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[1]Institute of Human Nutrition and Food Science, Christian-Albrechts-University Kiel
[2]Institute of Animal Nutrition and Physiology, Christian-Albrechts-University Kiel

Ochratoxin A-induced cytotoxicity in liver (HepG2) cells: Impact of serum concentration, dietary antioxidants and glutathione-modulating compounds*

Christine Bösch-Saadatmandi[1], Christoph Hundhausen[1], Laia Jofre-Monseny[1], Ralf Blank[2], Siegfried Wolffram[2], Gerald Rimbach[1]

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Abbreviations

BSO, buthionine sulfoximine; CAT, catechin; DMSO, dimethyl sulfoxide; DTNB, dithio-bis-nitrobenzoic acid; EGCG, epigallocatechin gallate; FCS, foetal calf serum; GSH, glutathione; IARC, international agency for research on cancer; NAC, N-acetylcysteine; NO, nitric oxide; NR, neutral red; OATP, organic anion-transporting polypeptide; OTA, ochratoxin A; PBS, phosphate buffered saline; QUE, quercetin; ROS, reactive oxygen species; ROSAC, rosmarinic acid; RPMI, roswell park memorial institute; α-TOC, α-tocopherol; α-TOC-P, α-tocopherol phosphate

Summary

Ochratoxin A (OTA) is a nephro- and hepatotoxic mycotoxin produced by various species of the genera *Aspergillus* and *Penicillium*. OTA is known to bind with high affinity to plasma proteins which may have a substantial impact on its bioavailability and, thus, on its toxicity. However, the underlying mechanisms of OTA-induced cellular toxicity have not yet been fully elucidated. It has been suggested that oxidative damage contributes to its cytotoxic effects. Dietary antioxidants such as vitamin E and polyphenols may therefore counteract OTA-induced cell death. Furthermore, compounds influencing the intracellular level of glutathione (L-gamma-glutamyl-L-cysteinylglycine, GSH), the most abundant thiol antioxidant in mammalian cells, may have an impact on OTA-induced cytotoxicity. In this study we investigated the effects of serum concentrations as well as different dietary antioxidants on the viability of OTA-exposed liver (HepG2) cells. Additionally, we determined the intracellular GSH-levels after incubation with OTA and N-acetylcysteine (NAC, a precursor of GSH) or buthionine sulfoximine (BSO, an inhibitor of gamma-glutamylcysteinyl synthetase). Incubation of human hepatoma cells (HepG2) for 24 h with increasing concentrations of OTA (0.25 - 50 µmol/l) in the presence of 0, 2.5, 5, or 10% foetal calf serum (FCS) resulted in a dose-dependent decrease in cell viability. Decreasing the serum concentrations in the cell culture medium led to increased cell mortality. Pre-treatment for 24 h with *RRR*-α-tocopherol (α-TOC), *RRR*-α-tocopherolphosphat (α-TOC-P), epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) at concentrations of 25, 50 and 100 µmol/l did not prevent OTA-induced toxicity. α-TOC-P, EGCG, and QUE even amplified the cytototoxic effects of OTA in HepG2. Supplementation with NAC and BSO in the presence of OTA did not substantially change cell viability, although BSO treatment resulted in depletion of cellular GSH. OTA treatment increased GSH levels at concentrations of 50 and 100 nmol/l, but decreased cellular GSH at the higher concentrations (250, 500, and 1000 nmol/l). The decrease in cellular GSH concentrations was less pronounced than for BSO. Our results indicate that the cytotoxicity of OTA in HepG2 cells, which is strongly dependent on the protein concentration in the cell culture medium, can not be prevented by pre-incubation with dietary antioxidants. Although cellular GSH levels are influenced by OTA incubation, mechanisms other than oxidative stress are likely to be involved in OTA-induced cell death in HepG2 cells.

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Introduction

The mycotoxin ochratoxin A (OTA), mainly produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, is the most toxic of the three known members of the ochratoxin family (ochratoxin A, B, and C). OTA was discovered and its chemical structure described in 1965 (VAN DER MERWE et al., 1965). Chemically, OTA consists of a dihydroisocumarin moiety joined by a peptide bond to 1-phenylalanine (Fig. 1). OTA can be found in many foods and beverages, *e.g.* cereal grains, coffee, and wine (FAZEKAS et al., 2002; STUDER-ROHR et al., 1995; VAN EGMONT et al., 1994). Due to its ubiquitous occurrence in foods, it is virtually impossible to completely avoid ingestion of OTA. Both in animals and humans, a wide range of toxic effects of OTA has been reported, including renal and hepatic toxicity (BENDELE et al., 1985), genotoxicity (CREPPY et al., 1985; BOSE and SINHA, 1994), neurotoxicity (MIKI et al., 1994; BRUININK et al., 1998), and immunotoxicity (HARVEY et al., 1992; PETZINGER and ZIEGLER, 2000). Severe human renal diseases, such as Endemic Nephropathy (BEN), chronic interstitial nephritis and karyomegalic interstitial nephritis, may be a result of continued exposure to OTA (SIMON et al., 1996). Moreover, the International Agency for Research on Cancer has classified OTA as a class 2B carcinogen (possible human carcinogen) (IARC, 1991).

