the literature.

AO/EB double staining is an inexpensive and reliable method for the evaluation of changes in the number of necrotic and apoptotic cells. Changes in the nuclear morphology of cells can be detected via fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. Therefore, many researchers recommended AO/EB as a dependable and correct method for distinguishing viable, apoptotic, and necrotic cells in culture.[29,30,42.] In the current study, DON treatment resulted in the degeneration and fragmentation of nuclei in DPSCs in a dose-dependent manner (Fig. 8).

CONCLUSION

We aimed to investigate the cytotoxic effects of DON, acetylated derivatives of DON (3ADON, 15ADON), and NIV on MSCs and L929 fibroblast cell line. Hereby we report the in vitro cytotoxic effect of DON on MSCs by providing a comparative evaluation between L929 stable cell line and undifferentiated DPSCs. DPSCs demonstrated markedly severe sensitivity to fusariotoxins in contrast to L929. We found that cell confluency is an important factor that should be considered in toxicity studies. Moreover, DON was able to induce apoptotic and necrotic cell death in DSPCs. Based on the findings of this study, the comparable exposure to mycotoxins

can be ranked as follows: cytotoxicity DON < MIX; treatment time 24 h < 48 h; affecting of cells L929 < DPSCs; confluency %50 < %80. We were not able to present mechanistic and molecular insights for the DON-induced effects observed in this study. In this study, we were unable to present mechanistic and molecular insights into the underlying mechanisms that lead to DON-induced toxicity. Further molecular experiments, such as gene expression and oxidative stress determinations, are required in this context. These findings could pave the way for future comparative toxicological studies on stem cells and stable cell lines.

Acknowledgements: This study was supported by the Scientific Research Project Center (BAP) of Istanbul University (Project number: 34442).

Informed Consent:
(It should be stated from whom the verbal or written consent was obtained.)
The teeth were collected under guidelines approved by the Istanbul University Medical Faculty Clinical Research Ethics Committee (No: 2019/455) and informed consent was obtained from the patients.

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TABLES

Table 1. The mean half-maximal inhibitory concentrations (IC_{50}) of DON and MIX on DPSCs and L929

	DON_{IC50} (µg/mL)		MIX_{IC50} (µg/mL)	
	24 h	48 h	24 h	48 h
DPSCs	7	0.5	7	0.25
L929	8	8	7	6

DPSCs: Dental pulp stem cells, L929: mouse fibroblasts cell line, DON: deoxynivalenol, MIX: B-trichothecene mix

Table 2. Cell viability percentages (%) of 50% and 80% confluent DPSCs treated with DON for 24 h and 48 h (mean ± SEM, n=3).

Concentrations of DON (µg/mL)	24 h		48 h	
Confluency:	50%	80%	50%	80%
0	100±0	100±0	100±0	100±0
0.25	90±1.7	85±2.6	65±0.5	68±0.5
0.5	74±1.7	83±1.2	50±1.2	68±1.7
1	70±1.7	85±1.5	38±1	69±1.2
2	67±2	87±0.3	39±1.5	65±2
4	59±2	83±0	41±0.5	64±2.2

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DPSCs: Dental pulp stem cells, DON: deoxynivalenol

