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Influence of deoxynivalenol on the oxidative status of HepG2 cells

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Abstract

This study was undertaken in order to investigate the effect of deoxynivalenol on the oxidative status and antioxidant defense of HepG2 cells. The HepG2 cells were exposed to 2.5 μM deoxynivalenol for 6, 12 and 24 hours. Higher ($p<0.01$) activities of the antioxidant enzymes such as superoxide dismutase and catalase were observed after 12 and 24 hours of mycotoxin exposure, while, for the same time points, the superoxide anion level returned to the control values. The present findings imply that the reactive oxygen species that are generated in HepG2 cells due to deoxynivalenol exposure were detoxified by the induction of antioxidant enzymes, such as superoxide dismutase and catalase. The glutathione levels and the activities of the enzymes involved in the metabolism of glutathione, glutathione peroxidase, glutathione-S-transferase and glutathione reductase, were decreased for all times of mycotoxin exposure. This finding suggests that the glutathione cell cycle was seriously perturbed by HepG2 exposure to 2.5 μM deoxynivalenol. The modulation of the two enzymes involved in NADPH synthesis, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase, were time-dependent. At 6 hours, the regeneration of NADPH was provided by isocitrate dehydrogenase, while at 12 hours both enzymes are increased and, finally, at 24 hours, only the activity of glucose-6-phosphate dehydrogenase was up-regulated. These data suggest that an incomplete adaptation of the antioxidant defense system of HepG2 cells occurs in order to minimize oxidative injury caused by deoxynivalenol.

Keywords**:** HepG2 cells, deoxynivalenol, antioxidant scavenging enzymes, superoxide anion, reduced glutathione

Introduction

Mycotoxins are fungal secondary metabolites commonly present in feed and food. Human and animal exposure occurs by ingestion of mycotoxin-contaminated products and can lead to serious health problems [1]. Various species of *Fusarium* may produce deoxynivalenol (DON) or "vomitoxin", which is a type B trichothecene, an epoxy-sesquiterpenoid. This mycotoxin is present predominantly in grains such as wheat, barley, oats, rye, and maize. The main effects of its low dietary concentration are anorexia and decreased growth, while higher doses induce vomiting, immunotoxic effects and alteration in

brain neurochemistry [2, 3]. DON has been tested in several mammals at different concentration. Short-term studies have evidenced that it produces atrophy of the thymus and spleen, in a high dose. Long-term studies described a decrease in the liver weight, nephrotoxicity, pulmonary edema and leucoencephalomalacia. [5, 6].

Direct DON administration into the embryonic eggs caused toxin accumulation in liver in a time-dependent manner, which was also accompanied by a notable accumulation of fat droplets [6]. In White Leghorn hens, this mycotoxin has been reported to accumulate at high levels in liver [7] and to increase the triglycerides and total lipid level in this organ [8]. Pregnant Landrace saws fed for 35 days with wheat naturally contaminated with DON and zealerone (ZON) presented histopathological changes in liver and spleen at the end of the pregnancy, whereas no significant changes were noticed in these tissues in their offsprings [9].

DON was reported to bind to the ribosomal peptidyl-transferase site. It inhibits protein and DNA synthesis and, as a consequence, cell proliferation decreased [10].

Human and animal cell lines have been used to study the mechanisms of *in vitro* toxicity of *Fusarium* mycotoxins. DON had a distinct effect on human primary hepatocytes, decreasing viability, protein content and albumin secretion in a dose-dependent manner and it was not metabolized by these cells [11]. In culture cells, the induction of the oxidative stress by DON has been shown [12, 13].

Oxidative stress occurs when the reactive oxygen species (ROS), formed during a variety of biochemical reactions and cellular functions, are not fully counteracted by the antioxidative mechanisms. ROS are promoting chain reactions in which the free radicals are passed from one macromolecule to another, resulting in extensive damage to cellular structures. Organisms have developed several cellular defence paths, which, under normal metabolic conditions, regulate the level of ROS and protect against the ill-effects of free radicals [14]. The defence system includes both low-molecular-weight free radical scavengers, such as the tripeptide glutathione (GSH) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) [15]. The induction of antioxidant enzyme activities has been used as a biosensor for ROS formation during oxidative assault of the cell [16].