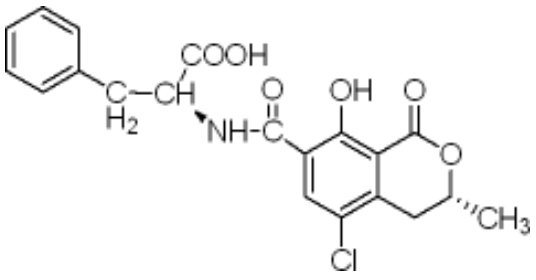


Fig. 1: Chemical structure of ochratoxin A

The toxicity of OTA is largely determined by its protein binding properties. In blood, 99% of OTA is bound to serum proteins (mainly albumin), which prolongs its systemic half-life, delays its elimination and may, therefore, amplify its toxicity (CHU, 1971; CHU, 1974). The exact mode of action of OTA toxicity is as yet unclear. The toxicity of OTA has been explained by three major mechanisms: 1.

inhibition of protein synthesis via inhibition of phenylalanine-tRNA synthetases (Creppy et al., 1979; Creppy et al., 1998), 2. inhibition of mitochondrial function (Moore and Truelove, 1970; Meisner, and Chan, 1974; Wei et al., 1985), and 3. generation of reactive oxygen species (ROS) which may oxidise DNA, proteins, lipids, and other macromolecules (Rahimtula et al., 1988; Omar et al., 1990; Schaaf et al., 2002; Domijan et al., 2005; Kamp et al., 2005). Therefore, antioxidants may counteract OTA-induced cytotoxicity. Recent studies have shown that supplementation with vitamin E or several polyphenols partly diminished the toxic effects of OTA (Schaaf et al., 2002; Baldi et al., 2004; Renzulli et al., 2004; Guerra et al., 2005). Furthermore, an involvement of the glutathione (GSH) system in OTA cytotoxicity, has been suggested. GSH (γ-glutamylcysteinylglycine) functions as a free radical scavenger, and serves as a nucleophilic co-substrate for glutathione transferases in the detoxification of xenobiotics. It has been shown that pre-incubation with N-acetylcysteine (NAC), a precursor of GSH, completely prevented OTA-induced cell death in kidney (LLC-PK1) cells (Schaaf et al., 2002).

The objective of the present study was to assess the impact of serum protein concentration in the cell culture medium and the effect of dietary antioxidants on OTA-induced cytotoxicity in HepG2 cells. Furthermore, we aimed at investigating whether modulation of cellular GSH levels affects OTA-induced cytotoxicity in human liver cells in culture.

Materials and methods

Cell culture and determination of OTA-cytotoxicity

The human hepatoblastoma derived cell line HepG2 was cultured in RPMI medium supplemented with 10% FCS, 2 mmol/l glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin under standard conditions (37°C, 5% CO_2). Confluent cells were harvested by trypsination and seeded at 1 x 10^5 cells per well in 24-well plates. The medium was changed every 48 h.

In dose-response experiments, HepG2 cells were exposed to increasing concentrations of OTA ranging from 0.25 - 50 µmol/l for 24 h. The FCS concentrations in the medium were 0, 2.5, 5 or 10%. In a separate experiment, HepG2 cells were incubated with OTA at concentrations of 10 - 1000 nmol/l to find out the highest non-toxic OTA concentration under serum-free conditions. The cytotoxicity of OTA was determined employing the neutral red (NR) assay (Valacchi et al., 2001). This test is based on the ability of viable cells to incorporate NR in lysosomes which is photometrically measured at 540 nm. Cell viability was expressed as percentage of solvent-exposed control (0.1% methanol) survival. For each incubation period, two independent experiments were performed in triplicate (n = 6).

Pre-incubation with test substances

To study the potential protective effects of vitamin E and polyphenols on OTA-induced cytotoxicity, cells were pre-treated with *RRR*-α-tocopherol (α-TOC), *RRR*-α-tocopherolphosphate (α-TOC-P), epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) (Fig. 2) for 24 h at concentrations of 25, 50, and 100 µmol/l (non-toxic concentrations for HepG2 cells, data not shown). 100 mmol/l stock solutions were prepared in dimethyl sulfoxide (DMSO), ethanol or an acetic acid:ethanol mixture (1:3) for the polyphenols, α-TOC and α-TOC-P, respectively. Stock solutions were further diluted in culture medium containing 10% FCS with a maximum solvent concentration of 0.1%.

