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Research Article

# Effects of the mycotoxin deoxynivalenol on human primary hepatocytes

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Toxic effects of the mycotoxin deoxynivalenol (DON) observed in animals range from diarrhea, vomiting, gastro-intestinal inflammation to necrosis of several tissues. In the last years, DON has been tested in hepatocytes of several animal species for its cytotoxicity. However, these tests are limited to the use of animal cells. No studies using human hepatocytes are available. Further investigations with the human hepatocellular liver carcinoma cell line HepG2 might be limited due to the disadvantages of cell lines (*e.g.* immortalization, tumor derivation, longtime cultivation) and do not necessarily reflect the response of normal human cells. In order to overcome this problem and to be closer to the human situation, we studied the effect of DON in human primary hepatocytes and compared these data to the effects in the HepG2 cell line. Cell viability, apoptotic and necrotic cell death, albumin secretion and metabolic activity were determined. It could be demonstrated that DON has a distinct cytotoxic effect on human primary hepatocytes. Viability, protein content and albumin secretion were reduced in a dose-dependent manner. The apoptotic key enzyme caspase-3 was activated, while LDH release occurred only after long incubation time due to a secondary necrosis. Furthermore, we studied the metabolism of DON using LC-MS/MS. DON was neither metabolized by primary hepatocytes cells nor by the HepG2 cell line.

**Keywords:** Apoptosis / Cytotoxicity / Deoxynivalenol / Human primary hepatocytes / Mycotoxin

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## 1 Introduction

The mycotoxin deoxynivalenol (DON) belongs to the large group of toxic secondary metabolites of *Fusarium* strains, like *Fusarium culmorum* or *Fusarium graminearum*. *Fusarium* molds infect cereals already during their growth, cause various plant diseases and contaminate grain with mycotoxins. Approximately 25% of the world's food crops are contaminated with mycotoxins [1]. Considering its rate of occurrence and mean concentration, DON appears to be one of the most important mycotoxins in cereal commodities [2]. Chemically, DON belongs to the trichothecenes, a category of structurally similar fungal metabolites, sharing a 12,13-epoxytrichothec-9-ene ring system as the basic chemical structure.

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**Abbreviations: BCA**, bicinchoninic acid; **CCK-8**, cell counting kit-8, cytotoxicity assay; **DON**, deoxynivalenol; **MTT**, 3-(4,5-Dimentylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid; **PNP**, *p*-nitrophenol

Toxic effects of DON were subject of various studies in the last years. The acute toxicity of DON differs between the animal species and the severity and duration of exposure. Its toxic effects range from diarrhoea, vomiting, gastro-intestinal inflammation, necrosis of the intestinal tract, the bone marrow and the lymphoid tissues. [1]. DON is not considered to be genotoxic, teratogenic or carcinogenic [3]. The “no observed adverse effect levels” vary between 0.04 and 0.38 mg/kg bw/day in different studies. Based on these studies a tolerable daily intake for DON of 1 μg/kg bw was established in 2002 [4].

The main toxic cellular effect of trichothecenes is the inhibition of protein synthesis. Apart from this primary toxic effect it is assumed that there are multiple other secondary cellular effects [5]. In summary, trichothecenes cause inhibition of protein, DNA and RNA synthesis, inhibition of mitochondrial function, have effects on cell division and membrane integrity and induce apoptosis.

The absorption, distribution, elimination and metabolism of DON after ingestion vary in the tested animal species. In general, DON is very poorly metabolized. The primary metabolite found in urine and feces of animals exposed to DON is the less toxic de-epoxy-DON [6]. It is produced by

intestinal or rumen microbe activity rather than by conversion in liver or other organs. In contrast no de-epoxy-metabolites were detected in human fecal incubation mixtures [6]. In liver or kidney DON can be conjugated to its glucuronide which is detected in several tissues, plasma and excreta of animals [1].

The cytotoxicity of DON in liver cells was already reported for several animal hepatocytes. Several effects in primary cultures of rat and porcine hepatocytes were assayed in the last years [7–9], but until now there are no data available concerning the effect of DON on human primary hepatocytes. Although DON has been tested for its cytotoxicity in the human hepatocellular liver carcinoma cell line HepG2 in the last years [10], these tests are limited to a cell line. Generally, the disadvantage of cell lines consists in the either chemical or genetical immortalization of the cells, the alteration of properties during longtime cultivation or – like in HepG2 cells – their tumor derivation. Thus, cells of cell lines may not reflect the response of primary cells. In order to be closer to the human situation *in vivo*, we investigated in this study the effect of DON in human primary cells. We assayed the effects of DON in human primary hepatocytes and at the same time in the HepG2 cell line and compared these data. In order to study cytotoxicity, mode of cell death and metabolism of DON in these cells, the parameters cell viability, protein content, albumin secretion, caspase-3-activity, LDH-release, and metabolic activity were measured, while the cells were exposed to different DON concentrations.

## 2 Materials and methods

### 2.1 Chemicals

DON was prepared in our laboratory according to the procedure described by Altpeter *et al.* [11]. DMEM, non-essential amino acids (NEA, 100 ×), penicillin/streptomycin/glutamine (100 ×) and fetal calf serum were obtained from Biochrom, HEPES from Roth Pharma (Karlsruhe, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany).

