



**SCIENTIFIC REPORT submitted to EFSA**

**Report on toxicity data on trichothecene mycotoxins HT-2 and T-2 toxins<sup>1</sup>**

**CT/EFSA/CONTAM/2010/03**

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## Abstract

T-2 and HT-2 toxin are secondary fungal metabolites belonging to the group of type A trichothecenes and are primarily produced by *Fusarium* species. Toxicity data on HT-2 toxin are very limited. As T-2 toxin is rapidly metabolised to HT-2 toxin and the acute toxicity of T-2 toxin and HT-2 toxin are within the same range, the toxicity of T-2 toxin *in vivo* is considered to include that of HT-2 toxin. T-2 toxin is rapidly absorbed via the oral and inhalation route. Dermal absorption is reported to be slow. T-2 toxin is rapidly distributed throughout the body and is rapidly metabolised. Glucuronide conjugates of the mother toxin and its metabolites are the major excretion products in urine and faeces. T-2- and HT-2 toxin are very toxic with oral LD50 values in rodents in the range of 5-10 mg/kg bw. T-2 is severely irritating to skin. The observed toxicity of trichothecenes is most likely a result of their ability to inhibit protein and - at higher doses - RNA and DNA synthesis, acting predominantly on actively dividing tissues such as bone marrow, lymph nodes, thymus and intestinal mucosa. In repeated-dose studies, T-2 toxin shows unspecific systemic effects such as body weight reduction and induces liver damage, reproductive toxicity, neurotoxicity as well as haematotoxic and immunotoxic effects. Recent findings in poultry are generally consistent with the effects observed in mammals. The most relevant study for risk assessment is a feeding study with pigs, in which a diet containing 0.5-3.0 mg/kg T-2 toxin led to immunosuppression in all dose groups (LOAEL 0.029 mg/kg bw/d).

**Key words:** T-2 toxin, HT-2 toxin, trichothecene, toxicity, hazard assessment

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## Background

Recent risk assessments on T-2 and HT-2 toxins have not been done at the European level and therefore it is expected that the European Commission may ask EFSA to assess the risks to human and animal health related to these substances in the near future. To carry out these risk assessments to the highest standards and in an efficient way scientific background information on T-2 and HT-2 toxins are needed.

Trichothecene mycotoxins can be present in plants and derived products thereof and therefore might be of concern for human and/or animal health. The trichothecene mycotoxins T-2 and HT-2 toxins are type A trichothecenes which in general are more toxic than those of type B trichothecenes (e.g. deoxynivalenol and nivalenol). Trichothecenes are known to be immunosuppressive compounds and to inhibit protein synthesis. They may cause neural disturbance, haemorrhages, skin irritation, vomiting, diarrhoea and reduced feed intake in experimental animals and livestock.

## Terms of reference

The expected outcome of the project NP/EFSA/CONTAM/2010/03 is a scientific report and it shall contain:

Collection of scientific information in the public domain on toxicity of trichothecene mycotoxins namely T-2 and HT-2 toxins. Scientific information shall be collected, compiled and synthesised by the contractor for T-2 and HT-2 toxins for the four areas listed below<sup>2</sup>. A scientific report including data on both mycotoxins is requested. This report shall outline the key findings in the four areas listed below, including a full reference list according to the EFSA citation standards.

Area 1: Data on toxicokinetics (absorption, distribution, metabolism, excretion), toxicity (such as acute, subacute, repeat dose toxicity, immunotoxicity, developmental and reproductive toxicity, neurotoxicity, carcinogenicity, genotoxicity, and other effects) in experimental and farmed animals

Area 2: Epidemiological data in humans, if applicable

Area 3: Data on biomarkers of exposure and effects, if applicable

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<sup>2</sup> It is expected that for the fulfilment of the tasks of this assignment, scientific information collected represent their gathering from different sources of the available public information (e.g. scientific literature, EU/National authorities' various sources, federations of industries, single industry). In any case the source of information must be indicated for each item. Even though priority shall be given to information from peer reviewed sources, this would not prevent not to mention other available data which could be considered relevant.

Area 4: Overview on health-based guidance values derived by international and national bodies in the previous risk assessments

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## Introduction

T-2 and HT-2 are secondary fungal metabolites belonging to the group of type A trichothecenes. This group shares some common structural features, such as a double bond between C9 and C10 and an epoxy group at C12 and C13 (see Figure 1). These mycotoxins are non-volatile, low molecular-weight sesquiterpene epoxides. They are not degraded during normal food processing. They are also stable at neutral and acidic pH and consequently, they are not hydrolysed in the stomach after ingestion (Rocha et al., 2005). Type A trichothecenes are of special interest because they are more toxic than the other food-borne trichothecenes (Miller, 2002).

T-2 toxin is produced primarily by *Fusarium species F. acuminatum*, *F. nivale*, *F. oxysporum*, *F. poae*, *F. sporotrichoides* and *F. solani*. T-2 toxin was also found in moulds belonging to other genera (*Trichoderma sp.*, *Myrothecium sp.*). *Fusarium* fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions but T-2 toxin and related mycotoxins are present worldwide. Weather conditions, grain defects and moisture content are the most important factors influencing T-2 toxin production. Temperature range for T-2 toxin production is wide (0°-32°C) with maximum production at temperatures below 15°C. Corn, wheat, barley, oat, and rye are the grains most frequently contaminated with this mycotoxin (Jaradat, 2005; SCF, 2001; Sokolovic et al., 2008).

The general structural formula of type A trichothecenes is shown in Figure 1 and identifiers of T-2 and HT-2 are given below.

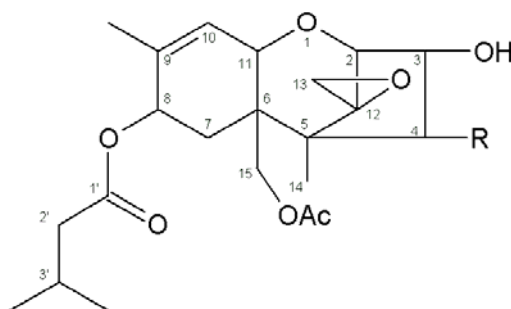


Figure 1: Structure of T-2 (R = OAc) and HT-2 (R = OH) toxin

### T-2 toxin

CAS no.: 21259-20-1

EC no.: 244-297-7

Substance name: (3 $\alpha$ ,4 $\beta$ ,8 $\alpha$ )-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol 4,15-diacetate 8-(3-methylbutyrate)

Molecular formula: C<sub>24</sub>H<sub>34</sub>O<sub>9</sub>

Molecular weight : 466.52 g/mol

### **HT-2 toxin**

CAS no.: 26934-87-2

Substance name: (3 $\alpha$ ,4 $\beta$ ,8 $\alpha$ )-12,13-epoxytrichothec-9-ene-3,4,8,15-tetrol 15-acetate 8-(3-methylbutyrate)

Molecular formula: C<sub>22</sub>H<sub>32</sub>O<sub>8</sub>

Molecular weight: 424.48 g/mol

There is little direct information available on the toxicity of HT-2 toxin alone. Existing data indicate that T-2- and HT-2 toxin induce adverse effects with similar potency. Furthermore, T-2 toxin is rapidly converted to HT-2 toxin in the gut. Therefore, the toxicity of T-2 toxin *in vivo* can be considered to include that of HT-2 toxin and the results of studies with T-2 toxin can be used to approximate the effects of HT-2 toxin (WHO, 2001).

## **1 Hazard identification and characterisation**

### **1.1 Toxicokinetics**

In general, toxicity of the trichothecene mycotoxins does not depend on metabolic activation (Wannemacher and Wiener, 1997). There are no human data available dealing with kinetics of T-2 or HT-2 toxin. However some *in vitro* data on T-2 toxin metabolites are available. Data on HT-2 toxin are very rare, but - as already mentioned - HT-2 is one of the major metabolites of T-2 toxin and it is generally accepted that HT-2 toxin will follow the same pathways as found for T-2 toxin.

#### **1.1.1 Absorption**

T-2 toxin is rapidly absorbed via the oral and inhalation route.

Radioactivity peaked in plasma of mice 30 minutes after oral administration of [<sup>3</sup>H] T-2 toxin (Doi et al., 2006). Single oral administration of 0.5 or 1 mg/kg T-2 toxin contained in diet to chicken resulted in 60-65% absorption within 120 minutes. Absorption was highest during time interval of 60-90 minutes after feeding (Reddy et al., 2004). Only limited data on absorption of HT-2 toxin are available. Oral application of HT-2 toxin to mini-piglets (single dose of 0.06 mg/kg bw) showed that it was rapidly absorbed and peaked in serum 1 h after administration (1.3  $\mu$ g/L). Serum concentration decreased thereafter (0.8  $\mu$ g/L, 4 h after

application; SCF, 2001). The penetration rate of T-2 toxin across human skin *in vitro* was described to be slow (Sudakin, 2003).

Radioactively labelled T-2 toxin was evident 4 h after intravenous (i.v.) application in the gastrointestinal tract (15-24%) and other tissues (4.7-5.2%, mainly muscle and liver) of pigs (SCF, 2001).

### 1.1.2 Distribution

Clearance of T-2 toxin itself from blood is very effective with a plasma half-life less than 20 minutes (SCF, 2001). After intravascular administration of a mixture of radioactive labelled and non-labelled T-2 toxin (0.15 mg T-2 toxin/kg bw, 1 mCi ) to pigs the plasma elimination phase half-life was 90 minutes for total radioactive residues (Corley, 1986; Doi et al., 2006). T-2 toxin tissue concentrations are consistently high in lymphoid organs. In spleen and mesenteric lymph nodes there are detectable amounts 3 h after intra-aortal administration to pigs (Doi et al., 2006). In experiments with pigs orally treated with [<sup>3</sup>H] T-2 toxin, muscles and liver retained the toxin for a longer time (18 h; 0.7% and 0.29-0.43%, respectively). The same effect was observed in other species, such as chicken and cows (Jaradat, 2005). Overall the lipophilic nature of toxins facilitates their passage through the blood-brain-barrier as well as their general entrance into all kinds of cells and organelles. T-2 toxin readily crosses the placenta (Doi et al., 2006).

### 1.1.3 Metabolism

T-2 toxin is rapidly metabolised in different species. For example in male cynomolgus monkeys 5 minutes after i.v. application of a single dose of radiolabelled T-2 toxin (14.6 µg/kg bw; 500 µCi/kg) already 22% of the T-2 toxin amount was found as metabolites in plasma and only 8% of the mother toxin were recovered from plasma after 24 h. Metabolic transformation observed in several species *in vivo* and *in vitro* (including human cells) are acetylation, deacetylation (via nonspecific carboxyesterase), hydroxylation (via Cytochrom P450 dependent enzymes), de-epoxidation (seen in rodents, swine and cattle), and glucuronide conjugation (see Figure 2) (Doi et al., 2006; Naseem et al., 1995; Wu et al., 2010).

C4 deacetylation of T-2 toxin to HT-2 by non specific carboxyesterase is the predominant metabolic transformation seen *in vitro* with human skin and human fibroblasts. After incubation of human skin with tritium-labelled T-2 toxin (74 ng/cm<sup>2</sup>) for 48 hours 71% of the radioactivity in the receptor fluid accounted for HT-2 toxin and 15% for T-2 toxin (Kemppainen et al., 1986). Unknown metabolites were tentatively identified as 3'-hydroxy T2, 3'-hydroxy HT-2, 3'-hydroxy T-2 triol, and 3-acetyl T-2 toxin. The pathway leading to 3'-hydroxy T-2 toxin is thought to be an activation pathway, as this metabolite was found to be even more toxic than T-2 toxin itself (Wu et al., 2010). Formation of 3'-hydroxy

metabolites was also found for HT-2 toxin and T-2 triol (see Figure 2, cf. to substances in the box). Transformation of T-2 toxin to HT-2 toxin was also observed in studies with human renal proximal tubule epithelial cells and normal human lung fibroblast (Königs et al., 2009). HT-2 was the only metabolite of T-2 toxin found in investigations with microsomal preparations of liver, kidney and spleen from various species (e.g. rat, sheep; SCF, 2001).

Biotransformation *in vivo* is mainly situated in the liver, but also in the intestine or blood plasma of rats, pigs, mice, chickens and cows. In ruminants metabolism particularly occurs in the rumen (Cavret and Lecoer, 2006). Based on HT-2, further metabolism led to a variety of products such as 3'-hydroxy HT-2, T-2 triol, 3'-hydroxy T-2 triol, 4-deacetylneosalaniol and subsequently T-2 tetraol. Starting from T-2 toxin 3'-hydroxy T-2 toxin was found as well in the above mentioned species (SCF, 2001; WHO, 2001; Wu et al., 2010). Moreover several de-epoxy metabolites from the underlined metabolites in Figure 2 (e.g. de-epoxy T2-tetraol), were found in rodents, swine and cattle (15-deacetylneosalaniol only in rodents, T-2 tetraol also in swine) (Wu et al., 2010). Phase II metabolism of T-2 toxin and its metabolites is characterised by the formation of glucuronide conjugates which account for the majority of the excreted metabolites (see below). Cats are lacking the ability to form glucuronide conjugates, which makes them extremely susceptible towards T-2 toxin toxicity (SCF, 2001; WHO, 2001).

In rats metabolism (in terms of conversion rates) *in vivo* was found to be dependent on route of application and time, but not on the applied dose. For example in rats treated with tritium-labelled T-2 toxin at 0.15 and 0.60 mg/kg bw higher relative rates of conversion to T-2 tetraol, HT-2 toxin, and de-epoxy tetraol and lower rates of conversion to 3'-OH-HT-2 were seen after intravenous than after oral administration and more de-epoxy 3'-OH-HT-2 was found in orally dosed animals than those treated intravenously (Pfeiffer et al., 1988; WHO, 2001; Wu et al., 2010).

