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Effects of the *Fusarium* toxin deoxynivalenol (DON) on protein synthesis, immunological parameters and DON-kinetics in the pig

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Zur Erlangung des Grades einer
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CONTENTS

ABBREVIATIONS

(cited in Introduction, Background and General Discussion)

3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol
ad lib.	*Ad libitum*
ANOVA	Analysis of variance
AP	Alkaline phospatase
APP	Acute phase protein
ASAT	Aspartate aminotransferase
ASR	Absolute synthesis rate
AUC	Area under the curve
BEA	Beauvericin
BML	Bundesministerium für Landwirtschaft
BrdU	5-Bromo-2´-deoxyuridine
BSA	Bovine serum albumin
bw	Body weight
C_0	Extrapolated serum concentration at time zero
CHX	Cycloheximide
CINC	Cytokine-induced chemo-attractant protein
CK	Creatinine kinase
Cl	Clearance
C_{max}	Maximal serum concentration
ConA	Concanavalin A
CRG	Cytokine-responsive gene
DAD	Diode array detector
DAS	Diacetoxyscirpenol
DFG	Deutsche Forschungsgemeinschaft
DLG	Deutsche Landwirtschafts-Gesellschaft
DM	Dry matter
DNA	Deoxyribonucleic acid
DOM	De-epoxy deoxynivalenol
DON	Deoxynivalenol
ED_{50}	Emetic dose of 50 %
EDTA	ethylene-dinitrilo-tetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ERK 1/2	Extracellular signal regulated protein kinase
F	Bioavailability
FAO	Food and Agricultural Organi-zation of the United Nations

FB	Fumonisin B
F-C	Fusarin C
FCS	Foetal calf serum
FSR	Fractional synthesis rate
FX	Fusarenon-X
GC/MS	Gas chromatography mass spectrometry
GfE	Gesellschaft für Ernährungs-physiologie
GLDH	Glutamate dehydrogenase
γ-GT	γ-Glutamyltransferase
HPLC	High performance liquid chromatography
HT-2	HT-2 toxin
IAC	Immuno-affinity column
IARC	International Agency for Research in Cancer
IC_{50}	Inhibiting concentration of 50 %
IFN-γ	Interferon γ
Ig	Immunoglobulin
ig	Intragastrical
IL	Interleukin
ip	Intraperitoneal
iv	Intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK 1/2	c-Jun N-terminal kinase
LD_{50}	Lethal dose of 50 %
LPS	Lipopolysaccharide
LW	Live weight
LOAEL	Lowest observed adverse effect level
MAPK	Mitogen activated protein kinase
MAS	Monoacetoxyscirpenol
MCP	Monocyte chemoattractant protein
ME	Metabolizable energy
MIP	Macrophage inhibitory protein
MJ	Mega Joule
MON	Moniliformin
MPE	Molar percent excess
mRNA	messenger RNA
MTBSTFA	N-(tert-butyl-dimethyl-silyl)-N-methyl-trifluoro-acetamide
MTP	Microtitre plate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW	Molecular weight
n.a.	Not analysed
n/a	Not applicable
n.d.	Not detectable, < detection limit
ns	Not significant
NEO	Neosolaniol

NIV	Nivalenol
NOAEL	No observed adverse effect level
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PCA	Perchloric acid
PHA	Phythaemagglutinin
PMN	Polymorphonuclear neutrophils
PP	Peyer´s Patches
ppb	Parts per billion (µg/kg)
ppm	Parts per million (mg/kg)
PSEM	Pooled standard error of means
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Rounds per minute
r^2	Stability index
RSD	Residual standard deviation
RT	Room temperature
sc	Subcutaneus
SCF	Scientific Committee on Food of the European Commission
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEM	Standard error of means
SI	Stimulation index
SSA	5-sulfosalicylic acid dehydrate
T-2	T-2 toxin
t-BDMS	Tertiary-butyldiemthylsilyl
TCA	Trichloroacetic acid
TEER	trans-epithelial electric resistance
TGF	Tumor growth factor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
tRNA	Transfer-RNA
tTDI	Temporary tolerable daily intake
$t_{1/2}$	Biological half-life
t_{max}	Time of maximum serum concentration
t_s	Secretion time
U	Units (enzymes)
V_d	Apparent volume of distribution
VDLUFA	Verband Deutscher Landwirt-schaftlicher Untersuchungs- und Forschungsanstalten
WHO	World Health Organisation
α-ZOL	α-zearalenol
β-ZOL	β-zearalenol
ZON	Zearalenone