Taking into account that the information concerning DON effects on a hepatic cell culture are limited, the aim of this study was to evaluate the antioxidant response in a human hepatoma HepG2 cell line exposed to this mycotoxin.

Materials and Methods

Chemicals

GIBCO® Dulbecco's Modified Eagle's Medium (DMEM), sodium pyruvate, foetal bovine serum, gentamicin (10 mg/ml), L-glutamine and vitamins solution (100X) were purchased from Invitrogen (Carlsbad, California, USA). Nicotinamide adenine dinucleotid phosphate disodium salt ($NADP^+$) and nicotinamide adenine dinucleotid phosphate reduced tetrasodium salt (NADPH) were supplied from Merck (Darmstadt, Germany). Phenylmethanesulfonyl fluoride (PMSF), 1,1,1-(hydroxymethyl)ethane (TRIS), sodium chloride and Nitrotetrazolium Blue Chloride (NBT) were purchased from Fluka (Milwaukee, USA). All the others chemicals used were of analytical grade and were supplied from Sigma (St. Louis, USA).

Deoxynivalenol determination

Deoxynivalenol level was measured according to the modified method of Rupp [17]. The cleaning-up was achieved with immunoaffinity collumns, using 2 ml of centrifuged culture medium previously passed through DONPREP column (R- Biopharm, Darmstadt, Germany). After washing the column with 5 ml ultra pure water, the elution was performed with 1.5 ml methanol. The elution solvent was removed and the mycotoxin was re-dissolved in 1 ml mobile phase. A volume of 100 µl sample was injected in the Aliance HPLC - separation system (Waters Corporation, Mildford Massachusetts, USA) and separated using RP Spherisorb analytical column 4.6x150 mm, 5µm spherical particle. The mobile phase consisted in acetonitrile-methanol-water (5:5:90 v/v) and the flow rate was 1 ml/min. UV detection was made at 220 nm. Data were analyzed with the Empower chromatography Data Software (Waters Corporation, Mildford Massachusetts, USA). The calibration curve used for processing the results had a linearity coefficient (r^2) 0.999922 and a correlation coeficint (r) 0,999961. The lowest limit of detection (LOD) was 0.02 µg DON/ml.

Cell culture and treatment

Hep G2 cells were maintained in DMEM, containing 3,7 g/L sodium bicarbonate, 4,5g/L D-glucose, 4,7g/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 mM L-glutamine, 0,1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin and 10% (v/v) foetal bovine serum. Cells were grown in 5% CO_2 at 37^{o}C. The cells were grown as monolayers in 75 cm^2 cell culture flasks coated with type I collagen. Cells were seeded at a density of $2,5x10^5$ cells/ml and incubated with DON at concentration of 1, 2.5 and 5µM and for different periods of time: 3, 6, 12 and 24 hours. Controls, with 0 µM DON were performed for each analysis.

Cell viability

The cell viability was determined by the MTT test [18]. The medium from each well was removed by aspiration, the cells were washed with 200 µl phosphate buffer solution (PBS)/well and then 50µl (1mg/ml) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added on each well. After 2 hours of incubation the MTT solution from each well was removed by aspiration. A volume of 50 µl isopropanol was added and the plate was shaken to dissolve formazan crystals. The optical density at 595nm, for each well, was then determined using a Tecan multiplate reader (Tecan GENios, Grödic, Germany).

Lipid peroxidation

The level of lipid peroxidation was measured via the 2-thiobarbituric acid (TBA) color reaction for malondialdehyde (MDA) by the modified method of Portoles et al. [18]. Cultured hepatocytes were previously homogenized in 1ml of 0.1 M saline phosphate buffer and sonicated on ice, at 40 V, 3 times for 30 seconds. Then, 0.375 ml of 40% (w/v) trichloroacetic acid (TCA) and 0.200 ml of 0.1M TBA was added to lysates. The samples were incubated at 90°C for 30 min. and then, 0.625 ml of distillate water were added. The samples were centrifuged for 10 minutes at 5000 rpm. The level of supernatants lipid peroxidation was determinated by the absorbance at 532 nm, using a MDA solution, freshly made by the hydrolysis of 1,1,3,3-tetramethoxy propane, as a standard. The results were expressed as nmoles of MDA per mg protein.

