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Original

Cytotoxic effect of Deoxynivalenol on the proliferation of the HepG2 cell line

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ABSTRACT

Objetive. To determine the cytotoxic effect and induction of apoptosis of Deoxynivalenol (DON) on the human hepatocarcinoma cell line (HepG2). **Materials and methods**. The HepG2 cell line was exposed to concentrations of 10, 25, 50 and 75 µM of lyophilized DON for 48 and 72 hours. Subsequently, the cytotoxic activity of DON was evaluated using the MTT assay. Finally, it analyzed the morphological changes of apoptosis in HepG2 cells by transmission electron microscopy, after treatment with 50 µM of DON for 48 hours. **Results.** DON, affects the metabolic activity and proliferation of HepG2 cells above 10 µM, compared to the control. The IC_{50} of DON on HepG2 cells, 42.8 µM SD±1.12 and 29.6 µM SD±3.1 at 48 hours and 72 hours of treatment, respectively. The morphological characteristics of apoptosis in HepG2 cells, such as nuclear and cellular fragmentation, invagination of the plasma membrane, and the formation of apoptotic bodies. **Conclusions.** DON is a cytotoxic agent in HepG2 cells that alters cellular metabolic activity, with a significant antiproliferative effect dependent on concentration and exposure time, and induces apoptotic cell death.

Keywords: *Fusarium* spp.; proliferation; toxicity (*Source: CAB*).

RESUMEN

Objetivo. Determinar el efecto citotóxico e inducción de la apoptosis de Deoxinivalenol (DON) sobre la línea celular de hepatocarcinoma humano (HepG2). **Materiales y métodos**. La línea celular HepG2 se expuso a concentraciones de 10, 25, 50 y 75 µM de DON liofilizado durante 48 y 72 horas. Posteriormente, la actividad citotóxica de DON se evaluó empleando el ensayo MTT (bromuro de 3-(4,5-dimetil-2-tiazolil) -2, 5-difeniltetrazolio). Finalmente, se analizaron los cambios morfológicos propios de la apoptosis en las células HepG2 por microscopía electrónica de transmisión, después del tratamiento con 50 µM de DON durante 48 horas. **Resultados.** DON, afecta la actividad metabólica y proliferación de las células HepG2 por encima de los 10 µM, en comparación con el control. La concentración inhibitoria media (CI_{50}) de DON sobre las células HepG2, fue de 42.8

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µM DE±1.2 y de 29.6 µM DE±3.1 a las 48 horas y 72 horas de tratamiento, respectivamente. Se observaron características morfológicas de la apoptosis en las células HepG2, como la fragmentación nuclear y celular, invaginación de la membrana plasmática y formación de los cuerpos apoptóticos. **Conclusiones.** DON, es un agente citotóxico sobre las células HepG2 que altera la actividad metabólica celular, con un efecto antiproliferativo significativo de manera dependiente a la concentración y al tiempo de exposición, e induce la muerte celular apoptótica.

Palabras clave: *Fusarium* spp.; proliferación; toxicidad (*Source: DeCS*).

INTRODUCTION

Deoxynivalenol (DON) is a type B trichothecene produced by *Fusarium* spp*.* (1), synthesized during the growth of *Fusarium graminearum* and *Fusarium culmorum* on cereal grains, such as wheat, barley and maize. Worldwide, trichotecene contamination is common in cereal grains, cereal by-products, human food and animal feed. This problem is becoming more frequent due to colonization by *Fusarium* spp., the practice of sowing without plowing and inadequate crop rotation, as well as changes in climate patterns in temperate regions (2,3,4).

Trichothecenes of low molecular weight (~200-500 Da) can spread rapidly in cells (5). The amphipathic nature of DON allows it to easily cross cell membranes and interact with ribosomes, mitochondria and endoplasmic reticulum (6). DON has multiple toxic effects at the eukaryotic cell level. For example, DON interacts with the enzyme peptidyl transferase in the ribosomal 60S subunit and triggers ribotoxic stress. Several studies suggest that it induces the production of free radicals, causing oxidative stress; mechanisms that cause cell damage, such as inhibition of protein, RNA, and DNA synthesis; mitochondrial function, cell viability, proliferation, cell differentiation, and membrane integrity are also affected, which eventually induce apoptosis or necrosis (3,6,7).

