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ORIGINAL ARTICLE

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

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ABSTRACT

Objectives: 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 pose a threat to human health. However, the effects of fusariotoxins on the viability and proliferation of stem cells have not been fully explored. We investigated the cytotoxic effects of deoxynivalenol (DON) and B-trichothecene mix (MIX) on mesenchymal stem cells (MSCs) and the L929 fibroblast cell line.

Materials and Methods: MSCs were isolated from the dental pulp tissue. The doubling time and viability of dental pulp stem cells (DPSCs) and L929 cells were determined using the MTT assay. The following doses of B-trichothecenes (0.25-16 µg/mL; 24 hours and 48 hours) were used to evaluate cytotoxicity. In addition, changes in the confluency-dependent response of DPSCs to DON toxicity were determined. Moreover, we investigated the effect of DON on cell death *via* acridine orange/ethidium bromide (AO/EB) double staining.

Results: A DON and MIX showed a dose- and time-dependent inhibitory effect on the proliferation of both cells. DPSCs exposed to DON for 48 hours (IC_{50} = 0.5 µg/mL) were found to be 16-fold more sensitive than L929 cells (IC_{50} = 8 µg/mL). Compared with 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 hours). 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).

Conclusion: This study reveals that undifferentiated MSCs are significantly more sensitive to DON than differentiated somatic cells (L929). Given 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 toxicants for both animals and humans. The Food and Agriculture Organization proposed that one-fourth of the global food crop is contaminated by mycotoxins.[1] Moreover, the International Agency for Research on Cancer and the World Health Organization called for measures to be taken against widespread mycotoxin contamination.[2] Type B-trichothecenes, produced mainly 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-DON (3ADON), 15-acetyl-DON (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

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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 their 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 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 *Fusarium graminearum* and *Fusarium culmorum*. Thus, regardless of the fact that DON is the most prevalent mycotoxin, the cytotoxic effects of NIV also should not be ignored. Several screening studies of *Fusarium*-contaminated food stuff and products showed that two or more mycotoxins frequently co-existed, and co-contamination of DON and NIV was predominant.[2,3,8] All of these studies demonstrate that DON and NIV contaminations are widespread worldwide 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] In addition, they inhibited RNA, DNA, and protein synthesis by binding to the 60S subunit of eukaryotic ribosomes and disrupting the activity of peptidyl transferase. 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] DON suppressed the activity of the Wnt/β-catenin signaling pathway, which affects stem cell fate during development and in adult tissues.[11,12] In addition, DON has a strong embryotoxic effect by inhibiting cell growth in embryonic stem cell lines.[13] Moreover, DON induced apoptosis and inflammation in intestinal cells by increasing ROS accumulation and activating the NF-κB and apoptotic signaling pathways.[14] In human gastric epithelial and intestinal cells, 3ADON had 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 MAPK inducer than 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, the co-occurrence of B-trichothecenes in foods and diets can cause more health problems than predicted.

Stable cell lines undergo morphological and genetic changes during transformation and/or multiple passage cycles. It can be argued that because of 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] Nevertheless, the effects of fusariotoxins on stem cells have received little consideration. Because 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 than 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. These 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, the L929 cell line was selected for comparative analysis of the cytotoxic effects of B-trichothecenes on MSCs.

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

MATERIALS AND METHODS

Isolation of the stem cells

Human DPSCs were harvested from the extracted human third molars of adult patients. Teeth were collected under guidelines approved by the İstanbul University Medical Faculty Clinical Research Ethics Committee (no: 2019/455, date: 29.03.2019), 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 hour at 37 °C in 5% CO_2 to generate a single-cell suspension, followed by centrifugation at 1000 rpm for 5 min. The cells were seeded on 10 cm^2 plates.

Cell culture conditions

The DPSCs were cultured in the following 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 the İstanbul Yeni Yüzyıl 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 the doubling time (td)

To determine the cell expansion efficiency and calculate the number of cells, DPSCs (passage 5) and L929 were seeded on a 24-well cell culture plate at a density of 5 x 10^3 cells/cm^2. DPSC and L929 cells were incubated for 7 and 4 days, respectively. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay on the 1st, 2nd, 3rd, 4th, and 7th days of culture. The growth curves were created by plotting the MTT assay absorbance values. The td 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 (OD), B - specific growth rate, and x 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. First, to eliminate the solvent effect, we exposed the cells to acetonitrile for 48 hours. The final concentration of acetonitrile (0.8 µg/mL), which existed in the solution for the highest DON concentration (8 µg/mL), was added to the cell media. Stock solutions of DON (100 µg/mL) and MIX (100 µg/mL from each mycotoxin) were added to the media at specified concentrations. The final concentration of the 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 and 48 hours, 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% compared 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 hours in the dark. After discarding the media, 400 µL DMSO was added to 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 using the following formula: Cell viability (%) = (OD sample/OD control) x 100. The percentage of cell inhibition was calculated using the formula: 100 - % cell viability.