### 2.2 Cell culture

The human hepatocellular liver carcinoma cell line HepG2 was cultured in DMEM, supplemented with 1% penicillin/streptomycin/glutamine, 1% non-essential amino acids and 10% fetal calf serum. The medium was changed three times a week and HepG2 cells were subcultured when cells reached a confluence of 90%.

Human primary hepatocytes plated in 96-well plates were purchased from Lonza/Cambrex BioScience (Walkersville, USA). They were cultured according to the manufactures instructions in hepatocyte culture medium (Bullet-Kit). Primary hepatocytes plated in 24-well plates were purchased from Primacyt Cell Culture Technology (Schwerin, Germany) and were cultured with manufacturer's medium. All cells were maintained at 37°C with 5% $CO_2$. Primary cells were cultured without serum. HepG2 cell medium was changed to serumfree medium 24 h before toxin exposure. A DON stock solution (10 mM in ACN) was diluted with serum free medium to concentrations ranging from 0.1 to 100 μM. Cells were incubated for 8, 24 or 48 h. Control cells were incubated with an equal solvent concentration.

### 2.3 Cytotoxicity assay (CCK-8)

Cytotoxicity was evaluated colorimetrically with the Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories (Tokyo, Japan) according to the manufacturer's instruction as described in [12]. Briefly, cells seeded on 96-well microplates were incubated with DON (0.1 – 100 μM). After toxin exposure, the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) solution was added. WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron carrier. The amount of the formazan generated by the activity of dehydrogenases in cells is directly proportional to the number of viable cells *per* well. The absorbance of the toxin-treated wells was compared with a solvent-treated control.

### 2.4 Albumin secretion

Albumin content in the medium was determined *via* an enzyme-linked immunosorbent assay (Human albumin ELISA quantitation kit, Bethyl laboratories, Texas, USA). The assay was performed according to the manufacturer's instructions. In brief, wells of MaxiSorb plates (Nunc, Denmark) were first coated with anti-human albumin antibody and then blocked with 1% BSA buffer. After washing, an aliquot of cell medium was transferred to the plate and incubated for 1 h. After incubation, the medium was discarded, wells were washed and the plate was incubated for 1 h with secondary detection antibody, anti-human albumin-horseradish-perxoidase, HRP. Then, wells were washed and incubated with HRP substrate solution (TMB/$H_2O_2$). The reaction was stopped after 30 min with 2 M $H_2SO_4$ and absorbance was measured at 450 nm. Albumin content in the medium was calculated by a calibration curve using human reference serum with known albumin concentrations ranging from 6.25 to 400 ng/mL.

### 2.5 Caspase-3 assay

For the determination of the caspase-3 activity, HepG2 and primary hepatocytes seeded in 24-well plates were incubated with DON. Caspase-3 activity was measured as described in [12]. Briefly, 30 μL of cell lysate were incubated with 30 μL

reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT) and 3 μL of caspase-substrate (80 μM DEVD-AFC) in a black 96-well microplate at 37°C for up to 15 h in the dark. The fluorescence of the cleaved product AFC was measured at 400 nm excitation and 505 nm emission wavelengths using a microplate reader (FLUOstar Optima, BMG Labtechnologies, Jena, Germany). Cleaved AFC was quantified by a calibration curve using known AFC concentration (0.32 – 12.8 μM) and was related to the protein content *per* well. Protein content of the cell lysates was determined as described in Section 2.6.

### 2.6 Protein content

The protein content was quantified with a bicinchoninic acid (BCA) assay kit from Sigma-Aldrich using BSA as standard. For 24-well plates, the exact procedure was described in [12]. In 96-well cell-culture plates, cell lysates were not harvested but directly mixed in the wells with 200 μL of BCA and 4% copper sulfate (50:1) and incubated at room temperature. The absorbance was measured at 560 nm in a microplate reader (FLUOstar Optima, BMG Labtechnologies). The protein content in the lysates was determined by a calibration curve with known BSA concentrations ranging from 50 to 500 μg/mL.

### 2.7 LDH-release

LDH-activity in the media was determined by photometric determination of $NAD^+$ from the LDH-catalyzed reaction of pyruvate and NADH to lactate and $NAD^+$ according to a standard protocol [13] in a scaled-down reaction batch. After 8, 24 and 48 h incubation, 40 μL of the cell medium was removed and mixed with 160 μL reaction buffer (100 mM HEPES, pH 7 supplemented with 0.14 g/L NADH, 1.1 g/L Na-pyruvate) in a 96-well microplate and incubated at 37°C in a microplate reader (FLUOstar Optima, BMG Labtechnologies). The absorbance was measured at 355 nm every minute and the LDH-activity was determined as mU/mL. The LDH release is expressed as LDH-activity in the medium compared to control cells. The LDH release of control cells was subtracted from the LDH release of samples.

### 2.8 Metabolism studies

#### 2.8.1 Metabolic activity

The cells in two wells of each 24-well plate were incubated with 100 μM *p*-nitrophenol (PNP). After 8, 24 and 48 h incubation, 90 μL of the medium was removed and mixed with 10 μL of 0.1 M NaOH. The absorbance of PNP and PNP-glucuronide was measured at 405 and 290 nm, respectively. PNP and PNP-glucuronide concentrations in the medium were determined *via* an calibration curve using known concentrations (5 – 100 μM).