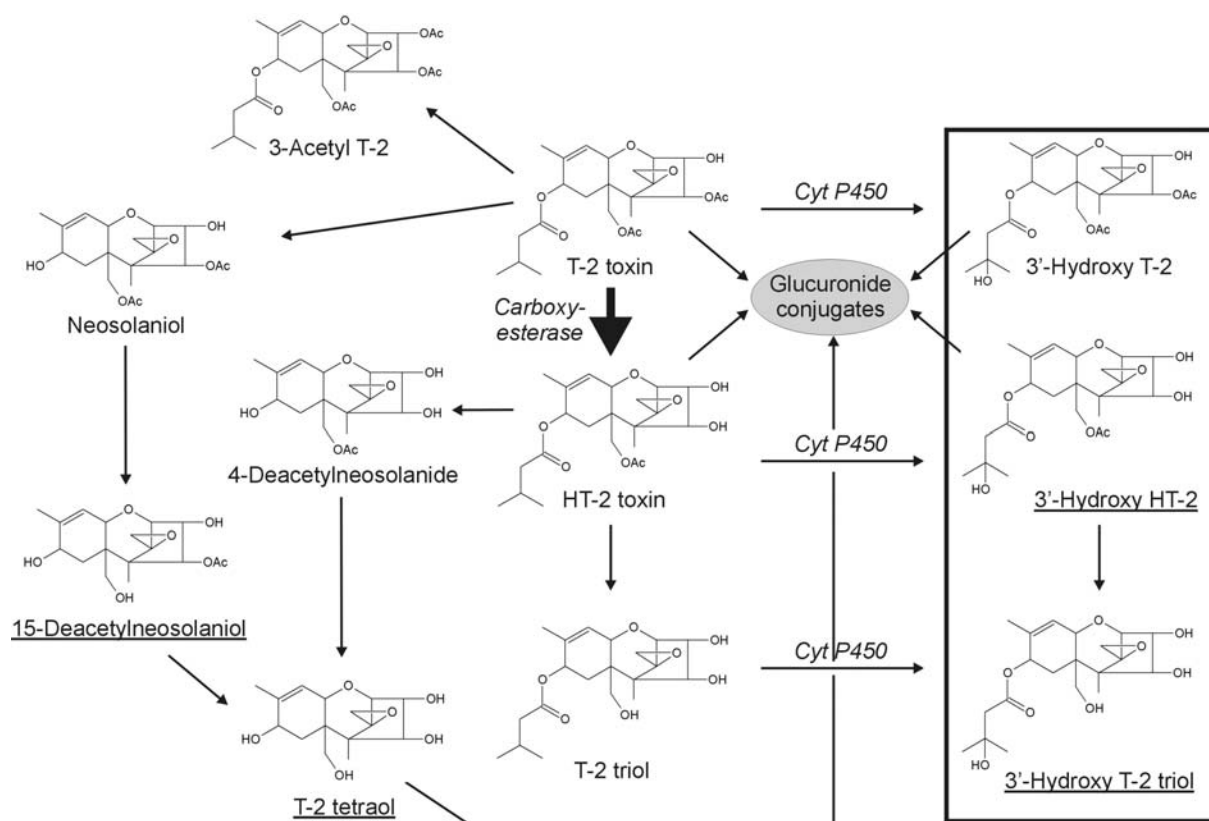


Figure 2: Metabolic pathways in animals and humans; data modified from (Dohnal et al., 2008)

#### 1.1.4 Excretion

In various rodent species (mice, rats, guinea pigs) excretion occurs via urine and bile, but the specific ratio of urinary to biliary excretion depends on the species (SCF, 2001; WHO, 2001). After intramuscular injection of radiolabelled T-2 toxin to guinea pigs (1 mg/kg bw) 75% of the administered dose was excreted via urine and faeces at a ratio of 4:1 over the first 5 days. Urinary excretion climaxed at 24 h and within 28 days 99% of the administered dose was eliminated (WHO, 2001).

In rats faecal excretion exceeded urinary excretion as observed with different doses and various routes of application. For example in rats orally treated with 0.15 or 0.6 mg T-2 toxin/kg bw/d 95% of the toxin was already excreted within 72 h. In the high dose group approximately 80% of T-2 toxin was excreted via faeces. The same is true administering 0.15 mg/kg bw intravenously (i.v.). After i.v. application metabolites were observed in urine

of rats for up to 5 days after exposure (SCF, 2001; WHO, 2001). In mice T-2 toxin and its metabolites were excreted in a ratio of 3:1 in faeces and urine (Eriksen and Alexander, 1998).

Oral administration of [<sup>3</sup>H] labelled T-2 toxin to a lactating cow led to excretion of toxin metabolites (HT-2, neosolaniol, 4-deacetylneosolaniol) via the urine, the bile and milk (Wu et al., 2010). Feeding of T-2 toxin to a sow (0.48 mg/kg bw/d for 220 days) resulted in 76 ng of T-2 toxin/g of milk (analysis was performed on treatment day 190; WHO, 2001). In ruminants milk contamination is depending on the ruminal metabolism (Cavret and Lecoecur, 2006). Due to bacteria present in the rumen which contribute to metabolism of ingested trichothecene toxins (Dohnal et al., 2008) concentrations found in milk of cow and sow were the same in spite of a four times higher exposure of the cow (Cavret and Lecoecur, 2006). In chickens the distribution pattern and excretion indicate that biliary excretion dominates (WHO, 2001). Single as well as repeated () oral application for 8 days of radiolabelled T-2 toxin to hens revealed transmission of the radiolabel into the eggs within 24 h after the application (maximum amount reached: 0.41% of administered dose in eggs white after repeated treatment), and rapidly decreased after dosing stopped (WHO, 2001).

The majority of the excretion products were glucuronide conjugates. For example 4 hours after pigs were treated i.v. with 0.15 mg [<sup>3</sup>H] T-2 toxin/kg bw 77% of the recovered radioactivity in bile and 63% in urine were glucuronide conjugates (Corley, 1986), indicating that T-2 toxin is subject to enterohepatic circulation. Enterohepatic circulation has also been observed in rats (Doi et al., 2006).

## **1.2 Biochemical mode of action**

The observed toxicity of trichothecenes is most likely a result of their ability to inhibit protein and at higher doses RNA and DNA synthesis, furthermore lipid peroxidation affecting cell membrane integrity and apoptotic effects were seen with T-2 toxin (Battilani et al., 2009; Rocha et al., 2005).

### **1.2.1 Effects on protein synthesis**

Various studies reported inhibition of protein synthesis in mammalian cell cultures treated with T-2 toxin *in vitro* (e.g. 0.01 ng/mL in suspension with rat hepatocytes gave 75% inhibition). *In vivo* inhibition of protein synthesis has also been shown in muscle, heart, liver, and spleen of rats which received a single intraperitoneal injection of 0.3, 0.75 or 2 mg T-2 toxin /kg bw. Furthermore intraperitoneal injection of 0.75 mg/kg bw per day for 3 or 7 days led to inhibited protein synthesis in mice cells obtained from bone marrow, spleen, and thymus (Eriksen and Alexander, 1998; SCF, 2001; Thompson and Wannemacher, 1990; WHO, 2001).

Disaggregation of the polysomes was reported in HeLa cells treated with T-2 toxin at concentration of 1-100  $\mu$ M (Liao et al., 1976). The toxin interacted with the centre of the

peptidyl transferase on the 60S ribosomal subunit and therefore inhibited transpeptidation of peptide-bond formation. However the ribosomes of a fungus called *Myrothecium verrucaria* were resistant to the T-2 toxin mediated protein inhibition. The sensitivity of eukaryotic cells towards T-2 toxin is probably due to direct interaction between the 60S ribosomal unit of the 80S eukaryotic ribosome and the T-2 toxin (Jaradat, 2005; WHO, 2001).

### 1.2.2 Effects on DNA and RNA synthesis

Both synthesis of DNA and RNA were inhibited by T-2 toxin in *ex vivo* cell cultures (bone marrow, spleen and thymus of mice, single or three or seven daily doses of 0.75 mg/kg bw) and *in vitro* (various cell lines; > 0.1 to ~10 ng/mL). For example total inhibition of DNA synthesis in phytohaemagglutinin-stimulated human peripheral blood lymphocytes could be observed using 8 ng/mL of T-2 toxin, an inhibition up to 80% was obvious using only 1.5 ng/mL (SCF, 2001; WHO, 2001).

### 1.2.3 Apoptosis

For T-2 toxin a variety of publications reported apoptotic effects *in vitro* (HL-60 cells: 10 ng/mL; Raw264.7 cells: 10 ng/mL; Jurkat cells: 10  $\mu$ M (also true for HT-2 toxin) as well as *in vivo* (10 mg/kg bw, e.g. in thymic and splenic lymphocytes, bone marrow and intestinal epithelial crypt cells of mice (SCF, 2001)). T-2 toxin exposure induces apoptosis in thymus and spleen of mice and rats after oral or intraperitoneal application. Thymus atrophy in mice was already seen after oral doses of 0.75 mg/kg bw. Apoptosis has also been observed in foetal tissues after *in utero* exposure (SCF, 2001; WHO, 2001).

Different theories for the mechanisms leading to apoptosis after T-2 toxin treatment are presented (Jaradat, 2005).

- a) DNA damage as secondary effect of protein synthesis inhibition or caused by oxidative stress (marked by depletion of glutathione levels and increased lipid peroxidation) can in turn activate mitochondrial apoptotic pathways.

Mitochondria and ROS seem to have a crucial role in T-2 induced apoptosis in human hepatoma cells (HepG2) and human cervix carcinoma cells (HeLa) *in vitro* (Bouaziz et al., 2009; Bouaziz et al., 2008). Various studies reviewed in Jaradat (2005) report the release of pro-apoptotic factors (e.g. cytochrome c) from mitochondria and increased activity of specific proteases executing this specific apoptotic program. After treatment of human cervical cancer cells with 10 ng T-2 toxin/mL oxidative stress was marked leading to DNA damage in a time dependent manner: 2 h after treatment mild diffusion of DNA; 4 h after treatment diffused DNA single strand breaks; 8 h after treatment granular appearance of cells indicating severe damage. Moreover the expression of p53, a pivotal apoptotic protein, was increased and other proteins involved in mitochondrial apoptotic pathway (e.g. Bax which mediates cytochrome c release from mitochondria, activation of procaspase-9; Chaudhari et al., 2009a). Doi et al.

(2006) reported that p-53-related apoptotic pathway seems to be the favourable cell death in haematopoietic and lymphoid tissues.

- b) Apoptotic cell death after induction of stress-activated protein kinase (SAPK/JNK) and mitogen activated protein kinase (p38/MAPK) either as secondary effect of protein inhibition or indirectly through lipid peroxidation with subsequent ROS production.

Trichothecenes induce ribotoxic stress response in skin (*in vitro* and *in vivo*) and macrophages (Doi et al., 2006; Li and Pestka, 2008). For example in rat keratinocyte primary cultures T-2 toxin mediated apoptosis. The expression of apoptosis related genes (*c-jun* and *c-fos*) and cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) mRNA was markedly elevated before the development of apoptosis (already detectable 0.5 h after onset of treatment) (Albarenque and Doi, 2005; Doi et al., 2006). Oral treatment of pregnant rats with 2 mg T-2 toxin/kg bw resulted in upregulated gene expression of *c-jun* in the rats liver and placenta as well as in the foetal liver, further suggesting the involvement of MAPK pathways finally inducing apoptosis (see section 1.3.5 (Sehata et al., 2005a; b)). According to Shinozuka *et al.* (2009) T-2 toxin induced damage to hepatocytes is mainly through oxidative stress followed by apoptotic cell death. Elevated expression levels of antioxidant genes such as hsp70 and SOD measured in the foetal brain of rats precede the apoptosis in the nervous system observed by Sehata et al. (2004a). Apoptotic cell death was observed in THP-1 cells (human acute monocytic leukemia cells, 3-6 h) administering 4  $\mu$ M of T-2 toxin and prior activation of p38 kinase, which was already evident after 15 minutes (Rakkestad et al., 2010).

Overall it seems that mechanisms of T-2 toxin induced apoptosis are still controversially discussed. Differences observed in various studies might be due to different experimental systems and T-2 concentrations applied. However, it is now generally believed that T-2 toxin and HT-2 toxin can induce apoptosis especially in high proliferating cell types. For example the atrophy seen in lymphoid organs (see above and details in section 1.3) is clearly assigned to apoptotic cell death. Moreover apoptotic cell death is confirmed in haematopoietic, lymphoid and gastrointestinal tissue. Dorsal skin and foetal tissue (e.g. central nervous and skeletal tissue) are affected too (Doi et al., 2006; Jaradat, 2005).

#### **1.2.4 Effects on membranes and lipid peroxidation**

In L-6 Myoblasts permeability of the cell membranes was affected *in vitro* at concentrations of 0.4  $\mu$ g/mL (SCF, 2001; WHO, 2001), furthermore phospholipid turnover in bovine platelets was changed (at 20  $\mu$ g/mL of T-2) as well as haemolysis of erythrocytes occurred *in vitro* (SCF, 2001). There is a minimum threshold dose observed for the haemolysis of platelets, since the effect occurs after exposure to 250 or 200  $\mu$ g T-2/mL but not at concentrations of 130  $\mu$ g T-2/mL (Eriksen and Alexander, 1998).

As T-2 toxin is an amphiphilic molecule it is thought to be taken up into the cells bilayer membrane and then induce lipid peroxidation by generating free radicals, thereby damaging cellular membranes. Lipid peroxidation therefore indicates oxidative stress in cells. Furthermore oxidative stress can be confirmed measuring levels of reactive oxygen species (ROS) or antioxidative molecules (such as glutathione (GSH)). Increased ROS levels with subsequent lowering of GSH levels and increased concentrations of malondialdehyde (MDA) were observed *in vitro* (with 10 ng T-2 toxin/mL on human cervical cancer cells) as well as *in vivo* (i.p. application of 11.2 mg/kg bw into mice; Chaudhari et al., 2009a; Chaudhari et al., 2009b). In another study MDA levels increased at 24 to 48 h after single dosing of mice with 1 to 6.25 mg of T-2 toxin/kg bw and decreased thereafter (Vilà et al., 2002). This is in line with findings in three species of fowl (ducks, chickens and geese). Animals were fed a diet containing T-2 toxin at concentrations of 0.2-0.6 mg/kg and elevated MDA concentrations were measured in liver homogenate and blood plasma (WHO, 2001). Lipid peroxides were increased in liver, spleen, kidney, thymus and bone marrow of rats after T-2 toxin treatment (single oral dose, 2 or 3.6 mg/kg bw; Rocha et al., 2005; SCF, 2001). Ascorbic acid,  $\alpha$ -tocopherol and selenium (substances which protect against free radicals) disclosed a protective effect against the T-2 toxin mediated lipid peroxidation; therefore the authors concluded that free radicals are involved (Eriksen and Alexander, 1998).