INTRODUCTION

Several *Fusarium* species are considered to be so-called field fungi as they infect wheat and maize principally before harvest in the northern temperate regions which results not only in a reduced crop yield by *Fusarium* head blight (scab), but also in the production of secondary metabolites, so-called mycotoxins. The trichothecene deoxynivalenol (DON) is of outstanding importance among these contaminants because of its frequent occurrence in toxicologically relevant concentrations worldwide (Bottalico and Perrone 2002, Logrieco et al. 2002, Placinta et al. 1999). Since the toxin accumulation depends strongly on environmental conditions such as temperature and humidity, a *Fusarium* toxin contamination can not be avoided completely. Moreover, DON can not significantly be removed during standard milling and processing procedures (Oldenburg et al. 2000, Lauren and Smith 2001). Therefore, exposure to this toxin is a permanent health risk assessment issue for both humans and farm animals.

Among farm animals pigs are particularly susceptible to DON, as they show overt signs of toxicity such as feed refusal, increased salivation and vomiting following DON ingestion (Rotter et al. 1996, Young et al. 1983). In addition to this rarely observed intoxication with high DON concentrations, the chronic exposure to lower amounts of DON is of major interest in DON-caused economical losses in animal production due to reduced feed intake and live weight gain. However, direct effects of DON on haematology, clinical-chemical parameters and immunity are as yet poorly defined since most investigations could not separate the effect of feed intake from DON contamination (Dänicke et al. 2001, Rotter et al. 1996).

Intragastrical application of radio-labeled pure DON indicated a rapid absorption, a widespread distribution, and slower elimination as well as a poor metabolism of this mycotoxin in pigs compared to that in more resistant ruminants (Prelusky et al. 1988). Nevertheless, the bioavailability and potential risk of a natural *Fusarium* contamination remains unclear, especially if one considers that DON from naturally contaminated material caused more adverse effects in animal studies than an application of pure toxin (Trenholm et al. 1994). The knowledge of bioavailability is of interest particular for wheat grain as this cereal is known for its frequent DON contamination and its importance in pig feeding.

Inhibition of protein synthesis on the cellular level is considered as the main toxic effect of trichothecenes. Here, the mycotoxin binds at the 60S ribosomal subunit and alters the conformation and activity of the peptidyl transferase (Ehrlich and Daigle 1987, Feinberg and

McLaughlin 1989). Therefore, it is often suggested that tissues with a high protein turnover or quickly proliferating cells, such as the liver or lymphocytes, respond especially sensitive to DON. However, effects of DON on protein synthesis have only been rarely examined, either with extremely high concentrations of pure toxin *in vitro* (ribosomes in cell-free systems or cell lines) or with a mouse model *in vivo* (Azcona-Olivera et al. 1995a, Ehrlich and Daigle 1987).

Furthermore, DON is known to either suppress or stimulate immunological parameters, for example, inhibition of lymphocyte proliferation with a concomitant increase of immunoglobulin and cytokine levels *in vitro* or *in vivo*, sometimes even at identical dosages (Bondy and Pestka 2000, Rotter et al. 1996, Pestka 2003, Pestka and Smolinski 2005). The phenomenon that protein synthesis inhibitors upregulate cytokine gene expression and secretion is called “superinduction” and may be due to inhibition of particularly labile translational repressor proteins (Efrat et al. 1984, Holt et al. 1988b, Zinck et al. 1995). However, it should not be overlooked that immunotoxicity studies have focused primarily on the mouse model with comparatively few investigations in domestic animals.