#### 2.8.2 Sample preparation

After 8, 24 and 48 h DON incubation (0.1 – 100 μM), the medium of 24-well plates was centrifuged at 13 000 rpm and used for LC-MS/MS analysis. Depending on toxin concentration, an aliquot was spiked with 15-$d_1$-DON (end-concentration: 160 ng/mL) as internal standard, evaporated with $N_2$ at 40°C, refilled to a defined volume and used for the quantitative determination of the DON-concentration in the cell medium using LC-MS/MS. 15-$d_1$-DON was synthesized according to [14], but is also commercially available at Sigma-Aldrich.

Quantitative evaluations were carried out *via* a calibration curve with standard solutions containing the same amount of the internal standard (160 ng/mL 15-$d_1$-DON) but different concentration of the analyte (15 – 180 ng/mL). The concentration ratios of DON to 15-$d_1$-DON were plotted against the peak area ratios and a quadratic regression curve was built to correct the spectral overlap [15, 16].

#### 2.8.3 Apparatus

Chromatographic separation was carried out by an Agilent G1312A binary pump. For sample injection an Agilent G1129A autosampler with G1330B ALS thermostat was used. LC-ESI-MS/MS analysis were conducted on a API 4000 QTrap tandem mass spectrometer system (Applied Biosystems, Foster City, USA) equipped with an Turbo V® ESI interface. Data acquisition and mass spectrometric evaluation were carried out with Analyst® 1.4 software (Applied Biosystems).

#### 2.8.4 MS analysis

For LC-ESI-MS/MS, chromatographic separation was carried out on an Phenomenex Synergi Fusion® column (150 × 2 mm, 4 μm) using a binary gradient. The gradient was the following with methanol as solvent A and water as solvent B: isocratic step for 1 min at 90% B, linear gradient to 0% B at 25 min followed by an isocratic step at 0% B for 5 min. The column was equilibrated for 10 min at the starting conditions. The flow rate was set to 0.2 mL/min and the injection volume was 10 μL. The measurements were performed with multiple reaction monitoring in the negative mode, selecting the transition 295 → 265 for DON and 296 → 265 for 15-$d_1$-DON. For ESI, the ion spray voltage was set at −4500 V. Nitrogen served as curtain gas (20 psi). The declustering potential, being the accelerating current from atmospheric pressure into high vacuum, was set at –80 V. The collision energy was adjusted at −16 V and the collision cell exit potential at −7 V.

### 2.9 Statistics

All measurements are given as mean values ± SEM. The significance of difference was determined by the unpaired Student's *t*-test. $p \leq 0.05$ was considered to be statistically significant. All experiments with HepG2 cells were per-

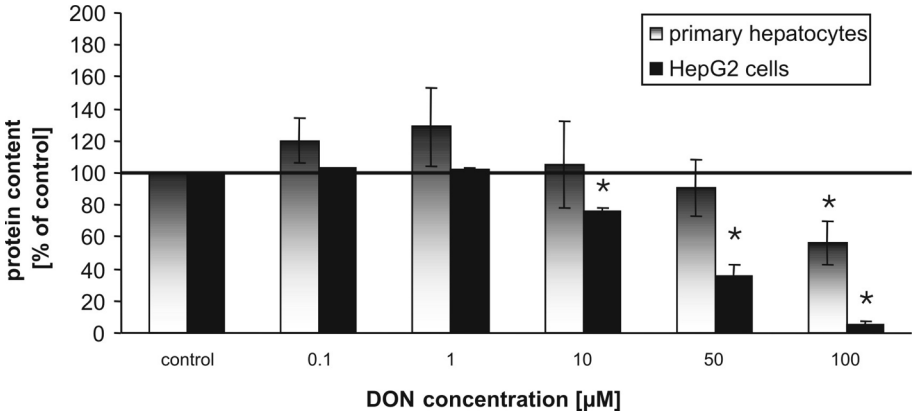



**Figure 1.** Concentration-dependent protein content (BCA assay) of human primary hepatocytes and HepG2 cells after 48 h exposure to the indicated DON concentrations; protein content as a percentage of control cells – protein content of control cells was defined as 100%; number of analyzed wells n= at least 16; mean ± SEM; * indicates significant differences from control ($p < 0.05$).

formed at least in three different cell passages using a minimum of four wells *per* group each time. Thus, at least 12 wells were used for each parameter. Protein content, albumin secretion and LDH release of primary cells were determined with cells of four different donors, using minimum four wells *per* concentration each time. Thus, at least 16 wells were used for each parameter. CCK-8 Assay, caspase-3-activation and metabolism studies were performed with cells of two different donors with at least eight wells for each parameter. The medium effective concentrations ($IC_{50}$ values) were calculated using SigmaPlot 8.0 according to [17].

## 3 Results

### 3.1 Cytotoxic effects

#### 3.1.1 Viability

Table 1 shows the calculated $IC_{50}$ values for primary hepatocytes and HepG2 cells, respectively, after treatment with DON concentrations between 0.1 and 100 µM for a period of 48 h determined with the CCK-8 assay. Both cell types showed a reduction of living cells with increasing DON concentrations in a dose-dependent manner. After treatment of primary cells with the highest DON concentration of 100 µM, the viability decreased to approximately 20%. In HepG2 cells the highest DON concentration of 100 µM led to a reduction in the viability down to 45% (figures not shown). The calculated $IC_{50}$ value for primary hepatocytes was 6.0 µM and therefore approximately 7-fold lower than the $IC_{50}$ value of 41.4 µM for the HepG2 cell line.