Fluorescence imaging of cell viability

Acridine orange (AO) penetrates both viable and non-viable cells and emits green fluorescence. Ethidium bromide (EB) is taken up only by non-viable cells and emits red fluorescence. After double staining with AO/EB, four types of cells can be detected according to fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei: (1) viable, (2) early apoptotic, (3) late apoptotic, and (4) necrotic.[29,30] Dye mixture (300 µg/mL AO and 300 µg/mL EB) was 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 filter for AO.

Statistical analysis

Cytotoxicity was expressed as the mean percentage change relative to the untreated control. The control values were set as 100% of viable cells. All experiments included three biological and three technical repetitions (n= 3). Statistical analysis was performed 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 treated cell groups. An unpaired *t*-test tested the statistical significance of the time-dependent variations. The results are presented as mean ± SEM (standard error of the mean) and *p* values show statistical significance: statistically significant ($p < 0.05$), very statistically significant ($p < 0.01$), and highly statistically significant ($p < 0.001$).

RESULTS

Morphology and growth characteristics of DPSCs and L929 cells

Within the first five 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 (Figure 1A). Although there were some variations among the cells from different tissue samples, all established cells exhibited similar growth characteristics and morphologies. The primary cultures reached confluency in approximately 10 days. Subcultures from all cells inclined 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 entire culture period (Figure 1B). L929 stable cell lines exhibited a healthy

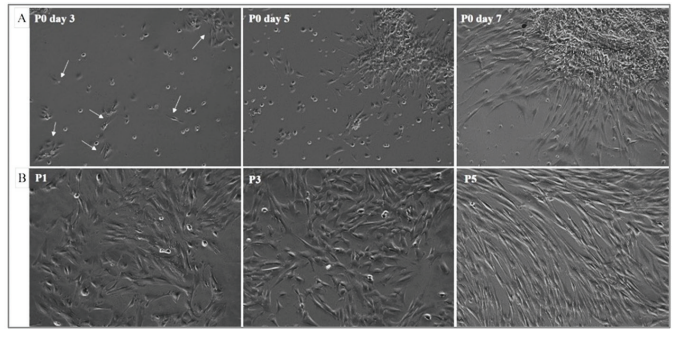

Figure 1. Morphological features of DPSCs. (A) Morphology of DPSCs at primary culture (P_0) investigated under an inverted optic microscope (Nikon Eclipse Ti-E). P_0: days 3, 5, and 7 of primary culture obtained from the dental pulp tissue. Small fibroblast-shaped cells (white arrows) and round-shaped cells appeared on day 3 of primary culture (P_0 day 3). (B) Morphology of DPSCs at different passages (P_1-P_5). Spindle-shaped cells became predominant in the culture after three passages. Original magnifications: x10

DPSC: Dental pulp stem cell

proliferation profile with a typical fibroblastic morphology (Figure 2).

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

Effects of DON and MIX on the viability of DPSCs and the 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 (Figure 4A). Acetonitrile exposure showed no significant effect compared with the untreated control (Figure 4B). Treatment with DON (8 µg/mL for 24 hours) reduced cell viability by almost 50% in both DPSC and L929 cell lines compared with the untreated controls. However, at lower concentrations, DON treatment for 48 hours showed a more pronounced toxicity in DPSCs (49.7% inhibition at 0.5 µg/mL) at lower concentrations compared with L929 (36.7% inhibition at 0.5 µg/mL). Likewise, after 24 hours of treatment with MIX at a concentration of 7 µg/mL, the survival rate of DPSCs decreased to 50%, and 48 hours of exposure reduced cell viability to 50% at a 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 hours. However, at longer exposures (48 hours), DON was found to be more toxic to DPSCs. As shown in Table 1, the IC_{50} value for DPSCs was 16-fold lower than that of L929 cells (0.5 µg/mL for DPSCs; 8 µg/mL for L929).