Cells were washed twice with PBS and incubated for another 24 h with 1, 5 or 15 µmol/l OTA in serum-free RPMI. Additionally, HepG2 were pre-incubated for 24 h with 0, 0.2, 0.5, 1, 2, or 4 mmol/l NAC or 0, 5, 25, 50, 100, or 500 µmol/l BSO. BSO and NAC were prepared as 100 mmol/l stock solutions in PBS. The pH of the NAC stock solution was adjusted to pH 7 with NaOH. Subsequently, cells were washed twice with PBS and incubated for 24 h with 1, 5 or 15 µmol/l OTA in FCS-free medium. Two or three independent replicates were performed in triplicate.

Determination of cellular glutathione levels

Total cellular GSH content was determined by the method of Griffith with minor modifications (Griffith, 1980). HepG2 cells were grown at a density of 9 x 10^5 cells per well in 6-well plates for 48 hours and incubated with OTA, BSO, or NAC at increasing concentrations. After 24 h, the incubation medium was aspirated; cells were washed with PBS and harvested by trypsination. Due to the limited cell material, cells of three wells were pooled together. Cell pellets were collected after centrifugation and stored immediately at -80°C. To measure glutathione concentrations, cell pellets were resuspended in lysis buffer (PBS, 0.05% Triton-X (w/v), 0.05 mmol/l EDTA) and homogenized. The cell suspension was treated with an equal volume of 10% sulfosalicylic acid (w/v) and separated by centrifugation (12,000 x g, 5 min, 4°C). 270 µL of the supernatant was mixed with 20 µL of a 150 mmol/l phosphate buffer (including 1% bovine serum albumin, pH 7.5) and 30 µL triethanolamin (50%). Sample (75 µL) was added to the reaction mixture containing 350 µL of 0.28 mmol/l NADPH and 50 µL of 6 mmol/l DTNB (5-5′dithio-bis-2-nitro-benzoic acid). The reaction was started by addition of 50 µl glutathione-reductase (10 U/ml) and the increase in absorption at 412 nm was followed for 180 min in 20 sec intervals. Each sample was measured in duplicate. Results were calculated by comparison of the difference in absorbance per minute with data from a standard curve generated with oxidised glutathione. The protein content of the cell suspension was determined with the commercially available BCA Protein Kit from Pierce. Three independent replicates were performed in duplicate.

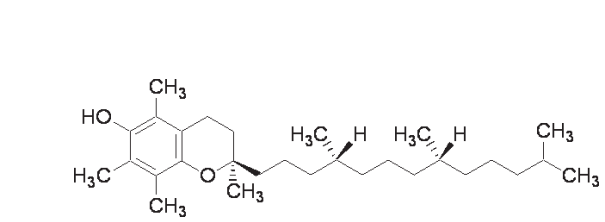
Statistical analysis

Data are presented as mean with standard deviation. The statistical program SPSS (version 13.0) was used to assess the effects of various treatments by analysis of variance (ANOVA) followed by the *post hoc* test Dunnett. In the case of inhomogeneous variances multiple t-tests were applied. Differences were considered significant if $p < 0.05$.

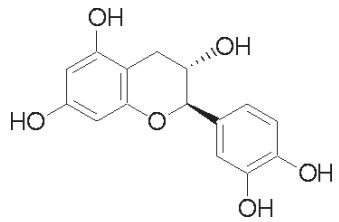
Results

Cytotoxicity of OTA

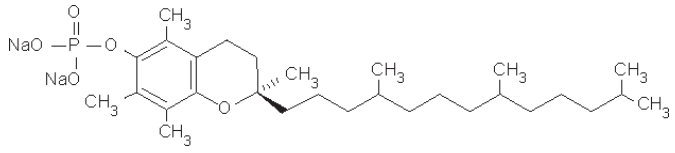
Incubation of HepG2 cells with concentrations of OTA ranging from 0.25 to 50 µmol/l for 24 h resulted in a dose-dependent decline in cell viability (Fig. 3). Decreasing FCS concentrations were paralleled by a marked increase in OTA-induced cytotoxicity, with the strongest effects at OTA concentrations between 0.25 and 15 µmol/l. In cells cultured in serum-free medium the strongest cytotoxicity was observed at OTA concentrations between 10 (100% viability) and 250 nmol/l (53% viability). However, in medium containing 5 or 10% serum, 24 h exposure to 1 µmol/l OTA did not lead to cell death at all, while 0 or 2.5% serum decreased cell viability to 52 and 87%, respectively. The minimum level of cell viability (approximately 40%) was reached upon incubation with 15 µmol/l OTA and did not