#### 3.1.2 Protein content

Protein content, indicating general cytotoxicity or changes in proliferation, is shown in Fig. 1. Cellular protein of primary hepatocytes and HepG2 cells after treatment with different DON concentrations for a period of 48 h was determined *via* the BCA assay. In the HepG2 cell line, the protein content was significantly decreased starting at a concentration of 10 µM. The highest DON concentration of 100 µM resulted in a protein content of only 10% compared to the control cells. In primary cells, the protein content was not significantly decreased up to a DON concentration of 50 µM. The incubation with the highest DON concentration of 100 µM led to a protein content of 56% compared to control cells (Fig. 1). Thus, with regard to the protein content – in contrast to the CCK-8 cytotoxicity assay – primary cells showed a lower sensitivity to DON than the HepG2 cell line.

**Table 1.** Calculated $IC_{50}$ values of primary hepatocytes and HepG2 cells after 48 h exposure to DON concentrations from 0.1 to 100 µM; $IC_{50}$ values result from the reduction in viability determined by the CCK-8 assay

| Cell type | $IC_{50}$ value |
|---|---|
| Primary hepatocytes | 6.0 ± 2.2 µM |
| HepG2 cell line | 41.4 ± 3.7 µM |

#### 3.1.3 Albumin secretion

Albumin secretion of hepatocytes, as a rate for the preservation of liver-specific functions, is shown in Fig. 2. Both cells had the ability to produce albumin. The average amount of produced albumin of primary hepatocytes (solvent treated control cells) was 8.3 ng/mL medium/h. The control cells of HepG2 secreted 11.4 ng albumin/mL/h. In Fig. 2, the albumin secretion of cells into the medium is depicted as a percentage of control cells after 8, 24 and 48 h treatment with DON concentrations ranging from 0.1 to 100 µM. In primary cells as well as in the HepG2 cell line the treatment with DON – already after 8 h – led to a sig-

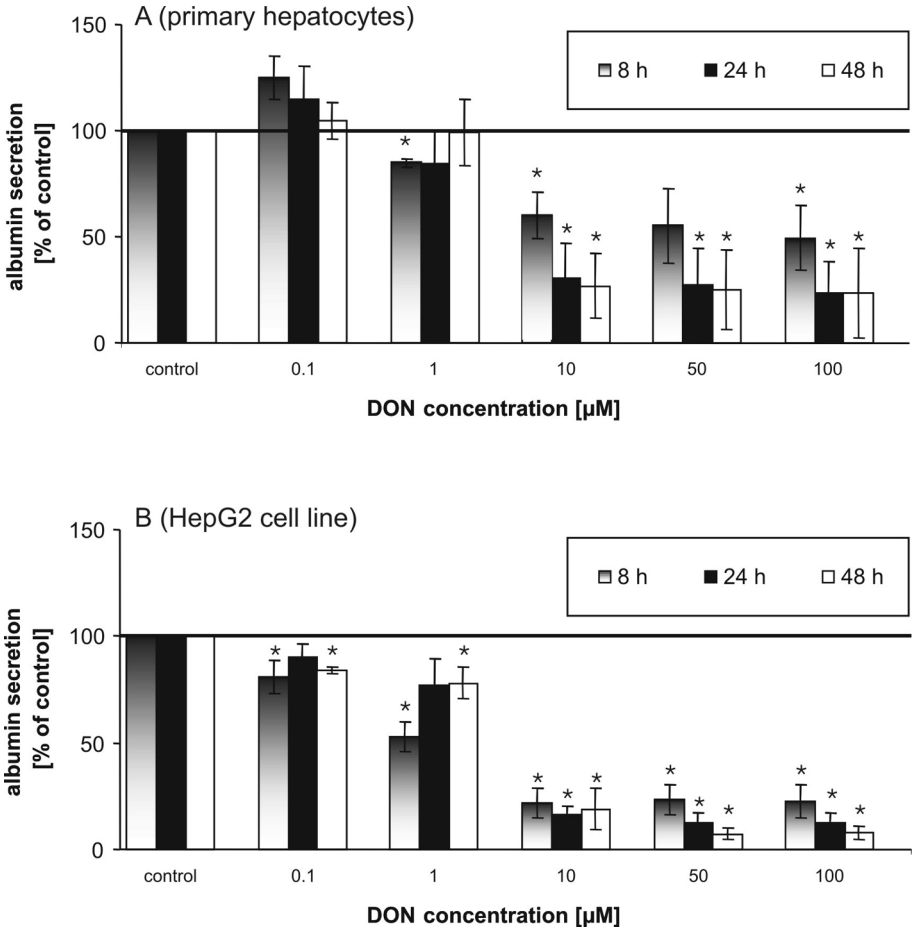



**Figure 2.** Concentration-dependent albumin secretion of human primary hepatocytes, A, and HepG2 cells, B, after 8, 24 and 48 h exposure to the indicated DON concentrations; albumin secretion in% of control cells, that were defined as 100%; number of analyzed wells n = at least 16; mean ± SEM; * indicates significant differences from control ($p < 0.05$).

nificant reduction of albumin secretion in a dose-dependent manner (Fig. 2). In the HepG2 cell line already the lowest DON concentration of 0.1 µM caused a significant reduction of albumin secretion (Fig 2B). These effects were not seen for primary hepatocytes. DON concentrations between 10 and 100 µM led to an intense reduction of the albumin secretion in both cell types, with a stronger reduction in HepG2 cells. With the highest DON concentrations (50 and 100 µM) the albumin secretion of HepG2 cells was between 10 and 20% of the control (Fig. 2B). In primary cells, the same DON treatment led to a less pronounced reduction of the albumin secretion down to 30 – 50% of control (Fig. 2A). Thus, with regard to albumin secretion, primary cells seem to be more robust than the HepG2 cell line.