BACKGROUND

1 Trichothecenes and deoxynivalenol

1.1 Mycotoxins and fungal growth

Mycotoxins are toxic, naturally occurring secondary metabolites produced mainly by the mycelial structure of filamentous fungi, so-called moulds, growing in agricultural products on the field or during storage. The worldwide contamination of cereals and related products with mycotoxins (25 % of the world´s crop production; Rotter et al. 1996) causes health risks for both farm animals and humans (D´Mello et al. 1999, Fink-Gremmels 1999, Placinta et al. 1999). The production of mycotoxins is not essential for the fungal growth or reproduction, but could be a "virulence factor" for some plant diseases and act against other microorganisms and higher organisms (Desjardins et al. 1993, Puschner 2002). Plant stressors such as draught or over-irritation, insect damage and pesticide exposure result in a higher susceptibility to fungal infection, whereas the production of mycotoxins may be due to stress or altered conditions for the fungus (Fink-Gremmels 1999, Oldenburg et al. 2000). The degree of fungal growth is influenced by various factors, for example temperature, humidity, rainfall during anthesis (flowering) and at crop harvest, soil treatment and crop rotation (Oldenburg et al. 2000). The presence of the toxigenic fungus in a food or feed commodity can indicate a potential hazard, but only the detection of the specific toxin is revealing, since (1) the present fungus is not obliged to produce the toxin; (2) the toxin may persist in the substrate while the fungus could have vanished; (3) the fungus could produce more than one toxin; and (4) one toxin could be produced by different fungus species (Fink-Gremmels 1999).

1.2 Fusarium species and deoxynivalenol

Trichothecene mycotoxins are produced by a range of different plant pathogenic fungi, of which the *Fusarium* family is the most important. Several taxonomically unrelated genera of fungi (*Cephalosporum*, *Myrothecium*, *Stachybotrys*, *Trichoderma*, *Trichothecium*, and *Verticimonosporium*) are also able to produce trichothecenes (Bamburg 1983, EFSA 2004, Krska et al. 2001). The most widespread and highly phytopathogenic species of these filamentous field fungi in northern temperate regions are *Fusarium graminearum (Gibberella zeae)* and *Fusarium culmorum*, which cause *Fusarium* head blight in wheat and *Gibberella* ear rot in maize (EFSA 2004, Placinta et al. 1999). The pattern and amount of mycotoxins varies between fungal genera and even within strains of one distinct fungal species (Table 1) as well as from year-to-year (Fink-Gremmels 1999, Gutleb et al. 2002).

Table 1 Toxigenic *Fusarium* species and associated mycotoxins from grains and maize in northern Europe (Bottalico et al. 1989, Chelkowski 1998, Logrieco et al. 2002)

***Fusarium* species**	**Occurrence**	**Phytopathogenicity**	**Mycotoxins**
F. graminearum	+++	+++	DON, 3-ADON, 15-ADON, NIV, DAS, FX, ZON
F. culmorum	++	+++	DON, 3-ADON, NIV, T-2, HT-2, NEO, ZON
F. crockwellense *(F. cerealis)*	+	+++	NIV, FX, ZON
F. avenaceum	++	++	NEO, FX, MON, ZON
F. subglutinans	+++	++	MON, BEA
F. moniliforme *(F. verticillioides)*	++	++	FB_1, FB_2, FB_3, F-C
F. poae	++	++	NIV, FX, DAS, MAS, T-2, HT-2
F. sporotrichioides	+	++	T-2, HT-2, NEO
F. tritrinctum	+	+	T-2, DAS, MON, ZON
F. proliferatum	+	+	FB_1, FB_2, FX, MON, BEA

+ - +++ = low – high; 3-ADON = 3-acetyl-DON, 15-ADON = 15-acetyl-DON, BEA = Beauvericin, DAS = Diacetoxyscirpenol, DON = Deoxynivalenol, FB = Fumonisin B, F-C = Fusarin C, FX = Fusarenon X, MAS = Monoacetoxyscirpenol, MON = Moniliformin, NEO = Neosolaniol, NIV = Nivalenol, ZON = Zearalenone

Deoxynivalenol (DON), a mycotoxin belonging to the Type B trichothecenes, is one of the most frequently detected mycotoxins in cereals and feeds causing economical losses in agricultural production and livestock (EFSA 2004, Rotter et al. 1996).