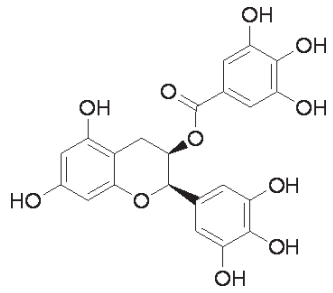
α-Tocopherol



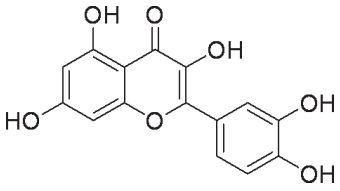
Catechin



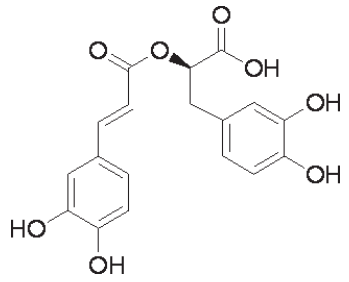
α-Tocopherol phosphate (disodium salt)



Epigallocatechin gallate



Quercetin



Rosmarinic acid

Fig. 2: Chemical structures of the antioxidants

decrease further even upon incubation with as much as 50 µmol/l OTA in FCS-free medium (Fig. 3).The highest non-toxic OTA concentration was 10 nmol/l (Fig. 4). Due to the higher bioavailability of OTA under these conditions, serum-free medium was chosen for further experiments.

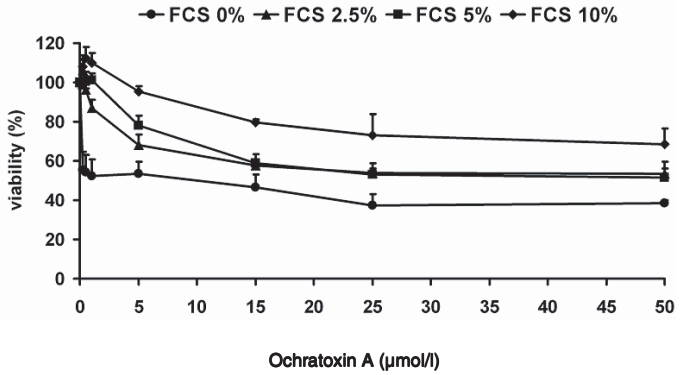


Fig. 3: Influence of foetal calf serum (FCS) concentration on the viability of HepG2 cells after 24 h incubation with ochratoxin A (0 - 50 µmol/l). Data are mean ± SD from two experiments in triplicate (n = 6).

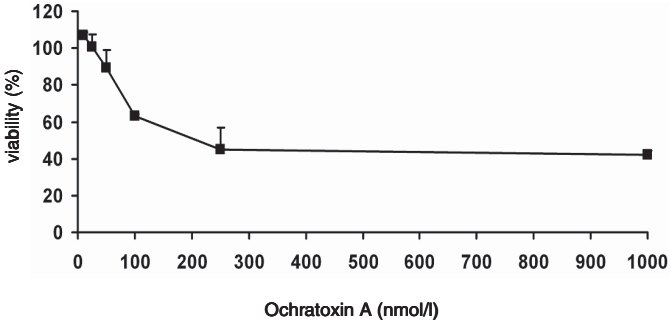


Fig. 4: Viability of HepG2 cells after 24 h incubation with ochratoxin A (0 - 1000 nmol/l). Data are mean ± SD from two experiments in triplicate (n = 6).

Pre-incubation experiments with antioxidants and glutathione-modulators

A 24 h pre-incubation of HepG2 cells with α-TOC, α-TOC-P, EGCG, QUE, CAT, and ROSAC at concentrations of 25, 50, or 100 µmol/l and a subsequent incubation with 1, 5, or 15 µmol/l OTA for 24 h did not prevent the cytotoxic effects of OTA (Fig. 5). Treatment with α-TOC-P, EGCG, and QUE even enhanced OTA cytotoxicity dose-dependently. No changes in cell viability were observed upon pre-

incubation with α-TOC, CAT and ROSAC. Cellular α-TOC levels remained unchanged in response to OTA treatment (data not shown), cellular concentrations of EGCG, QUE, CAT, and ROSAC were not determined.

To further investigate the involvement of cellular glutathione levels in OTA-induced cytotoxicity, additional pre-incubations with the glutathione modulators BSO and NAC were carried out. Incubation with NAC did not protect OTA-treated cells from cytotoxicity (Fig. 6A). Pre-incubation with 500 µmol/l BSO decreased cell viability by 10%, while lower concentrations of BSO were without effect (Fig. 6B).

Effect on cellular glutathione levels

As shown in Fig. 7A, incubation with BSO for 24 h resulted in a substantial and dose-dependent depletion of cellular glutathione levels in HepG2 cells. Concentrations as low as 5 µM decreased cellular GSH by about 40% compared to untreated controls. A complete GSH-depletion was observed at 100 µmol/l BSO. Supplementation with NAC did not increase cellular GSH (data not shown).

Incubation with OTA decreased GSH levels at concentrations of ≥ 0.25 µmol/l (Fig. 7B). 1 µmol/l OTA led to an approximately 30% decrease in cellular GSH as compared to controls. In contrast, lower concentrations (0.05 or 0.1 µmol/l) of OTA resulted in a moderate increase in GSH levels in HepG2 cells.