### 3.2 Apoptotic cell death

Figure 3 shows the caspase-3-activity as a sign for an apoptotic cell death. Caspase-3 activity was measured in human primary hepatocytes and HepG2 cells at different DON concentrations after an incubation time of 48 h. DON triggers an increase in caspase-3 activity in both cell types. In the HepG2 cell line the caspase-3 was not activated up to a concentration of 50 µM DON, whereas primary cells already showed an increased activity with the lowest DON concentration of 0.1 µM. The highest caspase-3 activation in HepG2 cells of 350% of control cells was reached with 100 µM DON and was therefore lower than in primary cells. In primary cells DON concentrations between 10 and 100 µM led to a caspase-3 activation of approximately 500% (Fig. 3).

### 3.3 Necrotic cell death

LDH release into the medium, as a sign for a necrotic cell death, is shown in Fig. 4. LDH release into the medium, compared to control cells, was measured after 8, 24 and 48 h treatment with different DON concentrations ranging

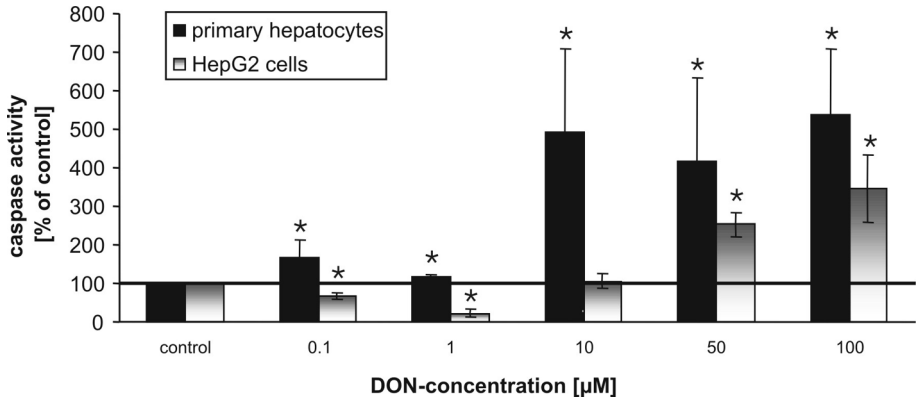
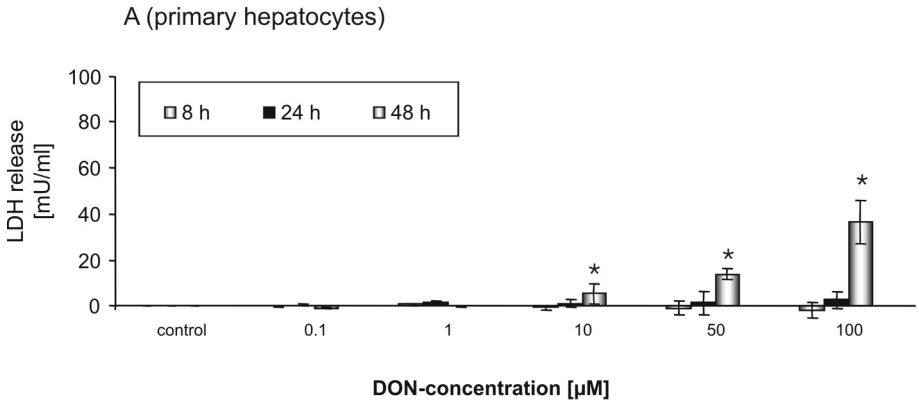



**Figure 3.** Concentration-dependent caspase-3-activity of human primary hepatocytes and HepG2 cells after 48 h exposure to the indicated DON concentrations; caspase-3-activity in% of control cells – caspase-3-activity of control cells was defined as 100%; number of analyzed wells n= at least 12; mean ± SEM; * indicates significant differences from control ($p < 0.05$).



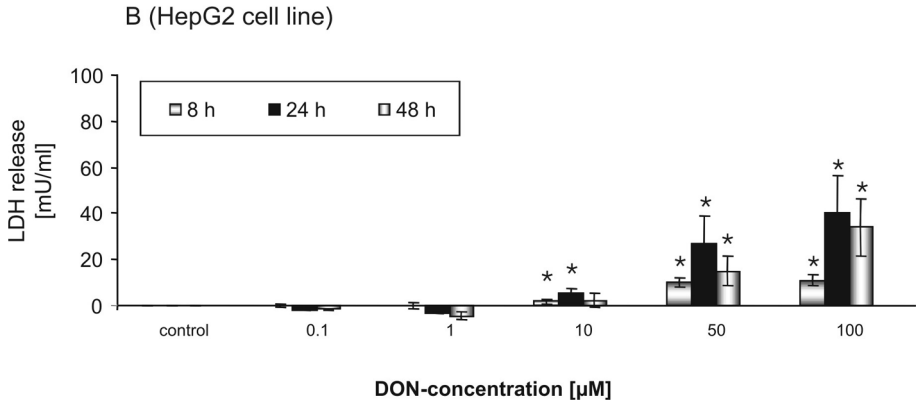




**Figure 4.** Concentration-dependent LDH release into the medium of human primary hepatocytes, (A), and HepG2 cells, (B), after 8, 24 and 48 h exposure to the indicated DON concentrations; LDH release represents LDH-activity in medium compared to control cells; number of analyzed wells n = at least 16; mean ± SEM; * indicates significant differences from control ($p < 0.05$).