Four different concentrations (0.25, 0.5, 1, and 2 µg/mL) of DON were tested for toxicity, and we also tested the toxicity of DON when combined with the B-trichothecene mix (MIX) (containing 0.25, 0.5, 1, and 2 µg/mL of DON). We found that both DON and MIX dramatically reduced the viability of DPSCs and L929 cells (Figure 5). However, at higher concentrations (1 µg/mL and 2 µg/mL), significant changes were not observed between DON and MIX treatments in DPSCs (Figure 5A), suggesting that DON caused the highest toxicity in MIX. Incubation with DON and MIX for 24 hours did not cause significant changes in cellular morphology (Figure 6). However, the morphology of DPSCs remarkably changed after 48 hours 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 fusariotoxins concentrations (› 2 µg/mL). DPSCs lost their spindle-shaped morphology and developed a 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.

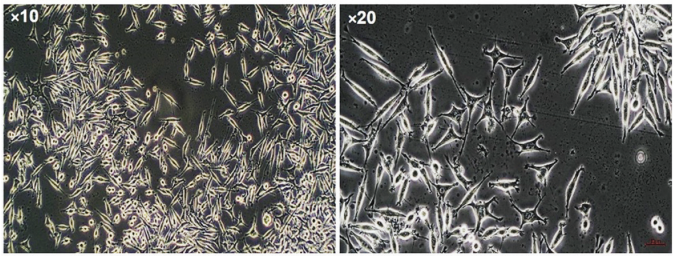

Figure 2. Morphological features of L929 cells. Morphology of L929 under an inverted phase contrast microscope. Representative images of L929 on the 5th day of culture. Cells exhibit spindle-shaped morphology. Original magnifications: x10 and x20

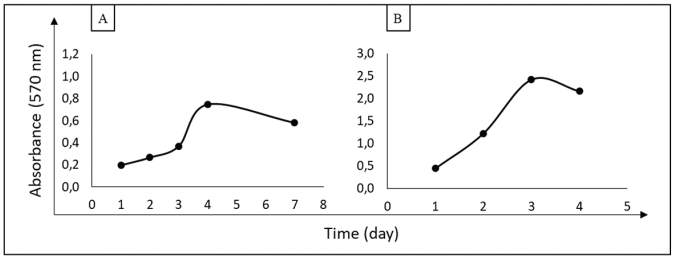

Figure 3. Growth curves of DPSCs (A) and L929 (B) cells

DPSC: Dental pulp stem cell

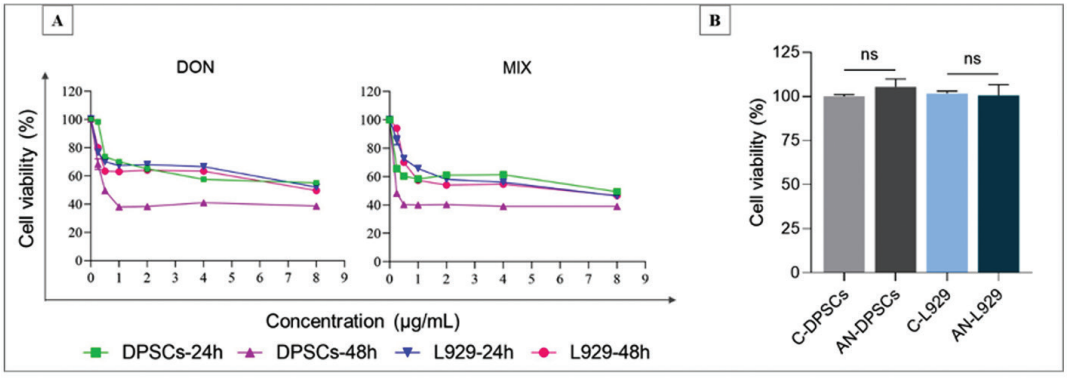

Figure 4. Cytotoxic effects of DON and MIX. (A) The curves demonstrate the dose-dependent toxic effect of DON and MIX (0.25-8 µg/mL) on DPSCs and L929 cells for 24 and 48 hours. (B) Effects of the DON solvent acetonitrile. To eliminate the solvent effect, both cells exposed to a concentration of acetonitrile (0.8 µg/mL) existed in solution for the highest DON (8 µg/mL)

Data are presented as the mean ± standard error of the mean, ns: Not significant, C: Control, AN: Acetonitrile, DON: Deoxynivalenol, DPSC: Dental pulp stem cell, L929: Mouse fibroblast cell line, MIX: B-trichothecene mix (DON, 3ADON, 15ADON, NIV)