Total antioxidant status (TAS) was reduced and a slight but not significant elevation in plasma and liver MDA content was seen in chickens fed a diet containing 10 mg/kg of T-2 toxin for 17 days (see also 1.3.7). T-2 toxin had no effects on glutathione peroxidase (GPx) activity (Frankic et al., 2006). In pigs (feeding of diet with 3 mg/kg of T-2 toxin, 14 days, see also 1.3.2) there were no changes in plasma and 24h urinary MDA excretion rates and GPx levels remained the same (Frankic et al., 2008).

Taken together there is still some inconsistency among the results shown for the T-2 toxin mediated effects on oxidative stress markers. While several studies showed increased MDA levels, others reported no changes. However, almost all studies which showed a drastic increase in oxidative parameters used high doses of T-2 toxin to cause acute short-term toxicity (Frankic et al., 2008).

### **1.2.5 Other effects**

T-2 toxin is reported to inhibit mitochondrial electron transport in yeast cells as well as intercellular communication through gap junctions in Chinese Hamster V79 cells (SCF, 2001). The latter finding was verified by single oral treatment of male mice with either 4 or 8 mg T-2 toxin /kg bw. Protein expression of connexin32, one of the major protein components of gap junctions, was decreased in liver starting 6 h after T-2 toxin treatment (Muto et al., 2002a).

Li et al (2006b) discussed that suppression of IFN- $\gamma$  (interferon gamma) by T-2 toxin is probably one factor responsible for the decreased anti-viral immunity in the presence of T-2 toxin. The suppressions of IFN- $\gamma$  may be due to increased IL-6 (interleukin 6) expression.

### 1.3 Toxicity in experimental animals and humans

#### 1.3.1 Acute toxicity

Most of the data on acute toxicity were obtained for T-2 toxin. For HT-2 only the mentioned LD50 values mentioned below are available.

Acute toxicity of T-2 toxin is quite high. **Oral LD50 values** in rodents are in the range of 5 to 10 mg/kg bw, with older animals being less sensitive in comparison to newborn animals and males being less sensitive than females. In addition, differences in strains, especially in mice, were observed. In pigs an oral LD50 of 5 mg/kg bw was reported. Median lethal dose after oral application of T-2 toxin in chicks was established to be between 2 to 6 mg/kg bw. For HT-2 toxin an oral LD50 in chickens of 7.2 mg/kg bw was reported (Jaradat, 2005; SCF, 2001; WHO, 2001). In a recent study an oral LD50 of 3.71 mg T-2 toxin/kg bw was determined for young male Fisher rats (95% CL: 2.69 – 5.01 mg/kg bw). Combined treatment with aflatoxin B1 resulted in similar LD50 values. Overall the results of combining these two toxins were described as additive by the authors (McKean et al., 2006).

**LD50 values** for T-2 and HT-2 toxin after **intraperitoneal** (i.p.) application to mice are in the dose range of 5 to 10 mg/kg bw, whereas rats were more sensitive to T-2 toxin (LD50 i.p. ranged from 0.9 to 2.2 mg/kg bw) compared to mice. **Subcutaneous, intravenous (i.v.) or intramuscular (i.m.)** application of both toxins resulted in LD50 values averaging just below 1 mg/kg bw for rats and around 6 mg/kg bw for mice. A study with pigs revealed a LD50 i.v. of 1.2 mg/kg bw and a study in monkeys established a LD50 i.m. of 0.65 mg/kg bw. The lowest value reported for acute toxicity of T-2 toxin after **inhalation** exposure was a **median lethal dose** of 0.035 mg/kg bw in male mice. Here, as seen after oral application, females are more susceptible to the T-2 toxin mediated effects than male mice (Jaradat, 2005; SCF, 2001; WHO, 2001).

Effects observed in various species after acute oral T-2 toxin treatment with doses ranging from 0.06 to 10 mg/kg bw include non-specific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages as well as necrosis of the epithelium of the stomach and intestine, bone marrow, spleen, testis and ovary. Cats appear to be exceptionally sensitive. The haematopoietic system is one of the main targets after acute T-2 toxin exposure (SCF, 2001). A detailed description is presented in section 1.3.4.

There were increased lipid peroxides in the liver of rats after single oral T-2 toxin treatment (3.6 mg/kg bw; SCF, 2001).

Acute effects in pigs and rats include disturbances of the circulatory system (hypotension and arrhythmia) which may be due to a central effect on blood pressure and catecholamine elevation. In more detail, early experiments on cultured myocardial cells revealed that a concentration of 250 µg/mL immediately decreased beat rate and amplitude. Using only 5 or 2.5 µg/mL the adverse effect on myocytes unravelled only after 24 h, followed by cell death after 48 h. Moreover the scientist could show that *in vivo* the coronary capillaries of rats were severely damaged and even often disrupted after T-2 toxin treatment. Single i.p. injection of 2 mg T-2 toxin/kg bw (as well as application of several smaller doses) into rats resulted in damage of endothelial cells, basement membrane changes, and changes of smooth muscle cells (SCF, 2001; WHO, 2001).

T-2 and HT-2 toxin are extremely potent skin irritants. Twelve to 24 hours after topical application of T-2 toxin onto the skin of rats vascular dilatation, oedema and mononuclear cell infiltration, with many degranulating mast cells, was observed in a dose dependent manner (SCF, 2001; WHO, 2001). In rats a threshold for irritating effects was reported to be 0.5 µg T-2 toxin/cm<sup>2</sup> (Sudakin, 2003).

Toxicity of T-2 toxin after dermal application to rats was also investigated by Albarenque et al. (2000; 2001a; 2001b). Rats received single applications of 5 µg T-2 toxin/cm<sup>2</sup> skin (purity of test item not reported). Histopathological investigations within 24 hours after application revealed acidophilic degeneration of basal cells in the epidermis characterised by shrinkage of cell body with acidophilic cytoplasm and pyknotic or karyorrhectic nuclei. These cells were apoptotic. In the dermis infiltration of inflammatory cells including mast cells was detected. Similar findings on skin morphology in mice treated with 10 µg T-2 toxin were reported by Nguansangiam et al. (2003).

T-2 toxin applied *in vitro* onto the tracheal epithelium of one day old chicks stopped ciliar movement at a concentration of 0.6-20 mg/L after 2 days and at 30-300 µg/L after 3 days. Therefore one of the most crucial barriers between an organism and its environment is disturbed in its functionality (Wijnands and van Leusden, 2000).

Further investigations performed in the last ten years on toxic effects of T-2 toxin after single exposure of different species (e.g. rats, mice) revealed effects on the immune and haematopoietic system, changes in gene and protein expression profiles and protein activities (such as oxidative stress genes/proteins or apoptosis inducing genes/proteins), DNA fragmentation, as well as maternal toxicity and foetotoxicity. Thereby confirmed the findings of previous studies, which brought forward new T-2 toxin related adverse effects and approached the biochemical pathways affected by T-2 toxin. The various studies are reported in more detail in the specific sections.

Effects of T-2 toxin on phase I and II metabolising enzymes after acute exposure have been investigated, *inter alia*, in mice, rabbits and pigs. Cytochrome P450 2E1 protein expression was significantly suppressed in liver of mice (n=5 examined per time point) 24 h and 48 h after treatment with 4 mg T-2 toxin/kg bw. Nevertheless CYP3A2 expression was not

affected. Total P450 contents were also decreased in the T-2 toxin treated animals, but this effect was not significantly different from control (Muto et al., 2002b).

Exposure of New Zealand white rabbits to 0.1, 0.25 or 0.5 mg T-2 toxin /kg bw/d by gavage for 5 days resulted in the death of 3/5 animals in the highest dose group. At 0.1 mg/kg bw/d no signs of toxicity were observed and metabolising enzymes were not affected. At 0.25 mg/kg bw/d animals had decreased body weight gain and moderate signs of toxicity. Furthermore total liver microsomal P450 content and the activities of aminopyrine and benzphetamine N-demethylases, pentoxyresorufin O-depentylyse, and glutathione S-transferases were decreased. T-2 toxin treatment had no effect on the activity of ethylmorphine and erythromycin N-demethylases, ethoxyresorufin and methoxyresorufin O-dealkylases, aniline hydroxylase and UDP-glucuronyltransferase. Protein expression of cytochrome P450 1A1, 1A2, 2A1 and 2B4 but not P450 2C3 and 3A<sub>g</sub> were decreased (Guerre et al., 2000).

Activities of metabolising enzymes were investigated in the liver of piglets 24 and 48 hours after single subcutaneous exposure to 0.3 mg T-2 toxin/kg bw. Enzyme activities of cytochrome P450 isoforms 1A2, 2E1, 3A4, and glutathione S-transferase towards cumene hydroperoxide were slightly increased (but not towards 1-chloro-2,4-dinitrobenzene). Protein levels of CYP1A2, 2E1, 3A4, glutathione S-transferase alpha and glutathione S-transferase M1-1 were increased at 24 h after application (Dong et al., 2008).

### **Observations in human cells**

Using two human cell lines in primary culture (human renal proximal tubule epithelial cells and normal human lung fibroblast) cytotoxic effects were observed and IC<sub>50</sub>-values for T-2 toxin as well as HT-2 toxin could be determined. For T-2 toxin IC<sub>50</sub>-values of 0.2 and 0.5 µM revealed the strongest cytotoxic effect on the cells, apoptotic cell death was already obvious at a concentration of 0.1 µM. HT-2 induced apoptosis at somewhat higher concentrations (> 1 µM) and showed slightly weaker cytotoxicity (IC<sub>50</sub>-values: 0.7 µM – 3 µM, Königs et al., 2009).

### **1.3.2 Repeated dose toxicity**

Toxicity after repeated exposure to T-2 or HT-2 toxin is characterised by signs such as weight loss, poor weight gain, bloody diarrhoea, dermal necrosis or beak and mouth lesions, haemorrhage and decreased production of milk and eggs as well as immunological effects. The following table gives an overview on the data on repeated dose toxicity in mammals as cited in former evaluations on T-2 and HT-2 toxins (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

Table 1 Summary of relevant studies on repeated dose toxicity of T-2 toxin in mammals (data from Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001)

Species	Application route	Doses (mg/kg bw/d)	Exposure time	N(L)OAEL* (mg/kg bw/d)	Effects	Reference
Subchronic toxicity						
Pig	Oral (diet)	0.03-0.09	3 weeks	0.03 (L)	10% reduction in feed intake at 0.03 mg/kg bw/d Lowered glucose, inorganic phosphorus and magnesium levels at doses from 0.06 mg/kg bw/d	(Rafai et al., 1995a)
Pig	Oral (diet)	0.03-0.13	3 weeks	0.03 (L)	Immunotoxicity	(Rafai et al., 1995b)
Pig	Oral (diet)	0.016-0.132	5 weeks	0.132	No haematological effects observed <sup>1</sup> , only non-significant tendency towards reduced feed intake	(Friend et al., 1992)
Pig	Oral (diet)	0.04-0.64	8 weeks	0.04 (L)	Reduced body weight gain <sup>2</sup>	(Weaver et al., 1978)
Minipig	Oral (gavage)	0.012, 0.06	7 weeks	0.06	No effects on susceptibility towards pathogens, clinical health, weight gain, haematology,	(Bernhoft et al., 2000)
Monkey	Oral (gavage)	0.1	4-5 weeks	0.1 (L)	Immunotoxicity, (as well as vomiting, haemorrhage, respiratory infection, death)	(Jagadeesan et al., 1982)

Species	Application route	Doses (mg/kg bw/d)	Exposure time	N(L)OAEL* (mg/kg bw/d)	Effects	Reference
Rat	Oral (diet)	0.25, 0.5, 0.75	4 weeks	0.25	Stomach lesions (similar to the lesions in mice, see chronic toxicity)	(Ohtsubo and Saito, 1977)
<b>Chronic toxicity</b>						
Mouse	Oral (gavage)	0.1	25 weeks	0.1 (L)	Papillomas	(Yang and Xia, 1988a)
Mouse	Oral (diet)	0, 1.5, 2.25	12 months	1.5 (L)	Reversible lesions in oesophageal region of stomach (hypoplasia, hyperkeratosis, acanthosis, papillomatosis with inflammatory-cell infiltration of the squamous epithelium)	(Ohtsubo and Saito, 1977)
Mouse	Oral (diet)	0.22, 0.45	71 weeks	0.22 (L)	Pulmonary and hepatocellular adenomas (m), epithelial hyperplasia in the forestomach (m/f) <sup>3</sup> , increased heart weight (m) at high dose	(Schiefer et al., 1987)
Rat	Oral (diet)	0.5 <sup>4</sup>	8 months	0.5	No effects	(Marasas et al., 1969)

m: male, f: female, n.d.: not determined

\*At the respective studies L indicates a LOAEL, were no NOAEL could be determined.

<sup>1</sup> analysed parameters: haematocrit, red blood cell count, cell volume, and haemoglobin concentration

<sup>2</sup> no haematological effects

<sup>3</sup> survival rate was constantly lower in control groups (62%) than in treatment groups (75% or more), analysed parameters with no effect: feed consumption, body weight gain, heart weights of females, different organ weights, haematological parameters

<sup>4</sup> four week alternating treatment; i.e. 4 weeks on T-2 toxin containing diet alternating with 4 weeks on control diet

In feeding experiments broiler chicks, turkey poults, mallard ducklings and ringneck pheasants all showed a decrease in body weight (bw) gain compared to the control groups (18 to 26% decrease, apparent at approx. 5 mg/kg of T-2 toxin in diet) as well as beak lesions (evident around 1 mg/kg of T-2 toxin in diet). In these studies concentrations in diets ranged from 0.2 to 16 mg/kg of T-2 toxin and feeding was continued for 19 to 24 days (WHO, 2001; Wijnands and van Leusden, 2000).

Oral administration of T-2 toxin on alternate days at a dose of 0.06, 0.08 or 0.1 mg/kg bw/d to cats resulted in death within 6 to 40 days. Prior effects seen were weight loss, emesis, anorexia, bloody diarrhoea and ataxia. Gross inspection revealed haemorrhages of the intestinal tract, lymph nodes and heart. Observed microscopic lesions were haemorrhages in the gut, lymph nodes, heart and meninges, necrosis of the gastrointestinal epithelium and decreased cellularity of the bone marrow. An inverse correlation between the mean survival time and the applied dose was found. The severity of the observed effects in cats is thought to be due to their limited ability to form glucuronide conjugates and therefore limited detoxification and excretion of the toxin (WHO, 2001).