from 0.1 to 100 µM. In primary cell medium as well as in HepG2 cell medium a significant increase in LDH release was detected starting at a concentration of 10 µM of DON. But in primary hepatocytes no increased LDH release was measurable after 8 and 24 h treatment with DON. Only the longest incubation time of 48 h led to a significant increase in LDH release above a concentration of 10 µM DON (Fig. 4A). In comparison, the LDH release of HepG2 cells was already significantly increased after 8 h treatment with a DON concentration of 10 µM (Fig. 4B). The highest DON concentrations of 100 µM led to a LDH release of approximately 40% after 24 h in this cell line. A similar increase in LDH release was reached in primary cells only after 48 h incubation.

## 3.4 Metabolism

### 3.4.1 Metabolic activity

Figure 5 shows the metabolic potency of primary hepatocytes (Fig. 5A) and HepG2 cells (Fig. 5B) to transform PNP to PNP-glucuronide. In the medium of primary hepatocytes as well as of HepG2 cells, a decrease of PNP and a concurrent increase of PNP-glucuronide content was measurable during an incubation time up to 48 h. In primary cell

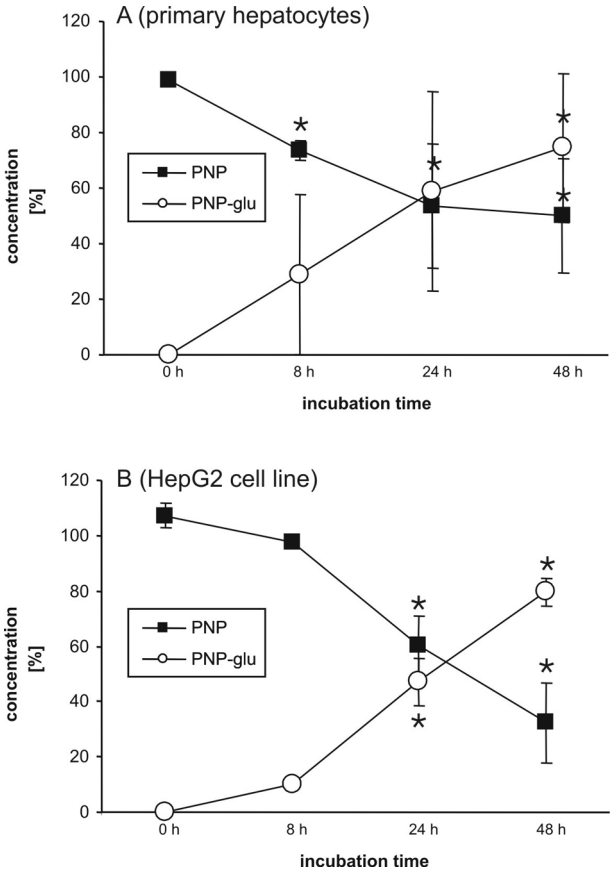



**Figure 5.** Time-dependent concentration of PNP and PNP-glucuronide (PNP-glu) during incubation in cell medium of human primary hepatocytes and HepG2 cells; * indicates significant differences from control ($p < 0.05$).

medium a significant metabolism of PNP was already detectable after 8 h of incubation. After 48 h incubation approximately 50% of the PNP were metabolized (Fig 5A). The metabolic activity of HepG2 cells was lower. No significant transformation of PNP was measured up to 8 h incubation (Fig. 5B). To exclude a chemical degradation of PNP, we also incubated PNP in medium without cells at 37°C and 5% $CO_2$. PNP was stable for at least 52 h (data not shown).

### 3.4.2 Recovery of DON

Table 2 presents DON concentrations in primary hepatocyte medium (Table 2A) as well as in HepG2 cell medium (Table 2B) after an incubation time of 8, 24 and 48 h. The results show, that after the incubation time approximately 100% of the DON amount originally added could be detected. Therefore nearly the entire amount of DON could be recovered so that no metabolism of DON by the cells occurred. Furthermore, several MS experiments were performed to analyze cell medium for metabolites (*e.g.* de-epoxy-DON and DON-glucuronide). Only traces of DON-glucuronide were detected in primary cell medium. De-epoxy-DON was not found, neither in primary cell medium nor in HepG2 cell medium. These experiments gave no hints for any further DON-metabolites.