Discussion

In line with previous studies our data indicate that OTA induces cytotoxicity in HepG2 cells in a dose-dependent manner (BALDI et al., 2004; RENZULLI et al., 2004; HUNDHAUSEN et al., 2005). More-

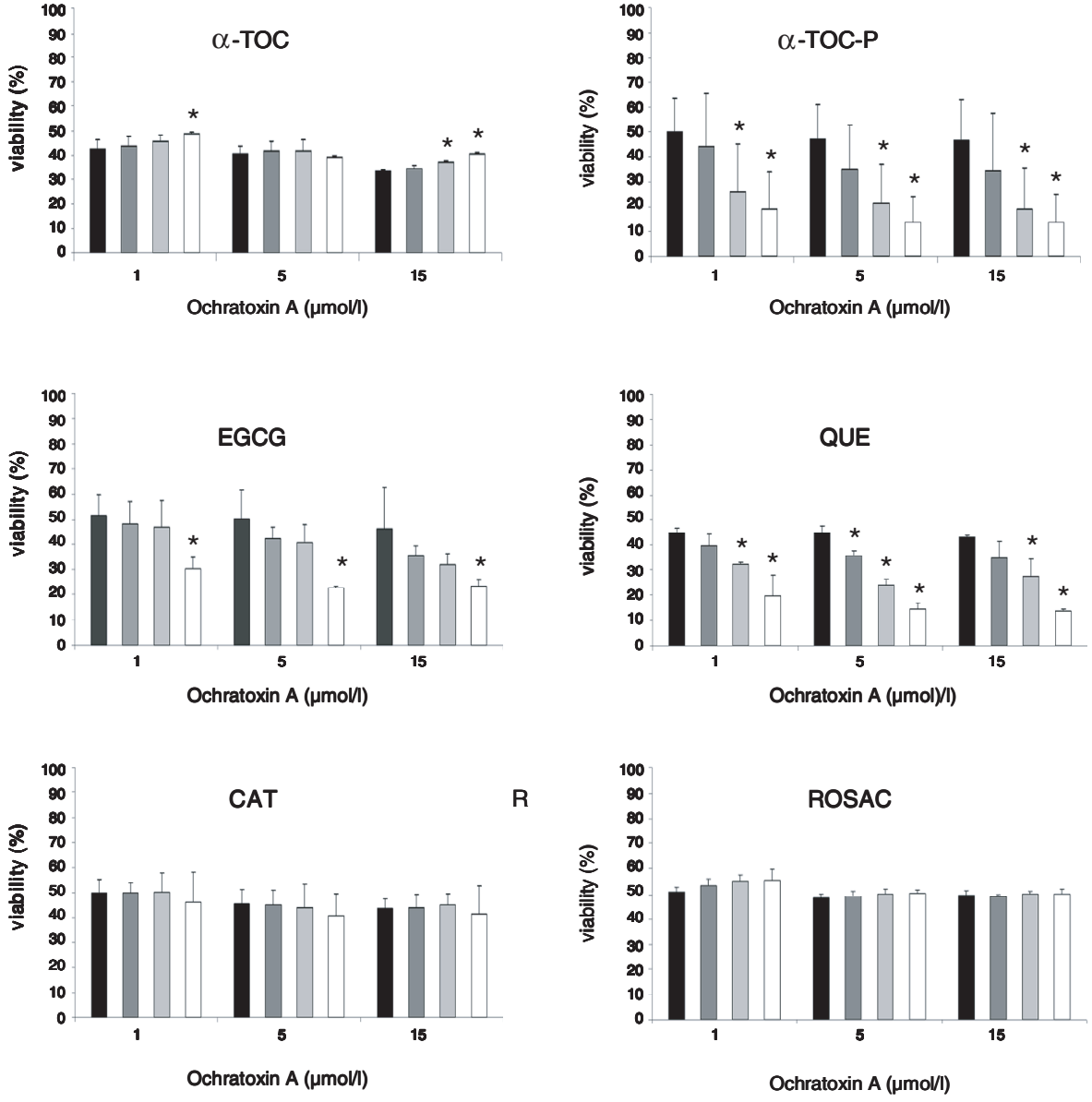


Fig. 5: Cytotoxicity of ochratoxin A (24 h, 1 - 15 µmol/l) in HepG2 cells after pre-incubation with *RRR*-α-tocopherol (α-TOC), *RRR*-α-tocopherolphosphate, epigallocatechin gallate (EGCG), quercetin (QUE), catechin (CAT), and rosmarinic acid (ROSAC) (24 h, 0 ■, 25 ■, 50 □, and 100 □ µmol/l). Data are mean ± SD from two experiments in triplicate (n = 6). *Indicates significant differences when compared to control ($p < 0.05$).