Feeding lambs with 0.3 or 0.6 mg T-2 toxin/kg bw/d for 21 days resulted in focal hyperaemia and dermatitis at the mucocutaneous junction of the commissure of the lips, diarrhoea, and immunotoxic effects. Calves treated orally (feeding of capsule) with 0.08, 0.16, 0.32, or 0.6 mg/kg bw/d for 30 days revealed various effects. The calf receiving the highest dose died on day 20. At all doses there were signs of mild enteritis and at doses above 0.32 mg/kg bw/d bloody faeces was seen. Moreover there were effects on the intestines and haematopoietic system noticed (WHO, 2001).

The data on toxicity of trichothecenes reported above are supported by the findings published in recent years, worth mentioning that almost all these data come from studies conducted with farmed animals. In large parts the new studies dealing with toxicity after repeated T-2 toxin treatment were set up as feeding studies in chicken (2 to 5 weeks, diet containing 0.5 to 13.5 mg/kg of T-2 toxin).

T-2 toxin and HT-2 toxin toxicity was investigated in 90 day old broiler cockerels (n=30). The cockerels were fed for 21 days with a diet containing 0.31 mg T-2 toxin and 0.26 mg HT-2 toxin /kg feed (mycotoxin content of feed analysed by HPLC). Based on the data on body weight and feed consumption a dose of 0.033-0.05 mg T-2 toxin and 0.03-0.04 mg HT-2 toxin/kg bw/d was calculated. Body weight gain and feed consumption were not affected in the exposed animals. MDA concentration of blood plasma, liver, heart and pancreas was increased in treated animals in comparison to controls, but this effect was not statistically significant. GPx activity was not affected in the treated animals. Treated animals showed increased content of reduced glutathione in blood plasma and heart and decreased content in liver and pancreas in comparison to the controls (only the effect in the heart was statistically significant). The authors discussed that the absence of clinical signs of mycotoxicosis might

be contributed to the antioxidant content of the diet (50 mg/kg vitamin E and 0.29 mg/kg selenium; Pál et al., 2009).

Another 17 days feeding study with male broiler chicks (0, 0.5, 1.5, 4.5, 13.5 mg/kg T-2 toxin in feed) reported a decrease in feed consumption and body weight gain (LOAEC = 4.5 mg/kg diet). Moreover DNA fragmentation as well as increased IgA-levels (but not IgG) were observed at the highest dose. Despite induction of DNA fragmentation no indications of oxidative stress induced by T-2 toxin were observed in this concentration range (Rezar et al., 2007).

Broiler chicks given a diet containing 2 mg/kg of T-2 toxin for 28 days revealed adverse effects on their performance (i.e. decrease in bw, increase in feed:bw gain ratio). This negative effect could be suppressed by adding “Mycofix” to the diet, this feed additive enzymatically inactivates the 12,13-epoxide ring of the mycotoxin (Diaz et al., 2005).

Feeding chickens with a diet containing 8.1 mg/kg T-2 toxin for 21 days resulted in significant decreases in the concentrations of selenium (by 32.2%), alpha-tocopherol (by 41.1%), total carotenoids (by 56.5%), ascorbic acid (by 43.5%) and reduced glutathione (by 56.3%) in the liver as well as a decrease in the hepatic activity of selenium-dependent GPx (by 36.8%). MDA concentrations were threefold increased in treated animals. The toxic effects of T-2 toxin could partially be prevented by inclusion of modified glucomannans or a combination of modified glucomannans with organic selenium (Dvorska et al., 2007).

Low dose feeding experiments in chicks (20 per dose group) and turkeys (12 per dose group) showed that concentrations up to 1 mg/kg of T-2 toxin (purity not reported) in diet (5 weeks, as well as the combinatory treatment of T-2 toxin with diacetoxyscirpenol up to 1 mg/kg) are not sufficient to induce decrease in body weight, feed efficiency or adverse effects on immunoglobulin formation after enteral or parenteral immunisation (bovine serum albumin or Newcastle disease virus). Mouth lesions as well as lesions of the intestines (e.g. decrease in surface area of villi in the jejunum and duodenum) were observed in animals receiving these low concentrations (Sklan et al., 2001; Sklan et al., 2003).

Pathological effects on broiler chickens were evaluated after feeding of 4 mg/kg of T-2 toxin in diet for 35 days. The birds revealed lesions in liver, lymphoid organs, proventriculus and intestines (Rajeev et al., 2003).

These histopathological alterations are confirmed by Krishnamoorthy and colleagues (2007) in their low dose feeding experiments with chicks (n=12). Feeding of a diet containing 0.5 mg/kg T-2 toxin (corresponding to 0.0625 mg/kg bw/d applying a feed factor of 8; no data on purity) for 28 days resulted in pathological effects on various organs, which were partially already detectable after 14 days (i.e. epithelial necrosis, crypt elongation, diphteric membrane formation and mononuclear cell infiltration in lamina propria, intestinal glandular fibrosis and hyperplasia, mononuclear cell infiltration in pancreas). This was also true for a combination

of T-2 toxin and chlorpyrifos (organophosphorus compound, 45 mg/kg in diet) tested in this study.

A 17 day feeding experiment with chicken (10 mg/kg T-2 toxin in diet) resulted in DNA fragmentation of spleen leukocytes (see also section 1.3.7). Furthermore, markers of oxidative stress were investigated. Whereas the total antioxidant status (TAS) was reduced and a slight but not significant elevation in plasma and liver MDA content could be found, no effects on GPx activity could be measured. Plasma liver enzyme level of aspartate aminotransferase (AST) was significantly decreased in the T-2 toxin group, but effects on alanine aminotransferase (ALT) and gamma glutamyl transpeptidase (GGT) were not conclusive (Frankic et al., 2006).

Toxicity of T-2 toxin to juvenile channel catfish was confirmed in a feeding study. Growth depression was noted regardless of concentration applied (feeding of diet with 0, 0.625, 1.25, 2.5, 5 mg/kg T-2 toxin for 8 weeks), poorer feed conversion was obvious in the highest dose group and in the three highest groups haematocrit values were adversely impaired as well as histopathological anomalies of stomach, head and trunk kidneys were visible. Haematocrit values in pair-fed were similar to those of the controls and significantly higher than those of fish receiving 1.25, 2.5, 5 mg/kg T-2 toxin in diet (Manning et al., 2003).

Summing up the new data in poultry, first effects after T-2 toxin treatment occur at concentrations in feed below 1 mg/kg and include lesions of the mouth and the intestine, which are believed to be systemic due to their development even after parenteral toxin application (Doi et al., 2006). Immunomodulatory effects are obvious in poultry at concentrations above 1 mg/kg in feed. Growth depression was observed in a dose range of 2 and 5 mg/kg in diet. Effects of T-2 toxin on the immune system and on the general performance at doses below 1 mg/kg in diet were only described in one study in Pekin ducks (see section 2.3.3; Rafai et al., 2000). DNA-fragmentation is only induced after high dose treatment (above 10 mg/kg). Effects on reproduction were seen in other species than fowl but only at concentrations above 15 mg/kg.

Several other studies on repeated dose toxicity report effects on metabolising enzymes and organ integrity in mammals after T-2 toxin exposure.

Male pigs (5 per group weighing about 11.4 kg at the start of the study) were exposed to feed containing 0.54, 1.3 or 2.1 mg/kg pure T-2 toxin (purity > 98%) for 28 days (Meissonnier et al., 2008). Effects on the immune system are reported in section 1.3.3. Body weight gain was reduced in all exposure groups, statistically significant only in the highest dose group (ca. -13%). T-2 toxin exposure had no effect on liver weight, lipid peroxidation, liver protein content or the plasma activities of liver enzymes ALP (alkaline phosphatase), ALT, and AST. No histopathological findings were observed in the liver and intestine, except a glycogenic overload in all groups including the control, which was accompanied by a slight interstitial inflammatory infiltrate in the mid and high dose group. T-2 toxin exposure reduced the activity of CYP1A related enzymes (ethoxyresorufin *O*-deethylation and benzo-*a*-pyrene

hydroxylation) and certain *N*-demethylases, but not UDP-glucuronosyl transferase and glutathione-S-transferase activity. Effects were already detectable at the lowest exposure concentration. Protein expression of cytochrome P450 1A, 2B, 2C and 3A and UDP-glucuronosyl transferase were not affected except reduced P450 1A protein expression in the highest dose group. The LOAEL in this study with respect on effects on metabolic enzymes is 0.54 mg/kg in diet (corresponding to about 30 µg/kg bw/d according to the authors).

In pigs which received a diet containing 3 mg/kg of T-2 toxin for 14 days no changes in plasma and 24h urinary MDA excretion rates and GPx levels were seen. The treatment resulted in a decrease of AST and ALT plasma levels compared to the control. Occurrence of DNA fragmentation in blood lymphocytes was increased as well as immunological effects were observed (see section 1.3.3) (Frankic et al., 2008).

T-2 toxin toxicity was investigated in Chinese hamsters which received 1 mg T-2 toxin/kg bw intragastrically twice a week for a period of three weeks. No effects on body weight, general health condition and histopathology of the liver, lung, kidney, spleen, pancreas, jejunum, thymus and testicles were seen at the end of the exposure period. Activity of ALP in the serum was significantly increased, but other biochemical indicators were not significantly influenced (ALT, AST, GMT, total and conjugated bilirubin, glucose and cholesterol). The leucogram revealed a significant increase of monocytes, but no other effects (Rajmon et al., 2001).

Male rats were subcutaneously exposed for 28 days to daily doses of 0.1 of LD50 (no further data). Time response of biochemical blood parameters and histological preparations of liver, heart and kidneys were monitored over the entire study period. Liver, heart and kidney showed severe damage up to necrosis. Increased activities of ALT (1.5-fold), AST (1.5-fold), LDH (lactate dehydrogenase; 3.8-fold), and creatine kinase (3.9-fold) were observed with maximum levels seen on days 14, 14, 21 and 21, respectively, while after 28 days their activities were significantly decreased (no further data). According to the authors these data indicate enzyme leakage from the damaged organs during the first three weeks of the experiment followed by a deprivation of the enzyme cellular stocks (Jovanovic et al., 2000).

Effects of T-2 toxin treatment on serum concentrations of ALT, AST and LDH were also observed in mice after single intraperitoneal injection of 5.61 or 11.22 mg T-2 toxin/kg bw (corresponding to 1 or 2 times the LD50). The data presented were somewhat ambiguous, results presented in tables were partly contradictory to the results discussed in the text (Chaudhari et al., 2009b). Shinozuka et al. (2009) reported increased plasma AST and ALT levels 48 hours after single oral application of 10 mg/kg bw T-2 toxin to mice.

Summarizing, T-2 toxin affects serum concentrations of liver enzymes in mammals, especially after application of high (i.e. generally toxic, near lethal) doses. Whereas an increase of liver enzymes often can be observed at early time points measurements at later time points often indicate a decrease of the enzyme concentrations, an effect probably due to a deprivation of the enzyme cellular stocks.

### 1.3.3 Immunotoxicity

The immune system is one of the main targets of T-2 toxin toxicity. After acute oral exposure severe damage to actively dividing cells in bone marrow, lymph nodes, spleen, thymus and intestinal mucosa has been observed. T-2 toxin like other trichothecenes can be both immunosuppressive and immunostimulatory depending on the dose and timing of exposure. Effects of T-2 toxin on both humoral and cellular immune response have been demonstrated in various studies (Bondy and Pestka, 2000).

Repeated exposure to trichothecenes increases the susceptibility to a diverse array of pathogens that include *Salmonella*, *Mycobacterium*, *Staphylococcus*, *Listeria*, *Toxoplasma*, and Herpes simplex virus (HSV-1). Effects were seen in rats, mice and chicken in a dose range of 0.5 to 5 mg/kg bw. Enhanced resistance to *Listeria* in the same dose range was observed after short-term preinoculation with T-2 toxin, whereas postinoculation with T-2 toxin results in immunosuppression. It was suggested that enhanced resistance is associated with increased migration of macrophages and elevated phagocytic activity, the latter effect may have been mediated by altered T regulatory cell activity (Bondy and Pestka, 2000; WHO, 2001).

T-2 toxin exposure induces apoptosis in thymus and spleen of mice and rats after oral or intraperitoneal application. Thymus atrophy in mice was already seen after oral doses of 0.75 mg/kg bw (LOAEL; SCF, 2001; WHO, 2001).

T-2 toxin exposure influences lymphocyte proliferation in the presence of mitogens *in vitro* and *in vivo*. Proliferation of spleen lymphocytes from mice in the presence of mitogens was enhanced at lower T-2 toxin concentrations (0.23 ng/mL in the presence of concanavalin A) and inhibited at higher T-2 toxin concentrations (1.2 ng/mL in the presence of concanavalin A; SCF, 2001; WHO, 2001).

A more detailed description of the studies published till 2000 is given in the reports of JECFA (WHO, 2001) and SCF (2001). Only the data obtained in pigs in the study from Rafai et al., (1995b), which was the central study for the derivation of the provisional maximum tolerable daily intake (PMTDI) and the temporary tolerable daily intake (t-TDI) is reported here in more detail to provide a better basis for comparison with studies published in the last decade.

