

Aus dem Institut für Tierernährung und Stoffwechselphysiologie  
der Christian-Albrechts-Universität zu Kiel

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# Influence of quercetin on Ochratoxin A toxicokinetics and toxicity in F344 rats

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## List of abbreviations

8-iso-PGF <sub>2α</sub>	8-iso-prostaglandine F2 alpha
α-TOC	alpha-tocopherol
ABC	ATP-binding cassette
AhR	aryl hydrocarbon receptor
AP-1	activator protein one
ARE	antioxidant responsive element
ATP	adenosine triphosphate
BRCP	breast cancer resistance protein
b.w.	body weight
Caco-2	human colon carcinoma cells
CYP	cytochrome P450 monooxygenase
ECH	erythroid cell-derived protein with CNC homology
EIA	enzyme immuno assay
EpRE	electrophile responsive Element
ERK	extracellular signal-reduced protein kinase
EROD	ethoxyresorufin-O-deethylase
FC	fold change
FLD	flourescence detection
GCLC	glutamate cysteine ligase, modifier subunit
GOI	gene of interest
GSH	glutathione
GSSG	glutathione disulfide
HKG	housekeeping gene
HEK	human embryonal kidney cells
HIF-1	hypoxia inducible factor 1
HNF4α	hepatocyte nuclear factor 4 alpha
HO-1	heme oxygenase 1
HPLC	high pressure liquid chromatography
JNK	c-Jun N-terminal kinase
Keap1	Kelch like ECH-associated protein 1
LOAEL	lowest observed adverse effect level
NOAEL	no observed adverse effect level

MAPK	mitogen activated protein kinase
MDA	malonyl dealdehyde
MDR1	multiple drug resistance transporter
MROD	7-methoxyresorufin-O-dealkylation
MRP2	multidrug resistance associated protein 2
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
Nrf2	nuclear factor-erythroid 2 p45-related
OAT	organic anion transporter
OAT-K	kidney specific OATP
OATP	organic anion transporting peptide
OH-OTA	hydroxy-OTA
OTA	ochratoxin A
OTA+Q	OTA + quercetin treated animals
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
TBARS	thiobarbituric acid reactive substances

# 1. Introduction

For the flavonoid quercetin multiple beneficial health effects were postulated, e.g. regarding the risk of fatal or non-fatal coronary artery diseases, the risk of lung cancer, the incidence of asthma and the impairment of pulmonary functions in chronic obstructive pulmonary disease, to name just a few (reviewed in 1-3). Until today, quercetin has been investigated intensively *in vivo* and *in vitro*, in order to understand its mechanism of action. The most popular mechanism for quercetin postulated is its antioxidative activity. Quercetin may reduce electrons from reactive oxygen species (4, 5) and is able to chelate free metal ions like  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ , which either act directly pro-oxidative or catalyze pro-oxidative processes (6-8). There is also evidence that quercetin derived antioxidative effects are attributed to modulating gene expression at the level of intracellular signaling pathways and transcription factors. One single transcription factor is able to regulate the gene expression of numerous enzymes. Hence, by modulating gene expression, quercetin influences biotransformation and other metabolic processes as well. Biotransformation of xenobiotics, like drugs or mycotoxins, is crucial for the exposition of tissues to those potentially harming substances. During biotransformation xenobiotics and endogenous lipophilic compounds are usually detoxified by increasing their hydrophilicity and their elimination via bile and urine.

The mycotoxin ochratoxin A (OTA) is produced by some species of the storage moulds *Penicillium* and *Aspergillus* as a secondary metabolite. Contaminated food and feed are mainly of vegetal origin and are found worldwide. OTA is primarily toxic to the kidney (nephrotoxic). For OTA, the popular mode of action postulated is a pro-oxidative effect. It has been assumed, that OTA generates reactive oxygen species (ROS) on the one side and down-regulates gene expression of antioxidative enzymes on the other side, resulting in oxidative stress.

Subject of this work was to examine whether dietary applied quercetin influences OTA toxicity in growing male F344 rats. According to our hypothesis, OTA derived oxidative stress might be diminished due to quercetin, when OTA and quercetin are co-administered. We also investigated, if quercetin influenced OTA toxicokinetics, because it was recently shown, that quercetin and OTA might compete for particular common efflux transporter at the level of the intestinal brush border membrane (9).

## 2. The mycotoxin ochratoxin A

Mycotoxins are produced by moulds as secondary metabolites on contaminated food and feed. Moulds are being distinguished in field fungi and storage fungi. Ochratoxins derive from some species of the storage fungi *Penicillium* and *Aspergillus* during inappropriate storage conditions. Naturally occurring ochratoxins are OTA, ochratoxin B and ochratoxin C. Chemically, OTA contains a derivative of isocoumarine (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin), also called ochratoxin  $\alpha$  (OT $\alpha$ ), linked to the acid group of L-phenylalanine by a peptide bond. Ochratoxin B is a dechloro derivative of OTA and has a ten times lower to almost no toxicity compared to OTA (10, 11). Ochratoxin C is the ethyl ester of OTA and can be formed during the metabolism of OTA, obtaining the same toxicity like OTA (12). Beside the main forms mentioned, more OTA metabolites occur in nature, for example, derivatives of OTA and ochratoxin B esterified with methyl and ethyl groups at the free acid group (10, 11), hydroxylated forms of OTA (OH-OTA), like (4R)-OH-OTA, (4S)-OH-OTA and 10-OH-OTA (13) and analogues of OTA, which contain other amino acids than phenylalanine, such as serine, hydroxyproline, lysine and tyrosine (14). With regard to toxic damages in animals and humans, OTA is of most concern. Hence, the following sections will focus on OTA.

### *OTA producing mould*

OTA production is dependent on various factors such as temperature, water activity ( $a_w$ -value), pH-value and other environmental parameters, which are crucial for the growth of OTA-producing mould. OTA was originally described as a metabolite of *Aspergillus ochraceus* (15). Other *Aspergillus* species have been described as OTA producers, of which only *A. carbonarius* plays a quantitatively substantial role. The only OTA producing *Penicillium* species is *P. verrucosum*. Each one of the three main OTA producers require environmental conditions for optimal growth, which are summarized in Table 1.

Table 1: Physical parameters for optimal growth of main ochratoxin A (OTA) producing mould

OTA producing mould	Range of temperature tolerance (°C, optimum)	Preferred water activity ( $a_w$ -value)	Preferred pH	Reference
<i>A. ochraceus</i>	8-37 (24-31)	0.95-0.99	3-10	(16, 17)
<i>A. carbonarius</i>	32-45 (40)	0.82	2-10	(18, 19)
<i>P. verrucosum</i>	0-31 (20)	0.80	2-10	(20, 16)

*A. ochraceus* was isolated from various foods and most commonly occurs in dried and stored food. These included, for example, dry beans and seeds, pepper, dried fruits and various nuts ( $a_w$  0.75-0.90) and also smoked salted dried fish ( $a_w$  0.75). *A. ochraceus* was also found in green coffee beans and their processed end-products like ready-to-drink coffee (21). To a lesser extent *A. ochraceus* was found in cereals and cereal products, cheese, spices, black olives and processed meat (16). About *A. carbonarius* relatively little is known. Its ability to produce OTA has been reported (22). *A. carbonarius* was mainly found on dried grapes and wine indicating that this species is the main source of OTA in those products. The capability of *P. verrucosum* to grow at lower temperatures affects its preferential distribution in geographical regions with a rather cool climate. Mainly grain is infested by *P. verrucosum* and as a consequence cereals and cereal products, including animal feed stuff, from Northern and Central Europe to Canada are contaminated with OTA. In a survey on Scandinavian farms, where pigs were suffering from nephritis, 67 of 70 barley samples contained high levels of *P. verrucosum*, of which 66 contained OTA (16). *P. verrucosum* also occurred on meat and cheese in Europe, but was not found in warmer regions or in other products than the above mentioned.

#### *Occurrence of OTA in contaminated food and feedstuff*

The "Joint FAO/WHO Expert Committee on Food Additives" (18) prepared a compilation of worldwide data available on OTA contamination of food and feed. The total number of samples was 23,167, of which 85 % were from Europe (Croatia, Denmark, Finland, France, Germany, Italy, Netherlands, Norway, Spain, Sweden, Switzerland and the UK), 7 % from South America (Brazil and Uruguay), 6 % from North America (Canada and USA), 1 % from Africa (Sierra Leone and Tunisia) and 1 % from Asia (Japan and Dubai). 1.4 % of all samples contained more than 5 µg/kg and 0.6 % more than 20 µg OTA/kg. For example, high average

concentrations were found in corn (7.5 µg/kg), moderate concentrations in dried grapes (2.3 µg/kg), instant coffee (1.4 µg/kg) and rye (1.2 µg/kg). Whereas low concentrations were found in wine (0.3 µg/kg), rice (0.06 µg/kg), animal products like meat, liver and sausage (0.05 µg/kg), and beer (0.03 µg/kg). The EU regulations for maximal allowance of OTA concentrations in food (23) are assessed in the range of 2-10 µg/kg for specified foodstuff, for example 10 µg/kg for dried grapes and instant coffee, 5 µg/kg for unprocessed grain, 3 µg/kg for processed grain (and products hereof) and 2.0 µg/kg for wine.

Feedstuff for animals usually contain mixtures of home-grown and imported crops, such as groundnut, palm kernels, cottonseed, copra and soya. Any mycotoxins present in these products will contaminate the final product (24). A number of 811 feedstuffs were examined and OTA was detected in both, home-mixed and commercially produced feedstuff. 12.8 % of home-grown cereals destined for pig-feeding were contaminated with OTA. Barley (< 5 mg OTA/kg) and wheat (< 2.7 mg OTA/kg) had OTA concentrations higher than the recommendation by the EU commission of < 0.25 mg/kg (25). In home-mixed pig-meal 12 % of the samples were contaminated with OTA (> 0.25 mg/kg) and only in 4 % of the commercially produced pig-feed OTA was detectable on a lower level than recommended. OTA was rarely detected in poultry (3 % positive for OTA, < 0.15 mg/kg) and cattle feeds (0.3 % positive for OTA, no results for concentration) (24). Transition of OTA from contaminated feedstuff to livestock and from produced animal products to the consumer ("carry over"), as well as the transition of OTA from contaminated plant products to the consumer must be considered as well. In fact, OTA was found in Danish pork in an investigation in 1979 (26). For human daily OTA intake, however, food of vegetal origin is of greater significance compared to animal derived food. More than 50 % of the daily intake of OTA stems from cereals and cereal products and about 30 % of red wine, coffee and beer (27). Poultry and pork accounted for only 4 % and thus contribute little to human OTA exposure. Meat of ruminants appears not to be important for human OTA intake, because OTA is reduced by more than 80 % to the less toxic OT $\alpha$  in the rumen of the animals. Hence, transition of OTA into dairy products is attributed no significance (27). Nevertheless, OTA contamination of dairy products like cheese may occur after production (16).

## ***2.1. Toxicokinetics and metabolism of OTA***

Generally, the extent of adverse effects of toxins depend on the route of exposition, dosage, bioavailability and biological half-life. Toxicokinetics of OTA involves absorption, distribution, metabolism and excretion. The following sections will focus on these aspects. The bioavailability of a substance is the pharmacological parameter, which is defined as that fraction of an administered dose, that reaches the systemic circulation in its original form. The oral bioavailability of OTA is 1.6 % in fish, 44 % in rat, 62 % in quail, 97 % in mouse, 57 % in monkey (28), 66 % in pig, 56 % in rabbit and 40 % in chicken (29, 30).

### *Absorption and secretion of OTA in the gastrointestinal tract*

After oral consumption, OTA may be absorbed by passive diffusion in the stomach and the small intestine. OTA is lipid-soluble in its non-ionized form, hence, pH of the chyme and polarity of OTA determine the extent of OTA absorption by passive diffusion. OTA has two ionisable groups, which can exist in the ionized or non-ionized form under physiological conditions (31). OTA is a weak acid with a dissociation constant (pKa) of 7.1. The non-ionized form will be mainly present in acidic solutions like in the stomach and will be partly absorbed by passive diffusion (32, 33). At neutral pH like in the the small intestine 50 % of OTA will be ionized (34). OTA is absorbed along the whole small intestine, whereas the highest capacity was found in the proximal jejunum (35, 36). Passive absorption of OTA is strongly influenced by the high binding affinity of OTA to plasma proteins, which generates an OTA gradient across the intestinal epithelial cells (35). Beside passive diffusion, active carrier systems are discussed to be involved in the intestinal absorption of OTA. The organic anion transport system (OAT, solute carrier family SLC22), organic anion-transporting polypeptides (OATP, solute carrier family SLCO, formerly SLC21), the large neutral amino acids transporter and the H<sup>+</sup>-dipeptide-cotransporter were investigated in this context. They were not involved in intestinal OTA transport as neither intracellular accumulation nor transepithelial transport of OTA was affected in the presence of specific substrates of those transporters in Caco-2 cells (37). The results of an *in vivo* study with rats confirmed, that OTA is not absorbed via the H<sup>+</sup>-dipeptide-cotransporter or OATP1-transporter in the small intestine (36).

Once absorbed into intestinal epithelial cells across the apical cell membrane, OTA may cross the basolateral membrane by passive diffusion and reach the blood or be actively transported back into the lumen (intestinal secretion) by efflux transporters. Thus, the amount of OTA

secreted by the whole intestine has been calculated to be similar to the amount secreted via bile (28).

Three transporters, belonging to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transport proteins, are discussed to be involved in OTA resecretion across the brush-border membrane (multidrug resistance associated protein 2 = MRP2, breast cancer resistance protein = BCRP, and P-glycoprotein = P-gp). They act as energy-dependent efflux pumps, decreasing cellular concentrations of drugs and toxins, for example (37). MRP2, BCRP and P-gp are expressed in brush border membrane of enterocytes, in hepatocytes (canalicular membrane) and in kidney tubulus epithelial cells (38-42). P-gp transports mainly cationic compounds (40), whereas MRP2 mediates the transport of amphiphatic anionic conjugates (glutathione (GSH), glucuronid and sulfate conjugates) as well as unaltered lipophilic substances (43). Substrates of BCRP are hydrophobic compounds and hydrophilic conjugated organic anions (40, 44). Only few studies have addressed OTA as a substrate for efflux transporters. In Caco-2 cells and human embryonal kidney cells (HEK), OTA was transported by MRP2 and BCRP (9, 37, 45) and it was discussed whether OTA is also a substrate for P-gp (37, 45). Under experimental conditions, mimicking the *in vivo* pH situation (lumen side pH 6.0, blood side pH 7.4), OTA was absorbed across Caco-2 cell monolayers. In another study with Caco-2 cell monolayers, it was shown that OTA was extensively secreted by these cells across the apical membrane. But addition of the MRP2 inhibitor indomethacin decreased secretion of OTA across the brush-border membrane which resulted in an increased absorption (37). The authors concluded, that due to the activity of MRP2 at the apical pole of intestinal epithelial cells, intestinal net absorption of OTA would be limited (37). In another experiment using Caco-2 cells, the effect of various polyphenols on OTA transport via MRP2 was examined (9). The cells were incubated with OTA (0.75 nmol/L, 7.5 nmol/L or 10  $\mu$ mol/L) together with 50  $\mu$ mol/L of chrysin, quercetin, genistein, biochanin A, catechin, epigallocatechin gallate, gallic acid, or resveratrol, respectively. Chrysin, quercetin, genistein, biochanin A and resveratrol increased OTA transport at the basolateral site and increased intracellular concentrations by 80-150 %, which was explained by a competitive inhibition of MRP2 by these polyphenols or their metabolites (9). Hence, an inhibition of the energy-dependent efflux pumps would result in a higher net absorption of OTA.

OTA not absorbed in its original form by the intestine, is metabolized to the less toxic OT $\alpha$  within the gastrointestinal tract. Phenylalanine is cleaved from OTA most likely by microbial



hydrolysis. Although *in vitro* studies revealed that OTA might be hydrolyzed by the pancreas enzymes carboxypeptidase A and chymotrypsin (29, 46, 47), investigations in rats treated with the antibiotic neomycin implicated, that the microbial flora plays a crucial role. After oral administration of the antibiotic, hydrolysis of OTA to OT $\alpha$  was clearly reduced as indicated by an increased OTA and a decreased OT $\alpha$  blood concentration and excretion, respectively, compared to controls (48). An extensive hydrolysis of OTA to OT $\alpha$  was also observed in rumen contents *in vitro* (49) and in caecum and colon contents of rats (48). In ruminants effective hydrolysis of OTA by the ruminal microflora is considered to be the cause for the high oral tolerance to OTA in ruminant species (50-52). The kidney and liver do not substantially contribute to the production of OT $\alpha$  from OTA (29).

#### *Tissue distribution of OTA*

Intestinally absorbed OTA is transported in the blood bound to serum proteins, mainly by albumin (30). This facilitates its passive absorption by generating a concentration gradient across the intestinal epithelial cells and explains the slow elimination of OTA from the body (53). Thus, a plasma "pool" of protein-bound OTA which can release OTA over a long period is formed (32). The biological half-life of OTA is species specific. It is 850 h in humans (54), 230 h in rats (55), 88.8 h in pigs and 4.1 h in chickens after oral application (30). The biological half-life of OTA was significantly longer than of its main metabolite like OT $\alpha$  (56). Tissue distribution of intravenously applied radioactive labelled OTA (<sup>14</sup>C-OTA) were conducted in mice and rats (57, 58). The highest amount of radioactivity was found in blood, kidney and liver and lowest concentrations in adipose tissue and brain (Table 2). The same ranking of OTA concentrations were found in pigs, chickens and rats (30, 59, 60). It was suggested, that reabsorption of OTA from the intestine into the circulation through enterohepatic circulation favours the systemic redistribution into different tissues (33, 57).

Table 2: Tissue content of OTA 1 hour after intravenous administration of 2.7 mg/kg b.w. <sup>14</sup>C-OTA depicted as percentage of applicated dose in the whole organ and calculated concentrations in blood ( $\mu\text{g/mL}$ ) and tissues ( $\mu\text{g/g}$ ) (58)

<b>Tissue</b>	<b>OTA content</b>	<b>OTA concentration</b>
blood	43.5	46.8
kidneys	1.2	16.9
liver	6.1	15.1
adipose tissue	1.8	2.8
brain	0.1	1.4

### *Biotransformation of OTA in the liver*

Uptake of OTA into hepatic cells is mediated by OATP1 (61). There, a part of OTA is metabolized by enzymes of the "biotransformation pathway". Because cytochrome P450 monooxygenases (CYP) play a central role in the biotransformation of xenobiotics (and endogenous metabolites as well) evidence for the involvement of CYPs in OTA metabolism will be subsequently illustrated in more detail. Investigations in liver microsomes of humans, pigs and rats indicate, that OTA is hydroxylated to (4R)- and (4S)-OH-OTA by CYPs (13, 62). In liver microsomes of rats, CYP1A1, CYP1A2, CYP2B1, CYP3A1 and CYP3A2 were involved in this transformation (63, 64). In contrast, in another study using rat liver microsomes, only low rates of (4R)-OH-OTA production by CYP1A1 and CYP3A4 were observed (62). In an *in vivo* study, conducted with Dark Argouti and Lewis rats over a period of two years, the expression of several CYPs before and after OTA administration by intragastric intubation (three times a week, 0.4 mg of OTA per kg body weight (b.w.)) was determined (65). In the liver, OTA induced CYP1A1, CYP1A2 and CYP2C11 expression in male animals of both strains, but only CYP3A1 was induced in dark Argouti and CYP2A2 in Lewis rats. In kidneys, CYP2B and CYP2C11 were induced in males of both strains and only CYP2A2 was induced in Lewis rats in response to OTA. Expression profiles of female Dark Argouti and Lewis rats differed from the males' ones (65). In addition, DNA adducts were found in the organs of OTA-treated animals in specific patterns and females had less DNA adducts than male rats of both strains. Based on these results, the authors concluded, that CYP1A1, CYP2A1, CYP2A2 and the female specific CYP2C12 were OTA-"detoxifying" enzymes, whereas CYP1A2, CYP2C11, CYP3A1, CYP2D, CYP2B and male specific CYP2C11 were "OTA-toxifying" enzymes (65).