Table 2 Deoxynivalenol (DON) concentrations in grains from Germany (update according to Oldenburg et al. 2000)

Cereal Type	Region	Year	Samples		Concentration		Reference
			N	Pos. (%)	Range (µg/kg)	Mean (µg/kg)	
Wheat	Baden-Württemberg	1987	84	96	4 - 20538	1692	Müller et al. 1997b
		1989	78	69	3 - 1187	152	
		1990	80	96	8 - 8969	595	
		1991	80	96	4 - 4627	359	
		1992	78	95	18 - 5412	335	
		1993	45	96	19 - 6165	391	
Wheat	Baden-Württemberg	1989	53	83	4 - 3464	303	Müller et al. 2001
		1990	54	87	4 - 15869	735	
		1991	57	93	4 - 2636	180	
		1992	52	88	14 - 1275	167	
		1993	60	77	16 - 1554	219	
Wheat	Bayern	1991	51	88	100 - 1200	420	Marx et al. 1995
			50	76	100 - 1000	490[3]	
Wheat	Schleswig-Holstein	1998	116	86	- 10800	2700	Reutter 1999
Wheat	Thüringen	1998	196	65	120 - 11660	1380[1]	Döll et al. 2002
Wheat	Baden-Württemberg	1998 2000	115	96	15 - 7730	471	Schollenberger et al. 2002
Wheat	Bayern	1999	73	67	50 - 3350		Janes et al. 2000
Wheat	Thüringen	1998		71	- 11080	610[2]	Kirchheim et al. 2002
		2000		55	- 4600	330[2]	
		2001		40	- 1900	270[2]	
Wheat	Sachsen-Anhalt	2000	8	25	- 400	260	Woese 2001
		2001	30	33	- 913	402	Woese 2002
Wheat	Schleswig-Holstein	2001	75	84	- 2400	170[2]	Reutter 2002
Wheat		2001	253	65	n/a n/a	272	Wolff 2003
		2002	261	84		239	

Table 2 (continued)

Cereal Type	Region	Year	Samples		Concentration		Reference
			N	Pos. (%)	Range (µg/kg)	Mean (µg/kg)	
Triticale	Schleswig-Holstein	1998	24	83	- 4710	990	Reutter 1999
Triticale	Bayern	1999	23	48	100 - 2100	n/a	Janes et al. 2000
Triticale		2001	258	79	n/a n/a	170	Wolff 2003
		2002	476	88		211	
Triticale	Thüringen	1998		75	- 13500	1550[2]	Kirchheim et al. 2002
		2000	n/a	67	- 5000	490[2]	
		2001		60	- 2700	760[2]	
Rye	Bayern	1991	50	40	100 - 1250	160	Marx et al. 1995
			50	56	100 - 500	430[3]	
Rye	Thüringen	1998	69	28	120 - 3090	450[1]	Döll et al. 2002
Rye	Sachsen-Anhalt	2000	2	50	- 130	130	Woese 2001
Rye	Sachsen-Anhalt	2001	27	7	- 150	140	Woese 2002
Rye		2001	189	58	n/a n/a	56	Wolff 2003
		2002	196	75		153	
Rye	Thüringen	1998		34	- 2940	230[2]	Kirchheim et al. 2002
		2000	n/a	6	- 960	370[2]	
		2001		24	- 340	160[2]	
Barley	Bayern	1999	84	87	100 - 3000	n/a	Janes et al. 2000
Barley	Thüringen	2000	n/a	23	- 850	150[2]	Kirchheim et al. 2002
		2001		51	- 950	160[2]	
Barley	Sachsen-Anhalt	2001	23	35	- 290	164	Woese 2002
Barley	Baden-Württemberg	1987	44	98	4 - 4764	400	Müller et al. 1997a
		1989	40	73	6 - 483	103	
		1990	47	72	2 - 300	74	
		1991	51	71	4 - 530	54	
		1992	58	90	4 - 486	42	