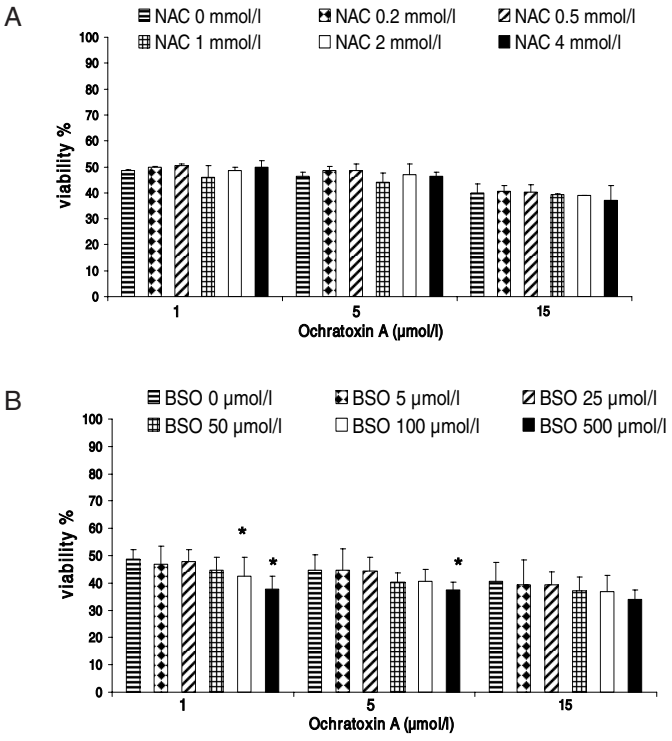


Fig. 6: Cytotoxicity of ochratoxin A (24 h, 1 - 15 µmol/l) on HepG2 cells after pre-incubation with N-acetylcysteine (NAC, A) (24 h, 0 - 4 mmol/l) or buthionine sulfoximine (BSO, B) (24 h, 0 - 500 µmol/l). Data are mean ± SD from two experiments in triplicate (n = 6). *Indicates significant differences when compared to control (Dunnett; $p < 0.05$).

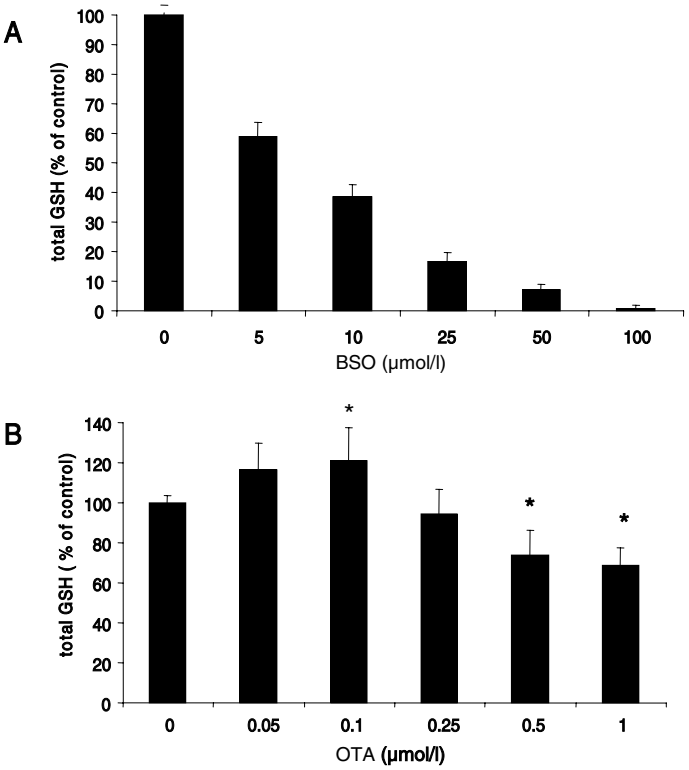


Fig. 7: Total glutathione (GSH, in % of control) after 24 h incubation with buthionine sulfoximine (BSO, A) or ochratoxin A (OTA, B) at the given concentrations. Data are mean ± SD from two experiments in duplicate (n = 4, A) or triplicate (n = 6, B). *Indicates significant differences when compared to control ($p < 0.05$).

over, we demonstrated a strong impact of serum protein concentration in the cell culture medium on the viability of OTA-exposed HepG2 cells. A decrease in serum concentration led to a dramatic increase in OTA-induced cell death in HepG2 cells. Importantly, under serum-free conditions even nanomolar OTA-concentrations resulted in a considerable loss of cell viability.

Concerning the protein binding properties of OTA, a high affinity of OTA for plasma proteins, both in animals and in humans, has been reported (RINGOT et al., 2006). In a wide range of species, including quail, mouse, rat and monkey, the fraction of free OTA in plasma was found to be less than 0.2% (HAGELBERG et al., 1989). Furthermore, it is well-known that OTA toxicity depends on its half-life in the body (O'BRIEN and DIETRICH, 2005), which in turn has been suggested to be influenced by binding of OTA to proteins (CHU, 1971; CHU, 1974). This is supported by findings that albumin-deficient rats had a 20- to 70-times faster OTA clearance from the systemic circulation than non-deficient rats (KUMAGAI, 1985).