#### *In vivo*

Four groups of seven-week-old pigs (10 animals per group) weighing about 9 kg were fed for three weeks with T-2 toxin. Feed concentrations were 0, 0.5, 1.0, 2.0 and 3.0 mg/kg of purified T-2 toxin (purity > 90%). This corresponds to an average daily intake of 0, 0.38, 0.81, 1.24 and 1.43 mg and a mean daily exposure concentrations of 0.029, 0.062, 0.105 and 0.129 mg/kg bw/d. Pigs were immunised with horse globulin on the first and fourth day of treatment. Blood samples were collected before the immunisation and at days 7, 14, and 21 and used to determine the antibody titre, the lymphoproliferative response to mitogens *in*

*vitro*, and the immune complex, cytotoxic reaction, and phagocytic activity of circulating granulocytes. Erythrocyte count, erythrocyte volume fraction, mean cell volume, haemoglobin concentration, leukocyte count and proportion of T lymphocytes were only investigated in blood samples drawn on day 21. Synthesis of antibodies towards horse globulin was reduced at all dose groups and at all time points (statistically significant except for the 1.0 mg/kg group at day 7). The number of red blood cells, the mean corpuscular volume and the haemoglobin concentration were significantly reduced in the two highest dose groups at day 21. Histopathology of thymus, spleen and mesenteric lymph nodes revealed a dose dependent depletion of lymphoid elements in the thymus and spleen, but in the lymph nodes this was difficult to quantify. Leukocyte counts and the portion of T lymphocytes were decreased in all exposure groups. Even at the lowest dose leukocyte count was reduced by 20%, the response to horse globulin was reduced by 29% and the blastogenic response of lymphocytes to PHA was reduced by 25%. Feed intake was dose dependently decreased. In the lowest dose group feed intake was reduced by 10 % without a significant change in weight gain in this group (Rafai et al., 1995b). The LOAEL in this study was 0.029 mg/kg bw/d. SCF and JECFA noted that potential confounding effects of differences in feed intake and weight gain on the end-points cannot be evaluated as pair-fed animals were not used in this study (SCF, 2001; WHO, 2001).

The results of Rafai et al. (1995b) were basically confirmed in a similar study performed recently (Meissonnier et al., 2008): Male pigs (5 per group weighing about 11.4 kg at the start of the study) were exposed to feed containing 0.54, 1.3 or 2.1 mg/kg pure T-2 toxin (purity > 98%) for 28 days. The animals were immunized with ovalbumin and subsequent humoral and cellular immune responses measured. Results obtained on metabolic enzyme activities and protein expression obtained from liver tissue samples are reported in section 1.3.2. Body weight gain was reduced in all exposure groups, but reached statistical significance only in the highest dose group. Histopathological investigation of the spleen did not reveal any toxic effects, no lesions were observed in the gastrointestinal tract (e.g. no erosion of the gastrointestinal epithelium, no cellular depletion of Peyer's patches. IgG and IgM plasma concentrations were not affected by T-2 toxin exposure. IgA concentrations were increased in the mid and high dose group. This effect was not statistically significant. Proliferation index of lymphocytes following mitogenic stimulation was unaffected by T-2 toxin. Production of anti-ovalbumin antibodies was reduced in the mid and high dose group on day 21 and 28, but not in the low dose group. The cell-mediated response against ovalbumin was numerically (but not statistically significant) reduced only in pigs of the mid and high dose group on day 28. The authors discuss that the lack of significance in the inhibition of the specific lymphocyte proliferation is probably due to the reduced number of animals used in comparison to Rafai et al. (1995b). The NOAEL in this study with respect on effects on the immune system is 0.54 mg/kg in diet (corresponding to about 30 µg/kg bw/d according to the authors). Investigation of pair-fed animals was also not performed by Meissonnier et al.

(2008). Dietary glucomannan supplementation (2 g/kg feed) partially prevented T-2 toxin toxicity (Meissonnier et al., 2009).

Bokkers et al. (2009) used the data from Rafai et al. (1995b) and Meissonnier et al. (2008) for a probabilistic risk assessment. They calculated a critical effect dose (CED) of 0.06 mg/kg bw/d based on the data from Meissonnier et al. (2008), critical effect size (CES) was a 5% increase of the plasma IgA concentration. CEDs of 0.02, 0.06 and 10.1 mg/kg bw/d were calculated from the data presented by Rafai et al. (1995b) using a CES of 5% decrease of white blood cell count, phagocytotic index or lymphocyte stimulation test, respectively.

Pigs (n=9) fed a diet containing 3 mg/kg of T-2 toxin for 14 days showed at the end of the treatment period a decrease in total serum IgG and IgA, but only the decrease of IgG was statistically significant (IgG: 0.49 and 0.39 AU; IgA: 0.38 and 0.30 AU in the control and T-2 toxin treated animals, respectively) (Frankic et al., 2008).

Nagata et al. (2001) detected in mice after single oral application of 10 mg/kg T-2 toxin apoptotic effects in Peyer patches, mesenteric lymph nodes and thymus within 24 hours after application. The degree of lymphocyte apoptosis was prominent in the thymus, moderate in the Peyer's patches and mild in the mesenteric lymph nodes.

Immunological effects of T-2 toxin in murine skin after dermal application were investigated by Nguansangiam et al. (2003). Ten micrograms T-2 toxin (purity not reported) were applied to the skin of one foot of mice and effects were scored after 1, 3, 5, or 7 days after application (n=5 mice examined per time point; no information on the extent of the treated area). Langerhans cell density was reduced by 20-35% in T-2 toxin treated footpad in comparison to the opposite control footpad. There was a marked decrease on day 1 which gradually recovered on days 3 through 7. Additionally, morphology of Langerhans cells was changed after T-2 toxin treatment: They showed a decrease in size with shortened and rounded dendritic processes. T-2 toxin treated footpad showed redness and swelling, desquamation of the keratinized epithelium, cellular necrosis, oedema and inflammatory cell infiltrate.

Balb/c mice received single intraperitoneal injections of 1.75 mg/kg bw T-2 toxin. Two hours later the animals were intranasally instilled with reovirus or saline vehicle. Ten days postinstillation virus plaque-forming responses and reovirus L2 gene expression were 10-fold higher in lungs of T-2 toxin treated animals compared to controls. T-2 toxin exposure increased bronchopneumonia and pulmonary cellular infiltration in reovirus-infected mice. Furthermore, T-2 toxin suppressed induction of IFN- $\gamma$  by reovirus, but enhanced production of IL-6 and MCP-1. T-2 toxin exposure also suppressed reovirus-specific mucosal IgA responses in lung and enteric tract, but potentiated serum IgA and IgG responses. Mice (n=12) received a single i.p. injection of 0, 0.02, 0.2, 0.5, 1.0 and 2.0 mg/kg bw T-2 toxin and were intranasally instilled with reovirus or saline vehicle two hours later. Lung RNAs were analysed 4 days after treatment for the presence of reovirus RNA. Clearance from reovirus from the lung was significantly inhibited at concentrations of  $\geq 0.2$  mg/kg bw, no effects were observed at 0.02 mg/kg bw (Li et al., 2006b).

Li et al. (2006a) also investigated the effects of T-2 toxin on enteric reovirus clearance. Mice received a single intraperitoneal injection of 1.75 mg /kg bw T-2 toxin, 2 h later mice were infected by oral gavage of reovirus. T-2 toxin treated animals had elevated intestinal plaque-forming viral titers after 5 days, failed to completely clear the virus from intestine by 10 days and had significantly increased virus L2 gene RNA levels in faeces. Dose-response analysis revealed that RNA levels were dose-dependently increased with statistical significant effects already observed at the lowest dose tested (0.05 mg/kg bw). Similar to the findings in the parallel study induction of IFN- $\gamma$  by reovirus was suppressed, and production of IL-6 was increased in the presence of T-2 toxin. Induction of reovirus-specific IgA in faeces was transiently suppressed as well as specific IgA and IgG<sub>2a</sub> in serum.

In a short publication (Sugita-Konishi et al., 2006) there is some evidence that a single exposure of mice *in utero* to T-2 toxin (doses not reported) leads to immunosuppressive effects in the F1 generation.

Morphometric changes of cardiac mast cells were investigated in rats acutely poisoned by T-2 toxin. Rats received a single subcutaneous dose of 0.23 mg/kg bw. Surviving animals were sequentially sacrificed on day days 1 to 28 after treatment, and a quantitative analysis of morphometric parameters (perimeter, area, roundness) of cardiac mast cells performed. No differences between treated animals and controls were observed with respect to hypogranular and hypergranular mast cells. However, degranulated mast cells of treated animals showed a significant increase in perimeter and area values while their roundness was decreased in comparison to control (Jacevic et al., 2007).

Single exposure of 28-day old unsexed broiler chicks (3 animals/group and time point) to 1 mg/kg T-2 toxin in diet caused statistically significant induction of apoptosis in thymus at 6, 12, 24 and 36 hours post-treatment (peak induction 24 h post-treatment). A slight increase of apoptotic cells was also observed in spleen, but this effect was not statistically significant (Venkatesh et al., 2005).

T-2 toxin in concentrations up to ca. 1 mg/kg in diet (range ca. 100 to 1000  $\mu$ g/kg) did not affect antibody production to antigens administered by enteral or parenteral routes in male poult or chicks (Sklan et al., 2001; Sklan et al., 2003).

Immunomodulatory effects were observed in an experiment with chickens exposed to 2.35 or 4.18 mg/kg T-2 toxin in feed for 14 days (Weber et al., 2006). At the low dose immunostimulatory effects were observed and at the high dose immunosuppressive effects occurred (endpoint: haemagglutination inhibition titres against Newcastle disease virus). Further investigations of the same group showed that oral application of vitamin E increased antibody formation against Newcastle disease in animals, an effect which could not be suppressed by exposure to 2.35 mg/kg T-2 toxin in diet (Weber et al., 2008).

Macroscopic examination of broiler chicks fed 4 mg/kg of T-2 toxin (~28 days) revealed atrophy of lymphoid organs (bursa of Fabricius, thymus and spleen) in histopathological

examinations these tissues showed lymphoid cell depletion due to lymphocytolysis (Nataraja et al., 2003).

Immunopathologic effects in unsexed broiler chicks (n=10) after exposure to 1 mg/kg T-2 toxin in diet for 28 days from the day of hatch were examined by Kamalavenkatesh et al. (2005). Chicken were vaccinated against Newcastle disease virus at 7 days of age. T-2 toxin exposure resulted in lymphocytolysis and lymphoid depletion in lymphoid organs, decrease in thymic CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, and decreased haemagglutination inhibition titres to Newcastle disease virus.

White Pekin duck broilers were fed for 49 days with T-2 toxin containing feed (0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 4 mg/kg purified T-2 toxin). Dermatotoxic oral lesions were seen in all dose groups within 2 days after start of the exposure which gradually disappeared at concentrations up to 2 mg/kg, but not at 3 and 4 mg/kg. After 7 weeks 0.4 mg/kg caused a severe decrease in weekly weight gain. At week 4 and later weekly weight gain was significantly reduced at 0.6 mg/kg. A uniformly depressed growth rate during the whole study was observed at doses of 1 mg/kg and greater. Feed intake was already depressed after 3 weeks with dietary levels of 0.6 to 4 mg/kg. In the 3 and 4 mg/kg dose group lymphocyte depletion in the spleen and bursa of Fabricius was evident. Non-specific as well as specific immunological response of lymphocytes to mitogens *in vitro* was impaired in all treatment groups but without clear dose response. Haematologic parameters were not affected (Rafai et al., 2000). Effects in this study occurred at somewhat lower doses as those described for poultry in section 1.3.2.

A 17 days feeding study with male broiler chickens (0, 0.5, 1.5, 4.5, 13.5 mg/kg T-2 toxin in feed) reported significantly increased DNA fragmentation in spleen leukocytes in the highest dose group. Serum IgA was increased at 13.5 mg/kg in diet, but no effects on serum IgG were observed (Rezar et al., 2007).

### ***In vitro***

Effects of T-2 toxin on cytokine production in mouse peritoneal macrophages and lymph node T cells *in vitro* was investigated by Ahmadi and Riazipour (2008). T-2 toxin reduced IL-1 $\beta$  in a dose dependent manner. IL-12 and TNF  $\alpha$  production were significantly increased in response to T-2 toxin concentrations in the range of 0.001 to 0.1 ng/mL, but reduced at concentrations at or above 1 ng/mL. In lymph node T cells IL-4 and IL-10 release was decreased in a concentration dependent manner at concentrations of 0.001 ng/mL or above. Reduction of IL-2 and IFN $\gamma$  was seen at concentrations  $\geq$  1 ng/mL.

Jaradat et al. (2006) reported that T-2 toxin inhibited mitogen stimulated chicken lymphocyte proliferation *in vitro* at concentrations of 1 ng/mL or higher. Proliferation was completely abolished at 10 ng/mL when the toxin was added at time zero, while it was decreased by 80%

when the toxin was added to the lymphocytes after 24 h. Addition of vitamin E provided considerable protection against T-2 toxin inhibition of lymphocyte proliferation.

### Observations in human cells

T-2 toxin inhibited the mitogen stimulated proliferation of human peripheral lymphocytes in several *in vitro* studies. T-2 toxin metabolites are less potent inhibitors, especially metabolism to 3'-OH HT2, T-2 triol and T-2 tetraol decreased *in vitro* toxicity (WHO, 2001).

These findings are supported by several studies published in the last decade:

T-2 toxin inhibits cell proliferation in human lymphoid cell lines of T (MOLT-4 cells) or B (IM-9 cells) lineage. Concentrations leading to a 50% inhibition of proliferation were 0.003 µg/mL in MOLT-4 cells and 0.00002 µg/mL in IM-9 cells. Fifty percent cytotoxic concentration was established to be 0.6 µg/mL in MOLT-4 cells and 0.2 ng/mL in IM-9 cells after 24 h incubation. Apoptotic cell death was determined in MOLT-4 cells after 4 h treatment with a T-2 toxin concentration of 0.01 µg/mL (Minervini et al., 2005).

Phytohaemagglutinin induced proliferation of isolated human peripheral blood lymphocytes was totally inhibited by T-2 toxin at a concentration of 10 ng/mL, but was not affected at 0.1 ng/mL. Early apoptotic cell death peaked at 8 h after treatment with 10 ng/mL of T-2 toxin. T-2 toxin had no direct influence on untreated lymphocytes. Further investigations revealed that T-2 toxin affected all subpopulations studied (CD4<sup>+</sup>, CD8<sup>+</sup>, CD45RA<sup>+</sup> and CD45RO<sup>+</sup>). T-2 toxin at sub-toxic concentrations (1 ng/mL) appeared to exhibit co-stimulatory properties to phytohaemagglutinin-stimulated cells (Vlata et al., 2005).

T-2 toxin is cytotoxic to human monocytes, monocytes in differentiation process into macrophages or dendritic cells, and to immature dendritic cells and macrophages. For example, only 2% of immature dendritic cells survived in the presence of 1 µM toxin. IC50 values after 24 h or 48 h exposure were determined to be 38 nM and 20 nM, respectively. In general, monocytes are more sensitive to T-2 toxin than differentiated cells. Monocyte differentiation into macrophages or dendritic cells is depressed in the presence of 10 nM T-2 toxin; this effect is less pronounced at 1 nM (Hymery et al., 2009; Hymery et al., 2006).