Reduced glutathione determination

The GSH level was assayed by the fluorometric method of Ghibelli [20], using a Glutathione Detection Kit (Chemicon, California, USA). In brief, 90 µl of cell lysate and 10 µl of prepared monochlorobimane (MCB) were added to a 96-well plate suitable for fluorometry and incubated 2 hours at room temperature away from light. The fluorescence

was read using a 380/460-nm (excitation/emission) filter set in a Tecan multiplate reader (Tecan GENios, Grödic, Germany). The GSH level was expressed as nmoles/cells number.

Determination of superoxide anion

The superoxide anion was assessed using the nitro blue tretazolium **(**NBT assay, which measures the conversion of NBT to blue formazan) [21]. The formazan formed after incubation of the cells with 0.5 mg/ml NBT, was solubilized by adding 300 ml of KOH/dimethylsulphoxide (DMSO) per well (mix 1 part of 2M KOH with 1.167 parts of DMSO just before use) and absorbance was measured at 520 nm using a Tecan microplate reader. Results were expressed as μmoles/cells number.

Enzymes activity assays.

The harvested cells were homogenized with 20 mM Tris/HCl buffer (pH 7.5), 0.2% Triton X-100, 0.5 mM PMSF and sonicated three times for 30 seconds. Total cell lysates were centrifuged at 3000 rpm, for 15 minutes at 4°C and aliquots of the supernatant were utilized for the subsequent enzymatic assays.

The total SOD (EC 1.15.1.1) activity was measured according the spectrophotometric method of Paoletti [22], based on NADPH oxidation. The method consists of a purely chemical reaction sequence which generates superoxide anion from molecular oxygen in the presence of EDTA, manganese (II) chloride and mercaptoethanol. The decrease in absorbance at 340 nm was followed for 10 min to allow NADPH oxidation. A control was run with each set of three duplicate samples and the percent inhibition was calculated as (sample rate)/ (control rate) ×100. One unit of activity was defined as the amount of enzyme required to inhibit the rate of NADH oxidation of the control by 50%.

The CAT (EC 1.11.1.6) activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm, according to the method of Aebi [23]. The CAT activity was calculated in terms of U/mg protein, where one unit is the amount of enzyme that catalyzed the conversion of one μmole H_2O_2 in a minute.

The total GPX peroxidase (EC 1.11.1.9) was assayed by Beutler [24] method, using H_2O_2 and NADPH as substrates. The conversion of NADPH to $NADP^+$ was followed by recording the changes in absorption intensity at 340 nm, and one unit was expressed as one μmole of NADPH consumed per minute, using a molar extinction coefficient of $6.22x10^3$ M^{-1} cm^{-1}.

The glutathione *S*-transferase (GSH) (EC 2.5.1.18) activity was assayed spectrophotometrically at 340 nm by measuring the rate of of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with GSH, according to the method of Habig et al. [25]. One unit of GST activity was defined as the formation of one μmole of conjugated product per minute. The extinction coefficient 9.6 $mM^{-1}cm^{-1}$ of CDNB was used for the calculation.

The GR (EC 1.6.4.2) activity was measured according to the method of Goldberg and Spooner [26], in 0.1 M phosphate buffer, pH 7.4 with 0.66 mM GSSG and 0.1 mM NADPH. One unit of GR activity was calculated as one μmole of NADPH consumed per minute.

All the enzymatic activities, calculated as specific activities (units/mg of protein) were expressed as % from controls.

The glucose 6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) activity was measured according to the method of Lohr and Walle [27], in 35 mM triethanolamine buffer with 0.5 mM $NADP^+$ and 0.67 mM disodium glucose-6-phosphate. One unit of G6PDH activity was calculated as one μmole of NADPH formed per minute.

The $NADP^+$-dependent isocitrate dehydrogenase (ICDH) (EC 1.1.1.41) activity was determined in 0.1 M triethanolamine buffer, 52mM NaCl, pH7.5 with 4.6 mM D,L-isocitrate

10 mM $NADP^+$ and 0.12 M $MnSO_4$. according to Bernt and Bergmeyer [28]. One unit of ICDH activity was calculated as one μmole of NADPH formed per minute.

All the enzymatic activities, calculated as specific activities (units/mg of protein) were expresed as % from controls.