Further research efforts to identify the "OTA toxifying" CYPs were conducted using transgenic cell models with human CYPs. In murine NIH/3T3 fibroblasts, which were transfected with recombinant human CYP1A1, CYP1A2, CYP2C10 or CYP3A4, a dose-dependent increasing mutation frequency of the cells was observed after incubation with OTA (66). However, no increased mutation rates were observed in cells expressing human CYP2D6 and CYP2E1 (67). In rabbit microsomes and in fibroblasts (NIH/3T3) expressing human CYP2C9, an increased DNA-adduct formation and loss of viability was observed, respectively (68, 69). It was postulated, that these "OTA-toxifying" CYPs generate OTA metabolites that are involved in DNA binding and renal damages of OTA (70-75). These bioactivated OTA metabolites, however, are not identified yet. The formation of OTA-derived

reactive quinones was suggested (76, 77). Thus, it was hypothesized, that OTA is activated by oxidation to the intermediate OTA-phenoxy radical and then the OQuinone/OHydroquinone redox couple is formed by oxidative dechlorination of OTA (78). In one study, in which rats were fed OTA by gavage, OHydroquinone was detected in urine (79) but not in liver and kidney microsomal fractions from rats and mice (62, 64).

A few studies investigated the conjugation of OTA by phase-II-enzymes. In an *in vivo* study with mice, treated with a single dose of radiolabeled [<sup>3</sup>H]-OTA, 28-68 % glucuronide and sulfate conjugates of OTA were found in bile. OTA-conjugates also have been found in liver (8-17 %) and in intestinal tissue (6 %) (33). Also in pigs fed with OTA contaminated feed, OTA conjugates were found in bile (80). In contrast, in liver microsomes of rats neither OTA nor (4R)-OH-OTA was a substrate for rat glutathione-S-transferase (62, 64).

Taken together, some CYPs, which generate OH-OTA, are considered as "detoxifying" enzymes for OTA (CYP1A1, CYP2A1, CYP2A2, CYP2A12), whereas other CYPs (CYP1A2, CYP2C9, CYP2C10, CYP3A4) are considered as "toxifying" enzymes. But in both cases, the evidence is inconsistent.

#### *Transport mechanisms of OTA in the kidney*

The kidney is the primary target organ for OTA derived toxic effects because OTA is accumulated there. It was suggested that OTA is excreted via tubular secretion in the proximal tubules rather than glomerular filtration, because of its high binding affinity to plasma proteins (28, 81). In toad kidney, transepithelial secretion of OTA was a dose-dependent saturable process with a  $K_m$  of 0.63  $\mu\text{mol/L}$  and OTA accumulated in the cells and tubule lumen in a concentration-dependent manner, which implicated active transport processes (Figure 1) (81). Indeed, proximal tubular secretion of OTA involves active transport across the basolateral as well as the apical membrane (81, 82). Organic anions as OTA are transported by OAT across the basolateral membrane into cells in exchange with dicarboxylates (e.g.  $\alpha$ -ketoglutarate, succinate). The investigation of basolateral OTA transport in isolated kidney tubuli of rabbits and proximal tubule cells of opossum pointed to the involvement of OAT (81, 83). In studies with *Xenopus laevis* oocytes expressing the basolateral isoform OAT1, this transporter mediated the uptake of OTA, showing that OTA is a substrate of OAT1 (84). Uptake of OTA via OAT1 was also found in murine proximal tubule cells (84). Other basolateral OAT isoforms, such as OAT3, were discussed in the transport of OTA across kidney tubule cell membranes during proximal tubular secretion (85-87). OTA transport

across the apical membrane into the proximal tubule is mediated by the already elucidated active efflux transporters MRP2, BCRP and possibly P-gp (see above) (45, 88).

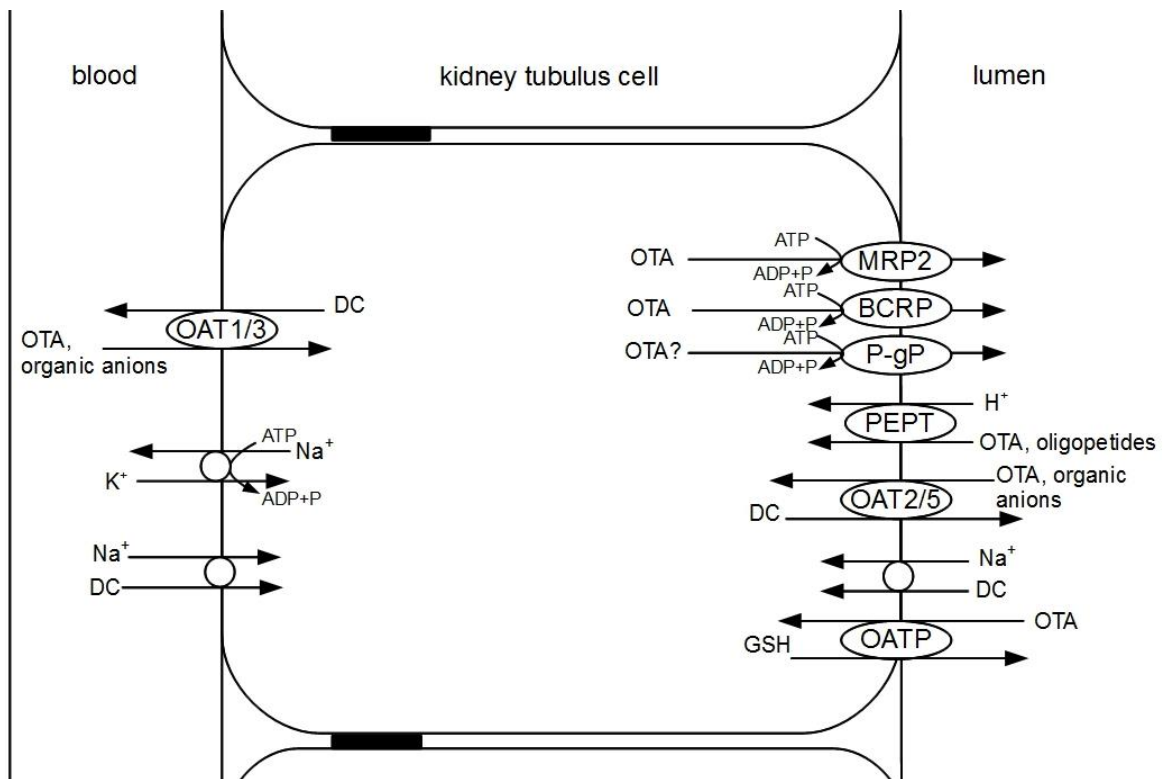


Figure 1: Schematic illustration of the transport systems involved in OTA transport across the membranes of kidney proximal tubulus epithelial cells. ADP, adenosine diphosphate; ATP, adenosine triphosphate; BCRP, breast cancer resistance protein; DC, dicarboxylate; GSH, glutathione; MRP2, multidrug resistance associated protein 2; OAT, organic anion transporter; OATP, organic anion transporting peptide; OTA, ochratoxin A; P, phosphate; PEPT, H<sup>+</sup>-dipeptide co-transporter; P-gp, P-glycoprotein (modified according to 89)

Whereas tubular secretion of OTA is limited to the proximal tubules, OTA is reabsorbed in the range of 75-90 % (90, 91) in all segments of the renal tubules, but via different mechanisms (Figure 2).

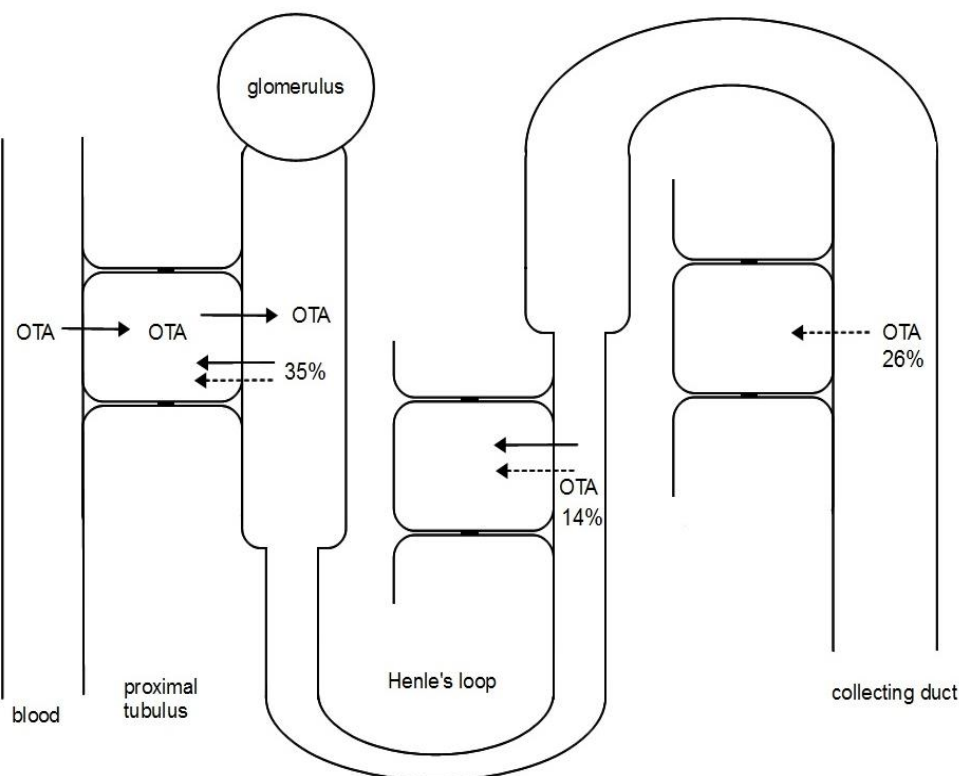


Figure 2: Schematic illustration of tubular secretion and reabsorption processes of OTA in renal tubules. The solid line stands for active transport processes, the dashed line for passive diffusion. The percentage values are the fraction of reabsorbed OTA in the respective segment under physiological conditions (91). OTA, ochratoxin A (modified according to 89).

In a study with rats using *in situ* micropuncture, OTA reabsorption in renal tubuli was examined at a concentration range of 0-0.05 mmol/L (90). 70 % of the infused OTA was reabsorbed in the proximal tubule in a partly saturable manner. 20 % of the infused OTA was reabsorbed in the distal tubule in a non-saturable manner. The reabsorption of OTA was pH-dependent. When the pH-value of the infusion solution was raised from 6.0 to 7.4, OTA reabsorption was reduced from 70 to 40 % in proximal tubule and from 20 to 10 % in distal tubule (90). The apical transporters involved in the reabsorption process appear to be the H<sup>+</sup>-driven dipeptide carrier in the proximal tubule and the kidney specific apical OATP (OAT-K1, OAT-K2) in the ascending limb of Henle's loop (91, 92). However, in distal tubule, OTA may be reabsorbed solely by non-ionic passive diffusion. The pH shift from 6.0 to 7.4 increased the dissociated fraction of OTA, which is not readily absorbed by passive diffusion due to its negative charge (90). Comparable results were achieved in other studies (91, 93, 94). For the entire tubule calculated, OTA reabsorption via H<sup>+</sup>-dipeptide co-transporter, OATP and non-ionic passive diffusion accounted for 30 %, 20 % and 20 %, respectively (94). The remaining part may be a yet unidentified pH-dependent mechanism. OAT2 and OAT5, which are

localized in the brush border membrane of tubulus cells might be involved (85-87). Despite the extensive OTA reabsorption, in rabbit renal proximal tubule cells, tubular secretion of OTA was eightfold higher than reabsorption and thus, net secretion occurred (82). However, OTA reabsorption and its binding to proteins prolongs the retention time of OTA in the renal cells, giving it opportunity to act deleterious (95).

Taken together, in kidney nephrons, OTA is excreted via proximal tubular secretion, because protein binding hinders glomerular filtration. The transport system involved at the basolateral membrane would be OAT1/3. OTA is transported into the lumen by the efflux transporters MRP2, BCRP and possibly OAT2/5 located at the apical membrane. OTA is reabsorbed in all tubule segments, probably mediated by the H<sup>+</sup>-dipeptide co-transporter and OATP (OAT-K1, OAT-K2) in the proximal tubuli and by passive diffusion in the distal tubuli.

#### *Total excretion of OTA and OTA-metabolites*

OTA is eliminated with bile and urine, each pathway contributing about 50 % to total excretion (56). OTA excreted via feces is derived from biliary and intestinal secretion, respectively, whereby the latter accounts for the minor fraction (29). 120 h after a high single oral dose of OTA (15 mg/kg b.w.), the excretion of OTA and its main metabolite OTα accounted for 57 % of the total dose administered to rats. In that study, total excretion was allocated to 34 % (OTα 23 %, OTA 11 %) and 22 % (OTA 11 %, OTα 12 %) to feces and urine, respectively (29). After a dose of 6.6 mg/kg b.w. (96), the main metabolite excreted was OTα (25-27 %, in urine and feces) followed by OTA in its original form (6 %) and (4R)-OH-OTA (1-1.5 %); after a dose of 0.5 mg/kg b.w. (55) the excretion of OTA and OTα was 14.7 % (6.3 % in urine and 8.4 % in feces) of the administered dose within 96 hours. OTA was the dominating form in feces (5.5 % vs. 2.9 %), whereas OTα was the dominating form in urine (4.2 % vs. 2.1 %).

## 2.2. Toxicity and toxicodynamics of OTA

OTA toxicity varies considerably between species and within a species it is dependent on diet, age and sex (31). Values for the acute oral toxicity of OTA (LD<sub>50</sub>) are shown in Table 3.

Table 3: Acute oral LD<sub>50</sub> doses for OTA in various species (31)

Species	Oral LD <sub>50</sub> (mg/kg b.w.)
mouse	46-58
rat	20-30
quail	17
guinea pig	8-9
rat neonatal	4
chicken	3.3
pig	1
dog	0.2

Whereas rats and mice are considerably tolerant with respect to the acute toxicity of OTA, pigs and dogs are highly sensitive. In all studied monogastric species OTA had dose-dependent nephrotoxic effects. In contrast, no LD<sub>50</sub> values were reported for ruminants because they are much more tolerant to OTA due to effective bacterial cleavage of OTA in the rumen (97).

Chronic toxicity of OTA was intensively investigated in pigs, because slaughtered pigs showed nephropathies after consuming OTA contaminated feed (98). Their kidneys were discoloured from usually red-brown to greyish-red, which was a result of fibrotic plaques in the interstitial gaps. Examinations of those kidneys revealed atrophic and ultra structural changes in tubules. The basal and brush border membranes were degenerated in proximal tubular epithelial cells (98). Controlled trials in pigs, using a daily oral intake of OTA (0.008 and 0.04 mg/kg b.w. per day) for 5 days (99), 3 months or 2 years (100) confirmed the above mentioned observations. In addition, an increased renal glucose excretion was found during the 2 year period of exposure to the mycotoxin (100). After 2 years of regular OTA intake (0.04 mg/kg b.w. per day), an advanced nephropathy without any renal failure was diagnosed (100). Similar results were observed in a study with young dogs. OTA was administered in capsules at doses of 0.1 or 0.2 mg/kg b.w. per day for 14 days. In those animals, no changes in renal function was observed, but their kidneys exhibited tubular necrosis and ultra structural changes in the proximal tubules. In addition, necrosis of lymphoid tissue of the thymus and the tonsils was discovered (101, 102). In studies with rats OTA-induced damages were dose-dependent; at doses of 15 µg/kg b.w. per day karyomegaly

of cells of the proximal tubules were observed and with increasing doses a decrease of the relative kidney weights and eosinophilia of the proximal convoluted tubules, rejection of proximal tubular cells, autolysis and changes in the rough and smooth endoplasmatic reticulum, thickening of the tubule membranes and growth retardation, respectively (103, 104). Long-term studies (90 days, 2 years) with mice and rats resulted in renal cell carcinoma and adenoma after daily OTA doses of 3.5 to 7 mg/kg b.w. (104, 105). The doses for the lowest observed adverse effect level (LOAEL) and no observed adverse effect level (NOAEL) are summarized in Table 4.

Table 4: Doses of ochratoxin A (OTA) for LOAEL and NOAEL in long-term studies on OTA toxicity (106), based on a compilation from (18)

<b>Species</b>	<b>Effect</b>	<b>Duration of studies</b>	<b>LOAEL (<math>\mu\text{g kg}^{-1}</math> b.w. <math>\text{day}^{-1}</math>)</b>	<b>NOAEL (<math>\mu\text{g kg}^{-1}</math> b.w. <math>\text{day}^{-1}</math>)</b>	<b>Reference</b>
Mouse	kidney tumours	2 y	4,400	130	(105)
Rat	karyomegaly of cells of proximal tubule	90 d	15	Not demonstrated	(103)
	kidney tumours	2 y	70	21	(104)
Pig	impaired renal function	90 d	8	Not demonstrated	(100)
	progressive nephropathy	2 y	40	8	(100)

The incidence of renal carcinomas and adenomas increased dose-dependently. Metastasis from renal and hepatic tumours was not observed. Rats and mice that died prematurely had kidney tumours, too (104, 105). Although the liver is often mentioned as a target organ for OTA toxicity, this organ was clinically effected only in severe cases and long term exposition as a liver tumour was manifested (104). In animal experiments, in which OTA clearly affected the kidney, the liver remained clinically unaffected (101, 107). However, it cannot be ruled out, that on molecular and/or metabolic level adverse changes will occur, although to a smaller extent than compared to kidney (108) and with less serious physiologic effects.

Taken together, the main target tissue for OTA is the kidney. Damage begins at the level of proximal tubule cell membranes and continues with karyomegaly of proximal tubule cells, necrosis, deposits of fibrotic plaques in interstitial gaps and leads to macroscopic alterations of the kidney and disordered renal function (nephropathy). High chronic doses culminate in a deteriorated general condition.