Cereal Type	Region	Year	Samples		Concentration		Reference
			N	Pos. (%)	Range (µg/kg)	Mean (µg/kg)	
Oat	Baden-Württemberg	1987		68	3 - 1480	135	Müller et al. 1998
		1989		77	4 - 563	130	
		1990	n/a	52	3 - 203	52	
		1991		49	8 - 857	219	
		1992		85	20 - 1224	302	
Oat	Bayern	1999	17	12	650 - 1720		Janes et al. 2000

[1] applied on dry matter; [2] median; [3] oecological cultivation; n/a, not applicable

Numerous investigations have demonstrated that DON can particularly be found in cereal grains following moisture at the time of flowering, which might result in so-called *Fusarium* years, for example 1998, with higher maximum and mean DON concentrations (Oldenburg et al. 2000; Table 2). On the other hand, it has to be noted that DON occurs simultaneously with other *Fusarium* mycotoxins, mainly Type B trichothecenes and zearalenone (ZON) (Müller et al. 2001, Placinta et al. 1999) since most of the *Fusarium* species have the ability to form more than one toxin, depending on genetic factors and various environmental conditions.

1.3 Structure and physical-chemical properties of trichothecenes and deoxynivalenol

Approximately 180 trichothecene derivates have been isolated and characterized (Pestka and Smolinski 2005). Chemically, trichothecenes are closely related, low-molecular-weight sesquiterpenoids and usually contain an epoxide ring at C-12 and C-13 and a double bond at position C-9 and C-10 which both are important for their toxicity (Desjardins et al. 1993, Ehrlich and Daigle 1987; Figure 1). Trichothecenes are divided into four groups (types A-D) according to their chemical properties and the fungi (Ueno 1977).

Fusarium species produce Type A and B trichothecenes, which are distinguished by the absence or presence of a carbonyl group at C-8 position, respectively. Figure 1 & Table 3 demonstrate the chemical structures of the major Type A (T-2 toxin and DAS) and Type B (DON and NIV) trichothecenes. The two other trichothecene categories are formed by other genera of fungi, e. g., *Myrothecium verrucaria*, and characterized by a second epoxide function at C-7,8 or C-9,10 (Type C) or a macrocyclic ring between C-4 and C-15 with two ester linkages (Type D) (Ueno 1985). However, Type C and Type D trichothecenes are not discussed any further, since they occur rarely in food and feed (Krska et al. 2001).