However, studies investigating the dose-response relationship between the protein concentration in the cell culture medium and OTA toxicity are lacking. For this reason, a major focus of the present study was to determine the impact of different FCS levels in the culture medium on OTA-induced cell death in hepatocytes. We observed that a decrease of serum concentration in the medium was paralleled by a dramatic increase in OTA toxicity, which may indicate strong protein binding of OTA, possibly linked with inhibited cellular uptake and, thus, lower toxicity in HepG2 cells. Consistently, a previous study revealed decreased neurotoxic effects of OTA by protein binding to bovine serum albumin in embryonic chick brain and neural retina cell cultures (BRUININK and SIDLER, 1997). On the other hand, protein binding alone does not always decrease OTA toxicity (STOJKOVIC et al., 1984). It has been suggested that binding of OTA to two small serum proteins (molecular mass 20.000 Da), being able to pass through the glomerular membrane, might explain the OTA-mediated nephrotoxic effect in mammals (STOJKOVIC et al., 1984).

Taken together, variations in the degree of protein binding as well as selective binding of OTA to distinct proteins may contribute to the often reported species-specific differences in OTA toxicity (DIETRICH et al., 2001; O'BRIEN et al., 2001; HEUSSNER et al., 2002).

In the present study, we investigated the potential cytotoxic effects of OTA at nanomolar concentrations. Previous studies have revealed that nanomolar OTA concentrations induced damage to renal and brain cells, due to an induction of apoptosis (GEKLE et al., 2000) and a loss of neuronal enzyme activity (MONNET-TSCHUDI et al., 1997). Our study, however, is the first to demonstrate that OTA causes cell death in hepatocytes at nanomolar concentrations, which is probably due to the absence of foetal calf serum (FCS) in the culture medium. Under serum-free conditions, incubation with 50, 100, or 250 nmol/l OTA for 24 hours resulted in an approximately 10, 40, or 60% decrease in viability of HepG2 cells, respectively. We observed toxicity with concentrations as low as 50 nmol/l OTA, which is within the range of OTA detected in human blood and serum. Between 6 and 26% of human blood and serum samples from the Balkan area contained OTA in the range of 1 - 35 ng/ml (2.5 - 88 nmol/l) and 1 - 40 ng/ml (2.5 - 100 nmol/l) (PETKOVA-BOCHAROVA et al., 1988), respectively, paralleled by an increased occurrence of the Balkan Endemic Nephropathie (BEN) (PETKOVA-BOCHAROVA et al., 1988). Considering the elimination half-life of OTA in humans of approximately 36 days (STUDER-ROHR et al., 2000), which is substantially

longer than that reported for other species – mice 40 h; rats 55-120 h (GALTIER et al., 1979; HAGELBERG et al., 1989); pigs 72-120 h (GALTIER et al., 1981); monkeys 820 h (KUIPER-GOODMAN and SCOTT, 1989) – it is apparent that OTA constitutes a higher risk in humans.

Our study also demonstrated that at least 45% of the cells were still viable after incubation with 50 µmol/l OTA for 24 h. In serum-free medium this maximum toxicity was reached at OTA concentrations as low as 0.25 µmol/l. Incubation with 1 - 50 µmol/l OTA did not further increase OTA-induced cell death in HepG2 cells. In accordance with our results it has been shown in LLC-PK1 cells that higher OTA-concentrations (high nmol/l to low µmol/l) or longer times of exposure (96 h) did not further decrease cell viability (DREGER et al., 2000). One hypothesis, which might explain the survival of a considerable number of HepG2 cells, suggests the existence of OTA carrier systems being saturated at low OTA-concentrations and, thus, not being able to increase intracellular OTA-transport at higher OTA-concentrations. Such OTA carriers, belonging to the family of human organic anion transporters, are well-known in proximal tubule cells from mice (JUNG et al., 2001; BABU et al., 2002). However, studies investigating the transport of OTA in liver cells are rare. It has been proposed, however, that the organic anion-transporting polypeptide (OATP) is involved in OTA-uptake in hepatocytes (KONTAXI et al., 1996).