**Table 2.** Concentration of DON in the cell medium after an incubation time of 8, 24 and 48 h calculated as a percentage of added DON concentration into the medium; determined with LC-MS/MS; mean ± SEM

| DON incubation concentration | 8 h DON concentration in medium (%) | 24 h DON concentration in medium (%) | 48 h DON concentration in medium (%) |
|---|---|---|---|
| A (primary hepatocytes) | | | |
| 0.1 μM | 98.5 ± 8.5 | 90.4 ± 13.2 | 118.2 ± 26.8 |
| 1 μM | 106.2 ± 2.2 | 95.6 ± 2.7 | 91.4 ± 1.3 |
| 10 μM | 91.8 ± 7.2 | 91.7 ± 8.8 | 85.6 ± 8.0 |
| 100 μM | 90.9 ± 2.3 | 90.4 ± 6.9 | 96.6 ± 9.7 |
| B (HepG2 cells) | | | |
| 0.1 μM | 92.4 ± 4.9 | 110.3 ± 19.1 | 119.2 ± 12.5 |
| 1 μM | 81.0 ± 26.5 | 92.1 ± 4.4 | 102.1 ± 5.5 |
| 10 μM | 103.3 ± 1.9 | 97.6 ± 2.3 | 99.9 ± 7.6 |
| 100 μM | 96.2 ± 2.0 | 94.0 ± 5.4 | 100.2 ± 9.5 |

## 4 Discussion

The toxic effects of DON on the liver have been studied in animals as well as in cell culture experiments. However, cells used in these studies were either of animal origin from different species or the established human hepatocellular liver carcinoma cell line HepG2. Thus, until now, no data exists about the effects of DON on human primary hepatocytes.

In our investigations using human primary hepatocytes and the human hepatocellular liver carcinoma cell line HepG2 it turned out that these cells behave differently, depending on the used assay. Overall, human primary cells as well as the HepG2 cell line showed a significant reduction in cell viability, protein content and albumin secretion when they were exposed to DON (Table 1 and Figs. 1 and 2). With regard to the CCK-8 assay, DON has a cytotoxic effect on both cell types, with primary cells being more sensitive. Concerning the calculated $IC_{50}$ values (Table 1) primary hepatocytes show a 7-fold higher sensitivity to DON than the HepG2 cell line. Cetin *et al*. have also tested the cytotoxicity of DON in HepG2 cells using the MTT test [10]. They reported an $IC_{50}$ value of 28 μM. In primary porcine hepatocytes DON also caused a dose-dependent reduction of viability with a strong decrease in viability in the range of 3 to 30 μM [7]. In experiments with primary rat hepatocytes, DON concentrations above 17 μM triggered cytotoxic effects [9]. Thus, in our investigations with the CCK-8 assay, human primary hepatocytes seem to be more sensitive to DON than the HepG2 cell line.

In contrast, the protein content, that gives a hint for general toxicity or changes in proliferation, was significantly decreased in HepG2 cells with lower DON concentration than in primary cells (Fig. 1). At lower concentrations the protein content of primary cells was even slightly increased. With regard to the protein content, primary cells show a 10-fold lower sensitivity to DON.

The determination of albumin secretion shows similar results (Fig. 2). In general, albumin synthesis of hepatocytes is a marker for liver-specific functions. Thus, a decrease in albumin production can give hints for the cytotoxicity of a substance. Albumin secretion into the medium was reduced in both cells in a time- and concentration-dependent manner, which is a sign for the disturbance of cellular functions by DON. With a DON concentration of 10 μM the albumin secretion was strongly reduced in both cells. However, in HepG2 cells this effect was more intense (Fig. 2B). Furthermore, in HepG2 cells the albumin secretion was already significantly decreased with the lowest DON concentration of 0.1 μM (Fig. 2A), which was not observed in primary cells. In primary hepatocytes, this concentration even led to a slight increase of albumin secretion in comparison to control cells. A significant reduction was only observed with higher DON concentrations. Mikami *et al.,* who determined albumin secretion of primary porcine hepatocytes, found that albumin secretion into the medium was slightly, but significantly, reduced at a concentration of only 30 nM DON. A strong reduction to approximately 16% of control was observed above 3 μM DON [7]. Thus, in comparison to human primary cells, porcine hepatocytes appear to be more sensitive to DON.

With regard to cell protein content and albumin secretion, primary cells seem to be more robust than the HepG2 cell line. For a significant reduction in cell protein content and albumin secretion higher DON concentrations are necessary in primary cells. With the lowest DON concentration of 0.1 μM the protein content as well as albumin secretion were even slightly increased in primary hepatocytes.

Due to different observations in our used cytotoxicity assays, we furthermore investigated the underlying mechanism. We analyzed the mode of cell death to see whether apoptosis or necrosis play a role in cell death of primary hepatocytes and the HepG2 cell line, respectively.

In our study, both cell types responded with a reduction of viability, protein content and albumin secretion, but we assume, that the underlying mechanisms of cell death are different. The capacity of DON to induce apoptosis in different cell lines and tissues has already been demonstrated by several authors. Induction of apoptosis by DON was reported for murine T, B, and Ig $A^+$ cells, for promyelotic cell lines (HL-60, U937, RAW264.7) [18], for Jurkat human T-lymphoid cells [19], for a human erythroleukemia cell line (K562) [20] and for human lung fibroblasts in primary culture [12]. In these reported studies the sensitivity to DON varies depending on the cell type. DON-induced apoptosis was also demonstrated in primary porcine hepatocytes and occurred through caspase-3-activation [7]. In our investigations with human primary hepatocytes, we also measured an increase in caspase-3 activation, a key enzyme in the apoptotic cell death. Above the lowest DON concentration of 0.1 μM, caspase-3 was activated with a strong increase at ≥10 μM DON (Fig. 3). These data are in close agreement with the results in primary porcine hepatocytes of Mikami *et al.* [7]. In comparison, in HepG2 cells the caspase-3 activation was different. At lower DON concentrations we measured a significant reduction of caspase-3 activation and a significant increase was not obtained up to 50 μM of DON. Furthermore, the activation in HepG2 cells was lower than in primary cells. We assume that these differences are due to the fact, that HepG2 cells are isolated from a liver carcinoma. In cancer cells the balance between proliferation and programmed cell death is often disturbed and cancer cell lines can show defects in apoptotic response [21]. Moreover, there are further differences between primary hepatocytes and the HepG2 cell line as determined by Wilkening *et al*. [22]. They showed that HepG2 cells are more robust with respect to the tested promutagens and that phase I enzyme expression and activity was very low in this cell line. They conclude that primary hepatocytes more closely reflect the situation in human liver [22].