A 50% inhibition of mitogen stimulated proliferation of human lymphocytes in the presence of 1-5 ng/mL T-2 toxin was reported by Meky et al. (2001).

In a human peripheral blood leukocytes cell line (HL-60 from a female AML patient) T-2 and HT-2 induced apoptosis in a concentration dependent manner 24 h after treatment with concentrations starting at 3.1 and 6.25 ng/mL, respectively (Holme et al., 2003).

To summarize, after acute oral exposure and in *in vitro* assays severe damage to actively dividing cells in bone marrow, lymph nodes, spleen, thymus and intestinal mucosa has been observed. Repetitive exposure to trichothecenes increases the susceptibility to a diverse array of pathogens. T-2 toxin exposure affects the humoral and cellular immune response, both

immunosuppressive and -stimulatory effects are observed, depending on time and dose of exposure. The most critical study for human risk assessment is the feeding study performed by Rafai et al. (1995b) in pigs. Toxic effects were observed at the lowest dose resulting in a LOAEL of 0.029 mg/kg bw/d. These data were basically confirmed in another study performed in pigs with similar design (Meissonnier et al., 2008), who also demonstrated a depression of the specific immune response. In contrast to Rafai et al. (1995b) Meissonnier et al. (2008) did not observe e.g. effects on spleen histopathology or leukocyte proliferation. Considering immunotoxicity the NOAEL in the study of Meissonnier et al. (2008) was 0.03 mg/kg bw/d. Both studies did not include pair-fed animals. The statistical power of the study of Meissonnier et al. (2008) is impaired in comparison to the study of Rafai et al. (1995b) due to the low number (n=5) of animals per dose group.

Similar effects like in mammals were observed in poultry. T-2 toxin feed concentrations of 1 mg/kg resulted in significant effects on the immune system. Applying a conversion factor of 8 for chicken (ECHA, 2008) this results in a dose of 0.125 mg/kg bw/d, which is about a factor 4 higher than the LOAEL in pigs.

#### **1.3.4 Haematotoxicity**

Effects of T-2- and HT-2 toxin on proliferation, differentiation and cytotoxicity of red blood cells, leukocytes and platelet progenitor cells from mice, rats and humans have been investigated *in vitro*. T-2- and HT-2 toxin inhibited proliferation and differentiation in concentrations between  $10^{-7}$  and  $10^{-10}$  M (0.05-50 ng/mL). Investigations with foetal liver haematopoietic precursor cells from mice revealed highly selective and nearly complete toxicity of T-2 toxin to a subpopulation of CD45R<sup>+</sup>B-lymphocytic cells (SCF, 2001).

T-2- and HT-2 toxin affects haematopoietic tissue *in vivo* of mice, rats, cats, rabbits, and guinea-pigs following acute exposure to one or more doses.

Single intramuscular injection of T-2 toxin at a dose of 0.65 mg/kg bw (LD<sub>20</sub> dose) caused death in 3/9 animals within 24 hours after treatment and two further animals died in the following days, but none of the 3 control animals died. At necropsy necrosis of lymphoid tissues and petechial haemorrhage of the colon and heart were observed. Treated animals showed transient leukocytosis and increased neutrophil and lymphocyte counts. Additionally, prothrombin time was prolonged and activities of coagulation factors were decreased. The latter effect was not due to vitamin K antagonism. After single subcutaneous application of T-2 toxin to mice reduced iron uptake into erythrocyte precursors in bone marrow and spleen and in circulating erythrocytes (LOAEL: 0.3 mg/kg bw; NOAEL: 0.17 mg/kg) was observed. *In utero* exposure of mice to doses  $\geq 1.2$  mg/kg bw affected haematopoietic cells in foetal livers (SCF, 2001; WHO, 2001).

Three male and two female adult rhesus monkeys were dosed with 1 mg T-2 toxin/kg bw (gavage) for 4 days. Dosage was reduced to 0.5 mg/kg bw on days 5-15 due to sings of

toxicity which were similar to those of alimentary toxic aleukia in humans, i.e. vomiting, apathy, weakness of the lower limbs. Signs of toxicity were more severe in males, which also developed petechial haemorrhages on the face, leukocytopenia, follicular atrophy of the spleen and lymph nodes and pneumonia. No bone marrow changes were detected at necropsy. All treated males died from respiratory failure and lung congestion between days 8 and 15 of treatment. After 30 days of recovery the two treated females and two additional males were treated for another 15 days with 0.1 mg/kg bw/d. These monkeys developed leukocytopenia and mild anaemia (Rukmini et al., 1980; SCF, 2001; WHO, 2001).

Pigs treated with feed containing 0, 0.5, 1.0, 2.0 and 3.0 mg/kg T-2 toxin (corresponding to 0, 0.029, 0.062, 0.105 and 0.129 mg/kg bw/d) for three weeks developed signs of haematotoxicity. A detailed description is given in section 1.3.3 (Rafai et al., 1995b).

Relevant studies on haematotoxic effects performed in the last decade are summarized in the following section. Studies, which investigated toxicity after repeated dose and also addressed haematotoxicity (Rafai et al., 2000) were reported in the correspondent sections:

Single oral administration of T-2 toxin (0.5 mg T-2 toxin/kg bw, into animals' crop) to chicken resulted in significant DNA damage of the peripheral blood leukocytes (see also 1.3.7) (Sokolovic et al., 2007).

Haematologic parameters (erythrocyte count, haemoglobin concentration, packed cell volume, mean cell volume of erythrocytes, lymphocyte count and count of the heterophils) were not affected in White Pekin duck broilers fed for 49 days with T-2 toxin containing feed (0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3 and 4 ppm purified T-2 toxin; Rafai et al., 2000).

Broiler chicks (n=30 per group) were exposed to feed with 0, 1, 2 or 4 mg/kg T-2 toxin for 42 days. Treated animals showed significant lower body weights and increase in feed conversion ratio. At 4 mg/kg, but not at the other dose groups, a significant reduction in haemoglobin values and packed cell volume was recorded. Other haematological parameters were not affected by T-2 toxin treatment. T-2 toxin caused a reduction in serum total protein and cholesterol levels and an increase in serum uric acid and LDH levels (Pande et al., 2006). The publication has some relevant shortcomings: e.g. no data on test-item purity, insufficient documentation of methods and result.

### **Observation in human cells**

The effect of T-2- and HT-2 toxin on red blood cell precursor proliferation and differentiation was investigated using burst-forming unit-erythroid colonies from human umbilical cord. The toxins caused decreased cell growth and different pigmentation (i.e. effects on haemoglobin synthesis). T-2 toxin also caused changes of the porphyrin or haemoglobin concentration however without clear dose response (WHO, 2001).

T2- and HT2-toxin transiently inhibited growth in granulocyte macrophage colony-forming cells harvested from umbilical cord at a concentration of 0.1 nmol/L (0.047 or 0.042 ng/mL, respectively). This effect was prolonged at 10 nmol/L (WHO, 2001).

A dose dependent inhibition of platelet aggregation was observed after incubation with T-2 toxin (5-500  $\mu\text{g}/10^9$  platelets) for 20 minutes (WHO, 2001).

Toxicity of T-2- and HT-2 toxin on megakaryocyte progenitors (CFU-MK) *in vitro* was investigated by Froquet et al. (2001). Proliferation was completely inhibited at  $10^{-8}$  M T-2 toxin and  $10^{-7}$  M HT-2 toxin. *In vitro* investigations on the effects of T-2 toxin on haemostasis parameters and the viability of circulating blood cells revealed that haemostasis parameters and erythrocytes were not affected at concentrations ( $10^{-5}$  and  $10^{-6}$  M) able to induce inhibition of haematopoietic progenitor proliferation, indicating that haematopoietic progenitors appear more sensitive to T-2 toxin than mature blood cells (Froquet et al., 2003).

In summary, T-2 toxin causes haematological perturbation manifested principally as leukopenia and disturbances of coagulation.

### **1.3.5 Developmental and reproductive toxicity**

In a two-generation study with CD-1 mice fed a semi-synthetic diet containing T-2 toxin at concentrations of 1.5 and 3 mg/kg (0.22 and 0.45 mg/kg bw/d) neither embryotoxicity nor foetotoxicity was observed. A small transient reduction in weight gain was observed in offspring of the high dose group (Rousseaux et al., 1986; SCF, 2001; WHO, 2001).

Developmental toxicity of T-2 toxin was investigated in several studies with mice after oral or intraperitoneal application. Intraperitoneal application of doses  $\geq 1$  mg/kg bw caused malformations (e.g. shortened or missing tails, deformities in the limbs, open eyes and retarded jaw development), foetal death, foetal body-weight loss and maternal toxicity. In further studies malformations were already observed at 0.5 mg/kg bw. Similar findings were reported after application via gavage. Thymic atrophy due to reduction in the number of foetal liver prolymphocytic cells and prothymocytes and suppression of humoral immunity have been reported in mouse foetuses from dams exposed to T-2 toxin from days 14-17 of gestation (1.2 or 1.5 mg/kg; Doi et al., 2008; SCF, 2001; WHO, 2001).

Investigations on developmental toxicity in Wag rats receiving T-2 toxin in the diet (2.4 and 10 mg/kg feed, equivalent to 0.1 or 0.4 mg/kg bw/d) or by daily intraperitoneal injections (0.1, 0.2 or 0.4 mg/kg bw) on days 14-20 of gestation did not reveal embryotoxic or teratogenic effects at doses not leading to maternal toxicity. Transient thymus atrophy was observed at all dose groups (SCF, 2001; WHO, 2001).

Pregnant mice were treated with a single oral dose of 3 mg T-2 toxin/kg bw on day 11 of gestation and the embryos were examined after 24 hours. Apoptosis was observed in some

layers of the central nervous system, caudal sclerotomic segment, caudal region of the tongue, trachea and facial mesenchyma (SCF, 2001).

These findings were confirmed in a further study with pregnant mice (n=10 per group) which were orally inoculated with 2 mg/kg bw T-2 toxin (purity not reported) or the vehicle propylene glycol at gestational day 8.5, 9.5, 10.5, 11.5, 12.5, 14.5, 15.5 or 16.5. Five mice per group were sacrificed 24 hours after inoculation and the foetuses were histopathologically examined. The remaining 5 animals were killed at gestational day 17.5 and the fetuses underwent skeletal examination. Apoptosis, detected as pyknotic or karyorrhectic cells, was detected in the central nervous system, peri-ventricular zone to subventricular zone, in chondroblasts and chondriocytes as well as in the thymus and renal subcapsular parenchyma. The number and region of apoptotic cells varied according to inoculation date. Few foetuses from dams which were treated at gestational day 13.5 or 14.5 and were killed at GD 17.5 showed skeletal abnormalities such as wavy ribs and short scapula (Ishigami et al., 2001).

Interaction of T-2 toxin with reproduction in rabbits and ewes were reported in WHO (2001), but the studies were criticised due to their poor description. Oral intubation of four heifers fed a diet that stimulated ruminal acidosis with 0.025 mg T-2 toxin/kg bw/d for 20 days after initiation of ovulation influenced follicle maturation and ovulation. A parallel experiment with similar study protocol also described influences of T-2 toxin on ovarian activity of ewes (Huszenicza et al., 2000; WHO, 2001).

T-2 toxin inhibited testosterone secretion in gerbil testicular interstitial cells *in vitro* with a median inhibitory dose of 0.02 ng/mL (WHO, 2001).

Exposure of hens with T-2 toxin in the diet (0.5 to 8 mg/kg for 8 weeks) decreased feed consumption, egg production and shell thickness, effects were statistically significant at the highest concentration. Furthermore, concentrations  $\geq 2$  mg/kg resulted in a lower hatchability of fertile eggs in comparison to that of the control group (WHO, 2001).

Studies on reproductive toxicity of T-2 toxin published in the last decade are reported in the following paragraph:

Pregnant Wistar rats received a single oral dose of 2 mg/kg bw T-2 toxin (purity not stated) at day 13 of gestation and were sacrificed 24 or 48 hours after treatment (n=3 per dose or control group and time point). Histopathological examinations were performed with the dams, placenta and foetuses. Single cell necrosis was observed in the thymus, spleen, liver, stomach, intestines, salivary glands and pancreas of the dams. Additionally fatty change of the liver was observed. One dam showed haemorrhage of the vagina at macroscopic examination as well as haemorrhage of the placenta and single cell necrosis of cytotrophoblasts at microscopic examination. An increase of single cell necrosis in the central nervous system of foetuses was observed at 24 hours after treatment. At 48 hours after treatment single cell necrosis of haematopoietic cells and hepatocytes was increased in the liver (Sehata et al., 2003).

In two further studies reported by Sehata et al. (2005b; 2004b) pregnant Wistar rats received a single oral application of 2 mg/kg bw T-2 toxin (purity not reported) on day 13 of gestation and were sacrificed at 1, 3, 6, 9 or 12 days after treatment (n=3 per dose or control group and time point). Histopathological investigation revealed an increased number of apoptotic cells in the liver, placenta and foetal liver; effects were maximal at day 6, 12 and 9-12 after treatment, respectively. Apoptosis was accompanied by increased expression of oxidative stress- and apoptosis-related genes, additionally decreased expression of lipid metabolism- and drug-metabolising enzyme-related genes was detected in these tissues. The authors concluded that T-2 toxin-induced toxicity in pregnant rats is due to oxidative stress followed by the activation of the MAPK pathway, finally inducing apoptosis. Additionally, increased expression of *c-jun* was consistently observed, which seems to play an important role in T-2 toxin-induced apoptosis (Sehata et al., 2005b). In a parallel study effects of gestational exposure to T-2 toxin on foetal brain was investigated. Histopathologically an increased number of apoptotic neuroepithelial cells in the telencephalon was detected. Microarray analysis revealed the expression of oxidative stress-related genes (heat shock protein 70 and haem oxygenase). Additionally expression of MAP-kinase related genes and other apoptosis – related genes (caspase-2 and insulin-like growth factor binding protein-3) was induced by T-2 toxin treatment. Data from microarray analysis were confirmed by real-time RT-PCR (Sehata et al., 2004a).