Protein concentration

The protein concentration, expressed as mg/mL, was determined by the method of Lowry et al. [29] using bovine serum albumine as standard.

Statistical analysis

All values were expressed as means of triplicate ± SD. The differences between control and deoxynivalenol-treated cells were compared by Student's test using standard statistical packages. The results were considered significant only if the p value was less than 0.05.

Results

DON is one of the most prevalent mycotoxin in human food [30]. The hepatocytes are the first cells affected by food contaminants. The HepG2 line was selected in our study taking into account that it maintains some properties of normal hepatic cells, which usually are mostly affected by toxins. [31].

Cytotoxicity induced by DON

The viability of HepG2 cells treated with a 1, 2.5 and 5μM DON, for different time, are presented in Figure 1. The results suggested that the cytotoxic effect of DON appeared to be time-and concentration- dependent. Treatment of the cells with three different concentrations for 3 hours each resulted in no significant loss of viability. Incubation of cells with 1μM of DON decreased the cell viability with 7% after 6 and 12 hours of exposure and with 22% after 24 hours. The concentrations of 2.5 μM and 5 μM diminished the cell viability with 25% after 6 and 12 hours and with 45% after 24h, respectively.

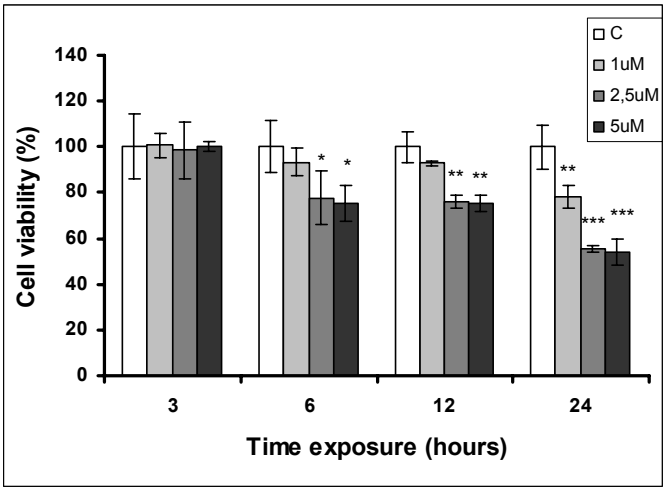

Figure 1. The viability of HepG2 cell treated with different concentrations of DON (1, 2.5 and 5 μM) for 3, 6, 12 and 24 hours**.** Data represent the means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls).

Taking into account that HepG2 cells are equally sensitive to 2.5 and 5 μM DON exposure, all the following experiments were done using the concentration of 2.5 μM at 6, 12 and 24 hours of exposure.

Variation of DON concentration in culture media

The DON levels in HepG2 cells, after different time of exposure, are shown in Figure 2. The concentration of DON in the HepG2 cell culture media has decreased by 66% after 6 hours of exposure and by 71.14% respectively 71.75% after 12 and 24 hours.

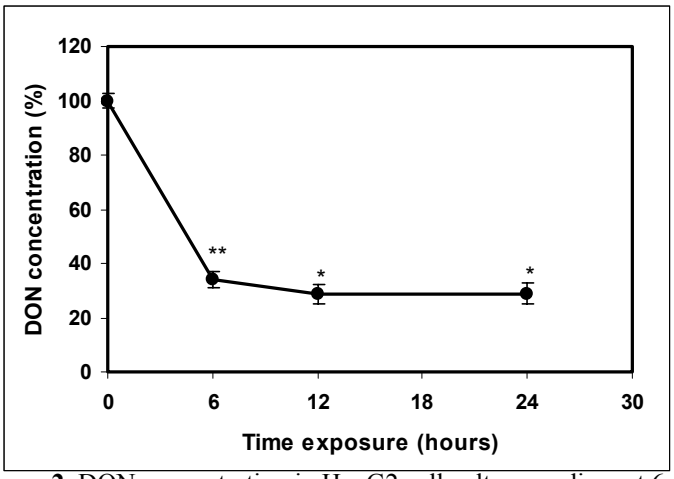

Figure 2. DON concentration in HepG2 cell culture medium at 6, 12 and 24 hours after 2.5 μM mycotoxin exposure. The values are calculated as means ± SD (n=3). (*$P < 0.05$, **$P < 0.01$ vs. controls).