Relevance of confluency and DON toxicity in DPSC culture

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

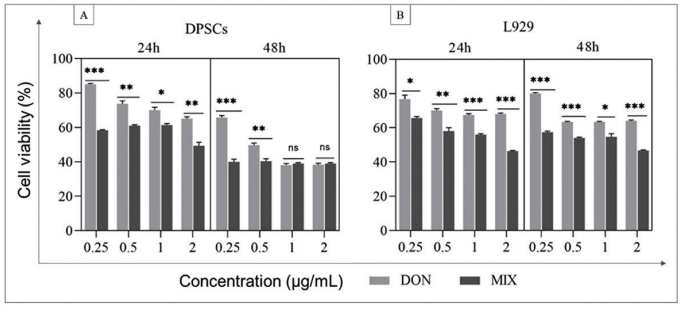

Figure 5. Comparison of the inhibitory effects of DON and MIX on (A) DPSCs and (B) L929. Stock concentrations for DON 100 µg/mL and for MIX 400 µg/mL that contain 100 µg/mL of each of the four mycotoxins (*i.e.,* 1 µg/mL of MIX contains 1 µg/mL of each of four mycotoxins)

Data are presented as the mean ± standard error of the mean, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: Not significant, DPSCs: Dental pulp stem cells, L929: Mouse fibroblast cell line, DON: Deoxynivalenol, MIX: B-trichothecene mix (DON, 3ADON, 15ADON, NIV)

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

	DON IC_{50} (µg/mL)		MIX IC_{50} (µg/mL)	
	24 hours	48 hours	24 hours	48 hours
DPSCs	7	0.5	7	0.25
L929	8	8	7	6

IC50: Inhibitory concentration, DON: Deoxynivalenol, MIX: B-trichothecene mix, DPSCs: Dental pulp stem cells, L929: Mouse fibroblast cell line

DON-induced cell death in the DSPC culture

Fluorescence microscopy 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 with 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 (48 hours) increased the rate of apoptotic and necrotic cells by 3.7 ± 0.3% (Figure 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).

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

Concentrations of DON (µg/mL)	24 hours		48 hours	
	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, SEM: Standard error of the mean, DPSCs: Dental pulp stem cells, DON: Deoxynivalenol

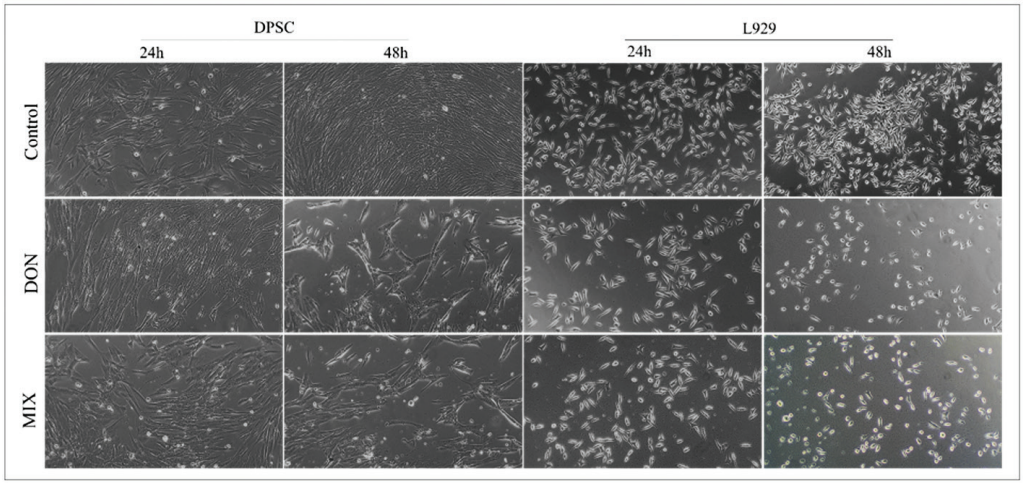

Figure 6. Mycotoxin-induced changes in cell morphology. Representative images showing phenotypic changes in DPSCs and L929 cells after treatment with DON or MIX at a concentration of 8 µg/mL for 24 and 48 hours

DPSCs: Dental pulp stem cells, L929: Mouse fibroblast cell line, DON: Deoxynivalenol, MIX: B-trichothecene mix

DISCUSSION

Analysis of recent data revealed that the most prevalent mycotoxins in contaminated food samples are DON, its acetylated forms (3ADON, 15ADON), and NIV. Plants infected by *Fusarium* spp. can produce conjugated forms of these mycotoxins called 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 their conversion to the main form, thereby increasing their toxicity.[32] Climate change and the increasing world population may impact the augmentation of mycotoxin contamination and distribution on a large scale in the upcoming years.[33]