Semen quality of bulls at a Finnish artificial insemination bull station (about 120 bulls) was impaired (low progressive motility and poor morphology, number of spermatozoa unaffected) after feeding of mouldy hay for about 14 weeks. The effect persisted until 5 month after withdrawal of the mouldy hay (Alm et al., 2002). Based on the results of a gas chromatographic analysis of the mycotoxin content of the hay and the hay consumption a very low exposure in the range of 117.5 µg T-2/day and 1425 µg HT-2/day was calculated. Possible contaminations of the hay with other mycotoxins cannot be excluded.

Yang et al. (2010) investigated the effects of T-2 toxin (purity not reported) on semen quality, fertility and serum testosterone concentration in mice (n=10 per group). Male mice were intraperitoneally injected with T-2 toxin (0, 5, 10, 15 mg/kg bw) on 7 consecutive days and then mated with untreated females. Males were sacrificed on day 9 and parameters analysed were semen quality, serum testosterone concentration. Females were killed on day 15 of gestation and the number of pregnant females, viable foetuses and foetal resorptions was recorded. In all dose groups a low pregnancy rate (no pregnancies in the highest dose group) and high foetal resorption rate were observed. A dose dependent reduction of testicular and cauda epididymal sperm counts, efficiency of sperm production and serum testosterone concentrations were observed at all dose groups (most effects differed already at the lowest dose group statistically significant from the controls). The number of abnormal spermatozoa was significantly increased and the number of spermatozoa with integrated acrosome was significantly decreased in all dose groups. The amount of live spermatozoa was significantly decreased in males of the 10 and 15 mg/kg bw group.

Caloni et al. (2009) reported that *in vitro* exposure of porcine granulosa cells to T-2 toxin affected steroid production and proliferation. Follicle stimulating hormone and insulin-like growth factor-1 induced estradiol production of porcine granulosa cells was completely inhibited at T-2 toxin concentrations in the range of 1 to 300 ng/mL, whereas 0.3 ng/mL T-2 toxin inhibited estradiol production by 40%. Progesterone synthesis was less sensitive to the inhibitory effects of T-2 toxin (no effects at 0.3 ng/mL, 30% inhibition at 1 ng/mL, complete inhibition at concentrations  $\geq$  30 ng/mL). Thirty ng/mL T-2 toxin significantly inhibited serum stimulated proliferation.

In a short publication (Sugita-Konishi et al., 2006) there is some evidence that a single exposure of mice *in utero* to T-2 toxin (doses not reported) leads to immunosuppressive effects in the F1 generation.

To summarize, T-2 toxin readily passes the placenta and is distributed to embryo/foetal tissues as reported in section 1.1. Maternal toxicity was observed in several studies with mice and rats. Treatment with a single dose of 2 or 3 mg T-2 toxin/kg bw resulted in apoptosis in lymphoic, haematopoietic and gastrointestinal tissues and liver, additionally, apoptosis of cytotrophoblasts in the placenta were reported. Induction of embryo/foetal death, foetal brain damage, and foetal bone malformation and impairment of the immune system was reported after *in utero* exposure. Additionally, T-2 toxin impairs testosterone synthesis and affects spermatogenesis.

### 1.3.6 Neurotoxicity

No data on neurotoxic effects are available for HT-2 toxin.

Feeding of rats or chicken with 0.1 to 22 mg T-2 toxin/kg bw/d changed neurotransmitter levels in their brains (noradrenalin, dopamine, serotonin, tryptophan, 5-hydroxy-3 indoleacetic acid, 3,4-dihydroxyphenylacetic acid). Effects on neurotransmitter regulation are heterogeneous. Depending on the study design and the brain region investigated increases or decreases were described. Neurotransmitter concentrations were affected even at the lowest doses tested (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001). Moreover intravascular application of T-2 toxin into pigs revealed a dramatic increase of norepinephrine and epinephrine concentrations in the brain (Jaradat, 2005). T-2 toxin application (i.p.; 1 mg /kg bw) increased transportation of mannitol over the blood-brain barrier of rats, whereas it had no effect on the uptake of dextran into the brain. Increased protein synthesis was observed in brain tissue after intraperitoneal application of 1 mg T-2 toxin/kg bw but not by intake of T-2 toxin in the diet. On the other side, monoamine oxidase activity was not affected by T-2 toxin given intraperitoneally but was decreased by administration of T-2 toxin in the feed at a concentration of 2.5 or 10 mg/kg, equal to 0.32 and 0.88 mg/kg bw per day (WHO, 2001).

Behavioural tests revealed reduced motor activity and performance in passive avoidance test for rats treated orally with a single dose of 2 mg/kg bw. No effect was seen in rats receiving only 0.4 mg/kg bw (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

Ishigami et al. (2001) and Sehata et al. (2004a; 2003) described neurotoxic effects in rats and mice exposed *in utero*. Single exposure to 2-3 mg T-2 toxin/kg bw induced apoptosis in several regions of the foetal brain.

Overall the effects observed in the nervous system are heterogeneous and occur at higher doses than e.g. immunotoxic effects. Therefore they are not considered to be critical for risk assessment.

### 1.3.7 Genotoxicity

Various *in vitro* and *in vivo* studies have been conducted to assess genotoxic potential of T-2 toxin and were described in former reviews on trichothecene mycotoxins. Only limited information is available for HT-2 toxin (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

In summary, there was no genotoxic activity observed in bacterial reverse mutation assays with *Salmonella typhimurium*, or in DNA repair assays with *Escherichia coli* and no mitotic crossing over or petite forward mutation in dividing yeast cells (*Saccharomyces cerevisiae*) was reported in the presence or absence of a metabolic activating system (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

DNA damage (DNA single-strand breaks) was noted *in vitro* with primary hepatocytes (only weak positive response), as well as in spleen and thymic lymphocytes of BALB/c mice (5 ng/mL for 2 hours). *In vivo* experiments showed that intraperitoneal application in BALB/c mice resulted in DNA single-strand breaks in spleen and thymus, but not in the liver (effects observed after 3 hours with 3 mg/kg bw). In another experiment DNA-fragmentation could be observed *in vitro* and *in vivo* in liver cells within 4 h after T-2 toxin application. This T-2 toxin induced effect was prevented by prior administration of antioxidants, indicating that formation of free radicals might be involved in DNA damage (Jaradat, 2005). As already mentioned in section 1.3.2 in a 17 day feeding experiment with chicken (10 mg/kg of T-2 in feed) DNA fragmentation of spleen leukocytes were found (Frankic et al., 2006). Moreover the same authors showed increased DNA fragmentation (by 27%) in blood lymphocytes of pigs after they were fed a diet containing 3 mg/kg of T-2 toxin for 14 days (Frankic et al., 2008). Another 17 days feeding study with male broiler chicks showed DNA fragmentation to occur when diet contained 13.5 mg/kg of T-2 toxin (Rezar et al., 2007).

In a recent publication chicken nucleated blood cells were established as cellular model for genotoxicity testing using the comet assay measuring DNA damage. Single oral administration of T-2 toxin (0.5 mg T-2 toxin/kg bw, into animals' crop, n=5; purity 98.4%),

with retrieval of whole blood samples 24 hours thereafter, resulted in significant DNA damage (Sokolovic et al., 2007).

Cytogenetic damage was found in examinations with *Drosophila* (sex-linked chromosomal loss assay) after 48 h (20 mg/kg in feed). Moreover cytogenetic damage was observed *in vitro* in Chinese hamster cells. Sister chromatid exchange (SCE) occurred at a concentration of 100 ng/mL with metabolic activation. Another experiment found SCE at 2300 ng/mL with and without metabolic activation. Chromosomal aberrations could be found with concentrations starting at 1 ng/mL as well as micronucleus formation was confirmed at 50 ng/mL in this cell type. Using human lymphocytes no sister chromatid exchange was found using 3 ng T-2 toxin/mL, yet chromosomal aberrations were already apparent at 0.1 ng/mL. *In vivo* intraperitoneal application of 3 mg/kg bw did not cause micronucleus formation in the bone marrow of Chinese hamsters. But chromosomal aberrations were obvious at lower concentrations in the same species (1.7 mg/kg bw, i.p.) as well as in mice (feeding experiment with 15 ng/kg bw for 12 weeks, effects in bone marrow cells), but no dose response of T-2 could be confirmed (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

A gene mutation assay with Chinese hamster V79 fibroblasts was positive with metabolic activation (100 ng T-2 toxin/mL, thioguanine selection (HPRT)). However, gene mutation experiments using *Drosophila melanogaster* (sex-linked recessive lethal mutation assay) gave inconclusive results (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

### **Observations in human cells**

There is only inadequate evidence for genotoxicity in humans. There were conflicting results in assays on unscheduled DNA synthesis (UDS). In primary cultures of human gastric epithelial cells no UDS was found after T-2 toxin treatment. Using HT-2 toxin with human fibroblasts no UDS was observed without metabolic activation, but at the highest dose tested (100 µg/mL) was a positive response in the presence of rat liver activation system. Applying T-2 toxin on human fibroblasts a positive outcome of the UDS assay was verified (5 ng/mL; Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001). DNA damage was observed after application of 4 µM T-2 toxin onto THP-1 cells (human acute monocytic leukemia cells, positive result in a comet assay; Rakkestad et al., 2010).

To summarize, genotoxicity tests using microorganisms were negative. All in all there are several *in vitro* and *in vivo* tests for genotoxicity which gave positive results for T-2 and HT-2 toxin. In particular, testing for clastogenic effects yielded positive results. Concentrations at which these effects occurred are known to also inhibit protein synthesis and DNA/RNA synthesis as well as inducing cytotoxicity, therefore it is assumed that the observed genotoxic effects may be most likely secondary.

### 1.3.8 Carcinogenicity

Carcinogenicity of T-2 toxin has been investigated in mice, rats and rainbow trouts after oral application. Additionally, initiation/promotion potential of T-2 toxin was investigated in mice after dermal application. There are no data on carcinogenic potential of T-2 toxin or HT-2 toxin in humans.

Mice (n = 50 per sex per dose) were fed a diet containing 0, 1.5 or 3 mg/kg purified T-2 toxin for 71 weeks (corresponding to 0, 0.22 or 0.45 mg/kg bw/day). Male mice showed elevated incidences for pulmonary adenomas in both dose levels compared to control (control 10%, low dose group: 15%, high dose group: 23.3%). Pulmonary adenocarcinomas were found in 2 control animals and in three high-dose male mice. Furthermore hepatocellular adenomas were increased in high dose males (21%) compared to controls (7%), but not in low dose males (6%). Differences were statistically significant in high dose male mice for both adenoma types compared to control ( $p < 0.05$ ). Epithelial hyperplasia in the forestomach was dose-related and statistically significant increased in males and females of all dose groups (SCF, 2001; Schiefer et al., 1987).

Forestomach papillomas occurred in 5/35 mice after oral treatment (gavage) with 0.1 mg T-2 toxin per kg bw per day (75 treatments, 3 times a week for 25 weeks). No papillomas were observed in 30 control animals (SCF, 2001; WHO, 2001; Yang and Xia, 1988a).

Occurrence of papillomas and carcinomas of the forestomach as well as tumours in other organs of rats was reported after prolonged gavage administration of T-2 toxin. In another study with rats which received T-2 toxin with or without additional application of nicotinamide increased incidences of pancreatic and other tumours were described. These studies in rats as well as one study in rainbow trouts (not reported) were judged as inadequate for evaluation by the IARC working group (IARC, 1993).

Hepatocarcinogenic properties of T-2 toxin were investigated in rats, either pre-treated with diethylnitrosamine or left untreated before giving T-2 toxin in doses of 2 or 5 mg/kg. There was no evidence for direct carcinogenicity of T-2 toxin nor for a carcinoma enhancing effect (Wijnands and van Leusden, 2000).

Tumour initiation/promotion potential of T-2 toxin was investigated in mice after dermal application.

A study was conducted either by dermal application of a) 100 µg 7,12-dimethylbenz[*a*]anthracene (DMBA) as tumour initiator, followed one week later by 3 times a week for 26 weeks with 0.5 µg T-2 toxin or b) 5 µg T-2 toxin for 6 days as tumour initiator, followed one week later by 3 times a week for 26 weeks with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or acetone. As a result in controls (DMBA treatment only) there was one mouse with papilloma. In treatment groups where T-2 toxin served as tumour promoter (conditions a, 45 mice), 8 mice with papillomas and one mouse with carcinoma was observed,

whereas when T-2 served as initiator agent (conditions b, 35 mice) no tumours occurred. Another study of the same authors with similar conditions revealed papillomas in two mice and skin carcinoma in one mouse treated with DMBA followed by T-2 toxin (22 mice). In contrast to the previous findings T-2 toxin treatment followed by TPA resulted in papillomas in 4 out of 21 mice treated (WHO, 2001; Yang and Xia, 1988a; Yang and Xia, 1988b). Two other studies reported a weak promoting activity of T-2 toxin on the DMBA induced mouse skin (Lindenfelser et al., 1974; Marasas et al., 1969; WHO, 2001).

Additionally promoting activity was also detected *in vitro* for T-2 toxin in a short term transformation assay using v-Ha-ras-transfected BALB/3T3 cells (Bhas 42 cells). T-2 toxin was active at very low concentrations (1-2 ng/mL) in culture medium (Sakai et al., 2007).

IARC classified T-2 toxin in 1993 into carcinogenicity group 3 because there were no data available on the carcinogenicity to humans and there is only limited evidence in experimental animals for the carcinogenicity of T-2 toxin (IARC, 1993).

#### **1.4 Epidemiological data**

There is evidence that alimentary toxic aleukia (ATA) is related to the presence of *Fusarium* species in grain; e.g. in cases of the disease occurring in the USSR since 1931. Subsequent analysis of suspected grain samples from a severe outbreak in 1944 identified T-2 and HT-2 toxin along with other fungal toxins. Extracts of the grain tested on the skin of animals were highly toxic. The clinical symptoms of affected humans during the outbreak were vomiting, abdominal pain and diarrhoea followed by leukopenia, bleeding from nose and throat, depletion of the bone marrow and fever. Pathological changes described as necrotic lesions of the oral cavity, oesophagus, and stomach were observed. In a high proportion of cases the disease was lethal (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

Two reports inform about outbreaks of toxicosis related to the consumption of trichothecene-contaminated grains such as wheat or rice in India (1987) and China (1993). Both cases involved about 100 persons and symptoms included were abdominal pain, nausea, dizziness, throat irritation, diarrhoea, blood in stools and vomiting. Symptoms developed within 1 h at the latest. Probing of the samples revealed 0.55-0.8 mg T-2 toxin per kg wheat flour in samples of India and 0.18-0.42 mg T-2 toxin per kg rice in China. Existence of other trichothecene toxins was verified in probes of India and could not be ruled out in the probes of China (Eriksen and Alexander, 1998; SCF, 2001; WHO, 2001).