Neither the toxicokinetics of OTA, nor its precise toxicity mechanisms have been established to date. Nevertheless, the involvement of reactive oxygen species (ROS) in OTA toxicity seems to be likely (RAHIMTULA et al., 1988; GAUTIER et al., 2001; SCHAAF et al., 2002). Therefore, one objective of our study was to counteract OTA toxicity by pre-incubation of HepG2 cells with various antioxidant test substances. We pre-incubated liver cells with 25, 50, or 100 µmol/l of the respective antioxidants. Concerning α-TOC these concentrations are similar to physiological plasma concentrations, which have been reported in the range of 25 - 40 µmol/l (HENSLEY et al., 2004), referring to flavonoid concentrations used in the cell culture experiments however exceeded physiological plasma concentrations (0.1 - 10 µmol/l) (MANACH et al., 2004). To avoid interactions between OTA and the test compounds in the culture medium, cells were washed twice with PBS before incubation with OTA. None of the test compounds prevented HepG2 cells from OTA-induced cell death. This is in contrast to studies reporting protective effects of antioxidants towards OTA-induced cell damage. For example, 24 h of pre-treatment with 50 µmol/l cyanidin-3-O-beta-glucopyranoside or 50 µmol/l rosmarinic acid inhibited the cytotoxicity of 10 µmol/l OTA in HepG2 cells by 25 and 35%, respectively (RENZULLI et al., 2004; GUERRA et al., 2005). Another experiment, also using OTA in serum-free medium, showed that three hours of pre-incubation with 1 nM α-TOC decreased OTA-induced cytotoxicity in bovine mammary epithelium cells by 10% (BALDI et al., 2004). The reasons for these conflicting results remain unclear, although cell-specific differences might be one plausible explanation.

We also found that EGCG, QUE, and α-TOC-P enhanced the toxic effects of OTA. α-TOC-P inhibits cell proliferation (OGRU et al., 2004) and modulates membrane fluidity (REZK et al., 2004). The latter might be the reason for a facilitated uptake of OTA into HepG2 cells, and thus, for the observed synergistic toxic effects of α-TOC-P and OTA. Moreover, it has been demonstrated that both EGCG and QUE are able to exert not only antioxidant, but also pro-oxidant activity, partly due to the formation of hydrogen peroxide (METODIEWA et al., 1999; ELBLING et al., 2005; FERRARESI et al., 2005), which might explain the increased cytotoxicity of OTA in HepG2 cells.

We further investigated the importance of the intracellular antioxidant glutathione for the prevention of OTA toxicity. Several studies report a depletion of glutathione by buthionine sulfoximine (BSO), often paralleled by either loss of cell viability or increased susceptibility to compounds exerting oxidative stress (WRIGHT et al., 1998; ANDERSON et al., 1999; LONG et al., 2000; ANDERSON and REYNOLDS, 2002; HONDA et al., 2004). However, it has been shown that HepG2 cells are able to survive BSO treatment, which may be explained by an up-regulation of Bcl-2, a protein preventing apoptosis (D'ALESSIO, 2004). In the present study we confirmed the resistance of HepG2 cells to BSO treatment and showed, additionally, that there were no synergistic effects of OTA and BSO on cytotoxicity in HepG2 cells. Although GSH was dose-dependently depleted by both BSO and OTA (Fig. 7), pre-treatment with BSO did not enhance cell death (Fig. 6). Furthermore, NAC did not increase cellular GSH levels (data not shown), and was not able to prevent HepG2 cells against the toxic effects of OTA (Fig. 6). Consistent with these findings are the results of a study with cultured rat embryonic cells demonstrating a decrease of GSH induced by OTA, but no protection due to exogenous GSH supplementation (HONG et al., 2000). On the other hand, it has been shown that NAC completely protected LLC-PK1 from OTA-induced cell death (SCHAAF et al., 2002).

Interestingly, we observed a biphasic effect of OTA on intracellular GSH levels in HepG2 cells. 50 and 100 nmol/l OTA increased cellular GSH to 116 and 121% compared to controls, respectively, while OTA at concentrations between 0.1 and 1 µmol/l dose-dependently decreased cellular GSH levels to approximately 70%. This effect may indicate that GSH is involved in the detoxification of OTA, possibly by directly binding to OTA as proposed by DAI and co-workers (2002). On the other hand, it has been shown for brain cells that OTA-exposure leads to an increased generation of nitric oxide (NO) (ZURICH et al., 2005), which in turn is detoxified through conjugation with GSH.

In conclusion, the presented data indicate a dose-dependent effect of foetal calf serum concentrations in the cell culture medium on OTA-induced cytotoxicity in HepG2 cells. Supplementation with dietary antioxidants did not counteract cytotoxic effects of OTA. Although cellular GSH levels are modulated by OTA, mechanisms other than oxidative stress are likely to be involved in OTA-induced cell death in HepG2 cells.

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Address of the authors:
Christine Bösch-Saadatmandi, Christoph Hundhausen, Laia Jofre Monseny, Gerald Rimbach[1], Institute of Human Nutrition and Food Science, Christian-Albrechts-University, Hermann-Rodewald-Strasse 6, D-24118 Kiel, Germany.
Ralf Blank, Siegfried Wolffram, Institute of Animal Nutrition and Physiology, Christian-Albrechts-University, Hermann-Rodewald-Strasse 9, D-24118 Kiel, Germany.
[1]Corresponding author: rimbach@foodsci.uni-kiel.de