FIGURES

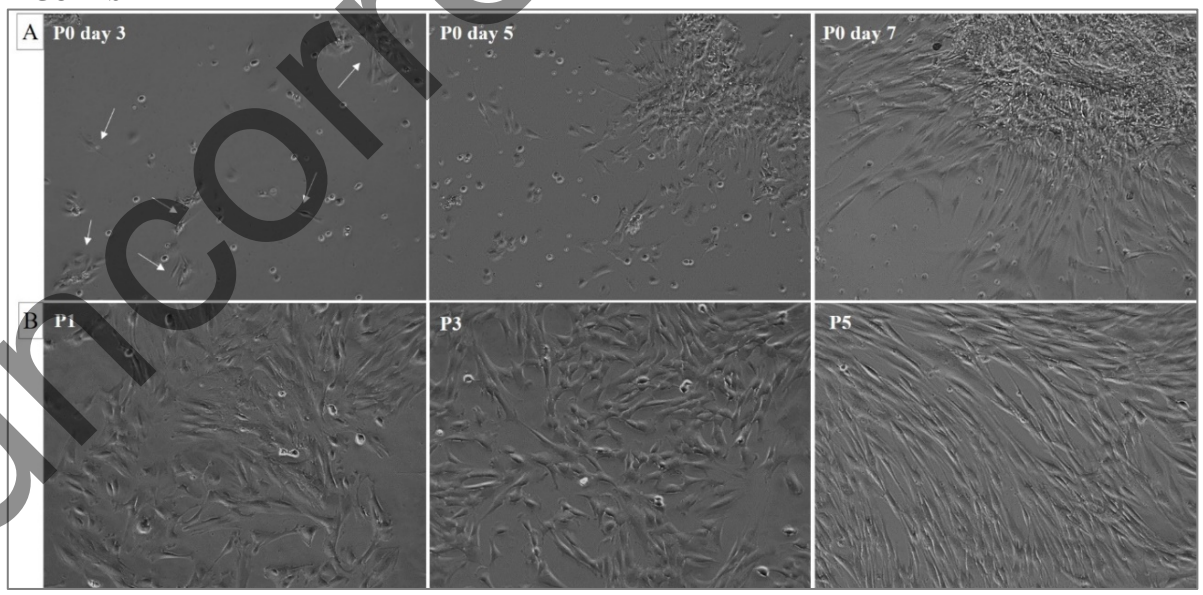

Figure 1. Morphological features of DPSCs. **A)** Morphology of DPSCs at primary culture (P0) investigated under an inverted optic microscope (Nikon Eclipse Ti-E). P0: days 3, 5, and 7 of primary culture obtained from dental pulp tissue. Small fibroblast-shaped cells (white arrows) and round-shaped cells appeared on day 3 of the

primary culture (P0 day 3). **B)** Morphology of DPSCs at different passages (P1-P5). Spindle-shaped cells became predominant in the culture after three passages. Original magnifications: ×10

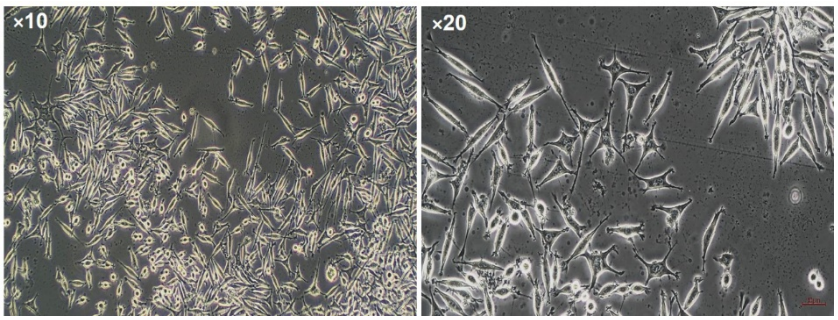

Figure 2. Morphological features of L929 cells. Morphology of L929 under an inverted phase contrast microscope. Representative images of L929 on the 5th day of culture. Cells exhibit spindle-shaped morphology. Original magnifications: ×10 and ×20

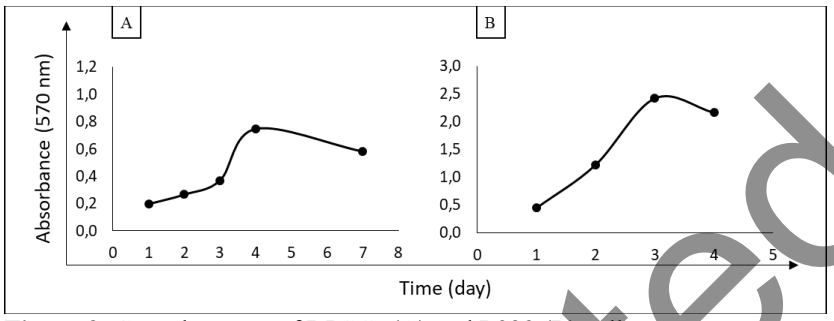

Figure 3. Growth curves of DPSCs (A) and L929 (B) cells.