The process of apoptosis advances through a complex cascade involving morphological, molecular and biochemical characteristics. Morphological characteristics of apoptosis include early events such as cell cytoplasmic contraction, decreased cell volume, and chromatic and nuclear condensation. Nuclear fragmentation and, consequently, DNA rupture, blister formation in the plasma membrane and the formation of apoptotic bodies digested by phagocytosis by neighboring cells or macrophages are presented below, which are considered late events in the apoptosis process. Furthermore, in the later stages of apoptosis, morphological characteristics include ultra-structural modification of cytoplasmic organelles, formation of vesicles with intracellular contents, rearrangement of the cytoskeleton and loss of contact with the extracellular matrix.

These morphological changes are due to characteristic molecular and biochemical events that occur in apoptotic cells. For example, initial biochemical events include the externalization of phosphatidylserine in most cells. This gives signals in apoptotic cells, causing them to be absorbed by macrophages and nearby cells, especially *in vivo*. Additional biochemical events involve the particular activation of proteolytic enzymes, including initiator and effector caspases, which hydrolyze structural proteins, as well as nucleases that cause DNA fragmentation at internucleosomal sites, on one hand, and the inactivation of repair enzyme systems, on the other. (8,9,10).

Primary cultures of bone marrow, epithelial, liver, lymphoid, kidney and lung tissue have been used in *in vitro* studies of DON toxicity. These cells are considered to be sensitive to DON (11) in addition to those that are part of the organs involved in the route of absorption such as the intestine and liver. Likewise, the HepG2 cell line, derived from human hepatocellular carcinoma, has been widely used in *in vitro* studies, as it maintains liver-specific functions and reflects the metabolism of toxins better than other cell lines. Studies of the effect of DON on HepG2 report that it affected cell viability when treated with a concentration of 0.9 µM. Similarly, in other cells of the intestinal epithelium, cell proliferation was inhibited after 24 hours of exposure to DON in a concentration range of 10-30 µM (1,12,13). Another study obtained inhibitory concentrations of DON for HepG2 cells of 1.0-15 µM, (14). Therefore, the objective of this research was to determine the cytotoxic effect of DON on the proliferation of the HepG2 cell line at different concentrations and treatment times.

MATERIALS AND METHODS

Mycotoxin. Lyophilization was performed using the Micotox standard for deoxynivalenol by HPLC in methanol (HPLC-DON-4), Micotox Ltda, Colombia. In summary, it started with the dialysis of the DON mycotoxin in a Tris buffer at 25 mM for 24 hours. The contents were then transferred to a Falcon tube, frozen for 4 hours and lyophilized for 24 hours.

HepG2 cells. The HepG2 (Human Hepatoblastoma Carcinoma) cell line was cultured in RPMI-1640 supplemented with 10% fetal bovine serum and a 0.05% antibiotic solution, to a confluence of 80%. The cells were then dissociated with trypsin 0.25% and seeded in 96-well plates, at a density of 20.000 cells/100 µl per well. The cells were left for 24 hours for cell adhesion and then, the DMSO (dimethyl sulfoxide) diluted DON mycotoxin was added at concentrations of 5, 10, 25, 50 and 75 µM. Changes in cell morphology and density were observed under the Nikon 37762 inverted optical microscope.

HepG2 cell proliferation. The effect of the DON mycotoxin on cell viability was determined using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). After 48 and 72 hours of treatment with DON, cell viability was evaluated by adding 100 µl of MTT (0.5 mg/ml) and incubated for 4 hours at 37°C, 5% CO_2 and 95% humidity. Subsequently, the plates were washed with PBS (phosphate buffered saline), and then 100 µl of acid isopropanol was added to solubilize the formazan crystals for 15 minutes under culture conditions and in gentle agitation for 5 minutes. Finally, the optical density was examined at 570 nm (Tecan Spectra classic spectrophotometer) of the solubilized solution (15) and the percentage of cell viability was calculated.