Toxicodynamics describes the actions and interactions of an exogenous compound like OTA within an organism. For the above described degenerative alterations of kidney tissue caused by OTA, several mechanisms were postulated.

At the beginning of OTA research, it was postulated, that OTA might interfere with amino acid metabolism of phenylalanine, because OTA contains a phenylalanine moiety. OTA was suggested to disrupt protein synthesis by interfering with the enzymes phenylalanine RNA synthetase and phenylalanine hydroxylase. Investigations in microorganisms, mammalian cells and *in vivo* studies proved the potency of OTA to inhibit these two enzymes, resulting in disturbed protein synthesis (109-111).

Another hypothesis is that OTA forms DNA adducts and causes genotoxicity. *In vivo* as well as *in vitro* a dose- and time-dependent formation of DNA adducts was detectable (75, 112-115). But the chemical structure of these adducts has not been elucidated yet and it is unclear if OTA is covalently bound to the DNA (34). Conflicting results were achieved in other studies, where the level of OTA-DNA adducts was below the limit of detection (62). The most common hypothesis is that OTA causes oxidative stress. Oxidative stress is known as an imbalance of the production of ROS, like superoxide anion radical ( $O_2^{\bullet-}$ ) and hydroxyl radical and their elimination through antioxidant systems. ROS attack lipids, proteins, nucleotides as well as amino acids or biogenic amides and affect redox sensitive mechanisms in cell metabolism like cell signaling and gene expression which lead to cell damage and apoptosis (34, 116). Various studies indicated, that OTA induces lipid peroxidation *in vitro* (116-121) and *in vivo* in rats (116, 119, 122-125). Some authors assume, that OTA complexes with  $Fe^{3+}$ , which enables the reduction of iron in the presence of the flavoprotein NADPH-CYP-oxidoreductase. The OTA- $Fe^{2+}$  complex may then generate hydroxyl radicals in the presence of oxygen, leading to lipid peroxidation in membranes (116-118). In another study, however, it was suggested that chelating of  $Fe^{3+}$  by OTA is not required for ROS generation (126). Irrespective of the chemical nature of OTA interaction with iron, the increased lipid peroxidation by OTA affects the permeability of the membrane for  $Ca^{2+}$  ions and affects the intracellular calcium homeostasis negatively. It causes an increased influx of calcium into the cells, a release from intracellular stores and consequently a disruption of calcium sensitive channels. A single high dose (10 mg/kg b.w.), or several smaller doses (0.5-2 mg/kg b.w.) per day of OTA led to an increased activity of the ATP-dependent calcium pump in the endoplasmic reticulum and the renal cortex cells (127). OTA, therefore, could interfere with

cellular functions that are under control of calcium, such as cellular signal transduction and neuronal functions.

The ability of potent antioxidant compounds to reduce OTA-induced lipid-peroxidation was investigated *in vitro* and *in vivo*. When vero monkey kidney cells were pre-treated with superoxide dismutase (SOD) and catalase in combination 4 h prior to OTA exposition, lipid peroxidation measured as malonyl dealdehyde (MDA) production was reduced, although not prevented completely (120). In Madin Darby canine kidney cells, vitamin A (all-*trans*-retinol) and vitamin E (racemic  $\alpha$ -tocopherol,  $\alpha$ -TOC) inhibited OTA-induced ROS production in a concentration dependent manner (128). *In vivo* studies in rats and mice confirmed, that the co-application of OTA and antioxidative compounds such as SOD, catalase (129), melatonin (124, 125) and cyanidin 3-O- $\beta$ -D-glucoside (130) resulted in a significantly reduced lipid peroxidation as measured by the production of thiobarbituric acid reactive substances (TBARS). Antioxidants such as retinol, ascorbic acid and  $\alpha$ -TOC, which are known to act as superoxide anion scavengers, prevented the induction of DNA damage by OTA in mice, given 2 mg/kg b.w. OTA by gastric intubation. In kidney, pre-treatment with  $\alpha$ -TOC decreased DNA adducts by 80 %, retinol by 70 % and ascorbic acid by 90 %, respectively (114).

#### *Impact of OTA on cell signaling and gene expression*

A new approach in understanding the molecular mechanisms of OTA toxicity is the investigation of OTAs influence on cell signaling and gene expression processes at tissue concentrations in nanomolar range (131). One cell signaling pathway, which is thought to be affected by OTA is the mitogen activated protein kinase (MAPK)-pathway. MAPKs are serine/threonine kinases and transduce signals from the cell membrane to the nucleus, finally influencing mRNA transcription. Physiologic activators of MAPK cascades are, for example, extracellular stimuli like growth factors, mitogens and inflammatory cytokines. Pathologic activators are oxidative stress, UV irradiation, heat and osmotic shock (132). Through the activated MAPK cascade, various cytosolic components can be affected. Main cellular reactions are inflammation, proliferation, differentiation and apoptosis processes. Three major MAPKs have been described: extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK (132). Several studies indicate, that OTA induced ERK (133) and JNK/p38 phosphorylation *in vivo* and *in vitro* (134-136). In most cases, ERKs seems to be linked to cell survival and JNK/p38 to inhibition of cell growth and apoptosis (137). How OTA actually mediates the activation of MAPK cascades, however,

has not yet been elucidated. Probably, ROS stimulates redox-sensitive signaling molecules, downregulates specific MAPK-phosphatases and modifies expression of various genes via transcription factors (132).

In recently conducted studies examining gene expression after OTA administration in rats, a regulation of several genes caused by OTA was observed. Downregulation of genes was the predominant effect and more genes in kidney than in liver were affected (108, 138-142). Genes encoding for proteins involved in energy metabolism, biotransformation, detoxification, transport and oxidative stress response were also downregulated (108, 138, 139, 141). In contrast, genes encoding proteins involved in acute phase response, inflammation, DNA damage, DNA repair, apoptosis and cancer were upregulated (108, 138, 139, 141, 143). A microarray study was conducted comparing gene expression profiles of cultured primary rat proximal tubular cells and tissues of male Wistar rats treated with each a low (5  $\mu\text{mol/L}$  and 1 mg/kg, respectively) or a high dose of OTA (12.5  $\mu\text{mol/L}$  and 10 mg/kg, respectively) for 24 or 72 h (141). Interestingly, gene expression profiles from the *in vivo* experiment described above differed from profiles obtained from cultured cells. Genes of metabolism, biotransformation and detoxification (e.g. CYP2D18) as well as transporter (OAT-K1, OAT) were downregulated *in vivo*, but not *in vitro* (141). In another work, male Fischer rats were administered a daily dose of 300  $\mu\text{g}$  OTA/kg b.w. per diet and after 7 days, 21 days, 4 months, 7 months and 12 months; thereafter, gene expression analysis of kidney and liver tissue were conducted (138). The early indicator gene for oxidative stress (144) - heme oxygenase 1 (HO-1, gene symbol *Hmox1*) - was upregulated in kidney but not in liver. In contrast to HO-1, genes of oxidative stress defence system were downregulated in kidney, like enzymes involved in glutathione synthesis (glutamate-cysteine ligase catalytic subunit, glutamate cysteine ligase, modifier subunit (GCLC), glutathione synthetase,  $\gamma$ -glutamyl transpeptidase). A similar study with male Fischer rats daily treated with OTA (500  $\mu\text{g/g}$  b.w., per gavage) was conducted. After 7 and 21 days, respectively, gene expression profiles were analyzed (108). This study confirmed the aforementioned findings. In detail, the genes of oxidative defence system, which were downregulated were GCLC, glutathione S-transferase, theta 2, microsomal glutathione S-transferase 1 and the extracellular SOD3. Lühe et al. (141) also found, that the transcription factor hypoxia inducible factor 1 (HIF-1), which indicated oxidative stress, was upregulated, but genes of the antioxidant system like catalase and glutathione synthetase were downregulated.

Regarding CYPs, the microarray studies revealed, that several CYPs (CYP2J4, CYP2C, CYP2D9, CYP2D26, CYP213, CYP2C39) were downregulated and none upregulated (108, 138). In a long term study by Pfohl-Leszkowicz et al. (65) investigating gene expression profiles of CYPs by the Western blot technique in Dark Argouti and Lewis rats after administration of OTA for 2 years (0.4 mg/kg b.w. by intragastric intubation three times weekly), it was shown, that CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2C11 and CYP3A were upregulated and only CYP2B was downregulated in liver of male Dark Argouti and Lewis rats. In the kidney, CYP2A2 and CYP2C11 were upregulated.

With respect to efflux transporters, the study of Marin-Kuan et al. (138) revealed a downregulation of MRP2 at all time points, whereas P-gp was downregulated by OTA after seven days, switching to upregulation at the subsequent time points (138). This agrees with findings of Arbillaga et al. (108). The ABC-transporter “multiple drug resistance transporter 1” (MDR1, gene *Abcb1a*), an isoform of P-gp, was downregulated after 7 and 21 days. Interestingly, transporter, involved in OTA secretion and reabsorption in kidney tubuli like OAT1 (gene *Slc22a6*), OAT-K1 (gene *Slco1a3/Slc21a4*), OATP (gene *Slco1a1/Slc21a1*) and the organic anion/cation transporter (gene *Slc22a12*) were downregulated by OTA (108, 138). Hence, OTA might affect its own toxicokinetics, resulting in a slowed elimination of OTA and a prolonged retention in the kidney, thus favouring its accumulation (138).

Many of the genes regulated by OTA share some regulatory elements. The common transcription factors involved are nuclear factor-erythroid 2 p45-related (Nrf2), hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$ ), nuclear factor-k B (NF $\kappa$ B) and the activator protein (AP-1). In pig kidney epithelial cells (LLC-PK1) OTA decreased the transactivation of the transcription factors AP-1 and Nrf2 (145). Consequently, mRNA levels and activities of SOD and glutathione-S-transferase decreased and intracellular levels of ROS increased. Further results from an investigation on OTA effects in Fischer rats (daily dietary intake of 300  $\mu$ g OTA/kg b.w.) and from *in vitro* experiments using proximal tubule epithelial cells of Osborne Mendel rats (NRK cells, 3 and 6  $\mu$ mol/L) indicated, that OTA inhibited Nrf2 activity, resulting in oxidative DNA damage. Pre-treatment of cell cultures with inducers of Nrf2 prevented the inhibition by OTA (142). Based on those results, it was suggested, that OTA causes and/or enhances oxidative stress by down regulating gene expression of antioxidative enzymes through inhibition of the transcription factor Nrf2, thereby diminishing the cellular defence system. Consequently, susceptibility of cells to oxidative damage increased (142, 146, 147). Another transcription factor, affected by OTA is HNF4 $\alpha$  (138). HNF4 $\alpha$  regulates genes,

encoding for proteins of biotransformation and transport proteins like OAT, OAT-K1 and OATP1. In contrast to Nrf2 and HNF4 $\alpha$ , OTA significantly increased the activity of NF $\kappa$ B in cell cultures (134, 148-150). The NF $\kappa$ B family includes six members, which can form homodimers or heterodimers, of which p50/p65 (NF $\kappa$ B1/RelA) is the predominant one. When binding to the inhibitory protein I $\kappa$ B $\alpha$ , the NF $\kappa$ B-dimer is sequestered and translocation to the nucleus is prevented. Genes targeted by NF $\kappa$ B are mainly involved in cell death and inflammation (151).

In conclusion, there is evidence, that toxic effects of OTA are centrally mediated by oxidative stress (Figure 3). Oxidative stress is increased because the cellular oxidant defence system is diminished by downregulation of gene expression on the level of the transcription factor Nrf2. In addition, the elimination of OTA is reduced due to downregulation of crucial transport proteins on the level of the transcription factor HNF4 $\alpha$ . Consequently, OTA accumulates in the proximal tubulus cells, ROS levels increase, oxidative damage like lipid peroxidation of cell membranes or damage of DNA occur. In an advanced phase, cell integrity is lost, cell proliferation, differentiation and apoptosis might be induced. This might be, in part, mediated by MAPK signaling cascades, which are activated by ROS. Finally, oxidative DNA damage might turn into mutations and result in cell transformation and tumour development after long-term exposure.

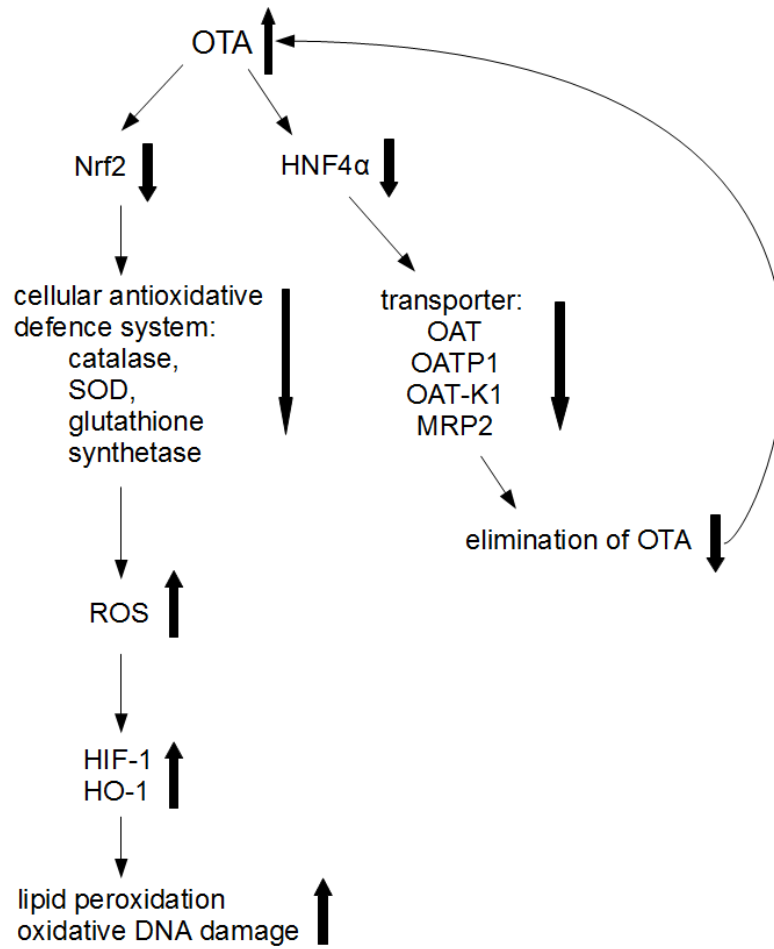


Figure 3: Schematic illustration of the correlations of OTA, its influence on gene expression and oxidative stress. ↑ increase, ↓ decrease. HIF-1, hypoxia-inducible factor 1; HNF4 $\alpha$  hepatocyte nuclear factor alpha; HO-1, heme oxygenase 1; NF $\kappa$ B nuclear factor-k B; OTA, ochratoxin A; ROS, reactive oxygen species; SOD, superoxid dismutase; Nrf2, nuclear factor-erythroid 2 p45-related.

### 3. Biological effects of the flavonol quercetin and its metabolites

Flavonoids are secondary plant metabolites which are divided into six main classes, namely flavones, flavonols, flavanones, flavanols (catechins), anthocyanidines and isoflavones (152). Quercetin is the most common flavonol, both with respect to its occurrence as well as its biological activities. In plants quercetin is extensively bound to sugar moieties. Due to glycosylation the hydrophobicity of the aglycone is increased and storage in plant cell vacuoles is enabled (153, 154). Quercetin is glycosylated with glucose, arabinose, galactose, glucorhamnose, rhamnose and xylose, as well as with glucuronic and galacturonic acid (152). The sugar moieties are bound via O- $\beta$ -glycosidic bond at the preferred position C-3, less often C-7 and seldom at the C-4' atom. Until now, about 200 different quercetin glycosides are known. They occur abundantly in onions (284-486 mg/kg) as isoquercetin (quercetin-3-O- $\beta$ -glucosid), spiraeoside (quercetin-4'- $\beta$ -glucoside), quercetin-3,4'-diglucoside, isorhamnetin-4'-glucoside and rutin (quercetin-3-O-glucorhamnoside) (155, 156). Other rich sources of quercetin are kale (110 mg/kg), french beans (32-45 mg/kg), broccoli (30 mg/kg) and apples (21-72 mg/kg) (155). In this chapter bioactivities of quercetin and quercetin metabolites will be described in more detail.

In the beginning, evidence for positive health effects of quercetin derived from epidemiological studies (157-161). According to those studies, a high flavonoid intake correlated with protective effects regarding the risk of fatal and non-fatal coronary artery diseases, the risk of lung cancer, the incidence of asthma and the impairment of pulmonary functions in chronic obstructive pulmonary disease (see reviews 1-3). Single flavonoids such as quercetin, were investigated intensively *in vivo* and *in vitro*. As the postulated common mechanism of these diseases is mainly the imbalance of production and elimination of ROS, which results in oxidative stress, the antioxidative properties of quercetin is thought to be of central importance.

#### *Antioxidative activity of quercetin and quercetin metabolites*

Several studies have shown, that quercetin is able to influence the redox status of cells. This might occur due to direct activity as radical scavenger, or indirectly, e.g. reduction of pro-oxidative metal ions, modulation of certain enzymes or regeneration of physiologic antioxidants (2, 8, 162). Quercetin may intercept electrons from reactive oxygen species (4, 5). For the ability to take part in one electron transitions the catechol (3',4'-

dihydroxy)-structure of the B-ring, the C2, C3-double bond in combination with the 4-keto-group and the hydroxyl groups in position 3, 5, and 7 are responsible (163-165). Furthermore, at least *in vitro* quercetin is able to chelate free metal ions like  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ , which either act directly pro-oxidative or catalyze pro-oxidative processes (6-8). Interactions between the hydroxyl group at the C3-atom and the 4-keto-group of the C-ring are made responsible for this chelation reactions (6). An indirect antioxidative action of quercetin is due to its ability to interact and regenerate physiologic antioxidants like vitamin E and C, at least *in vitro* (166). In vitamin E deficient rats, dietary quercetin intake (100 mg/kg diet) for 10 weeks did not significantly affect markers of lipid peroxidation, while in the same study, quercetin was very effective in reducing lipid peroxidation in hepatic microsomes (167). However, in a feeding trial with pigs, receiving a low vitamin E diet (7 mg/kg), supplementation of quercetin (10 mg/kg b.w.) ameliorated oxidative processes as shown by reduced concentrations of TBARS and 8-iso-prostaglandine  $\text{F}_{2\alpha}$  (8-iso-PGF $_{2\alpha}$ ) and exerted a moderate vitamin E sparing effect (168).