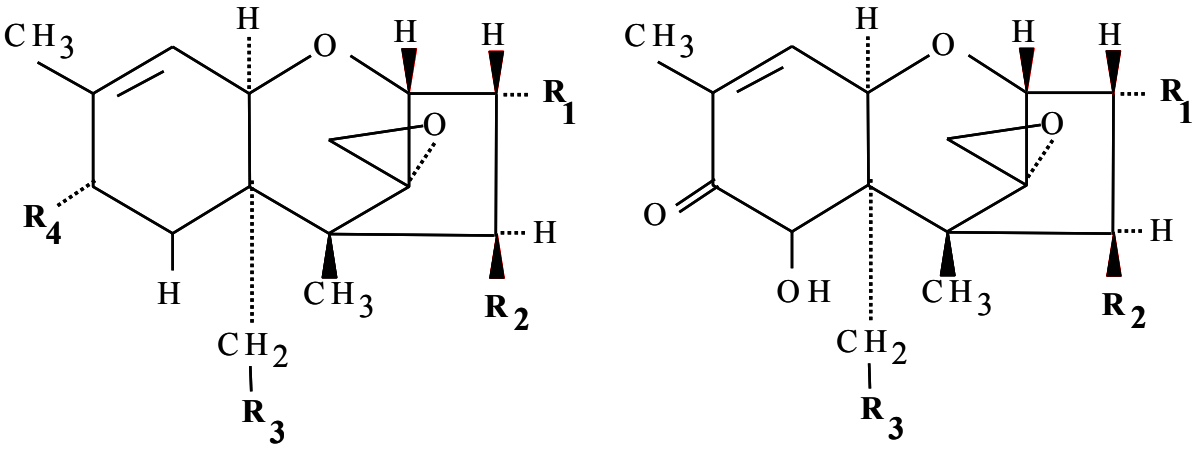


Figure 1 Chemical structures of Type A and B trichothecenes. Substituents R1 – R4 are given in Table 3

Table 3 Chemical structures of substituents R1 – R4 of Type A and B trichothecenes

Toxin	Abbr.	R1	R2	R3	R4
Type A					
T-2 toxin	T-2	OH	OCOCH	OCOCH	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	HT-2	OH	OH	OCOCH	$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpenol	DAS	OH	OCOCH	OCOCH	H
Type B					
Nivalenol	NIV	OH	OH	OH	
Deoxynivalenol	DON	OH	H	OH	
3-acetyl-deoxynivalenol	3-ADON	OCOCH	H	OH	
15-acetyl-deoxynivalenol	15-ADON	OH	H	OCOCH	
Fusarenon X	FX	OH	OCOCH	OH	

The trichothecene skeleton is chemically stable in heat as well as at neutral and acidic pH values, and the 12,13 epoxid ring sustaines nucleophilic attacks (Krska et al. 2001, Lauren and Smith 2001, Ueno 1987). Thus, trichothecenes are not degraded during normal food

processing and are not hydrolyzed in the stomach after ingestion (Lauren and Smith 2001, Scott 1990). Furthermore, most of the trichothecenes are soluble in solvents like acetone, chloroform and ethylacetate, but highly hydroxylated trichothecenes, for example DON and NIV, are also soluble in more polar solvents like acetonitrile, methanol, ethanol and water (Ueno 1987).

Deoxynivalenol (DON; 12,13-epoxy-3α,7α,15-trihydroxy-trichothec-9-ene-8-one) was first isolated by Japanese scientists (Morooka et al. 1972) as 'Rd-toxin'. Shortly thereafter Yoshizawa and Morooka (1973) described the chemical structure and termed it as '4-deoxy-nivalenol'. In the same year, the same compound was named 'vomitoxin' by Vesonder et al. (1973), who isolated it from a maize batch that induced emesis in pigs. The chemical and physical data of deoxynivalenol were summarized by the IARC (1993) (Table 4).

Table 4 Physical-chemical properties of deoxynivalenol (Reference: IARC 1993)

	Description	Empirical formula	Molecular weight	Melting point
DON (CAS No. 51481-10-8)	White needles	$C_{15}H_{20}O_6$	296.32	151-153 °C

It was suggested that the lower toxicity of DON may be due to the oxygen substituents in the A-ring (C-8 keto and C-7 hydroxyl) which make this side of the molecule more hydrophilic compared to more potent trichothecenes which lack substitution in the A-ring or have esterified hydroxyls. Furthermore, replacement of the C-4 hydroxyl (NIV) with hydrogen (DON) would not appreciably alter the steric access to the trichothecene, but would leave the epoxide more exposed (Ehrlich and Daigle 1987).

1.4 Relevance of DON for human and animal nutrition

In general, Type A trichothecenes tend to be more toxic than Type B, but toxicological ranking order depends on the test system used (Rotter et al. 1996). However, DON is one of the most abundant and important trichothecenes in food and feed, because of its worldwide frequent occurrence in toxicological relevant concentrations and the insufficient elimination during milling and processing procedures (IARC 1993, Müller et al. 1997a,b, 2001, Placinta et al. 1999, Rotter et al. 1996). Since *Fusarium* species are ubiquitous, a total prevention of a *Fusarium* infection and the contamination with trichothecenes seems to be unlikely. Therefore, it can be predicted that food and feed are always contaminated with toxins to a

greater or less extent, and with increasing accuracy of analysis (lower detection limits) DON can be detected in more and more cases (nearly 100 %).