The effect of the mycotoxin on HepG2 cells was then expressed as inhibition percentage values using the following formula:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{Mean absorbance of treated cells}}{\text{Mean absorvance of the control}}\right)\right] \text{ x } 100$$

The inhibitory concentration, i.e., the concentration of mycotoxin required to inhibit 50% of the growth in HepG2 cells (IC_{50}), was measured by calculating the concentration and percentage inhibition curves. The hyperbolic non-linear model was selected for data adjustment. Non-linear regression analysis was used to obtain the model estimators by least squares, using the inhibitory dose as a response variable and the concentration of DON evaluated as a regression variable. This model was used for its great utility in this type of assays and for having the lowest Akaike information criterion (AIC) (16).

Transmission electron microscopy. 3 x 10^6 HepG2 cells were seeded in well plates for 24 hours to allow cell adhesion, then 50 µM of DON was added for 48 hours. The cell monolayer was then dissociated with 0.25% trypsin, and the cell suspension was washed with PBS and centrifuged at 2.000 rpm for 10 minutes. The cell precipitate was set for 1 to 2 hours in a 3:3 mixture (3% glutaraldehyde-formaldehyde in a 0.1 M cacodylate buffer, pH 6.3). The cell suspension was then centrifuged at 2.000 rpm for 10 minutes, washed with the cacodylate buffer and then post-set for 3 to 12 hours in a 1% osmium tetroxide solution prepared in the same cacodylate buffer. After this time, the cell precipitate was washed with buffer solution and dehydrated in ascending concentrations of ethyl alcohol followed by propylene oxide, subsequently infiltrated and included in epoxy resin. Ultrafine 90-nanometer sections were prepared from the precipitate of the included cells, which were tested with lead citrate and uranyl acetate for the ultra-structural analysis of apoptosis in the transmission electron microscope (H-7000, Hitachi).

Statistical analysis. Statistical analysis was performed using Graph Pad Prism software to compare differences between control means and mycotoxin-treated groups at 48 and 72 hours. Data were analyzed with one-way ANOVA and Dunnett's multiple comparison test.

RESULTS

Effect of DON on the metabolic activity of HepG2. Metabolic activity was observed in the ability of HepG2 cells to form formazan crystals. HepG2 cells that were not treated with DON showed high metabolic activity due to the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in formazan crystals (Figure 1A); while, as the DON concentration increased, this metabolic activity decreased in HepG2 cells (Figures 1B and 1C).

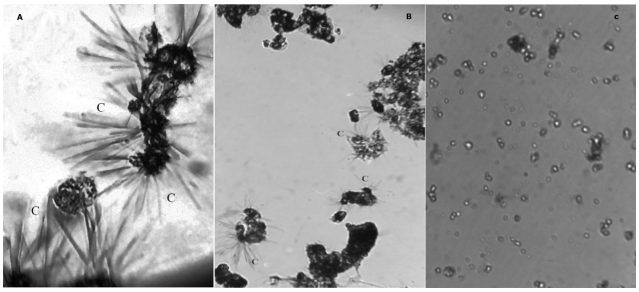

Figure 1. **a.** Untreated HepG2 cells. **b.** HepG2 cells treated with a mean concentration (25 µM) of DON. **c.** HepG2 cells treated with a high concentration (75 µM) of DON. Increase: A: 400X B and C: 200X. C= Formazan crystals. Nikon 37762 inverted optical microscope.

Cytotoxic effect of DON on HepG2 cells. The cytotoxic effects of DON on HepG2 cell proliferation are shown in Figure 2. Cell proliferation was inhibited in a concentration-dependent manner after treating HepG2 cells with DON in the range of concentrations from 0 to 75 µM for 48 and 72 hours. A significant effect of DON on cell proliferation was observed from 10 µM (75.37% ±4.0 and 74.01% ±0.66) compared to the control (98.2% ±0.05), respectively ($p<0.0001$). Consequently, the higher the concentration of DON (75 µM), the percentage of proliferation was significantly reduced to 33.39% ±0.0 and to 23.73% ±3.0, with a decrease in cell proliferation and metabolic activity for the formation of formazan crystals (Figure 1C). In turn, the results show an IC_{50} value of 29.6 µM ±3.1 after 72 hours of exposure, as opposed to the IC_{50} of 42.82 µM ±1.2 after 48 hours of exposure to DON.