After reaching the systemic circulation, quercetin is mainly present as conjugated metabolites. Antioxidative activity of these metabolites might not be consistent with the antioxidative activity of quercetin aglycone. In general, the antioxidant activity of the aglycone is clearly reduced by its conjugation. It was shown, that conjugated quercetin metabolites inhibit  $\text{Cu}^{2+}$  induced lipid peroxidation by half the magnitude compared to the quercetin aglycone (169). Nevertheless, after adaptation to a 0.2 % quercetin diet, total antioxidant status in plasma of rats was significantly increased (170). With respect to various quercetin conjugates, it was shown, that quercetin-7-glucuronide and quercetin-3-glucuronide are more efficient as antioxidants to reduce LPL-oxidation than are isorhamnetin-3-glucuronide or quercetin-3'-sulfate (171, 172). In addition, quercetin-4'-glucuronide is a potent inhibitor of lipoxygenase (173). Methylated conjugates like isorhamnetin (3-methylquercetin), 3'-O-methyl and 4'-O-methyl quercetin showed strong antioxidant activity (174-176). Compared to numerous *in vitro* studies, only few *in vivo* studies were conducted (167, 177-180). In humans, the beneficial health effects of quercetin were confirmed, but only one study was able to show improved *in vivo* oxidative stress markers (180) and another study showed increased plasma antioxidant capacity *ex vivo* (178).

There is evidence that in addition to the above mentioned properties, quercetin's antioxidative effects are attributed to modulating gene expression. Thus, it was shown, that quercetin is able to increase the expression of the rate limiting enzyme of glutathione synthesis (181).



Glutathione is the most important endogenous cellular antioxidant and exists in the thiol-reduced (GSH) and disulfide-oxidized (GSSG) form. It acts as the main cellular redox buffer and a shift in the ratio of GSH:GSSG may influence sensitive cysteinyl residues in polypeptides (182). Glutathione is required for various cellular functions including scavenging of free radicals, reduction of peroxides or conjugation of electrophilic compounds (182, 183). It is postulated, that quercetin interacts with proteins of cellular signaling cascades and with *trans*-active elements of gene expression (transcription factors) (182). Many genes involved in endogenous antioxidant defence of cells contain the same specific DNA sequences at the 5'-flanking promoter region called *cis*-active element, which is recognized by a specific transcription factor (182). Antioxidant enzymes, such as HO-1, NADPH:quinone oxidoreductase and  $\gamma$ -glutamyl cysteine synthetase are regulated by the transcription factor Nrf2. Nrf2 belongs to the family of basic region-leucine zipper transcription factors. It binds to the *cis*-active antioxidant responsive element (ARE) or the electrophile responsive Element (EpRE) located in the promoter region of target genes (184). It was shown, that quercetin induces Nrf2 related gene expression (185-188) and it was suggested, that quercetin deblocks this transcription factor from its inhibitor protein Kelch like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1). As an underlying mechanism of these quercetin activities it was hypothesized that the pro-oxidative activity of quercetin, which was shown *in vitro* under certain conditions, might be responsible (189, 190). It is suggested, that quercetin is oxidized either enzymatically or spontaneously into a quinone, that can be reduced back to parental molecules meanwhile ROS would be generated (191). This generation of ROS by quercetin is thought to occur at a subtoxic level for cells, but to be strong enough to activate cellular defence mechanism mediated by Nrf2. Keap1 might sense ROS via thiol modification of its cysteinyl residues, which changes the conformation and releases Nrf2 from the complex. In addition, the degradation of Nrf2 by the 26S proteasome, which is supported by Keap1, is diminished and Nrf2 is stabilized (182, 184). In addition, phosphorylation of the Nrf2/Keap1 complex, triggered by kinase signaling pathways might be another possibility for the release of Nrf2 from the complex. The involved signaling pathways are considered to be MAPK, ERK, and phosphatidylinositide 3-kinases (PI3K). Their activation is required for the nuclear translocation of Nrf2 triggered by antioxidants (184). In addition, quercetin is a dietary protein kinase inhibitor (182). The inhibitory activity of quercetin is associated with its ability to compete with the binding of ATP to the nucleotide binding site of the kinases (192). It was shown *in vitro*, that quercetin reduced

phosphorylation of the kinases Akt and ERK at concentrations ranging from 10 to 30  $\mu\text{M}$  (193). Also, quercetin was able to reduce the kinase activity of PI3K to approximately 20 % of control values (194). In a study investigating the potency of 3'-O-methyl quercetin and 4'-O-methyl quercetin to protect cardiomyoblasts against  $\text{H}_2\text{O}_2$ -induced oxidative stress, quercetin aglycone but not its O-methylated forms modulated the cellular signaling pathways PI3K/Akt and ERK1/2 and counteracted  $\text{H}_2\text{O}_2$  cell damage. In this study, 3'-O-methyl quercetin and 4'-O-methyl quercetin were also able to reduce intracellular ROS production but not by modulating PI3K/Akt and ERK1/2 signaling pathways (175).

Other transcription factors modulated by quercetin resulting in antioxidant cellular defence are AP-1 and HIF-1. AP-1 is a dimeric transcription factor and belongs to the basic leucine-zipper family. The protein families Jun<sup>1</sup>, Fos<sup>2</sup>, ATF<sup>3</sup> and Maf<sup>4</sup>, again have numerous members which dimerize. The AP-1 transcription complex binds to the TPA (12-O-tetradecanoyl phorbol 13-acetate)-response element (TRE) or cyclic AMP response element. Regulating stimuli are pro-inflammatory cytokines, growth factors, oxidative stress and tumour promoters. Target genes of AP-1 are involved in cell growth and cellular stress (184). Signaling pathways, that regulate AP-1 activity are the major MAPK and PI3K pathways. Beside the ability to interact with these signaling pathways, quercetin is thought to inhibit AP-1 DNA binding, through inhibiting the JNK/stress-activated protein kinase (195-197). At low concentrations (< 50  $\mu\text{M}$ ), quercetin increased AP-1 activity in a prostate cancer cell line, but at high quercetin concentrations AP-1 was inhibited. This indicates that regulation of basal AP-1 activity by quercetin depends on its concentration (184). HIF-1 belongs to the basic helix-loop-helix protein family, too, and consists of two subunits (HIF-1 $\alpha$  and HIF-1 $\beta$ ). The subunit HIF-1 $\alpha$  is expressed constantly, but triggered to 26S proteasome for ubiquitination after hydroxylation of certain proline residues (198, 199).

If the oxygen concentration in cells decreases, HIF-1 $\alpha$  degradation is diminished due to decreased activity of the oxygen dependent prolylhydroxylases. HIF-1 $\alpha$  translocates to the nucleus and binds with HIF-1 $\beta$  to the HIF-1 responsive element. Target genes of HIF-1 are numerous and are involved in inflammation, regeneration of matrix, cell proliferation, differentiation, apoptosis, cell migration and adhesion. In *in vitro* studies, it was shown, that quercetin induced HIF-1 in a concentration and time-dependent manner (200-203).

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<sup>1</sup>jun proto-oncogene, <sup>2</sup>FBJ osteosarcoma oncogene, <sup>3</sup>activating transcription factor, <sup>4</sup>v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog

The effect of quercetin was not due to its pro-oxidative property, but rather due to the chelation of  $\text{Fe}^{3+}$  ions, which are essential cofactors for the prolylhydroxylases. As a consequence HIF-1 $\alpha$  is stabilized by quercetin and translocates to the nucleus (200-204).

#### *Effects of quercetin on CYP1A1 and CYP1A2*

Another metabolic pathway affected by quercetin is the biotransformation pathway. There are some *in vivo* (205-207) and several *in vitro* (208-215) studies indicating that quercetin modulates expression and activity of phase-I and phase-II-enzymes as well as of efflux transporter. Because the present study focuses on the phase-I-enzyme CYP1A and the efflux transporters, these will be considered in more detail subsequently.

Isoforms of CYP1A are expressed in the liver (CYP1A2) and the intestine (CYP1A1). Treatment of breast cancer cells (MCF7) with quercetin (1-20  $\mu\text{mol/L}$ ) resulted in a concentration- and time dependent increase in the amount of CYP1A1 mRNA (208). Quercetin also caused a concentration and time dependent increase in CYP1A1 enzyme activity as measured with the ethoxyresorufin-O-deethylase (EROD) activity assay (208). In Caco-2 cells, quercetin acted as an inducer of CYP1A1 expression on the mRNA as well as protein level at a high concentration of 100  $\mu\text{mol/L}$  and an incubation time of 48 h (209). Genes of the CYP enzymes contain xenobiotic responsive elements in their promoters and may be regulated in part by the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor (216). Flavonoids such as quercetin have a basic chemical structure with some poly aromatic hydrocarbons in common. Therefore, it is plausible, that quercetin interacts with cellular defence systems such as phases I detoxification enzymes (182, 217, 218). Quercetin binds directly to the AhR (208, 219) and induces gene expression of CYP1A1/2 and other proteins of the biotransformation pathway (210). The dose-dependent inducing effect of quercetin on the CYP1A1 activity, measured with the EROD assay was confirmed in Caco-2 cells (211). But in contrast to previous results quercetin did not affect the CYP1A1 mRNA concentration. The authors of that study suggested, that quercetin was not a AhR ligand in intestinal cells and that it rather acted on a posttranscriptional level. However, in yeast cells, quercetin inhibited the bioactivation of a carcinogenic heterocyclic amine (3-amino-1-methyl-5H-pyrido[4,3-b]indole) with an  $\text{IC}_{50}$  of 0.35  $\mu\text{mol/L}$  (220). In human hepatocytes (Hep G2 cells), which were treated with 10  $\mu\text{mol/L}$  benzo[a]pyrene for 18h in the presence and absence of quercetin, the decrease in CYP1A1 enzyme activity indicated that

quercetin inhibited benzo[a]pyrene induced CYP1A1 expression through blocking the AhR (212).

In a study using a perfused rat liver model, quercetin exerted an inhibiting effect on CYP1A2 activity (221). Quercetin also inhibited CYP1A2 in a recombinant CYP1A2 enzyme preparation with an IC<sub>50</sub> value of 7.5 µmol/L (213). Quercetin was not potent inducer of CYP1A2 in S9 fractions of rat liver (222). In Chinese hamster V79 cells expressing rat CYP1A2, 10 µmol/L quercetin also inhibited rat CYP1A2 (214). In transfected HepG2 cells quercetin inhibited mouse CYP1A2 and human CYP1A2, determined with the 7-methoxyresorufin-O-dealkylation (MROD) assay (215).

Taken together from the studies conducted so far, at least quercetin aglycone exerted an inducing effect on CYP1A1 in the intestinal epithelial cells by binding to the transcription factor AhR, but may act as a competitive inhibitor when co-applied with substrates of CYP1A1. In liver, quercetin aglycone inhibited CYP1A2 activity.

#### *Effects of quercetin on MRP2, BCRP and P-gp*

In numerous studies interactions of quercetin with ABC transporters, mainly P-gp, were investigated. Early studies demonstrated that quercetin efficiently inhibited the transport activity of P-gp in a dose-dependent manner (223, 224). However, some studies indicated stimulating effects of quercetin on P-gp (225). It was demonstrated, that at low concentrations (10µmol/L) quercetin decreased, while at high concentrations (50 µmol/L) it increased the steady-state accumulation of the P-gp substrate vincristine in mouse brain capillary endothelial cells (MBEC4 cells) (226). Similar effects were achieved *in vivo*. In mice, quercetin decreased vincristine concentrations in brain at a concentration of 0.1 mg/kg b.w., but significantly increased it at a concentration of 1 mg/kg b.w. (226). The explanation for this biphasic effect was, that low concentrations of quercetin indirectly stimulate P-gp by increasing P-gp phosphorylation while high concentrations of quercetin inhibit P-gp (226). However, a majority of the more recent studies have indicated that quercetin has an inhibitory activity on P-gp mediated transport. For example, co-administration of quercetin and moxidectin subcutaneously, increased moxidectin bioavailability in lambs (227). Quercetin also increased the oral bioavailability of paclitaxel in rats (207, 228) and the oral bioavailability of digoxin in pigs (229). The inhibitory effect of quercetin on P-gp is explained with interaction at different binding sites of P-gp. A biologically active ABC transporter protein contains two transmembrane domains and two ABCs or nucleotide binding

domains (230). Transmembrane domains are the substrate-binding sites and/or substrate translocation pathways through the membrane to the efflux substrates. Nucleotide binding domains are located at the inner side of the cell, participating in ATP binding and hydrolysis being the driving force for active transport (231). Inhibition of either transmembrane domains or nucleotide binding domains may cause the impairment of P-gp function. As quercetin itself is a P-gp substrate (229, 232), it was postulated that quercetin may competitively or noncompetitively interact with other substrates for substrate-binding sites of P-gp. Quercetin and rutin were shown to directly interact with the ATP-binding site and the substrate-binding site, leading to inhibition of ATP-binding and ATPase activity and consequently inhibition of P-gp function and efflux of xenobiotics (233). In addition, quercetin is capable of decreasing P-gp expression in a dose-dependent manner (234). It was also shown, that quercetin inhibited the up-regulation of P-gp protein and mRNA in response to hyperthermia involving a heat shock factor (235). A recent study confirmed these findings. At a concentration of 12  $\mu\text{mol/L}$  quercetin inhibited the mRNA synthesis and also the transport activity of P-gp in a pancreatic carcinoma cell line (236). It was suggested, that the mode of action could be the inhibition of heat shock factor DNA-binding activity (237) or downregulation of the beta-catenin signaling pathway (238).

However, regarding the interaction of quercetin with P-gp *in vivo*, it should be considered, that most of these P-gp substrates are also substrates for CYP3A. It was shown, that quercetin is also capable to inhibit CYP3A. Hence, a distinct correlation of quercetin effects on CYP3A and P-gp activity in the above-mentioned studies is difficult (239). Nevertheless, for the physiologic outcome, *in vivo* results are more predictive than *in vitro* experiments. Furthermore, the concentration of quercetin aglycone in the systemic circulation would not be high enough for significant P-gp interaction and the quercetin glucuronide and sulfate conjugates may not interact with P-gp because these metabolites are organic anions and not preferred substrates of P-gp (239). Anyway, the aglycone which may be released in the intestine from the natural glycosides, could be present in concentrations high enough to inhibit intestinal P-gp.

Quercetin also affects the efflux transporter MRP2 and BCRP. The conjugated metabolites of quercetin (glucuronides and sulfates) are MRP2 substrates (42, 240). Since these metabolites can reach a high concentration in the systemic circulation after supplementation, they could possibly inhibit MRP2 resulting in decreased biliary or renal excretion of other MRP2 substrates (239). In fact, quercetin conjugates inhibited the transport of OTA in Caco-2 cells at

a concentration of 50  $\mu\text{mol/L}$  (9). In recombinant membrane vesicles of the insect cell line Sf9, quercetin inhibited transport of the dye calcein mediated by MRP2 to 35 % (241). A mixture of quercetin metabolites (84 % 7-O-glucuronosyl quercetin and 10 % 3-O-glucuronosyl quercetin, 1 % quercetin aglycone, some other minor conjugates) exerted a strong inhibitory effect of 95 %. In contrast to the inhibiting effects reported in the above mentioned studies, quercetin induced MRP2 mRNA synthesis in Caco-2 cells at high concentrations of 50  $\mu\text{mol/L}$  and 100  $\mu\text{mol/L}$  (242), but not in hepatocytes (HepG2) (243). The gene expression of the *Abcc2*-gene encoding for MRP2 is regulated by Nrf2 (244). As elucidated above, quercetin interacts with this transcription factor, hence it might be a possible mode of action.

Quercetin can interact directly with BCRP and modulate its transport activity. It was observed that quercetin down-regulated the beta-catenin signaling pathway, which lead to a decreased expression of BCRP at the blood-brain barrier (238). It was also observed, that quercetin inhibited BCRP-mediated transport of mitoxantrone in human lung carcinoma cells (NCI-H460) at a concentration of 50  $\mu\text{mol/L}$  (245) and in epithelial breast cancer cells (MCF7) at a concentration of 30  $\mu\text{mol/L}$  (246). In contrast, quercetin induced BCRP mRNA synthesis at a concentration of 25  $\mu\text{mol/L}$  in Caco-2 cells (247). Further tests indicated, that the induced BCRP protein was functionally active. The authors suggested a regulation via AhR-dependent signaling pathways.

Taken together, quercetin mainly exerts inhibiting effects on ABC transporters via different mechanisms, namely binding to ATP-binding sites, competitive inhibition, down-regulating  $\beta$ -catenin signaling cascade. At high concentrations, however, especially in intestinal cells (Caco-2 cells) an induction of MRP2 and BCRP gene expression through the transcription factors Nrf2 and Ahr, respectively, appears to be possible.

## 4. Materials and Methods

### 4.1. Animals and treatment

#### *Experiment 1: Influence of quercetin on the toxicokinetics of OTA*

Eighteen growing male F344 Fisher rats (Charles River Lab., Sulzfeld, Germany) were acclimatized for a period of seven days to the conditions of the animal housing facility (room temperature  $22 \pm 2^\circ\text{C}$ ,  $55 \pm 5\%$  relative humidity, 12 hour dark/light cycle). During the adaptation period, a basic diet (see below) and tap water were offered for *ad libitum* intake. For the feeding trial of 6 days, the animals were randomly divided into two groups (OTA and OTA+quercetin (Q)-treated group,  $n = 9$ , each) with an average initial body weight of  $202.0 \pm 8.1$  g and  $204.0 \pm 10.1$  g, respectively. The animals were housed individually in metabolism cages. The diets were fed restrictively ( $\sim 70\%$  of *ad libitum* intake, 13 g/d) to ensure similar OTA intake in both feeding groups. Feces and urine were completely collected daily. Urine was acidified to pH 2-3 with  $10\%$  (v/v)  $\text{H}_3\text{PO}_4$  during collection. At the end of the feeding period, animals were anesthetized with isofluran (Abott GmbH, Wiesbaden, Germany) and euthanized by exsanguination. Blood was collected in EDTA-containers (Monovette®, Sarstedt, Nümbrecht, Germany) and plasma was immediately prepared by centrifugation (10 min,  $4000 \times g$ ,  $4^\circ\text{C}$ ). In addition, tissue samples of liver, kidney, *musculus quadrizeps* and brain were taken. Fecal and urinary samples, blood plasma and tissues were stored at  $-20^\circ\text{C}$  until analyses.

#### *Experiment 2: Influence of quercetin on the mechanisms of OTA-toxicity*

Forty-eight growing male F344 Fisher rats (Charles River Lab., Sulzfeld, Germany) were acclimatized for a period of seven days to the conditions of the animal housing facility (room temperature  $22 \pm 2^\circ\text{C}$ ,  $55 \pm 5\%$  relative humidity, 12 hour dark/light cycle). The basic diet and tap water were provided for *ad libitum* intake during the adaptation period.

For the experimental period of 23 days, the animals were randomly divided into four groups (control, OTA, quercetin, and (OTA+Q)-treated group,  $n = 12$ , each) with an average body weight of  $161.3 \pm 10.8$  (control),  $163.9 \pm 10.0$  (OTA),  $162.5 \pm 8.9$  g (quercetin), and  $162.2 \pm 10.3$  g (OTA+Q), respectively. The animals were housed in pairs in macrolon cages on filter paper, which was changed daily. During the trial, feed and water consumption was measured daily and feed and water was also replaced daily; body weight was measured weekly. After a period of 24 days the animals were anesthetized with isofluran (Abott GmbH,

Wiesbaden, Germany) and euthanized by decapitation. Blood samples were collected in lithium-heparin container (Monovette®, Sarstedt, Nümbrecht, Germany) and blood plasma was immediately prepared by centrifugation (10 min, 4000 x g, 4 °C). Tissue samples of liver, kidneys, *musculus quadrizeps*, brain and small intestine were taken immediately and were shock frozen in liquid nitrogen within 5 minutes to avoid RNA-degeneration. Plasma and tissue samples were stored at -80 °C until analyses.