Although many researchers have investigated the toxic effects of *Fusarium* mycotoxins on various cells using transformed stable cell lines, more information is needed on the effects of these mycotoxins on stem cells.[11,34,35] Currently, 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.[22]

Table 3. Acridine orange/ethidium bromide results of DPSCs treated with DON (mean ± SEM, n= 3)

Concentrations of DON (µg/mL)	Viable cells (%)	Apoptotic/necrotic cells (%)
0	98±0.3	1.7±0.3
0.6	98±0.0	2.0±0.3
1.2	96±0.3	3.7±0.3
2.4	83±1.8	16.7±1.8
4.8	50±2.0	50.3±2.0

DPSCs: Dental pulp stem cells, DON: Deoxynivalenol, SEM: Standard error of the mean

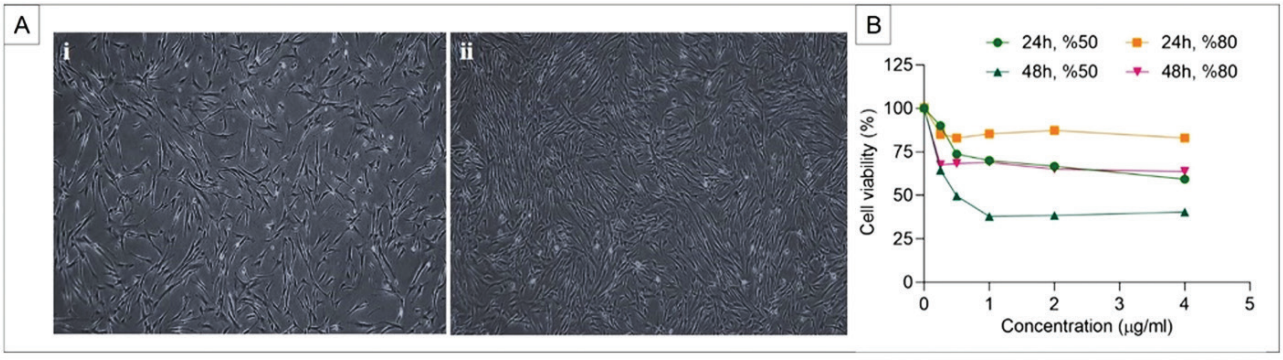

Figure 7. Effect of cell density on toxicity. (A) Confluence of DPSCs. Representative images from cultures with i) 50% and ii) 80% density of cells. Images taken by an inverted optical microscope were analyzed for confluence using ImageJ software. Original magnifications: x4 (B) Cell viability curves showing the effect of DON on DPSCs at two different confluences (50% and 80%)