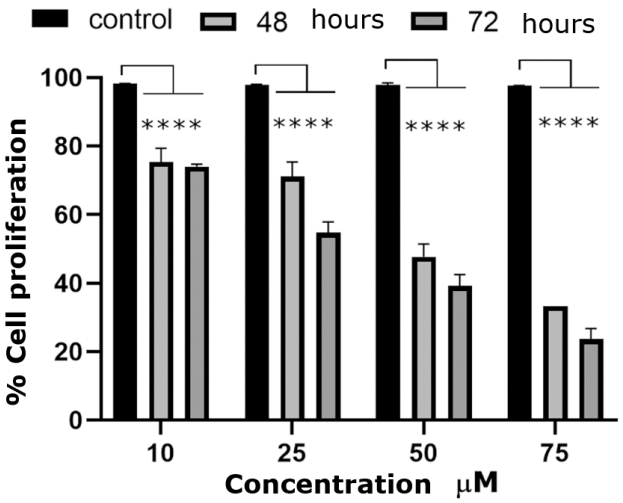

Figure 2. Proliferation of HepG2 cells treated with a concentration in the range of 0 to 75 µM of DON. Each bar represents the mean of ±(n=3). ****$p<0.0001$ vs. ANOVA control.

Apoptosis of HepG2 cells by DON. Changes were observed in cell morphology upon analysis of the apoptosis process in HepG2 cells by exposure to DON (50 µM) for 48 hours by transmission electron microscopy. Cells treated with DON showed fragmentation of cell and nuclear content, the formation of protuberances in the plasma membrane, apoptotic bodies, alterations of cell morphology and therefore, loss of cell-to-cell contacts, which are typical of apoptotic cells. In turn, in the control, the nucleus maintained its rounded shape with the presence of a defined nucleolus, the nuclear and plasma membrane remained intact, and a preserved cell content and epithelial morphology in HepG2 cells were observed (Figure 3 A, B and C).

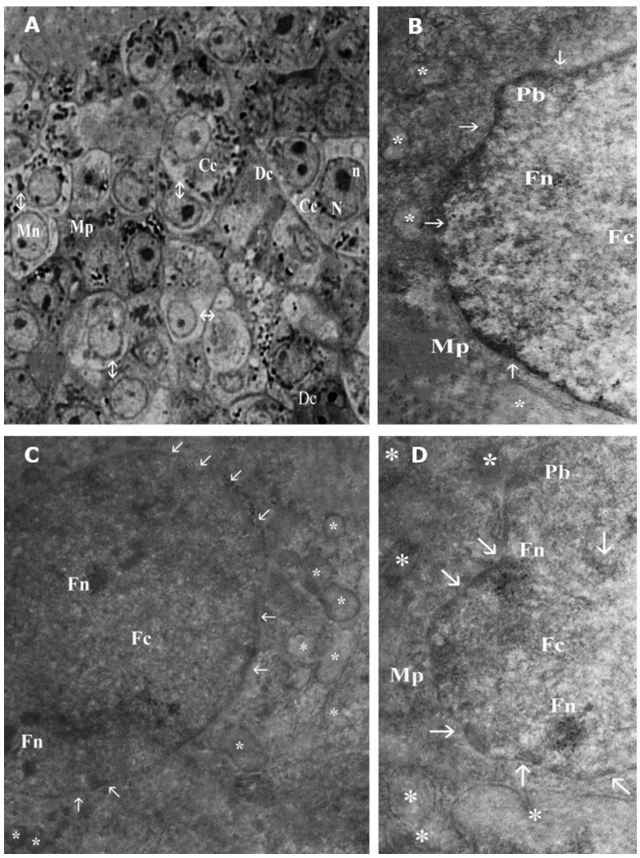

Figure 3. Transmission electron microscopy of HepG2 cells, exposed to 50 µM of DON. A. Negative control, B - D. Cells exposed to 50 µM of DON. Increase: A:4000X B-D: 6500X. Nm = Nuclear membrane, Pm=Plasma membrane, Cc=Cell content, Cd=Cell division, N=Nucleus, n=Nucleolus, Pb=Protuberance, Nf=Nuclear fragmentation, Cf=Cell fragmentation, *=Apoptotic bodies, →=Plasma membrane invaginations, double-headed arrow= Cell-to-cell contact. Transmission electron microscope (H-7000, Hitachi)

DISCUSSION

The cytotoxicity of DON has been studied in both animal models and cell cultures because mycotoxins are natural contaminants in food and are a risk factor for human and animal health. This study reports the cytotoxic effect of different DON concentrations on the HepG2 cell line through *in vitro* assays.