All animal experiments were approved by the Ministry of Agriculture, the Environment and Rural Areas of Land Schleswig-Holstein, Germany (V 312-72241.12-25 (49-4/09)).

#### 4.2. Diets

The experimental diets were based on a synthetic, balanced, flavonoid-free, powdered diet for adult rats (C1000, Altromin, Lage, Germany) containing (as fed basis) 58.2 % carbohydrates, 17.6 % proteins, 5.1 % fat, 4.5 % fiber and 14.6 MJ/kg metabolisable energy. Because a high dietary vitamin E concentration might mask quercetin derived antioxidative effects, the vitamin E content of the diet was reduced to 30 mg/kg, covering the physiological demand. The experimental diets contained OTA and/or quercetin (as quercetin dihydrate, Roth GmbH & Co. KG, Frankfurt, Germany) as depicted in Table 5. As source for OTA, OTA-contaminated wheat (2.5 mg OTA/g) was used, which was produced by inoculation of wheat with *Aspergillus ochraceus* NRRL 3174 as previously described (248). The dietary concentration of OTA was calculated to achieve a daily intake of 0.6 mg/kg b.w., a dose which has been used in several previous toxicokinetic studies and was not associated with any short-term morbidity and mortality (249). The supplementation level of quercetin was chosen according to the *in vitro* studies by Sergent et al. (9) in which only ratios of quercetin:OTA higher than 5:1 caused increased OTA absorption and accumulation.

Table 5: Supplementation of the experimental diets

Supplement (g/kg)	Control	Quercetin	OTA	OTA+Q
quercetin	-	0.100	-	0.100
OTA	-	-	0.010	0.010

OTA, ochratoxin A; OTA+Q, OTA and quercetin fed group



### **4.3. Analysis of OTA and OT $\alpha$**

Because daily amounts of feces per animal were too small for analysis, feces obtained during the 6 days feeding period were pooled per animal, freeze-dried (-80°C, 25 h; Beta I, Martin Christ GmbH Co. KG, Osterode, Germany) and homogenized in a mortar before analysis. Extraction of OTA and OT $\alpha$  from blood plasma, feces, urine and tissues was performed as described previously (27). For sample preparation, diluted plasma samples (0.5 mL of plasma added to 1.5 mL of 145 mM NaCl solution), or 250 mg of freeze-dried feces (acidified to a pH of 2.3 with 6 M HCl, Sigma-Aldrich, Munich, Germany), or 0.5 mL of thawed urine samples, respectively, were mixed with 10 mL of a solution containing 0.05 M HCl and 100 mM MgCl<sub>2</sub> (Sigma-Aldrich, Munich, Germany). After the addition of 10 (plasma and urine) or 15 mL (feces) chloroform (Mallinckrodt Baker, Griesheim, Germany), respectively, the mixtures were shaken for 30 min. Samples were centrifuged at 10,000  $\times$  g for 15 min (plasma) or 30 min (feces and urine) at 2 °C. After removal of the upper water layer by suction (KNF Neuberger Laboport®, Trenton, NJ, USA), deionized water was added to the chloroform fractions (plasma and urine samples 2 mL, feces samples 3 mL). Samples were shaken for 5 min, and centrifuged at 4000  $\times$  g for 10 min at 2 °C. After removal of the water layer, 4 mL (plasma samples) or 5 mL (urine or feces samples, respectively) of the chloroform fraction were evaporated until dryness (SpeedVac AES 1010, Savant Instruments, Holbrook, NY, USA). Prior to high pressure liquid chromatography (HPLC) analysis, the samples were reconstituted in 1 mL (plasma, urine) or 2 mL (feces) of methanol (Mallinckrodt Baker, Griesheim, Germany). For extraction of OTA from tissue samples, one whole kidney (approx. 0.7 g), 1 g of liver, 1 g of muscle, or 0.7-1 g of brain were homogenized (Potter S, B. Braun Melsungen AG, Melsungen, Germany) in 5 mL of 0.1 M sodium bicarbonate solution and were added by 5 mL of 0.1 M sodium bicarbonate solution, 500  $\mu$ L of 2.25 M *ortho*-phosphoric acid (Carl Roth GmbH & Co. KG, Frankfurt, Germany) and 12 mL chloroform. The samples were shaken for 30 min and centrifuged for 30 min at 10 000  $\times$  g at 2 °C. After removing of the upper water layer, the chloroform layer was washed with 1.5 mL of deionized water and constantly shaken for 10 min before centrifugation at 4000  $\times$  g for 10 min at 2 °C. Five milliliters of the chloroform layer were evaporated until dryness. Prior to HPLC analysis, the samples were reconstituted in 1 mL of methanol. All samples were determined in double. OTA and OT $\alpha$  contents of tissues, urine and feces were determined by HPLC with fluorescence detection as described earlier (27).

The HPLC system (Waters, Eschborn, Germany) consists of an autosampler (Waters 717 plus Autosampler), gradient pump (Waters 600E Multisolute Delivery System), a fluorescence detector (Waters 474 Scanning Fluorescence Detector), a degasser (Waters In-line Degasser) and a column oven (Millipore/Waters TCM Column Oven System). A sample aliquot of 25  $\mu$ L was injected by the autosampler. The separation of ochratoxins was performed by a 250 mm  $\times$  4.6 mm (I. D.) 4  $\mu$ m Nova-Pak-C18-column (Waters) with an upstream 20 mm  $\times$  3.9 mm (I.D.) 4  $\mu$ m Nova-Pak-Precolumn (Waters). The flow rate was 1.5 mL/min with an oven temperature at 40 °C. The fluorescence detection was carried out at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. To facilitate the separation of ochratoxins, a gradient elution was used. The fluids were “solvent A” (acidified ultrapure water with H<sub>3</sub>PO<sub>4</sub> to pH 2.1) and “solvent B” (methanol/isopropanol 90/10, v/v). The following gradient was programmed: minute 0 to 12: 50 to 25 % solvent A, minute 12 to 12.1: 25 to 10 % solvent A, minute 12.1 to 17.0: 10 % solvent A, minute 17.0 to 17.1: 10 to 50 % solvent A, minute 17.1 to 22: 50% solvent A. Of each, the missing part of 100 % consisted of solvent B.

The standards were prepared according to the AOAC method 970.44 (250). After calibration of the spectrophotometer with kalium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>), OTA was solved in ethanol. Based on the molar absorption coefficient of 5500/mol/cm at 330 nm for OTA, the OTA concentration was determined spectrophotometrically. Afterwards, the standards were evaporated until dryness and methanol was added until concentrations in the range of 15.48-173.14 nmol/l were adjusted. The identification of OTA was based on the retention times of the standards. The retention time for OTA in this method was 11.5 minutes. The quantification of OTA was calculated by the integration of the area under the curve of the OTA-peak (Millenium Chromatography Software, Waters, Eschborn, Germany). The absolute value of the area under the curve was divided through the regression factor, which was determined using the calibration curve of the standards. The result was the OTA concentration of the examined sample. For each sample run (with a maximum of 42 samples) an own calibration curve was created. The minimum detectable level for both OTA and OT $\alpha$  was 1 ng/mL in plasma, urine and feces and 1 ng/g in tissue samples. Recoveries were estimated on the basis of recovery of OTA and OT $\alpha$  from spiked samples and were 95 % for plasma, urine and feces and 88 % for liver, kidney, brain and muscle. OT $\alpha$  concentrations were stated as OTA equivalents (molecular weight of OTA/molecular weight of OT $\alpha$  (403/256)  $\times$   $\mu$ g of

OTA) and total OTA concentrations were calculated as the sum of OTA and OTA equivalents.

#### ***4.4. Analysis of $\alpha$ -TOC***

Concentrations of  $\alpha$ -TOC in plasma, liver, kidney, muscle and brain were determined by HPLC with fluorescence detection (251).  $\alpha$ -TOC concentrations were quantified using authentic  $\alpha$ -TOC (Merck KGaA, Darmstadt, Germany) as an external standard. All measurements were performed in duplicate.

For precipitation of proteins, diluted plasma samples (100  $\mu$ L), or tissue samples (100 mg) were mixed with 2 mL of a 1% ethanolic ascorbic acid solution (w/v, Sigma-Aldrich GmbH, Steinheim, Germany) and added to 800  $\mu$ L (plasma) or 700  $\mu$ L (tissue) of ultrapure water (Merck KGaA, Darmstadt, Germany). For tissue samples only, 300  $\mu$ L of saturated potassium hydroxide solution (56.5 g KOH ad 50 mL) were added, the samples incubated for 30 min (70 °C) in a shaking waterbath and cooled down in an ice-bath. To all samples 50  $\mu$ L of a 0.1 % ethanolic BHT-solution was added (w/v, Merck KGaA, Darmstadt, Germany) to prevent oxidative changes.

To extract  $\alpha$ -TOC, each sample was mixed with 2 mL hexane (Merck KGaA, Darmstadt, Germany) for 30 seconds manually and centrifuged for 5 minutes (2000 x g, 4°C, 5810 R, Eppendorf, Hamburg, Germany). 1 mL of the hexane phase was removed with a pipette, transferred into an Eppendorf-cup and vaporised at room temperature until dryness (Savant SpeedVac® System AES 1010, Global Medical Instrumentation, Minnesota, USA). The residue was solved in 250  $\mu$ L of a methanol-water mix (98:2, v/v) and directly used for HPLC.

The HPLC-system used was manufactured by Jasco (Gross-Umstadt, Germany) and consisted of a pump (PU-1580), autosampler (AS-2057 Plus) and a fluorescence detector (FP-1520). 40  $\mu$ L of each prepared sample was injected into the HPLC system. The stationary phase consisted of a Waters Spherisorb ODS-2 column (100 x 4,6 mm, particle size 3  $\mu$ m, Sigma-Aldrich GmbH, Taufkirchen, Germany). The mobile phase was a methanol-water mix (98:2, v/v) and had a flow rate of 0.8 mL/min. The column was tempered at 25 °C in a column oven. The fluorescence photometric determination of the  $\alpha$ -TOC concentration occurred at an excitation wavelength of 296 nm and an emission wavelength of 325 nm. For determination of  $\alpha$ -TOC concentrations, a standard curve was calculated. A defined mass of

DL- $\alpha$ -TOC (Merck KGaA, Darmstadt, Germany) was solved in 20 mL of ethanol and successively diluted with a 0.005 % methanolic BHT-solution (w/v). Over the studied concentration range of 1.08 to 21.60  $\mu\text{g } \alpha\text{-TOC/mL}$  was a linear relationship between the concentration and the  $\alpha$ -TOC peak area (Figure 4). Analysis of chromatographic data was performed using the Borwin Chromatography Software (Version 1.5, Jasco, Gross-Umstadt, Germany).

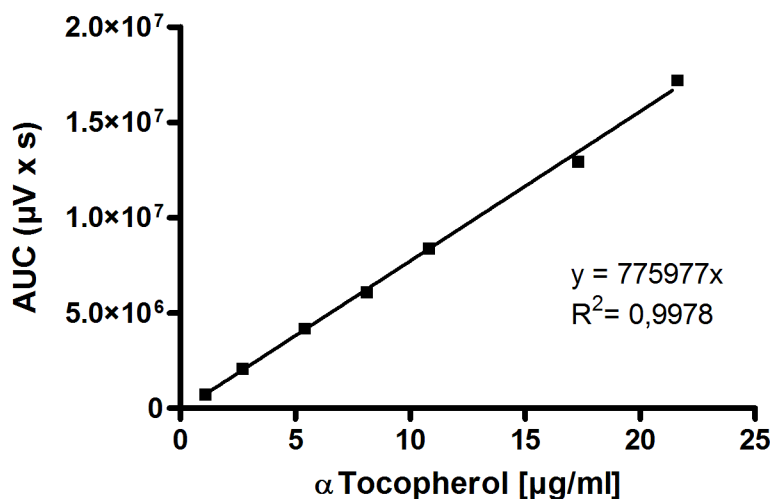


Figure 4: Exemplary standard curve for the determination of  $\alpha$ -TOC concentrations in plasma and tissues;  $R^2$ , coefficient of determination.

#### 4.5. Analysis of 8-Iso-PGF<sub>2 $\alpha$</sub>

Isoprostanes are prostaglandin-like compounds and consist of numerous isomers. 8-Iso-PGF<sub>2 $\alpha$</sub>  is an 8-epimer of prostaglandin F<sub>2 $\alpha$</sub>  and is produced *in vivo* by non-cyclooxygenase dependant mechanisms like peroxidation from arachidonic acid (252). It is used as an *in vivo*-marker of oxidative stress. *In vivo* lipid peroxidation was assessed by measuring the plasma concentrations of 8-iso-PGF<sub>2 $\alpha$</sub>  with an enzyme immuno assay (EIA, Direct 8-Iso-Prostaglandin F<sub>2 $\alpha$</sub> , Assay Designs, Michigan, USA) according to manufacturer's protocol. All measurements were performed in triplicate. This assay is based on the principal of competitive binding. 8-Iso-PGF<sub>2 $\alpha$</sub>  in the sample (or standard) competes with a defined amount of 8-Iso-PGF<sub>2 $\alpha$</sub> -phosphatase conjugates for a limited number of 8-Iso-PGF<sub>2 $\alpha$</sub> -specific binding sites of an antibody complex (which consists of primary and secondary antibodies) localised on a microtiter plate.

For detection of total 8-Iso-PGF<sub>2α</sub>-concentration in the sample, which includes free and esterified 8-Iso-PGF<sub>2α</sub> with phospholipids, 200 μL of blood plasma were incubated with 50 μL of a 10 mol/L sodium hydroxide solution at 45 °C in a water bath, added to 50 μL hydrochloric acid (12.1 mol/L) and centrifuged (4 °C, 20.800 x g). The supernatant was removed and adjusted to pH = 7 with a sodium hydroxide solution (2 mol/L). Of each sample, 50 μL were used for the EIA. The concentration range covered from the standard curve was from 160 to 1,000,000 pg/mL (Figure 5).

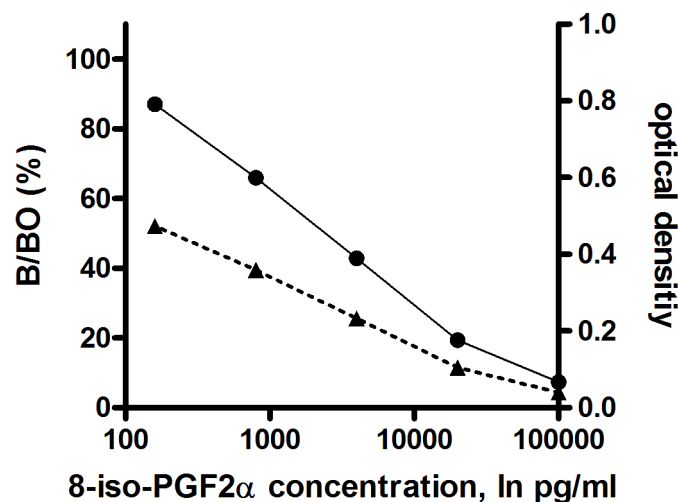


Figure 5: Exemplary Standard curve for the determination of 8-iso-PGF<sub>2α</sub> in blood plasma; bound 8-iso-PGF<sub>2α</sub> (% B/BO), ▲; optical density, ●

#### ***4.6. RNA isolation, real-time quantitative RT-PCR and calculation of relative mRNA concentrations***

Of five animals per group, frozen tissues (liver, kidney, proximal jejunum) were ground in a mill cooled with liquid nitrogen. Total RNA was isolated from tissues according to manufacturer's protocols (Qiagen, Hilden, Germany) using the RNeasy® Lipid Tissue Mini Kit for intestinal and hepatic tissue and the RNeasy® Fibrous Tissue Mini Kit for kidney tissue. For hepatic and intestinal tissues, samples of 35-40 mg were homogenized in QIAzol Lysis Reagent (1000 μL) for 15-90 s with the Ultra Turrax (Micra D1, ART Prozess- & Labortechnik GmbH & Co. KG, Müllheim, Germany). The samples were left to rest for 5 min at room temperature. 200 μL of chloroform was added and gently swung for 15 s. After 2-3 min rest period at room temperature, the homogenate was centrifuged (15 min, 4°C, 12000 x g). The upper, clear (aqueous) phase was transferred into new Eppendorf-cups (600 μL).

Ethanol (70 % v/v, 600  $\mu$ L) was added and immediately mixed with a vortex-mixer. For purification of RNA, 700  $\mu$ L homogenate was transferred on a mini-column in a 2 mL collection tube and centrifuged (15 s, 14 000 x g, room temperature). The flow-through was dropped and this step repeated. Afterwards, 350  $\mu$ L RW1 buffer was injected on the mini column and centrifuged (15 s, 14 000 x g, room temperature). The flow-through was dropped, 500  $\mu$ L RPE buffer was added to the column and again centrifuged (15 s, 14 000 g, room temperature). Again, the flow-through was dropped and the step was repeated with a longer period of centrifugation (2 min, 14000 g, room temperature). The mini column was moved onto a new collection tube and centrifuged again to remove residues of buffer from the column (1 min, 14000 x g, room temperature). To elute the RNA from the silica-gel membrane, 40  $\mu$ L RNase-free water was injected directly on the silica-gel membrane, incubated for 10 min and centrifuged (2 min, 8000 x g). To achieve a higher total RNA concentration, this step was repeated.

For kidney tissue, samples of 30  $\mu$ g were lysed in a guanidine-isothiocyanate buffer. After dilution of the lysate, the sample was treated with proteinase K. Debris was pelleted by centrifugation and 600  $\mu$ L of 70 % v/v ethanol was then added to the cleared lysate. Kidney tissue sample preparation was then processed as described for hepatic/intestine tissue, skipping the addition of chloroform. The mRNA containing eluates were stored at -70 °C.

mRNA concentrations in the samples were quantified with a spectrophotometer. The extinction of 100  $\mu$ L of defined diluted eluate was measured at 260 nm and 280 nm. The concentration calculated by the extinction at 260 nm ( $OD_{260} \times 40 = \text{mRNA concentration } [\mu\text{g/mL}]$ ) was in the range of 3 to 7  $\mu\text{g mRNA}/\mu\text{L}$  with an average of  $4.4 \pm 1.0 \mu\text{g}/\mu\text{L}$ . The purity of RNA isolation was checked by the ratio  $OD_{260\text{nm}}/OD_{280\text{nm}}$ . The ideal ratio for RNA isolation is 2. Lower ratios indicate a contamination with proteins and phenols. A ratio of 1,7 was accepted but samples with lower ratios were dismissed.