Intracellular superoxide anion production

The total level of superoxide anion in HepG2 cells treated with 2.5 μM DON was measured during 24 hours (Table 1}. A distinct significant increase, 148.4% of control, was noticed after 6 hours, whereas after 12 respectively 24 hours, this concentration returned to the control values.

Intracellular lipid peroxidation

Our experiments had showed a decrease of MDA level in HepG2 cells exposed to 2.5 μM DON (Table 1). The most significant changes were recorded after 6 and 12 hours of DON exposure, when MDA levels were decreased by 20.6% and 13.5%, respectively.

Intracellular GSH depletion

DON induced a depletion of intracellular GSH in a time-dependent manner (Table1). After 6 h of exposure, the GSH content was diminished by only 10% and these changes were not significant from the statistical point of view. Nevertheless, a decrease in the intracellular GSH content was observed after 12 and 24 h by about 36.2%, and 34.7%, respectively.

Table 1. Effects of DON on superoxide anion, lipid peroxidation and GSH in HepG2 cells[1]. Values are means ± SD (n=3)

Parameter	Control	Time of DON exposure (hours)		
		6	12	24
Superoxide anion (μmoles/cells number)	100±9.3	148.4±9.7**	107.6±10.3	97.8±8.7
Lipid peroxidation (nmoles MDA/mg protein)	100±9.2	79.4±6.3**	86.5±9.4*	94.1±8.8
GSH (nmoles/cells number)	100±7.5	89.4±8.7	63.8±5.5*	65.3±6.7*

Means significantly from controls at * $p<0.05$ and ** $p<0.01$.

DON induced changes in NADPH generating enzymes

The specific activities of two enzymes implicated in NADPH generation, G6PDH and ICDH are shown in Figure 3. A decrease with 30% of specific activity of G6PDH after 6 hours of exposure was noticed, whereas longer exposures of 12 and 24 hours were associated with an increase of about 42% and 30.9%, respectively. The variation profile of ICDH specific activity was the opposite of that of G6PDH. An increase of this activity with 35% and 46% was recorded after 6 and 12 hours, respectively, whereas, after 24 hours, a sharp decrease by 75% was noticed.

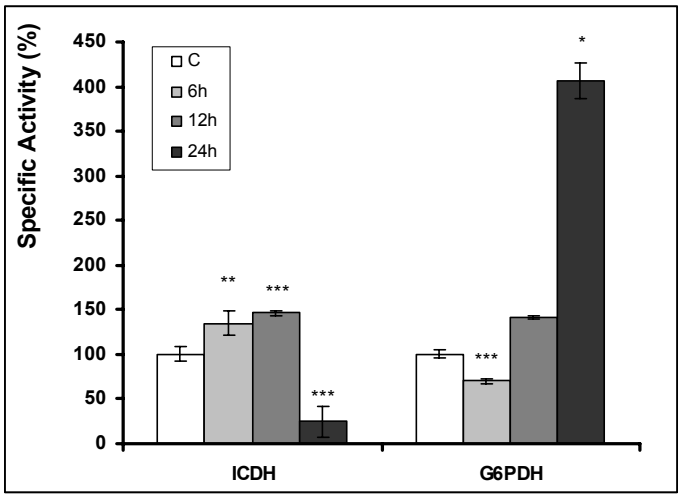

Figure 3. Effects of DON exposure for 6, 12 and 24 hours on ICDH and G6PDH activities in HepG2 cells. The values are means ± SD (n=3). (*$P < 0.05$, **$P < 0.01$ vs. controls).

The antioxidant scavenging enzymes

The antioxidant scavenging enzymes include SOD, CAT, GPX, GR and GST, enzymes which contribute directly or indirectly to ROS elimination. The variation of SOD and CAT specific activity in HepG2 cells treated with 2.5 μM DON showed a progressive increase (Figure 4). For these enzymes, after 12 and 24 hours of exposure, the increases were distinctly significant, respectively very significant. In the case of SOD, its level was 118%, 128.67% and 302% compared to control, after 6, 12 and 24 h of exposure, respectively, whereas CAT levels were enhanced by 74.4% and 156.6% after 12 and 24h of intoxication, respectively.