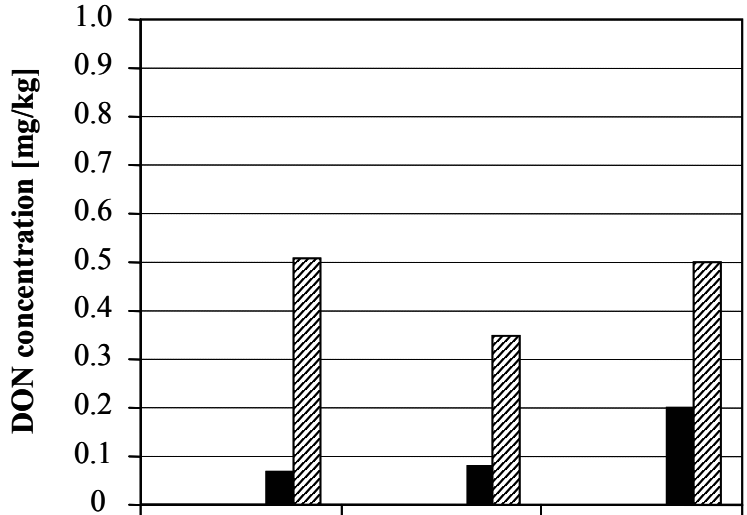
Table 5 Orientation values for critical concentrations of deoxynivalenol and zearalenone in diets of swine, cattle and chicken (mg/kg diet, based on 88 % dry matter, Reference: BML 2000)

Species	Animal category	Deoxynivalenol	Zearalenone
Swine			
	prepubertal female breeding pigs	1.0	0.05
	fattening pigs and breeding sows	1.0	0.25
Cattle			
	pre-ruminant	2.0	0.25
	female cattle/cow	5.0	0.5
	fattening cattle	5.0	-[1]
Chicken			
	laying hen, broiler	5.0	-[1]

[1]at present standard of knowledge no orientation values required

Low DON concentrations in processed food products including wheat flour, bread, breakfast cereals, noodles, baby and infants foods, beer and related products were recently reported (JECFA 2001, Schollenberger et al. 1999, Sudakin 2003). However, the most data on trichothecene contamination is derived from grains and grain products destined for human consumption (EFSA 2004). Therefore, it could be suggested that poorer quality grain is probably diverted to animal feed which would probably result in a higher incidence and higher level of DON (EFSA 2004). Additionally, it has to be taken into account that DON was shown to be concentrated in by-products, such as bran, that often serve as animal feed (EFSA 2004). As cereal crops are commonly contaminated with DON and the proportion of cereals in pigs diets are relatively high compared to the high roughage intake of ruminants (EFSA 2004), it can be assumed that pigs are frequently exposed to DON contaminated diets.

Furthermore, swine were described to react especially sensitive to DON, as they showed feed refusal and emesis after dietary DON exposure (Vesonder et al. 1973). This is also reflected by the orientation values of critical concentrations of deoxynivalenol and zearalenone in diets for farm animals published by the German Federal Ministry of Nutrition, Agriculture and Forestry (BML 2000; Table 5).



	2001	2002	2003
N	175	329	472
% > d.l.	60	56	75
Min.	<0.025	<0.005	<0.005
Max.	1.9	1.2	2.3
Median	0.07	0.08	0.20
90 %	0.51	0.35	0.50

Figure 2 Survey of DON concentrations in complete mixed commercial pig diets according to the German Official Control Program (> d.l., above detection limit, orientation value of 1 mg DON/kg, Reference: Meng et al. 2006)

In any case, an examination of complete mixed commercial pig diets according to the German Official Control Program (Meng et al. 2006) revealed that this critical concentration of 1 mg/kg was indeed exceeded by the maximal DON concentration observed, but the median values and even the 90 % percentile are below this critical concentration of DON (Figure 2).