The integrity of mRNA was checked using an Agilent 2100 bioanalyzer with the RNA6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, USA). This analytical method is based on the principal of electrophoresis. 1  $\mu$ L of diluted sample, containing about 250 ng RNA were injected into the wells of a RNA nano chip (Eukaryote Total RNA Nano, Agilent Technologies, Santa Clara, USA) after a gel-dye mix was transferred into the wells. The RNA was separated electrophoretically with long fragments moving slower than small fragments. The Agilent 2100 bioanalyzer assigns a RNA integrity number (RIN) based on the detected fluorescence signals and allows an estimation of the integrity and degradation of a

RNA sample. A RIN of "10" stands for a perfect RNA sample without any degradation products, whereas "1" marks a completely degraded sample. The labels in-between are used to indicate progressing degradation states of the RNA sample. In our study, samples with a RIN of at least 8 were used for further mRNA concentration analyses.

For determination of relative mRNA concentrations, One-step real-time quantitative RT-PCR with SYBR green detection was performed using the iCycler device (BIO-RAD, Munich, Germany). All reagents necessary including validated primer sets were purchased from Qiagen (Hilden, Germany, Table 6). Reaction parameters were: 30 min at 50 °C (reverse transcription), 15 min at 95 °C (hold, 3-step cycling), 15 s at 94 °C (denaturation), 30 s at 55 °C (annealing), and 30 s at 72 °C (extension, 40 times). All measurements were performed in duplicate. PCR data was processed with the iCycler software which specified the threshold cycles ( $C_T$ -values). The  $C_T$ -value is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The  $\Delta\Delta C_T$ -method was used to calculate the relative changes of mRNA concentrations (fold change = FC) of the genes of interest (GOI) compared to the housekeeping gene (HKG) according to the following formula (253):

$$FC = \frac{(E_{GOI})^{\Delta C_{T_{GOI(control-treated)}}}}{(E_{HKG})^{\Delta C_{T_{HKG(control-treated)}}}}$$

$C_T$ : threshold cycle;  
 FC: fold change;  
 E: efficiency of PCR reaction;  
 GOI: gene of interest;  
 HKG: housekeeping gene

For controls, the dividend and divisor are the same for each sample, resulting in a ratio = 1 and consequently no standard deviation occurs. Thus, these values are intended to set a point of reference. Genes were considered up- or downregulated if FC was  $\geq 1.3$  and  $\leq 0.7$ , respectively. For all GOI analyzed in liver, 18S-rRNA, which is extensively expressed, was used for normalization as HKG. For intestine and kidney, a HKG expressed at low mRNA concentrations was necessary to achieve comparability to our GOI. Commonly used reference genes with low expression in studies with rats are *Alas1*, *Gusb*, *Tbp*, *Tubb4* and *Hprt1* of which only the latter being expressed in rat intestine. *Hprt1* (hypoxanthine-guanine

phosphoribosyl-transferase) was used in one study involving OTA-treatment (254) and in studies involving quercetin- treatment (255, 256). Therefore, we decided to use Hprt1 as HKG in the tissues kidney and intestine.

Table 6: Specification of primers used for qRT-PCR, provided by Qiagen

<b>Title of gene product</b>	<b>Gene symbol (<i>rattus norvegicus</i>)</b>	<b>Product name</b>	<b>Catalogue number</b>
MRP2	Abcc2	Rn_Abcc2_3_SG QuantiTect Primer Assay	QT00365722
p-gp	Abcb1b	Rn_Abcb1b_1_SG QuantiTect Primer Assay	QT00194719
BCRP	Abcg2	Rn_Abcg2_1_SG QuantiTect Primer Assay	QT00183127
Hprt1	Hprt1	Rn_Hprt1_2_SG QuantiTect Primer Assay	QT00365722
SOD	Sod1	Rn_Sod1_1_SG QuantiTect Primer Assay	QT00174888
HO-1	Hmox1	Rn_Hmox1_1_SG QuantiTect Primer Assay	QT00175994
CYP1A1	Cyp1a1	Rn_Cyp1a1_1_SG QuantiTect Primer Assay	QT01082018
CYP1A2	Cyp1a2	Rn_Cyp1a2_2_SG QuantiTect Primer Assay	QT01082011
18sRNA	Rn18s	Rn_Rnr1_1_SG QuantiTect Primer Assay	QT00199374

#### **4.7. Statistical analysis**

For statistical analysis of the first experiment data were processed with a three-factorial (OTA, quercetin, time) analysis of variance (ANOVA) and Tukey's multiple comparison test for the parameter daily OTA-concentrations in urine, using SAS statistical analysis software (version 9.2, Institute Inc., Cary, NC, USA) and for all other parameters (feed and water consumption, body weight gain, OTA concentrations in tissues and feces) by Student's t-test with Graphpad Prism 4 (version 4.01, GraphPad Software, Inc., La Jolla, CA, USA).

For the second experiment, data of feed consumption and body weight gain were performed with a three-factorial (OTA, quercetin, time) ANOVA and Tukey's multiple comparison test using SAS. Data of  $\alpha$ -TOC and 8-iso-PGF<sub>2 $\alpha$</sub>  analysis were processed with a two-factorial (OTA, quercetin) ANOVA and relative mRNA concentrations (FC) with one-factorial (treatment) ANOVA and Tukey's multiple comparison test using Graphpad Prism 4 (Version 4.01, GraphPad Software, Inc., La Jolla, CA, USA). Differences between means were considered significant at  $p < 0.05$ .



## 5. Results

### 5.1. Influence of quercetin on toxicokinetics of OTA

All animals appeared to be in good health during the course of the first experiment. Macroscopic observation at autopsy showed no obvious abnormalities. Table 7 depicts average daily feed and water consumption and body weight gain of the animals during the experiment.

Table 7: Average daily feed and water consumption, and body weight gain of rats fed diets containing 10 mg/kg diet ochratoxin A (OTA) only, and 100 mg/kg diet quercetin (OTA+Q) additionally, over an experimental period of 6 days. Values are means  $\pm$  SD (n = 9).

	OTA	OTA+Q
Feed consumption (g)	12.7 $\pm$ 0.2	12.5 $\pm$ 0.4
OTA consumption ( $\mu$ g)	137.1 $\pm$ 2.2	130.0 $\pm$ 4.2
Water consumption (mL)	13.8 $\pm$ 1.0	13.3 $\pm$ 0.9
Body weight gain (g)	1.9 $\pm$ 0.5	1.7 $\pm$ 0.7

Because feed was provided restrictively, daily feed consumption did not differ significantly during the 6 days in both feeding groups (Figure 6A). Average daily water consumption slightly increased during the experiment in both groups equally from 12 to 14 mL ( $p < 0.05$ , Figure 6B).

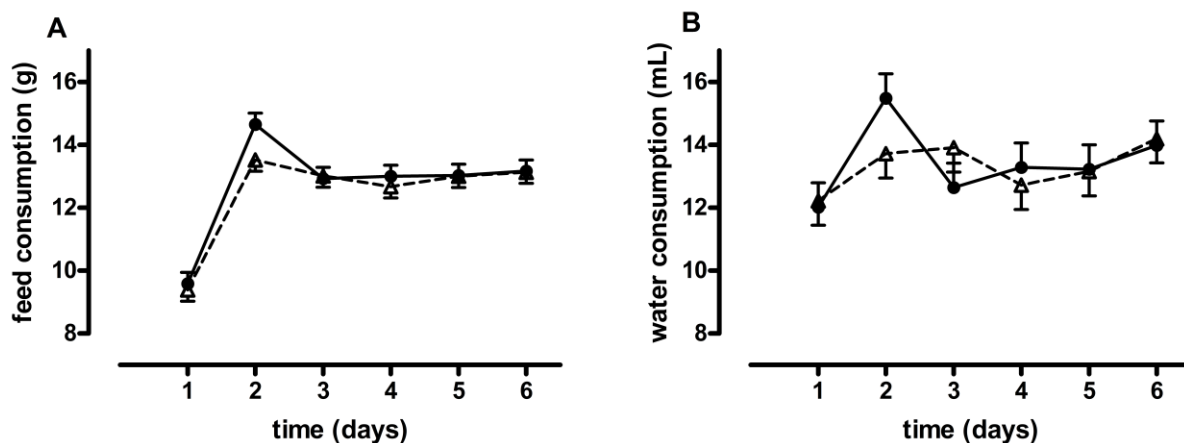


Figure 6: Daily feed (A) and water (B) consumption of rats fed diets containing 10 mg/kg diet ochratoxin A (OTA ●) only, and 100 mg/kg diet quercetin (OTA+Q  $\Delta$ ) additionally, over an experimental period of 6 days. Values are means  $\pm$  SEM (n = 9).

Plasma and tissue samples were analyzed for their total OTA concentrations, calculated as the sum of OTA and OT $\alpha$  equivalents (Table 8). The highest concentration was measured in plasma and in descending order in kidney, liver > muscle > brain. With the exception of blood plasma, which contained 0.2 % of total OTA as OT $\alpha$ , no OT $\alpha$  was detected in the other tissues investigated.

Table 8: Total OTA concentrations in plasma and tissues (expressed as sum of OTA and OTA-equivalents of OT $\alpha$ ) of rats fed diets containing 10 mg/kg diet ochratoxin A (OTA) only, and 100 mg/kg diet quercetin (OTA+Q) additionally, over an experimental period of 6 days. Values are means  $\pm$  SD (n = 9).

	<b>OTA</b>	<b>OTA+Q</b>
Plasma [ $\mu\text{g/mL}$ ]	11.27 $\pm$ 2.07	10.91 $\pm$ 2.38
Kidney [ $\mu\text{g/g}$ ]	1.44 $\pm$ 0.22	1.42 $\pm$ 0.15
Liver [ $\mu\text{g/g}$ ]	1.12 $\pm$ 0.31	1.94 $\pm$ 0.14
Muscle [ $\mu\text{g/g}$ ]	0.48 $\pm$ 0.09	0.53 $\pm$ 0.07
Brain [ $\mu\text{g/g}$ ]	0.13 $\pm$ 0.02	0.14 $\pm$ 0.02

Excretion of OTA in urine and feces are shown in Table 9. Daily excretion of OTA and OT $\alpha$  via urine increased during the experiment (Figure 7). Total OTA excreted via urine was mainly in the form of OT $\alpha$  (93 %) with only a small part (6-7 %) appearing in its unmetabolized form. There was no significant difference between the feeding groups. Daily excretion of total OTA via feces could not be calculated on an individual base, because fecal samples obtained during the experiment were too small. Hence, daily samples were pooled for each animal over the experimental period of 6 days. Like in urine, also in feces OT $\alpha$  accounted for 94 % of total OTA excreted, the remaining part being excreted as unchanged OTA. Again, no significant differences between the groups were obtained. Based on the sum of urinary and fecal excretion of total OTA, a retention of the mycotoxin within the body was calculated as 70 % of OTA intake. Here, again, no significant differences were found between the control and quercetin treated group.

Table 9: Excretion of OTA, OT $\alpha$  (expressed as OTA-equivalents) and total OTA in urine and feces of rats fed diets containing 10 mg/kg diet ochratoxin A (OTA  $\square$ ) only, and 100 mg/kg diet quercetin (OTA+Q  $\blacktriangle$ ) additionally, after an experimental period of 6 days expressed in percent of the consumed amount. Values are means  $\pm$  SD (n = 9).

	OTA	OTA+Q
	<b>Urine</b>	
OTA	1.1 $\pm$ 0.2	1.0 $\pm$ 0.1
OT $\alpha$	15.7 $\pm$ 1.7	14.4 $\pm$ 2.4
Total OTA	16.8 $\pm$ 1.8	15.4 $\pm$ 2.5
	<b>Feces</b>	
OTA	1.1 $\pm$ 0.4	0.9 $\pm$ 0.2
OT $\alpha$	13.3 $\pm$ 1.4	14.0 $\pm$ 4.2
Total OTA	14.2 $\pm$ 1.5	15.0 $\pm$ 4.4
Sum of total OTA urine + feces	30.0 $\pm$ 1.5	29.5 $\pm$ 5.1

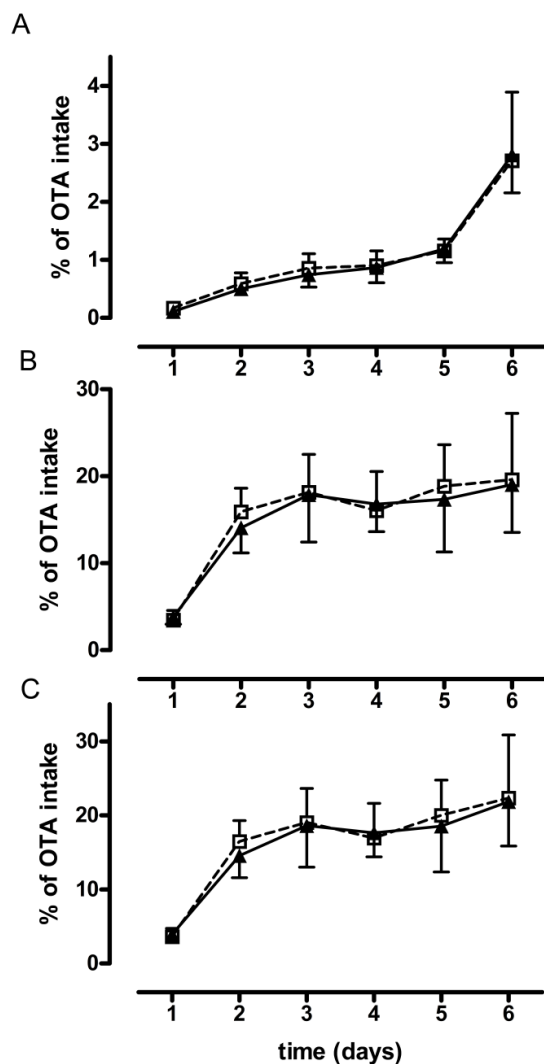


Figure 7: Daily excretion of OTA (A), OT $\alpha$  (B, expressed as OTA-equivalents) and the sum of both (C, total OTA) in total urine volume of rats fed diets containing 10 mg/kg diet ochratoxin A (OTA  $\square$ ) only, and 100 mg/kg diet quercetin (OTA+Q  $\blacktriangle$ ) additionally, depicted as % of daily oral OTA-intake over an experimental period of 6 days. Values are means  $\pm$  SD (n = 9).

## ***5.2. Influence of quercetin and OTA on feed consumption and body weight***

During the second experiment, feed consumption decreased significantly in the groups fed OTA (Table 10 and 11). This was already obvious after the first 7 days, where feed intake was reduced by about 20 % in both, the OTA and OTA+Q treated groups compared to controls. After 21 days, suppression of feed intake was even more pronounced compared to controls at the same time (26 and 27 % for OTA and OTA+Q treated group, respectively). At the end of the trial (days 22-24), average feed intake was significantly reduced by 8 % in the quercetin treated group, by 43% in the OTA treated group, and by 54 % in the OTA+Q treated group, compared to controls.

Like feed consumption, body weight gain of animals fed OTA and OTA+Q was dramatically reduced during the trial. Because in the OTA+Q treated group, even body weight loss was observed in some animals during the last days, this experiment was terminated ahead of schedule. At the end of the trial, the average bodyweight compared to the initial ones were +18 %, +17 %, +5 % and +4 % for the groups control, quercetin, OTA and OTA+Q, respectively.

Table 10: Average feed intake and body weight gain of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means  $\pm$  SD (n = 12). Values differ significantly (p < 0.05, ANOVA), if small letters differ within a row or capital letters within a column, respectively.

Time (days)	Control	Quercetin	OTA	OTA+Q
<b>Feed intake (g)</b>				
1- 7	16.5 $\pm$ 1.2 <sup>a,A</sup>	16.8 $\pm$ 1.1 <sup>a,A</sup>	13.3 $\pm$ 0.6 <sup>b,A</sup>	13.2 $\pm$ 0.6 <sup>b,A</sup>
8-14	15.8 $\pm$ 0.4 <sup>a,A</sup>	16.3 $\pm$ 0.8 <sup>a,A</sup>	12.7 $\pm$ 0.8 <sup>b,A</sup>	12.9 $\pm$ 1.1 <sup>b,A</sup>
15-21	15.6 $\pm$ 1.4 <sup>a,A</sup>	15.8 $\pm$ 1.2 <sup>a,A</sup>	11.5 $\pm$ 0.6 <sup>b,B</sup>	11.4 $\pm$ 1.4 <sup>b,B</sup>
22-24	16.5 $\pm$ 1.4 <sup>a,B</sup>	15.2 $\pm$ 2.0 <sup>b,B</sup>	9.5 $\pm$ 1.4 <sup>c,C</sup>	7.6 $\pm$ 1.2 <sup>d,C</sup>
<b>Body weight gain (g)</b>				
1- 7	4.1 $\pm$ 0.6 <sup>a,A</sup>	4.3 $\pm$ 0.8 <sup>a,A</sup>	2.2 $\pm$ 0.4 <sup>b,A</sup>	2.3 $\pm$ 0.8 <sup>b,A</sup>
8-14	3.2 $\pm$ 0.4 <sup>a,B</sup>	3.1 $\pm$ 0.9 <sup>a,B</sup>	1.0 $\pm$ 0.5 <sup>b,B</sup>	0.8 $\pm$ 0.8 <sup>b,B</sup>
15-21	2.6 $\pm$ 0.7 <sup>a,B</sup>	2.6 $\pm$ 0.6 <sup>a,C</sup>	0.1 $\pm$ 0.7 <sup>b,C</sup>	-0.2 $\pm$ 0.7 <sup>b,C</sup>
22-24	3.1 $\pm$ 0.8 <sup>a,B</sup>	2.1 $\pm$ 0.9 <sup>b,B</sup>	-2.3 $\pm$ 2.8 <sup>c,D</sup>	-2.5 $\pm$ 2.9 <sup>c,D</sup>

Table 11: P-values of the factors ochratoxin A (OTA), quercetin, time and their interaction effects, explaining the share of variances for average feed intake and body weight gain of rats fed a basic diet, solely or containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin or both over a period of 24 days.

	OTA	quercetin	time	OTA x quercetin	OTA x time	Q x time	OTA x quercetin x time
Feed intake	< 0.0001	0.6056	< 0.0001	0.3395	< 0.0001	0.3063	0.9446
Body weight gain	< 0.0001	0.2794	< 0.0001	0.8747	< 0.0001	0.4935	0.6017

### 5.3. Influence of quercetin and OTA on oxidative parameters

To determine possible differences with respect to the oxidative status between the feeding groups, concentrations of 8-iso-PGF<sub>2α</sub> in plasma and α-TOC in plasma, liver, kidney, *musculus quadriceps* and brain were measured.

Regarding plasma concentrations of 8-iso-PGF<sub>2α</sub> no significant differences were found between the feeding groups (Figure 8).