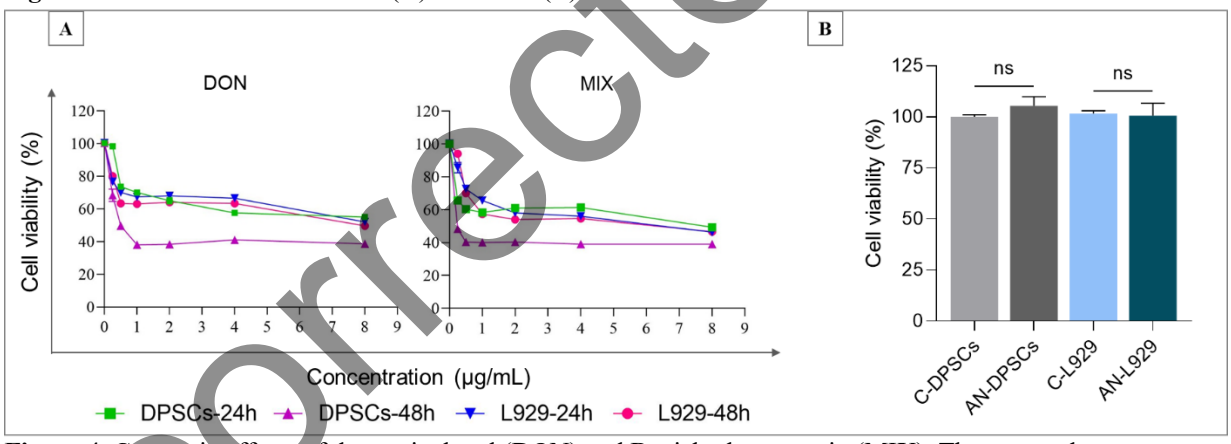

Figure 4. Cytotoxic effects of deoxynivalenol (DON) and B-trichothecene mix (MIX). The curves demonstrate the dose-dependent toxic effect of DON and MIX (0.25-8 µg/mL) on DPSCs and L929 cells for 24 h and 48 h. B) Effects of DON solvent acetonitrile. To eliminate the solvent effect both cells exposed to concentration of acetonitrile (0.8 µg/mL) existed in solution for the highest DON (8 µg/mL). C: control; AN: acetonitrile. Data were presented as the mean ± SEM; ns: not significant.

DPSCs: Dental pulp stem cells, L929: mouse fibroblasts cell line, MIX: B-trichothecene mix (DON, 3ADON, 15ADON, NIV)