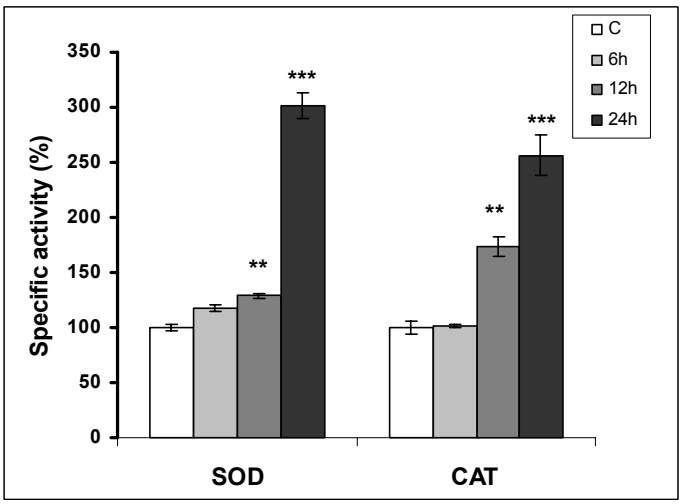

Figure 4. SOD and CAT specific activities in HepG2 control cells and in DON treated cells. Data represent the means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls).

An alteration in specific activities of the enzymes involved in GSH metabolism was recorded after HepG2 treatment with 2.5 μM DON (Figure 5). Our data showed that GR specific activity decreased after 6, 12 and 24 hours of exposure with 39%, 90% and 85%, respectively, compared to control. For GPX, the specific activity has diminished with 23%, 87% and 85% after 6, 12 and 24 hours of treatment, respectively. In addition, the total GST specific activity dropped by 60%, 53% and 50% compared to control, at 6, 12 and 24 hours after DON administration.

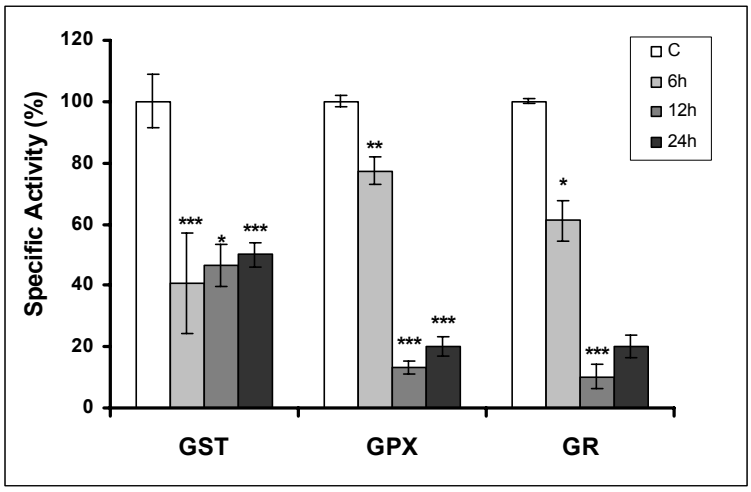

Figure 5. Effects of 2.5μM DON exposure on the GST, GPX and GR activities in HepG2 cellsValues are means ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. controls).

Discussion

The present study was designed in order to investigate the effects of DON on various biological parameters of the HepG2 cell line, a useful model of human liver cells [32]. Our results demonstrate that the concentration of 2.5μM DON is as toxic as the 5 μM one. This observation corresponds to the reduction in viability caused most probably due to non-specific inhibition of transcription and/or translation [33]. The cytotoxic concentration of DON found in our study is in agreement with the others toxic concentrations, ranging from 0.34 to 6.8 μM, reported in a variety of other cell types [9, 11, 13, 34, 35].

In our experiment, the first six hours of exposure were the most important in the acummulation of DON in Hep G2 cells. It is possible that this mycotoxin crosses these cells through the tight junctions. The experiments on the Caco-2 cells, a widely used *in vitro* model of human intestinal barrier suggested that DON crosses the intestinal mucosa by a paracellular pathway through the tight junctions although some passive transcellular diffusion may not be ruled out [36, 37]. The results of this study suggested that after an absorption of about 70% of this mycotoxin, the cells exhibit a saturation process.