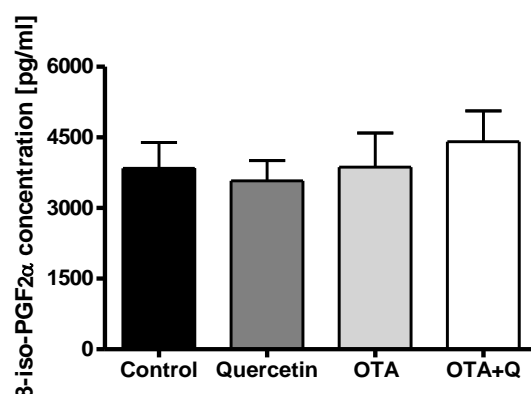


Figure 8: Concentrations of 8-iso-PGF<sub>2α</sub> [pg/mL] in blood plasma of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means ± SEM (n = 12), p > 0.5, ANOVA.

Analysis of α-TOC concentrations in plasma revealed significantly diminished concentrations in the groups OTA and OTA+Q down to 55 % and 44 %, respectively, compared to controls (Table 12). Because feed intake of animals in these groups OTA and OTA+Q was significantly reduced (see above), a regression analysis between the variables “feed intake” and “plasma α-TOC concentration” was conducted (Figure 9). Plasma α-TOC concentration was highly correlated with feed intake ( $r^2 = 0.77$ ,  $p < 0.0001$ ).

There were no significant differences in α-TOC concentrations in liver. In kidneys, the α-TOC concentrations in the OTA- and OTA+Q-treated group were significantly elevated by 47 % and 65 %, respectively, compared to controls. In muscle, α-TOC concentrations were significantly increased in the groups quercetin (+87 %) and OTA (+80 %), whereas it was not significant in the OTA+Q-treated group (+51 %), compared to controls. In brain, quercetin supplementation lead to a 17 % higher α-TOC concentration compared to controls, which was decreased down to 8 % by the combination of OTA with quercetin.

Table 12:  $\alpha$ -tocopherol concentrations in tissues of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means  $\pm$  SD (n = 12). Means are significantly different, if letters differ in a row (ANOVA,  $p < 0.05$ ) and means with two letters are not significantly different to means with the correspondent ones.

	Treatment <sup>1</sup>				Effect <sup>2</sup>		
	Control	Quercetin	OTA	OTA+Q	OTA	quercetin	OTA x quercetin
Plasma	14.4 $\pm$ 1.4 <sup>a</sup>	13.0 $\pm$ 2.5 <sup>a</sup>	7.9 $\pm$ 3.9 <sup>b</sup>	6.3 $\pm$ 1.9 <sup>b</sup>	< 0.0001	0.0529	0.8372
Liver	89.7 $\pm$ 36.2 <sup>a</sup>	86.4 $\pm$ 64.7 <sup>a</sup>	95.5 $\pm$ 57.4 <sup>a</sup>	91.1 $\pm$ 34.4 <sup>a</sup>	0.7909	0.7203	0.9675
Kidney	80.9 $\pm$ 26.4 <sup>a</sup>	90.3 $\pm$ 19.9 <sup>a,b</sup>	118.5 $\pm$ 35.3 <sup>b,c</sup>	133.2 $\pm$ 17.8 <sup>c</sup>	< 0.0001	0.1154	0.7279
Muscle	31.6 $\pm$ 13.8 <sup>a</sup>	59.0 $\pm$ 20.4 <sup>b</sup>	56.9 $\pm$ 9.6 <sup>b</sup>	47.8 $\pm$ 24.6 <sup>a,b</sup>	0.1840	0.0868	0.0011
Brain	170.1 $\pm$ 9.5 <sup>a</sup>	199.3 $\pm$ 12.8 <sup>b</sup>	175.6 $\pm$ 17.1 <sup>a</sup>	182.9 $\pm$ 17.3 <sup>a</sup>	0.2036	< 0.0001	0.0126

<sup>1</sup> concentrations in [ $\mu\text{g/mL}$ ] for plasma and [ $\text{ng/mg}$ ] for the other tissues, respectively.

<sup>2</sup> p-value

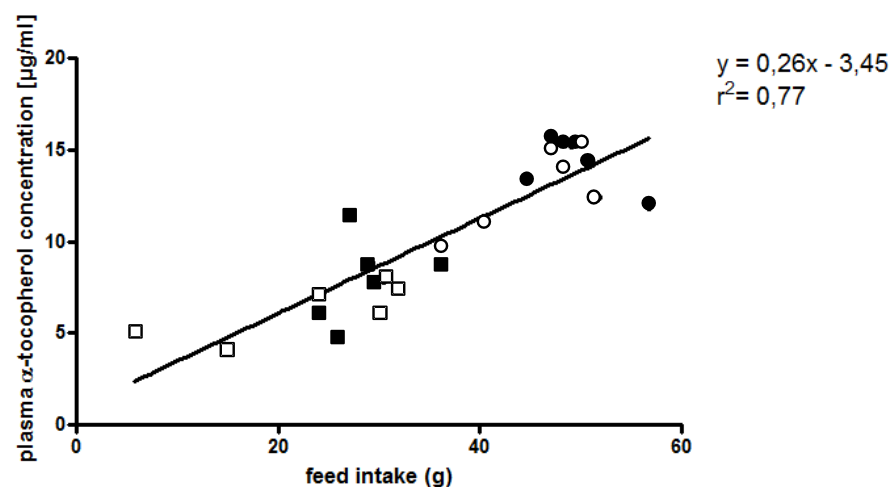


Figure 9: Correlation of feed intake and  $\alpha$ -TOC concentration in plasma of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means  $\pm$  SD (n = 12).  $\bullet$  control,  $\circ$  quercetin,  $\blacksquare$  OTA,  $\square$  OTA+Q;  $r^2$ , coefficient of determination,  $p < 0.0001$ , regression analysis.

To characterize the oxidative status of the animals, relative mRNA concentrations of HO-1 (gene *Hmox1*) and Cu<sup>2+</sup>/Zn<sup>2+</sup>-SOD (gene *Sod1*) were measured in liver and kidney. The concentrations of HO-1 mRNA in liver and of SOD mRNA in kidney were below the detection limit. However, SOD mRNA was detected in liver and HO-1 in kidney tissue (Figure 10). In liver, relative SOD mRNA concentration was significantly higher in all treatment groups (FC = 1.3, 1.4 and 1.6 for quercetin, OTA and OTA+Q, respectively) compared to the control. In kidney, the relative HO-1 mRNA concentration was significantly higher in OTA (FC = 2.4) and OTA+Q (FC = 1.7) compared to controls. A significant difference was also found between the groups OTA and OTA+Q (FC = 1.7). Relative HO-1 mRNA concentrations of OTA+Q were not significantly different compared to the group quercetin (FC = 1.4).

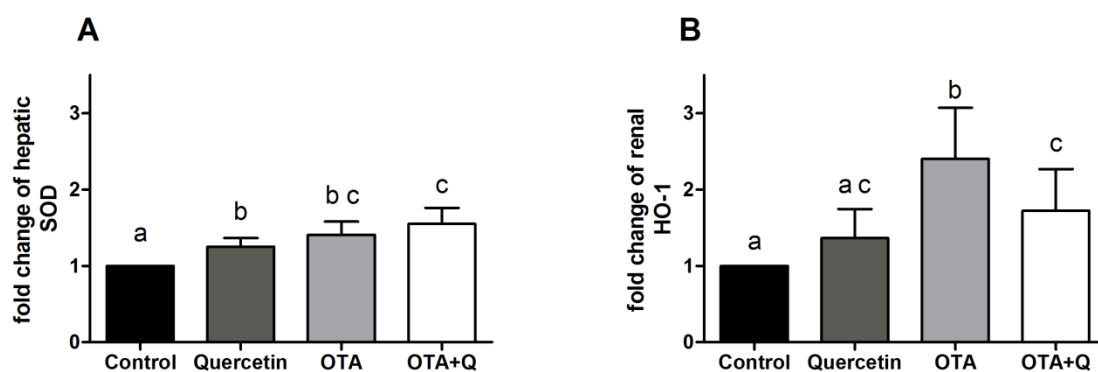


Figure 10: Relative mRNA concentrations depicted as fold change of hepatic SOD (A) and kidney HO-1 (B) of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means  $\pm$  SD (n = 5) with  $1 \pm 0$  for control. Significant differences were tested with ANOVA,  $p < 0.5$ .



#### 5.4. Influence of quercetin and OTA on relative mRNA concentrations of MRP2, BCRP, P-gp, CYP1A1 and CYP1A2

The relative mRNA concentrations of the efflux transporters MRP2 (gene *Abcc2*), BCRP (gene *Abcg2*) and P-gp (gene *Abcb1b*) were investigated in small intestine (proximal jejunum), liver and kidney. Relative mRNA concentrations of the monooxygenases CYP1A1 (gene *Cyp1a1*) and CYP1A2 (gene *Cyp1a2*) were examined in small intestine and liver, only. In intestinal tissue, P-gp and CYP1A2 mRNA were not detectable. BCRP, MRP2 and CYP1A1 mRNA concentrations were transcribed on a detectable level. Group comparisons were conducted and are shown in Figure 11. Compared to controls, relative mRNA concentrations of MRP2 and BCRP were significantly higher in the groups OTA+Q (FC = 2.2) and OTA/OTA+Q (FC = 1.6 and 1.8), respectively. No significant differences between the groups were found for CYP1A1.

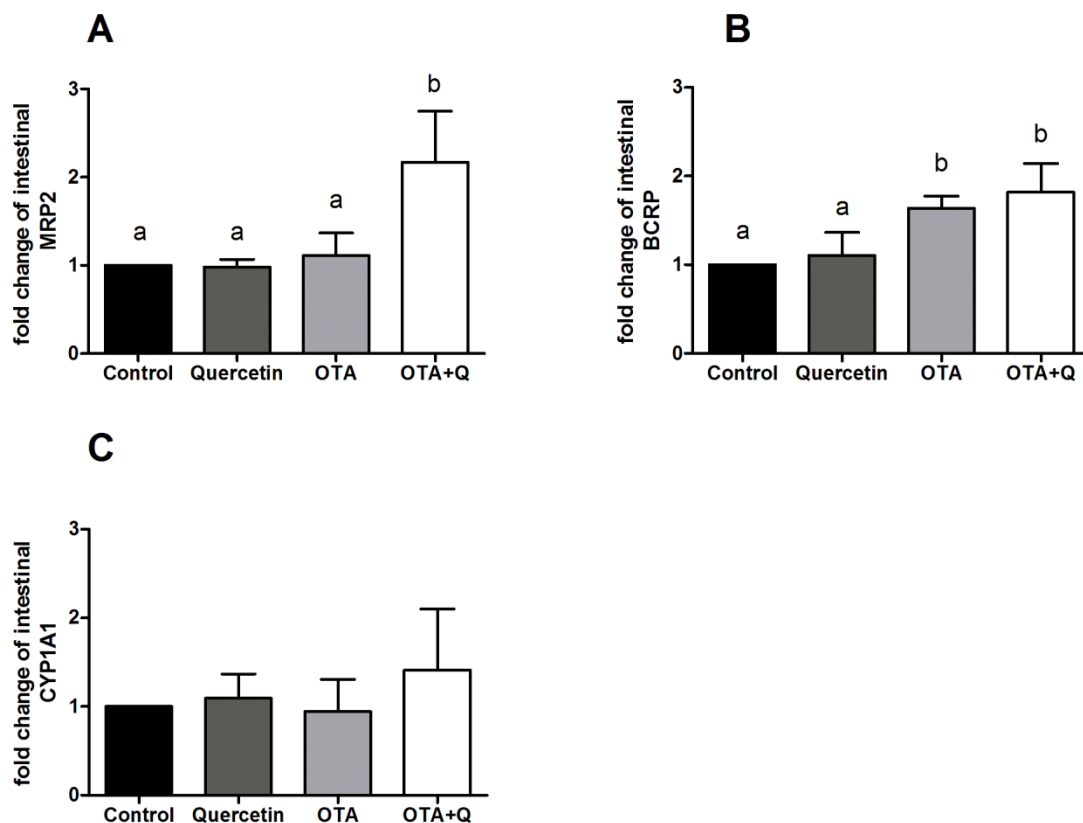


Figure 11: Relative mRNA concentrations depicted as fold change of MRP2 (A), BCRP (B) and CYP1A1 (C) in proximal jejunum of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means  $\pm$  SD (n = 5) with  $1 \pm 0$  for control. Significant differences were tested with ANOVA,  $p < 0.5$ .

In liver samples, CYP1A1, P-gp and BCRP mRNA were not detectable, whereas MRP2 and CYP1A2 mRNA were detectable (Figure 12). Relative mRNA concentrations of MRP2 and CYP1A2 differed statistically significant in the quercetin-treated group (FC = 1.1) and OTA as well as OTA+Q group (FC = 1.1 for both groups), respectively, compared to controls.

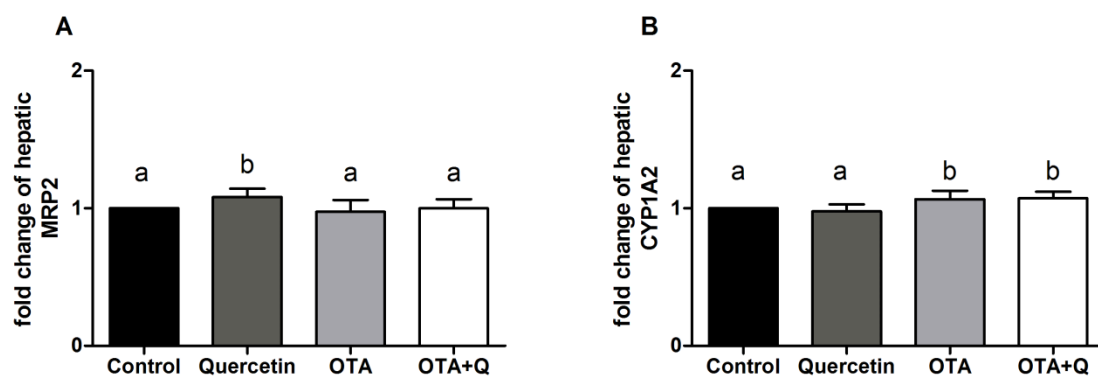


Figure 12: Relative mRNA concentrations depicted as fold change of MRP2 (A) and CYP1A2 (B) in liver of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means (n = 5) with  $1 \pm 0$  for control. Significant differences were tested with ANOVA,  $p < 0.5$ .

In kidneys, MRP2 and BCRP mRNA were detectable in all groups (Figure 13). In all three treatment groups relative MRP2 mRNA concentrations were significantly decreased compared to controls (FC = 0.8, 0.5 and 0.3 for quercetin, OTA and OTA+Q, respectively). The relative BCRP mRNA concentration was decreased in OTA+Q treated animals only (FC = 0.6), compared to controls. P-gp mRNA was barely expressed at detection level in kidney of controls ( $C_T$  29.9) and quercetin fed animals ( $C_T$  30.4). In the groups OTA and OTA+Q  $C_T$ -values were markedly earlier ( $C_T$  25.8 and 24.9, respectively). Hence, relative mRNA concentrations of P-gp increased in the groups OTA (FC = 2) and OTA+Q (FC = 1.6), compared to the control group. However, results of relative mRNA concentrations of MRP2 and BCRP should be interpreted carefully, because the HKG Hprt1 also differed with treatment to different extent.

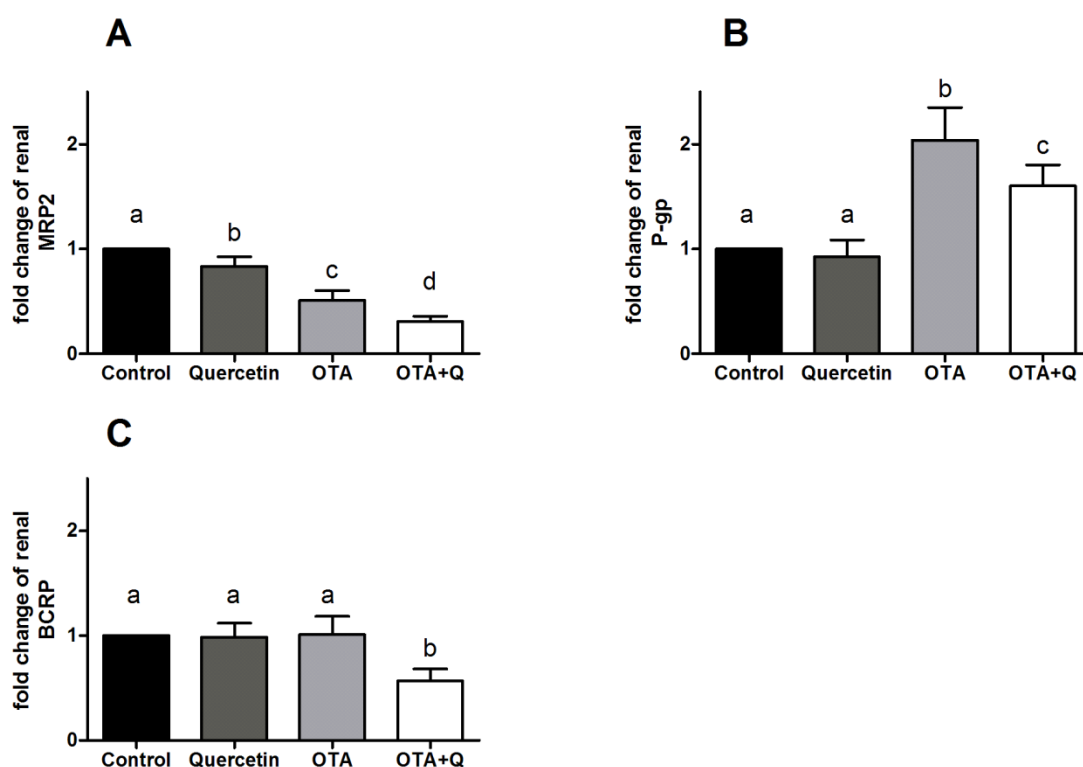


Figure 13: Relative mRNA concentrations depicted as fold change of MRP2 (A), P-gp (B) and BCRP (C) in kidney of rats fed a basic diet (control) containing either 10 mg/kg diet ochratoxin A (OTA) or 100 mg/kg diet quercetin (Quercetin), or both (OTA+Q) over a period of 24 days. Values are means (n = 5) with  $1 \pm 0$  for control. Significant differences were tested with ANOVA,  $p < 0.5$ .

## 6. Discussion

The aims of our study were to investigate the influence of the plant-derived flavonol quercetin on OTA toxicokinetics as well as toxicodynamics. This is based on the facts, that on the one hand quercetin may influence mechanisms of OTA metabolism and transport and thus may influence OTA toxicokinetics. With respect to the toxicodynamics of OTA, on the other hand, pro-oxidative mechanisms are discussed and major biological activities of quercetin are thought to be attributable to quercetin's antioxidative properties.

In the first experiment, we examined quercetin's influence on OTA toxicokinetics *in vivo*, because it was previously shown, that the co-application of polyphenols and OTA reduced the OTA efflux transport across Caco-2 cell membranes and consequently increased the intracellular OTA concentration. It was hypothesized, that *in vivo* competitive inhibition of efflux transporter by polyphenols may increase intracellular OTA concentrations in the intestinal epithelial cells and thus driving OTA absorption into the blood across the basolateral membrane, increasing the systemic bioavailability of OTA (9). It was also demonstrated *in vitro*, that net absorption of OTA into Caco-2 cells was limited due to resecretion of OTA via MRP2 and BCRP (37, 45, 88). Hence, changes in the activity of these efflux transporters by inhibiting or promoting agents might influence intestinal OTA absorption.