The MTT assay is a measure of the mitochondrial metabolism of cells, so results suggest that exposure to DON affects this cellular mechanism in HepG2 cells, given the decrease in the formation of formazan crystals as DON concentrations increased. Also, several authors report that exposure to DON causes metabolic alteration in several cell lines. These studies show that exposure of human embryonic kidney cells (Hek-293) to DON concentrations >7.5 µM significantly reduces cell viability, decreases NADH levels and increases ROS, which may be related to mitochondrial dysfunction (17).

Cytotoxicity results showed a 25.31% decrease in HepG2 cell proliferation by ±0.96 at the lowest DON concentration (10 µM). However, other studies report a 25% decrease in HepG2 cells due to exposure to 1 µM DON (1). Furthermore, the proliferation of intestinal epithelial cells is inhibited at DON concentrations between 10-30 µM (3), suggesting a lower sensitivity of the cells tested in this study. The difference in results may be due to the purity of the DON standard tested in each case. In this study, a standard DON in ethanol was used, which was lyophilized prior to treatment of HepG2 cells.

In relation to the IC_{50} value of 42.82 µM 1.2 obtained after 48 hours of exposure of HepG2 cells to DON, these results differ with the current reports of IC_{50} from 3.0 to 4.15 µM after 48 hours of exposure, using HepG2 cell lines and adenocarcinoma of the human colon (CaCo-2) and cytotoxic neutral red and MTT assays (14, 18). For the exposure time of 72 h, the results of IC_{50} obtained from 29.6 µM ±3.1, by contrast to these results, showed that the values of IC_{50} obtained in other research after 72 hours of DON treatment of HepG2 cells were 2.53 µM ±0.21, 4.30 µM ±0.36 to 9.30 µM using the MTT and Red Neutral assay (14,19). However, although IC_{50} concentrations differ between authors due to the various experimental conditions evaluated, as well as the purpose of each study, type of cells, exposure time, concentration range and cytotoxic assay used, it is agreed that the effect of DON on the HepG2 cell line is dependent on concentration and time.

Morphological changes in HepG2 cells exposed to DON, identified by electron microscopy, included fragmentation of cell and nuclear content, invaginations of the plasma membrane and formation of apoptotic bodies, as well as alterations of cell morphology and therefore, loss of cell-to-cell contacts (Figure 3: B-D). These findings indicated that the cytotoxicity of DON in HepG2 cells is characterized by apoptotic cell death. These results coincide with reports observing that epithelial cells of human liver tissue (L-02) treated with DON showed morphological changes typical of apoptotic cells, with the appearance of irregular shaped nuclei and fragmented chromatin, accompanied by the increase of oxidative stress with the generation of ROS and the decrease in mitochondrial membrane potential, with toxic effects on the inhibition of protein and nucleic acid synthesis and induction of apoptosis (20). Other studies report apoptotic lesions in liver tissues of pigs due to exposure to DON, revealing a condensation of chromatin and apoptotic bodies (21); similarly, exposure to DON of human colon cancer cells HT-29 causes nuclear fragmentation and the presence of apoptotic bodies (22).

In conclusion, DON had a cytotoxic effect on HepG2 cells that altered cellular metabolic activity, with a significant marked antiproliferative effect dependent on concentration and exposure time. Consequently, it induces apoptotic cell death in HepG2 cells, characterized by morphological changes such as fragmentation of cell and nuclear content, invaginations of the plasma membrane and formation of apoptotic bodies, as well as alterations of cell morphology, resulting in loss of cell-to-cell contact. This may imply that the metabolic alteration generated by DON activates cellular mechanisms, possibly through the mitochondrial pathway, that trigger apoptosis.

Conflict of interests

All authors declare that there is no conflict of interest for the publication of this manuscript.

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