A disease called scabby grain toxicosis affecting humans and farmed animals was reported from Japan and Korea occurring during 1946-63. Most common clinical symptoms were as described in cases above. Toxicosis was an acute and reversible effect and none of the cases was lethal. Investigations on the suspected cereals were limited to detection of *Fusarium* species but no analysis of the toxin content was performed (WHO, 2001).

Accidental contamination of the skin with crude extracts of fungal cultures or T-2 toxin containing solutions resulted in grave effects on skin. There was severe irritation, loss of sensitivity, and desquamation. The contact material contained T-2 toxin, however the impact on the observed effects of other compounds within this material could not be ruled out (WHO, 2001).

No later studies than those reported above on human mycotoxicoses in the context with T-2 and HT-2 toxin exposure were identified in the literature.

#### 1.4.1 Biomarkers

Up to now there are no established methods for the determination of biomarkers of exposure or effects. Exposure to these mycotoxins is generally determined by analytical determination of contents in food items. Gas chromatography analysis is the method of choice for the determination of type-A trichothecenes coupled with mass spectrometry, electrochemical derivatisation or flame ionization detection (WHO, 2001). Detection of trichothecene mycotoxins in human blood using gas chromatography has also been reported (Sudakin, 2003).

### 1.5 Health based guidance values

Health based guidance values were derived for the group of T-2 and HT-2 toxin as the latter one is a metabolite of T-2 and the observed effects could, at least partially, be attributed to the metabolite *in vivo*.

In 1998 the Working group on fusarium toxins of the **Nordic Council of Ministers** derived a temporary tolerable daily intake (t-TDI) of **0-0.2 µg/kg bw per day** for the sum of T-2 and HT-2 toxins on the basis of possible **carcinogenic effects** and applying a safety factor of 1000 onto the NOAEL for tumourigenic effects. The authors stated that they believe that this t-TDI would protect from immunotoxic effects, thought to be the additional critical effects evoked by T-2 toxin, too (Eriksen and Alexander, 1998).

Later on several regulatory authorities agreed that the critical endpoints of T-2 and HT-2 toxins are **immunotoxicity** and **haematotoxicity** which both were evident in several species under different circumstances.

A provisional maximum tolerable daily intake (PMTDI) was set by the **Joint FAO/WHO Expert Committee on Food Additives (JECFA)** in 2001 on the basis of effects observed in a 3 week study with pigs which revealed haematological effects (Rafai et al., 1995b). The observed LOAEL of 0.029 mg/kg bw and day was considered to be near to the NOAEL, as the effects are very subtle, reversible and there were no such effects in other studies on pigs reported even at slightly higher doses. Using a safety factor of 500 (considered the fact that

only a LOAEL was available from a short term study) a group PMTDI of **60 ng per kg bw per day** was derived for T-2 toxin and HT-2 toxin, alone or in combination.

In line with these explanations is the combined t-TDI for the sum of T-2 toxin and HT-2 toxin set by the **Scientific Committee on Food** of the European Union in 2001 (SCF, 2001). The overall evaluation of the SCF concluded that this **t-TDI of 60 ng/kg bw** would also protect against other chronic, subchronic and reproductive effects seen up to the evaluation date.

## 2 Summary and conclusions

The toxicity of HT-2 toxin is only scarcely investigated. Most data on toxicity are available for T-2 toxin. Because T-2 toxin is rapidly metabolised to HT-2 toxin and the acute toxicity of T-2 toxin and HT-2 toxin are within the same range the toxicity of T-2 toxin *in vivo* is considered to include that of HT-2 toxin and the results of studies with T-2 toxin are used to approximate the effects of HT-2 toxin.

T-2 toxin is rapidly absorbed via the oral and inhalation route. Dermal absorption is reported to be slow. Once ingested, the toxin is rapidly distributed throughout the body and passes the blood-brain barrier and the placenta. It is also transmitted into eggs and milk. T-2 toxin is extensively metabolised. C4 deacetylation of T-2 toxin to HT-2 is the predominant metabolic transformation. Further metabolites are, *inter alia*, 3'-hydroxy HT-2, T-2 triol, 3'-hydroxy T-2 triol, 4-deacetylneosolaniol and subsequently T-2 tetraol. The pathway leading to 3'-hydroxy T-2 toxin is thought to be an activation pathway because this metabolite was found to be even more toxic than T-2 toxin itself. T-2 and HT-2 toxin as well as their metabolites are mainly excreted as glucuronide conjugates via urine and faeces within several days. The ratio of urinary to faecal excretion varies from species to species.

The observed toxicity of trichothecenes is most likely a result of their ability to inhibit protein and - at higher doses - RNA and DNA synthesis. Furthermore, lipid peroxidation affecting cell membrane integrity and apoptotic effects were seen with T-2 toxin. Actively dividing tissues, such as bone marrow, lymph nodes, thymus and intestinal mucosa, are most susceptible to the action of T-2 toxin. The effects most relevant for risk assessment are general toxicity, haematotoxicity and immunotoxicity.

T-2 and HT-2 toxin are highly toxic after acute exposure with oral LD50 values in rodents in the range of 5-10 mg/kg bw. The lowest value reported for acute toxicity of T-2 toxin after inhalation exposure was a median lethal dose of 0.035 mg/kg bw in male mice.

Effects observed in various species after acute oral T-2 toxin treatment with doses ranging from 0.06-10 mg/kg bw include non-specific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages as well as necrosis of the gastrointestinal epithelium, bone marrow, spleen, testis and ovary. T-2 and HT-2 toxin are extremely potent skin irritants. In rats, a threshold for irritating effects of 0.5 µg T-2 toxin/cm<sup>2</sup> was reported.

Longer-term investigations with poultry revealed mouth lesions as well as lesions of the intestines (e.g. decrease in surface area of villi in the jejunum and duodenum) at feed concentrations < 1 mg/kg. Decreased weight gain and reduced feed intake were observed at 0.4 mg/kg and 0.6 mg/kg, respectively, in Pekin duck broilers after repeated exposure. At 0.2 mg/kg, effects on the immune system were already detectable. In another study, immunopathologic effects in broiler chicks were reported after exposure to 1 mg/kg T-2 toxin for 28 days. In a study with broiler cockerels fed a diet containing 0.31 mg/kg T-2 toxin and 0.26 mg/kg HT-2 toxin (corresponding to a dose of 0.033-0.05 mg T-2 toxin and 0.03-

0.04 mg HT-2 toxin/kg bw/d) for 21 days, no clinical signs of mycotoxicosis were observed. However, the diet was supplemented with relevant amounts of vitamin E and selenium.

The findings in poultry are generally consistent with the effects observed in mammals. But they are of limited value for the evaluation of toxicity to humans due to certain limitations of the studies (e.g. high concentrations of antioxidants in the diet, no data on feed consumption and feed efficiency).

A two-generation study with CD-1 mice fed a semi-synthetic diet containing T-2 toxin at concentrations of 1.5 and 3 mg/kg (0.22 and 0.45 mg/kg bw/d) found neither embryotoxicity nor foetotoxicity. Maternal toxicity was observed in several studies with mice and rats: treatment with a single dose of 2 or 3 mg T-2 toxin/kg bw resulted in apoptosis in lymphoid, haematopoietic and gastrointestinal tissues and liver. Additionally, apoptosis of cytotrophoblasts in the placenta was reported. Induction of embryo/foetal death, foetal brain damage and foetal bone malformation as well as impairment of the immune system was reported after *in utero* exposure. Furthermore, T-2 toxin impairs testosterone synthesis and affects spermatogenesis.

Based on the available information, neurotoxic effects are not considered to be critical for risk assessment because they are heterogeneous and occur at higher doses than e.g. immunotoxic effects. For example, feeding of rats or chicken with 0.1 to 22 mg T-2 toxin/kg bw/d changed neurotransmitter levels in their brains.

The haematologic/immune system is the main target of T-2 toxin toxicity. T-2 toxin, like other trichothecenes, can be both immunosuppressive and immunostimulatory depending on the doses and timing of exposure. Effects of T-2 toxin on the humoral as well as the cellular immune response have been demonstrated in various studies. T-2 toxin exposure increases susceptibility to a diverse array of pathogens.

Investigations with non-human primates (rhesus monkeys), which were treated for 15 days with 0.1 mg T-2 toxin/kg bw/d, revealed leukocytopenia and mild anaemia. Most relevant in this context is the study of Rafai et al. (1995b) with pigs fed a diet containing 0, 0.5, 1.0, 2.0 and 3.0 mg/kg of highly purified T-2 toxin for three weeks. Immunotoxic effects were already observed in the lowest dose group resulting in a LOAEL of 0.029 mg/kg bw/d. These data were basically confirmed by the study of Meissonnier et al. (2008) with a NOAEL of 0.03 mg/kg bw/d. The study of Rafai et al. (1995b) is still considered the most relevant one for human risk assessment of T-2 toxin.

Genotoxicity tests using microorganisms were negative. There are several *in vitro* and *in vivo* tests for genotoxicity, which gave positive results for T-2 and HT-2 toxin. In particular, testing for clastogenic effects yielded positive results. Concentrations at which these effects occurred are known to also inhibit protein synthesis and DNA/RNA synthesis as well as inducing cytotoxicity. Therefore, it is assumed that the observed genotoxic effects may be most likely secondary.

IARC classified T-2 toxin in 1993 into carcinogenicity group 3 because there were no data available on the carcinogenicity to humans and there was only limited evidence in experimental animals. Up to now, no additional data were reported addressing the tumourigenic potential of the fungal toxins.

Relevant repeated-dose studies are summarised in Table 2.

Overall, the toxicity of T-2 toxin is extensively investigated but valid subchronic or chronic toxicity studies are lacking. Existing data reveal that T-2 toxin is a very potent toxin that primarily affects the immune and haematopoietic system. These findings are consistently reported in several species including mammals and poultry.

*Fusarium* fungi produce several trichothecene mycotoxins with common chemical structure and similar mode of action (Pestka, 2008; Rocha et al., 2005). Possible combination effects should therefore be considered in the risk assessment of T-2- and HT-2 toxin.

Table 2 Relevant studies for risk assessment of T-2- and HT-2 toxin

Study design	Critical effect	N(L)OAEI (mg/kg bw/d)	References
Mouse, two-generation study, diet	No embryotoxicity or foetotoxicity	0.45 (NOAEI)	Rousseaux et al., 1986
Monkey, 15 days, gavage	Leukopenia	0.1 (LOAEI)	Rukmini et al., 1980
Pig, 3 weeks, diet	Reduced leukocyte and lymphocyte count, reduced synthesis of antibodies towards horse globulin, histopathologic changes of thymus, spleen and mesenteric lymph nodes	0.029 (LOAEI)	Raffai et al., 1995b
Pig, 28 days, diet	Reduced anti-ovalbumin antibodies No effects on liver enzymes and liver histopathology Effects on drug metabolising enzymes	0.030 (NOAEI) about 0.1 (NOAEI) <sup>a</sup> 0.030 (LOEI)	Meissonnier et al., 2008
Chicken, 21 days, diet	No clinical signs of mycotoxicosis	0.06 (NOAEI)	Pal et al., 2009
Chicken, 28 days, diet	Pathological effects on various organs (e.g. epithelial necrosis, crypt elongation, intestinal glandular fibrosis and hyperplasia)	0.5 mg/kg diet (LOAEI) <sup>b</sup>	Krishnamoorthy et al., 2007
White Pekin duck broilers, 49 days, diet	Impairment of immune system, not dose dependent Decrease in weekly weight gain Decreased feed intake	0.2 mg/kg diet (LOAEI) <sup>c</sup> 0.4 mg/kg diet (LOAEI) <sup>d</sup> 0.6 mg/kg diet (LOAEI) <sup>e</sup>	Rafai et al., 2000

a) Estimated on the basis of body weight and feed consumption data of pigs as presented in the study of Rafai et al., 1995a.

b) Chicken were fed a diet containing 0.5 mg/kg T-2 toxin. This corresponds to a dose of 0.0625 mg/kg bw/d applying a feed factor of 8 (ECHA, 2008).

c) 0.2 mg/kg diet corresponds to a dose of 0.025 mg/kg bw/d applying a feed factor of 8.

d) 0.4 mg/kg diet corresponds to a dose of 0.05 mg/kg bw/d applying a feed factor of 8.

e) 0.6 mg/kg diet corresponds to a dose of 0.075 mg/kg bw/d applying a feed factor of 8.

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## Glossary / Abbreviations

µg	Microgram
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATA	Alimentary toxic aleukia
bw	Body weight
CED	Critical effect dose
CES	Critical effect size
CL	Confidence limit
DMBA	7,12-dimethylbenz[ <i>a</i> ]anthracene
DNA	Desoxyribo-nucleic acid
DON	Deoxynivalenol, another mycotoxin
EFSA	European Food and Safety Authority
<i>F.</i>	<i>Fusarium</i>
FAO	Food and Agricultural Organization
GD	Gestational day
GGT	Gamma glutamyl transpeptidase
GPx	Glutathione peroxidase
GSH	Glutathione
HeLa cells	Human cervix carcinoma cells
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
IC50	Median inhibitory concentration
IFN	Interferon

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Ig	Immunoglobulin
IL	Interleukin
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kg	Kilogram
L	Litre
LD50	Median lethal dose
LDH	Lactate dehydrogenase
LOAEL	Lowest observed adverse effect level
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
mg	Milligram
mL	Millilitre
ng	Nanogram
NOAEL	No observed adverse effect level
PHA	Polyhydroxyalkanoates
PMTDI	Provisional maximum tolerable daily intake
RNA	Ribo-nucleic acid
ROS	Reactive oxygen species
SCE	Sister chromatid exchange
SCF	Scientific Committee on Food of the European Union
<i>sp.</i>	Species
TAS	Total antioxidant status
TNF	Tumour necrosis factor
TPA	12- <i>O</i> -tetradecanoylphorbol 13-acetate
t-TDI	Temporary tolerable daily intake
UDS	Unscheduled DNA synthesis
WHO	World Health Organization