On the one hand polyphenols like quercetin might inhibit OTA resecretion by intestinal epithelial cells and in turn might increase systemic availability of OTA. Thus, it was shown that quercetin had an inhibitory effect on the activity of BCRP and P-gp *in vitro* (207, 245, 246). On the other hand, substances promoting the activity of efflux transporters could reduce the systemic availability of OTA by stimulation of resecretion of the mycotoxin. To this regard, , quercetin induced the expression of MRP2, BCRP and P-gp. In Caco-2 cells (242, 247, 257). Obviously, those *in vitro* studies appears to be contrarious, and only *in vivo* studies are helpful to define the overall effect and its physiological importance. In rats, MRP2, BCRP and P-gp are expressed at the apical membrane of intestinal epithelial cells, hepatocytes and renal proximal tubulus cells and hence these organs may be primary sites of interaction between OTA and quercetin. If quercetin affects either of these OTA-relevant transporters, tissue distribution and excretion of OTA and OT $\alpha$  should be affected. In addition, we hypothesized that intestinal CYP1A1 and hepatic CYP1A2 detoxify OTA by hydroxylation to OH-OTA (63, 64), which is rapidly eliminated, and stimulation of these enzymes by quercetin would contribute to a decrease of OTA concentration in plasma and tissues.

Results of our first experiment revealed, that about 30 % of the orally fed OTA was excreted. The remaining 70 % of OTA were absorbed and distributed into the tissues, primarily kidneys and liver, where the highest concentrations of OTA were found aside from plasma. Tissue distribution and concentrations of OTA as reported in this work are in good agreement with earlier findings (56, 64). Quercetin treatment, however, did not affect concentrations and tissue distribution of OTA. In addition, no significant differences in OTA and OT $\alpha$  excretion were found between the OTA and OTA+Q treated group. Thus, the hypothesis, that co-administration of quercetin and OTA may delay OTA excretion due to mutual inhibition of both substances could not be substantiated by our findings. The hypothesis, that quercetin decreases OTA concentrations in plasma and tissues by stimulating CYPs and thereby increasing OTA hydroxylation and elimination, could also not be substantiated by our results. Intestinal CYP1A1 and hepatic CYP1A2 relative mRNA concentration analyses revealed no detectable expression of CYP1A1 and only a 1.1-fold change of CYP1A2, which is not considered as an upregulation. However, regarding OTA toxicokinetics we can not exclude that an enhanced expression of cellular exporters like BCRP, MRP2 or P-gp by quercetin, which has been shown in cell culture studies (226, 242, 247) could have overcome a competitive inhibition between OTA and quercetin or more likely, quercetin conjugates, resulting in a zero net effect. To verify this, relative mRNA concentrations of the efflux transporters MRP2, BCRP und P-gp after oral uptake of quercetin and OTA were examined in our second study. Intestinal BCRP was upregulated 1.6 and 1.8-fold compared to controls in the groups OTA and OTA+Q, respectively, pointing to the possibility that OTA might be resecreted across the apical membrane mainly by BCRP. Only when quercetin was co-applied with OTA, intestinal MRP2 was upregulated 2.2-fold (OTA+Q), compared to controls. These finding could be explained with an adaptation of apical transport capacity to the higher substrate availability, thus compensating competitive inhibition between both substrates. Probably, the transport capacity of the MRP2 in the apical membranes of intestinal epithelial cells was sufficient when either one of the substrates quercetin or OTA were fed solely, because no increase of MRP2 mRNA concentration was observed in the respective feeding groups

Regarding the efflux transporters in other tissues, our results revealed, that in liver, P-gp and BCRP were not detectable and MRP2 was not regulated by OTA or quercetin.

In contrast, MRP2 was downregulated 0.5-fold in response to OTA in kidney. On the one hand, downregulation of this transporter might lead to accumulation of OTA in proximal

tubulus cells resulting in adverse pro-oxidative effects as elucidated below; on the other hand, P-gp expression was induced in response to OTA, implicating again a compensatory mechanism for the diminished MRP2 transport capacity. Downregulation of MRP2 and upregulation of P-gp at the same time after OTA-administration in rats was also observed by Marin-Kuan et al. (138) in a recently conducted study investigating the mRNA concentration profiles in response to OTA in F344 rat kidneys under similar conditions as we used (0.3 mg/kg b.w., 21 days). The OTA induced upregulation of P-gp might implicate an OTA transport by P-gp in renal proximal tubulus cells. To date, OTA transport mediated by P-gp was only studied in Caco-2 cells, where it was not a substrate for P-gp (45). But it has to be noticed, that in contrast to *in vivo*, cultured cells sometimes show declines in special transport capacities (258). However, the supplementation of quercetin, which itself did not influence MRP2, BCRP or P-gp expression significantly, downregulated MRP2 in addition to the OTA-effect. Furthermore, only in this treatment group (OTA+Q) renal BCRP was downregulated 0.6-fold and the induction of P-gp was decreased from 2 to 1.6-fold compared to OTA fed solely. This could be partly explained by the reported inhibiting action of quercetin on P-gp expression (234, 236). However, as mentioned in chapter 5.4. results and interpretation of renal MRP2 and BCRP expression must be regarded carefully.

Taken together, whereas *in vitro*, a competitive inhibition between OTA and quercetin with respect to resecretion resulting in intracellular accumulation of OTA was observed (9), *in vivo* results indicate adaptive mechanisms concerning the expression and or activity of the involved transporters on a molecular level. In a comparative study addressing the mRNA concentration profiles from renal cell cultures and rat proximal tubulus cells in response to OTA, especially proteins involved in biotransformation processes were regulated *in vivo*, but not *in vitro* (141). These contradictory results could be explained by the fact that cell cultures, as usually used in studies, lack the whole complexity of hormonal and immunological influences as present in the intact organism (141).

In our second trial, the influence of quercetin on OTA toxicodynamics was examined. The daily consumption of a chronically toxic OTA dose with the feed clearly reduced feed intake and body weight gain in OTA-treated animals as observed in another study (259). Quercetin, which itself did not substantially influence feed consumption and body weight gain added to the OTA-containing feed, however, did not amend the adverse effects of OTA. The toxic effects were hypothesized to be the consequence of OTA-derived oxidative stress.

In order to assess the oxidative status of the experimental animals, we measured 8-iso-PGF<sub>2α</sub> in plasma and α-TOC concentrations in plasma and tissue samples of liver, kidney, *musculus quadriceps*, and brain as well as relative mRNA concentrations of HO-1 and Cu<sup>2+</sup>/Zn<sup>2+</sup>-SOD in liver and kidney. Because 8-iso-PGF<sub>2α</sub> concentrations in blood plasma did not differ between the feeding groups, no evidence was obtained for an increased lipid peroxidation in the OTA-treated animals. Comparable findings were made by Gaultier et al. (259), who did not find evidence for lipid peroxidation determined as MDA concentrations in rat's plasma after an exposure to OTA (2 g/kg b.w., 24 h). In addition to 8-iso-PGF<sub>2α</sub> we measured the α-TOC concentration. Alpha-TOC functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions; hence, a decrease of α-TOC in tissues usually implicates a shift in oxidative balance to oxidative stress (260). In our trial, plasma α-TOC concentrations in both OTA-treated groups were significantly decreased compared to control and the quercetin-treated group. On a first glance, this could be interpreted as an increased oxidative stress caused by OTA leading to an enhanced consumption of α-TOC. More likely, however, this finding can be explained by the reduced feed consumption in the OTA-treated animals as a result of OTA-related toxicity, rather than an enhanced oxidative stress caused by the mycotoxin. In addition, quercetin again did not prevent the OTA effect on plasma α-TOC concentration.

In kidneys significantly higher α-TOC concentrations were recorded in those animals solely treated with quercetin or with OTA. Whereas the increased α-TOC concentrations in the quercetin-fed group may indicate an vitamin E-sparing effect (168), the increased α-TOC concentrations in response to OTA are in contrast to results published by Gautier et al. (259), who found a 22 % decrease of α-TOC in the kidney after OTA exposure. These equivocal results may be attributable to different duration of the trials (24 d vs. 24 h). Possibly, in our trial time was given for a redistribution of α-TOC to tissues with higher need for vitamin E in response to OTA. This could be triggered by a receptor mediated uptake of α-TOC, like the phospholipid transfer protein or the scavenger-receptor BI, which were involved in α-TOC uptake into extrahepatic tissues (261, 262). In fact, in a study with rats examining the relationship between hydrazine-induced oxidative stress and α-TOC concentrations in tissues, it was reported, that in those tissues in which a significant increase of oxidative stress was measurable, also the α-TOC concentrations were significantly increased and plasma concentrations decreased (263). Based on a positive correlation between tissue α-TOC concentration and local oxidative stress, our result of kidney α-TOC concentration could even

be interpreted as an evidence for pro-oxidative OTA-effects in this organ. This would make sense regarding our results of mRNA analyses. As an early indicator of a cellular answer to oxidative stress, relative mRNA concentrations of HO-1 as well as Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD were determined in liver and kidney. HO-1, an enzyme of heme catabolism, cleaves heme to form biliverdin. It responds immediately to changes in cellular redox potential caused by multiple agents like ROS generating systems, xenobiotics or changes in oxygen tension (144). The cytosolic Cu<sup>2+</sup>/Zn<sup>2+</sup>-dependent SOD protects cells from ROS by catalyzing the disproportionation of superoxide anion radicals into molecular oxygen and hydrogen peroxide (264). Hence, in the case of oxidative stress, HO-1 and Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD expression could be expected to be increased. The gene expression analyses of our data revealed an 2.4-fold upregulation of HO-1 in kidneys of OTA-fed animals, which indeed could indicate oxidative stress. This is in accordance with the studies by Marin-Kuan et al. (138) and Gautier et al. (259), where elevated HO-1 expressions in rat kidneys were found after OTA exposure. Concerning expression data of the renal efflux transporters in the present study (see above), the downregulation of MRP2 might have contributed to OTA accumulation in renal proximal tubulus cells resulting in pro-oxidative effects and in turn stimulating HO-1 expression. Although P-gp was induced in parallel, this could not (fully) compensate for the decreased transport capacity due to the reduced activity of MRP2. Co-administration of quercetin and OTA decreased the upregulation of HO-1 from 2.4 fold (OTA) to 1.7 fold (OTA+Q). This could be interpreted, that quercetin only partially reduced OTA-derived oxidative stress. Because quercetin appearingly downregulated all three efflux transporters (MRP2, BCRP, and P-gp) in the kidney, a reduction of oxidative stress by quercetin due to higher OTA secretion was probably not the underlying mechanism. In addition, because Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD was not detectable in kidneys of all experimental animals, this enzyme most likely did not contribute to the reduction of oxidative stress. Hence, other quercetin-derived mechanism not directly addressed in this study must be considered. Nevertheless, our results obtained from the *in vivo* investigations confirms a very recently conducted *in vitro* study, in which quercetin ameliorated OTA-induced oxidative stress in vero monkey kidney cells by activation of the Nrf2 transcription factor (265).

In liver,  $\alpha$ -TOC concentrations were not significantly different between all feeding groups and HO-1 was not expressed on a detectable level in this tissue, but Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD was upregulated in response to quercetin (1.3-fold), OTA (1.4-fold) and the combination of both (1.6-fold). These results are in accordance with studies of Gautier et al. (259) and Marin-Kuan



et al. (138). Those authors did not find altered  $\alpha$ -TOC concentrations in liver (259) and although HO-1 was detected in the livers of rats in those studies it was not reported to be upregulated (138, 259). Hence, no evidence was obtained for a pro-oxidative effect of OTA in liver. Because of the elevated relative mRNA concentrations of  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD in both OTA-fed groups, it could be possible in our study, that the upregulation of this antioxidative enzyme prevented the manifestation of OTA-induced oxidative stress. Quercetin itself increased relative  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD mRNA concentrations (1.3-fold) like reported before (266) confirming the stimulation of endogenous antioxidative systems. The stimulating effects of quercetin on  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD expression combined with the OTA-induced effects on this enzyme might interact synergistically, resulting in a stronger upregulation of  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD when OTA and quercetin were co-applied.

Taken together, we hypothesized that quercetin would decrease OTA-induced pro-oxidative effects. Our results show that OTA indeed induced oxidative stress in the kidney, as measured by increased  $\alpha$ -TOC concentrations (redistribution of vitamin E due to higher need) and relative HO-1 mRNA concentrations. Quercetin was able to decrease relative HO-1 mRNA concentrations and thus may act as an antioxidant. In liver, no evidence for oxidative stress was obtained, but probably defence mechanism like the  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD prevented the manifestation of oxidative stress. Our findings further implicate, that after 24 days of trial, induction of oxidative stress by OTA in F344 rats was at an early stage. Clearly, toxic effects of the chronically fed dose of OTA used in our study were feasible when body weight gain of the experimental animals decreased. At an early stage of OTA intoxication, the kidney is the first organ to be affected (100) and HO-1 is an early indicator for oxidative stress (144). The liver is only affected after long-term and severe intoxication (104).

## 7. Summary

Subject of this work was to examine if dietary applied quercetin influences OTA toxicokinetics and toxicity in growing male F344 rats. We stated the hypothesis, that co-administration of quercetin and OTA may increase delay of OTA excretion due to a competitive inhibition of both substances during elimination. In addition, we hypothesized, that the potent antioxidant quercetin diminishes OTA derived oxidative stress. Therefore, two experiments were conducted.

In the first one, the two experimental groups (n = 9) received OTA added to the feed (10 mg/kg diet, daily intake of 0.6 mg/kg b.w.) of which one was supplemented with quercetin (100 mg/kg diet), additionally. OTA and the main metabolite OT $\alpha$  were measured in daily collected urine and feces, and in plasma, kidney, liver, brain and *musculus quadriceps*. Our results revealed, that 30 % of the totally consumed OTA was excreted with feces and urine mainly in the form of OT $\alpha$ . In the tissues, highest OTA-concentrations were found in plasma and in descending order in kidney/liver, muscle and brain. No significant differences were found between the animals who received OTA only, or OTA and quercetin.

In the second experiment four groups (n = 12) received the basic diet (Control), supplemented with either 100 mg/kg quercetin (Quercetin) or 10 mg/kg OTA (OTA) or both. Feed consumption and body weight gain were measured. Animals receiving OTA suffered from reduced body weight gain due to reduced feed intake. Additional supplementation of quercetin did not improve this findings. Oxidative parameters were measured in plasma and in tissues. Our results on 8-iso-PGF<sub>2 $\alpha$</sub> ,  $\alpha$ -TOC and relative mRNA concentrations of HO-1 and SOD indicate that OTA induced oxidative stress in the kidney and quercetin was able to decrease relative HO-1 mRNA concentrations, but not any other parameter for oxidative stress we measured. In liver, no evidence for oxidative stress was obtained, but probably defence mechanism like Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD prevented the manifestation of OTA-induced oxidative stress. Our results on relative mRNA concentration analysis of the efflux transporter indicated an apical OTA transport mediated by intestinal BCRP and when OTA and quercetin were co-administered by MRP2. Also, renal P-gp was induced by OTA implicating P-gp mediated OTA secretion in the kidney.

We concluded, that in respect of OTA toxicokinetics *in vivo* the competitive inhibition of intestinal resecretion and renal tubular secretion by quercetin and OTA, as shown *in vitro*, was overcome by the upregulation of intestinal and renal efflux transporters and that quercetin was able to diminish OTA-induced pro-oxidative effects on molecular level.

## 8. Zusammenfassung

Ziel dieser Arbeit war es zu untersuchen, ob im Futter verabreichtes Quercetin die Ochratoxin A (OTA)-Toxikokinetik und Toxizität bei adulten männlichen F344 Ratten beeinflusst. Es sollte einerseits geklärt werden, ob Quercetin zu einer Verzögerung der OTA Exkretion aufgrund von kompetitiver Hemmung während der Elimination kommt. Andererseits sollte untersucht werden, ob das Antioxidans Quercetin den pro-oxidativen Effekten von OTA entgegenwirken kann. Es wurden zwei Versuche durchgeführt, in denen eine Basisdiät ohne oder mit Supplementierung von OTA (10 mg/kg Diät, tägliche Aufnahme von 0.6 mg/kg Körpergewicht) und/oder Quercetin (100 mg/kg Diät) verfüttert wurde ("Kontrolle", "OTA", "OTA+Q", bzw. "Quercetin"). Im ersten Versuch wurde die Toxikokinetik von OTA in den Gruppen OTA und OTA+Q (n = 9) untersucht. 30 % der insgesamt aufgenommenen OTA-Menge wurde mit dem Urin und dem Kot ausgeschieden, hauptsächlich in Form des Hauptmetaboliten OTa. In den Geweben wurde die höchste Konzentration im Plasma und in absteigender Reihenfolge in der Niere/Leber, Muskel und im Hirn gefunden. Es wurden keine signifikanten Unterschiede zwischen den Gruppen gefunden. Im zweiten Experiment wurden alle vier Versuchsgruppen (n = 12), Kontrolle, Quercetin, OTA und OTA+Q, miteinander verglichen. Tiere die OTA bekamen hatten eine reduzierte Gewichtszunahme aufgrund reduzierter Futteraufnahme. Eine zusätzliche Supplementierung von Quercetin änderte diesen Befund nicht. Die Ergebnisse der Gewebeanalysen oxidativer Parameter, 8-iso-PGF<sub>2α</sub>, α-TOC und relativer mRNA Konzentration von HO-1 und SOD, zeigten, dass OTA das frühe Indikatoren für oxidativen Stress HO-1 in der Niere hochregulierte und dies gemeinsam mit einer erhöhten α-TOC Gewebekonzentration auf pro-oxidative Effekte schließen lässt. Die Zugabe von Quercetin verminderte die HO-1 Expression. In der Leber wurde kein Hinweis für oxidativen Stress gefunden. Möglicherweise verhinderten die durch OTA und Quercetin stimulierten zelleigenen antioxidativen Abwehrmechanismen wie SOD die Manifestation von oxidativem Stress. Die mRNA-Konzentrationsanalysen der Effluxtransporter weisen darauf hin, dass die Hochregulation der intestinalen Transporter BCRP und MRP2 und des renalen P-gp eine kompetitive Hemmung, wie sie *in vitro* demonstriert wurde, kompensierten.

Aus den Ergebnissen kann geschlossen werden, dass Quercetin die Toxikokinetik von OTA nicht beeinflusst, weil *in vivo* eine Hochregulation von Effluxtransportern eine kompetitive Hemmung der intestinalen sowie der renalen tubulären Sekretion kompensierte und dass Quercetin in der Niere in der Lage war, von OTA verursachte prooxidative Effekte auf molekularer Ebene zu vermindern.

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# Lebenslauf

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## Promotion

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## Hochschulstudium

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