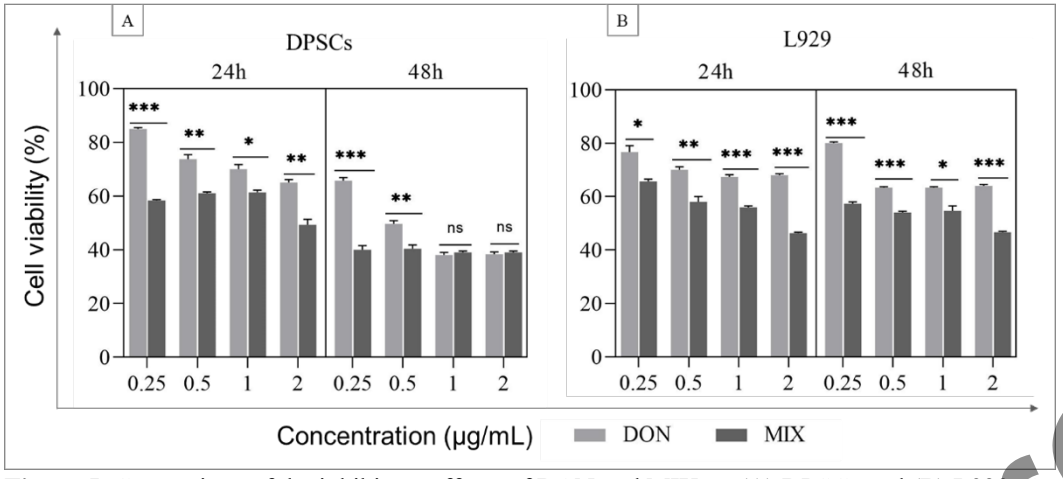

Figure 5. Comparison of the inhibitory effects of DON and MIX on **(A)** DPSCs and **(B)** L929. Stock concentrations for DON 100 µg/mL and for MIX 400 µg/mL that contain 100 µg/mL of each of four mycotoxins (i.e.,1 µg/mL of MIX contains 1 µg/mL of each of four mycotoxins). Data were presented as the mean ± SEM; ns - not significant, *p<0.05. **p<0.01. ***p<0.001.
DPSCs: Dental pulp stem cells, L929: mouse fibroblasts cell line, DON: deoxynivalenol, MIX: B-trichothecene mix (DON, 3ADON, 15ADON, NIV)

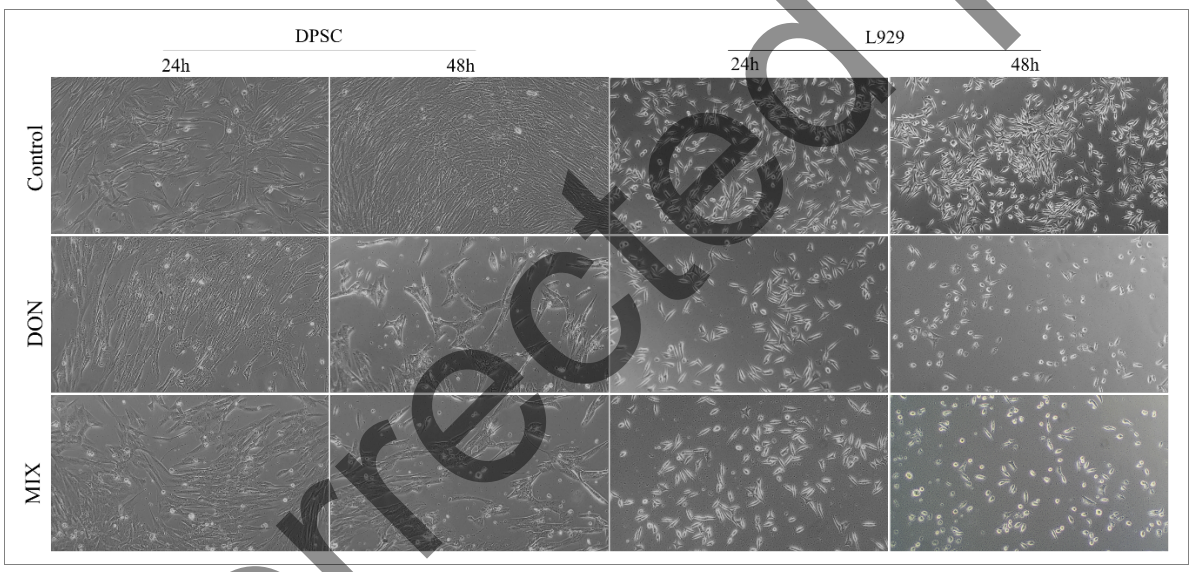

Figure 6. Mycotoxin-induced changes in cell morphology. Representative images showing phenotypic changes of DPSCs and L929 cells after treatment with deoxynivalenol (DON) or B-trichothecene mix (MIX) at the concentration of 8 µg/mL for 24 h and 48 h.