DPSCs: Dental pulp stem cells, DON: Deoxynivalenol

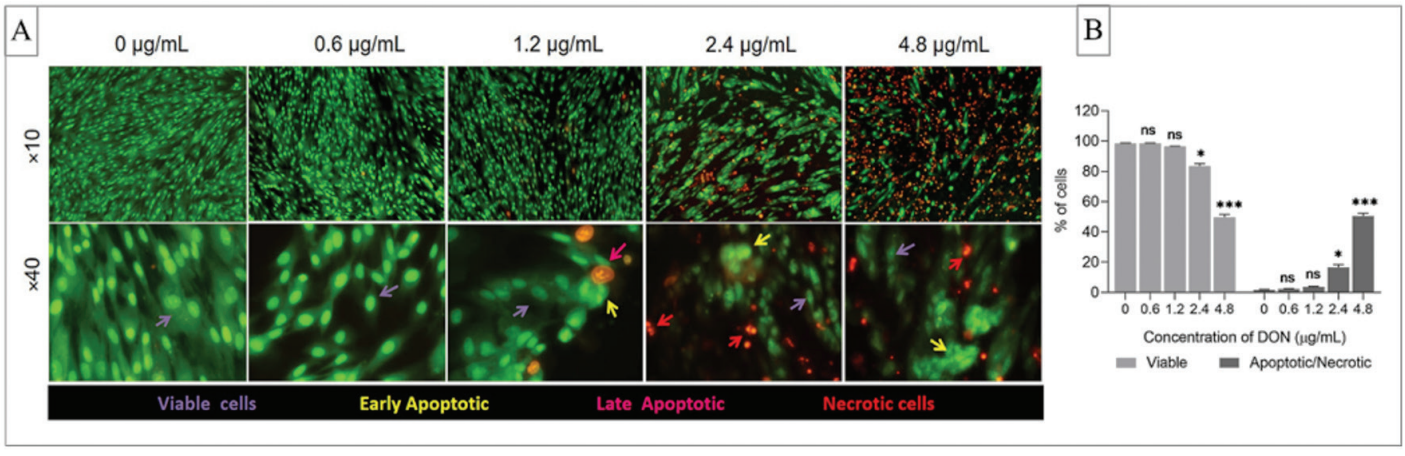

Figure 8. Acridine orange/ethidium bromide double staining of DPSCs 48 hours after treatment with different concentrations of DON. (A) Representative images of viable, apoptotic, and necrotic cells are shown with the arrows in the corresponding colors. Viable cells have uniform bright green nuclei with 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: x10 and x40; (B) Percentage of viable and apoptotic/necrotic cells, statistical assessment according to control (0 µg/mL)

*$p < 0.05$, ***$p < 0.001$, ns: Not significant, DPSCs: Dental pulp stem cells, DON: Deoxynivalenol

In contrast to 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 dental pulp, which 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, and in addition to their 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 cell morphology and growth patterns in subsequent passages. In addition, the td of cells were in accordance with the data reported by Rajendran et al.[36] for DPSCs, and for L929 by Kubat et al.[37] The inhibitory effect of *Fusarium* toxins on the proliferation of both cells (24 hours and 48 hours) was evaluated *via* MTT assay. The results showed that fusariotoxins affected both cell types in a dose-dependent and time-dependent manner (Figure 4A). Similar to our findings, Lee et al.[22] demonstrated that exposure to chemicals was more toxic to stem cells than to terminally differentiated fibroblasts. Identically, several groups reported that differentiated cells were less sensitive to mycotoxins (DON, 3ADON, 15ADON, and DON3G) than their proliferative counterparts.[19,24] We determined the concentrations of DON and MIX that led 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. We observed that DON-induced toxicity at 24 hours (7 µg/mL) was much lower than that at 48 hours (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 td (32 hours) of DPSCs compared with that of fibroblasts (td: 19 hours).

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

Cell confluency enormously affected the response of cells to the tested toxins. Several groups have reported that subconfluent cultures responded vigorously to the toxic effects of a similar number of chemicals or drugs compared with confluent cell cultures.[38,39] Data in the literature suggest that the main differences between cell confluences originate from proliferative capacity, motility, and intercellular cell-cell adhesion contacts. In addition, confluent cells excrete extracellular matrix components that inhibit cell proliferation, make cells quiescent, and induce growth arrest.[40] In this context, we evaluated the relationship between the confluence 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. Thus 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 evaluating changes in the number of necrotic and apoptotic cells. Changes in the nuclear morphology of cells can be detected *via* fluorescence emission and chromatin condensation in the stained nuclei. Therefore, many researchers have recommended AO/EB as a reliable and correct method for distinguishing viable, apoptotic, and necrotic cells in culture.[29,30,41] In the current study, DON treatment resulted in the degeneration and fragmentation of nuclei in DPSCs in a dose-dependent manner (Figure 8).

CONCLUSION

We investigated the cytotoxic effects of DON, acetylated derivatives of DON (3ADON, 15ADON), and NIV on MSCs and the L929 fibroblast cell line. Here, we report the *in vitro* cytotoxic effect of DON on MSCs by providing a comparative evaluation between the 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 induced 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 hours <48 hours; affecting of cells L929 < DPSCs; confluency 50%<80%. In this study, we could not provide mechanistic and molecular insights into the underlying mechanisms that lead to DON-induced toxicity. Further molecular experiments, such as gene expression and oxidative stress determination, are required in this context. These findings could pave the way for future comparative toxicological studies of stem cells and stable cell lines.

Ethics

Ethics Committee Approval: Teeth were collected under guidelines approved by the İstanbul University Faculty of Medicine Clinical Research Ethics Committee (approval no: 2019/455, date: 29.03.2019).

Informed Consent: Informed consent was obtained from the patients.

Authorship Contributions

Surgical and Medical Practices: Ö.Y.G., I.S., Concept: G.A., I.S., Design: G.A., Data Collection or Processing: I.S., G.A., C.K., Analysis or Interpretation: I.S., G.A., C.K., Literature Search: I.S., G.A., C.K., Writing: I.S., G.A., C.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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