LDH release, as a sign for necrotic cell death, was not observed in primary hepatocytes during incubation for up to 24 h. Hence, shorter DON incubation had no effects on membrane integrity and indicates that DON does not induce cell injury in primary cells directly by damaging the cell membrane. The release of LDH into the medium was significantly increased only after an incubation of 48 h (Fig. 4). We assume that this measured LDH release after 48 h is based on a secondary necrosis. Apoptotic cells can undergo secondary necrosis, which appears in *in vitro* systems, where there were no scavengers for apoptotic cells, *e.g.* macrophages. Mikami *et al.* have seen similar effects in primary porcine hepatocytes and came to the same conclusion [7]. In comparison, in HepG2 cells a significant LDH release was already observed after 8 h. Unlike in primary hepatocytes, DON showed a necrotic activity in HepG2 cells. Thus, necrosis appears to play a role in the cell death of HepG2 cells. Cell membrane damage is also one of the suggested mode of cytotoxic action of DON. Effects of trichothecenes on cell membranes and impaired cell membrane functions were already observed in myoblasts, rat erythrocytes and human proximal tubule cells in primary culture [12, 23 – 25]. Necrosis was also reported in several tissues like liver, spleen, kidney and bone marrow of mice [1]. But it is assumed that these effects of DON are secondary effects after the initial inhibition of protein synthesis.

Several investigations showed that de-epoxy-DON is the primary metabolite that was found in urine and feces of animals exposed to DON. In addition, in liver or kidney DON

can be conjugated to its glucuronides, which were found in several tissue, plasma and excreta of animals [1]. Due to the fact, that the liver is the main organ for metabolic reactions of xenobiotics, we investigated the ability of primary hepatocytes as well as of HepG2 cell line to metabolize DON. To verify that the cells have a metabolic activity we incubated both cell types with PNP, a substrate for phase II metabolism. The decrease of PNP concentration with an simultaneous increase of PNP-glucuronide concentration demonstrated, that the cells are able to perform phase-II metabolism reactions like glucuronidation (Fig. 5). In primary cells, already after 8 h a significant metabolic activity was measured. To detect any metabolism of DON by the liver cells, we quantified DON in the cell medium after a certain incubation time. In case of a metabolism, DON would be transformed or degraded by the cells which would result in a reduced concentration of DON in the medium. Possible metabolites (*e.g.* de-epoxy-DON, DON-glucuronide) would be released into the medium and could be detectable therein.

In the cell medium of primary hepatocytes (Table 2A) as well as of the HepG2 cell line (Table 2B) nearly the whole amount of DON was recovered after different incubation times. Besides, we performed several mass spectrometric experiments that showed only traces of the mentioned metabolite DON-glucuronide in primary cell medium. De-epoxy-DON was neither detected in primary cell medium nor in HepG2 cell medium. Due to recovery close to 100% and due to the fact, that only traces of DON-glucuronide were detected in the medium, we conclude that DON is not metabolized by human hepatocytes. Cetin *et al*. [10] assumed that the low sensitivity of HepG2 cells to DON – in comparison to other cell lines – is based on the conversion of DON to the less toxic de-epoxy metabolite by the cells. Our results did not give any hints for this assumption. But our findings are in close agreement with results of hepatic microsomal preparations. In liver microsomes of rats and pigs no metabolites of DON could be detected [26].

In conclusion, we demonstrated for the first time the effects of DON on human primary cells and compared these data to the effects in the human hepatocellular liver carcinoma cell line HepG2. We could show that the effects of DON in human primary hepatocytes differ from those in the HepG2 cell line. DON is cytotoxic for human primary hepatocytes as well as for the HepG2 cell line. The cells respond differently depending on the investigated parameters. Especially with regard to the mechanism of cell death, the cells show a different behavior. Human primary hepatocytes die primarily by apoptosis whereas in HepG2 cells necrosis seems to predominate in mode of cell death. Apoptotic cell death has also been described for primary porcine hepatocytes.

Given the fact that the recovery of DON in the medium was close to 100% and no metabolites were detectable in the cell medium, we conclude that DON is neither metabolized by human primary hepatocytes nor by the human hepatocellular liver carcinoma cell line HepG2.

Our results obtained with human primary hepatocytes clearly prove the toxicological potential of DON on humans. We could confirm the cytotoxic effects of DON on the liver, which were measured hitherto only in animal studies, animal hepatocytes and in the human liver carcinoma cell line HepG2. Because of the differences between primary cells and the HepG2 cell line, we recommend the use of human primary cells for toxicological studies.

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