DPSCs: Dental pulp stem cells, L929: mouse fibroblasts cell line

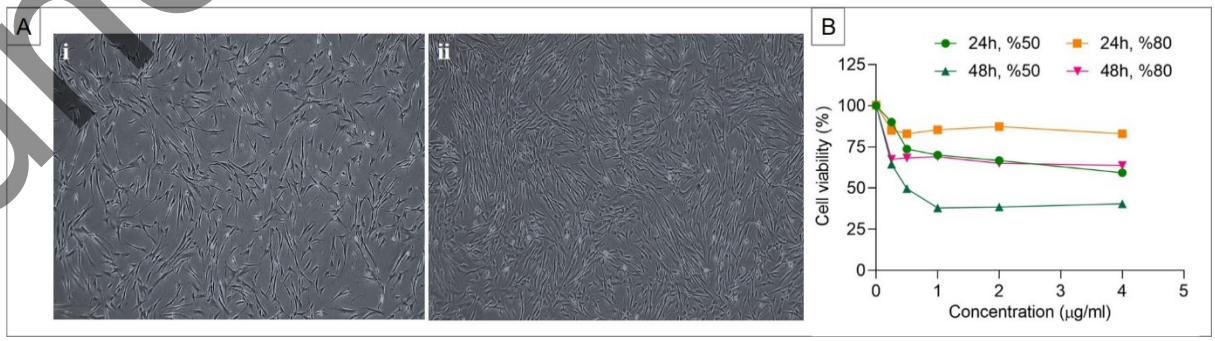

Figure 7. Effect of cell density on toxicity. A) Confluency of DPSCs. Representative images from cultures with **i)** 50% **ii)** 80% density of cells. Images taken by an inverted optical microscope are analyzed for confluence

using ImageJ software. Original magnifications: ×4 B) Cell viability curves display the effect of DON on DPSCs in two different confluencies (50% and 80%).
DPSCs: Dental pulp stem cells, DON: deoxynivalenol

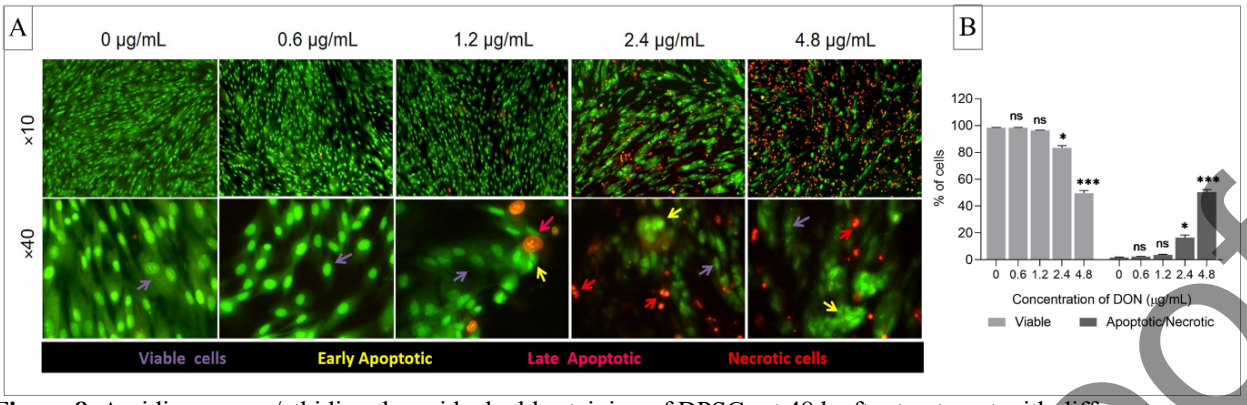

Figure 8. Acridine orange/ethidium bromide double staining of DPSCs at 48 h after treatment with different concentrations of DON. A) Representative images for viable, apoptotic, and necrotic cells were shown with the arrows in corresponding colors. Viable cells have uniform bright green nuclei with an organized structure. Early apoptotic cells with irregularly structured green nuclei and chromatin condensations are visible as bright green patches, fragments, or apoptotic bodies. Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have uniformly orange to red nuclei with an organized structure. Original magnifications: ×10 and ×40; B) Percentage of viable and apoptotic/necrotic cells, statistical assessment according to control (0 µg/mL); ns = not significant, *p<0.05, ***p<0.001.
DPSCs: Dental pulp stem cells, DON: deoxynivalenol