The maintenance of the cytosolic pool of NADPH is crucial for the cellular redox balance. The most important enzyme for NADPH synthesis is G6PDH but other enzymes, such as $NADP^+$-dependent malate dehydrogenase and ICDH can alternatively produce this co-enzyme. In our study, the specific activities of two NADPH regenerating enzyme, G6PDH and ICDH, were investigated. The variation of the activities of these enzymes was correlated with the superoxide anion level and, indirectly, with SOD specific activity. Previous studies

showed that DON has the same effect as phlorizin on cellular glucose uptake, inhibiting Na^+-D-glucose cotransport [38]. As a result, at 6 hours of DON exposure, the rate of the first enzyme of the pentose phosphate pathway, G6PDH, decreased and the level of NADPH formed is lowered with 30%. This decline of NADPH was compensated by the increase of ICDH specific activity with 35%, this enzyme being an alternative cellular source of NADPH. The reduced nucleotide can be oxidazed in the presence of molecular oxygen, via NADPH oxidase, a highly expressed enzyme in HepG2 cells [39], which generates superoxide anion, a substrate for SOD. After six hours the SOD activity was unchanged and the superoxide anions accumulated in the HepG2 cells previously exposed to DON.

A different response was observed at 12 hours after DON treatment. The G6PD, ICDH and SOD activities increased and the level of superoxid anion decreased. Even if DON is known as an inhibitor of gluconeogenesis [40] in the case of a long exposure, it is possible that for short periods of time (12 to 24 hours), an increase in the rate of this metabolic pathway could occur, creating a high level of NADPH. The superoxid concentration decreased because the SOD activity increased and, on the other hand, the resulting NADPH could be used for maintaining catalase in its active form, this enzymatic activity being distinct and very significantly increased compared to the control, after 12 and 24h of treatment.

Previous studies [10] showed that mycotoxins may posses the capacity of inducing lipid peroxidation, but T-2 toxin, another trichothecene, did not enhance the MDA liver level in broiler chicken [41]. Recent experiments [42] showed that simultaneous exposure of chicken to DON and ZON generated an increased in the liver MDA level. In our experiments, the increases in the activities of the first antioxidant enzymes, SOD and CAT after 12 and 24 hours of DON exposure diminished the possibility of hydroxyl radicals production and initiation of lipid peroxidation in HepG2 cells.

To search for biochemical adaptation of HepG2 cells to DON exposure, we quantified the levels of GSH and the activities of the enzymes involved in GSH metabolism. Reduced glutathione plays an important role in the detoxification of ROS in human cells and is a reaction partner for the detoxification of xenobiotics. Glutathione is a co-substrate of GPX, enzyme which reduces hydrogen peroxide to water. Reduction of the oxidized form of glutathione (GSSG) and regeneration of GSH is accomplished by the enzyme GR. The family of GSTs contains enzymes that are capable of multiple reactions with a multitude of substrates in order to detoxify the endogenous compounds, such as peroxidised lipids, as well as the metabolism of xenobiotics [43]. The depletion of GSH content, determined starting with six hours, and the decreases in GPX and GST activities recorded, suggested an oxidative stress induced in HepG2 cells by 2.5μM DON. The deep decrease of GR after 6, 12 and 24 h could be due to the utilization of NADPH in other cellular reaction and, as a consequence, the GSH/GSSG ratio and GSH cell cycle are seriously perturbed. It is very possible that, during the 24 hours exposure to 2.5 μM DON of HepG2 cells, the GSH synthesis is slightly affected, whereas higher periods of treatment would have a huge impact on this process, favoring the orientation towards apoptosis.

In conclusion, 2.5 μM DON exposure on the HepG2 cell line induced an increase of G6PD, ICDH, SOD and CAT specific activities, alongside a decrease in GSH content and GST, GPX and GR activities. It seems that HepG2 cell line has the capability to counteract, in a certain measure, the oxidative stress induced by this mycotoxin at the concentration used in our experiment during 24 hours of exposure. Although the MDA level appears to be decreased, taking into account the decrease in the GSH concentration and the GSH-depending enzymes, it is possible that after longer periods of 2.5 μM DON administration, the HepG2 cells become fully exposed to oxidative stress.

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