Furthermore, it has to be kept in mind that the co-occurrence of DON together with zearalenone and other trichothecenes, such as nivalenol, 3-ADON, 15-ADON and FX, is common. However, it is rarely possible to assess how many different toxins are present at what levels in specific samples (EFSA 2004). Therefore, DON has developed to an "indicator toxin".

2 Toxicity of deoxynivalenol

The Scientific Committee on Food (SCF 1999, 2002) derived a tolerable daily intake (TDI) of DON for humans of 1 µg/kg body weight after multiplying a no observed adverse effect level (NOAEL) of 0.1 mg DON/kg LW, resulting from one long-term study in mice, with a safety factor of 100. However, it has to be emphasized that this TDI of DON is not based on its molecular mode of action, but only on growth data of mice. In addition, the orientation value of maximal 1 mg DON/kg in pig diets (BML 2000) was also derived primarily from performance of animals. Therefore, it seemed necessary to investigate the effect of DON on its principal mode of action, which is ascribed to be protein synthesis inhibition.

2.1 Mode of action

2.1.1 Protein synthesis inhibition

Trichothecenes are well-known inhibitors of the protein synthesis. They bind to the 60S subunit of eukaryotic ribosomes and impair the function of the peptidyl transferase (Feinberg and McLaughlin 1989). The inhibitory ability requires an intact 9,10-double bond and the C-12,13-epoxide functionality (Ehrlich and Daigle 1985, Wei and McLaughlin 1974). Reduction of the epoxide ring (de-epoxidation) results in the loss of any apparent toxicity (Feinberg and McLaughlin 1989). Depending on the substituents, trichothecenes inhibit either the initiation or the elongation and termination step of the protein synthesis (Carter and Cannon 1977, Ehrlich and Daigle 1987). Increase of the amount of free ribosomes (60S + 40S) compared to polyribosomes (80S) was observed by initiation inhibitors (I-Type), while elongation (or termination) inhibitors (E-Type) stabilize polyribosome profiles (Figure 3; Cundliffe et al. 1974, Schindler 1974). Highly substituted trichothecenes such as T-2 can bind only at peptidyl transferase centers in which a nascent peptide of less than three or four amino acids is present, whereas ribosomes containing longer amino acid chains can continue the process of elongation on the mRNA (“run off”) (Cannon et al. 1976, Carter and Cannon 1977). On the other hand, small trichothecenes can bind to most or all peptidyl transferase centers and prevent polypeptide chain elongation (Ehrlich and Daigle 1987). Whereas most of the trichothecenes predominantly inhibit the initiation, DON is an inhibitor of elongation (Figure 3; Ehrlich and Daigle 1987). But the trichothecenes that mainly inhibit the peptide chain initiation are several orders of magnitude more potent than are those that affect peptide chain elongation (Ehrlich and Daigle 1985).

In vitro protein synthesis of EL-4 T-cells, a murine lymphoma cell line, using [^{3}H]leucine was slightly increased at low DON concentrations (10 and 25 ng/ml), but was inhibited at higher

DON doses (Dong et al. 1994). However, cycloheximide (CHX) appeared to be a more potent protein synthesis inhibitor, as the inhibitory concentration of 50 % (IC_{50}) were 280 ng DON/ml and 55 ng CHX/ml (Dong et al. 1994). Accordingly, IC_{50} of [^{3}H]leucine incorporation were 444 and 252 ng DON/ml in Vero cells and rat spleen lymphocytes, respectively (Thompson and Wannemacher 1986). However, the minimum inhibitory concentration required to inhibit protein synthesis in cells is much higher than that in cell-free systems (Khachatourians 1990). This difference might be due to toxin uptake into the cell and/or metabolism of mycotoxin by cytosolic enzymes (Thompson and Wannemacher 1986). Furthermore, it has to be noted that several trichothecenes, which were weak inhibitors of protein synthesis *in vitro*, showed high toxicities in a whole animal lethality test *in vivo*, indicating that the *in vitro* cell response is not always a good indicator of toxicity in whole animals (Thompson and Wannemacher 1986, 1990).

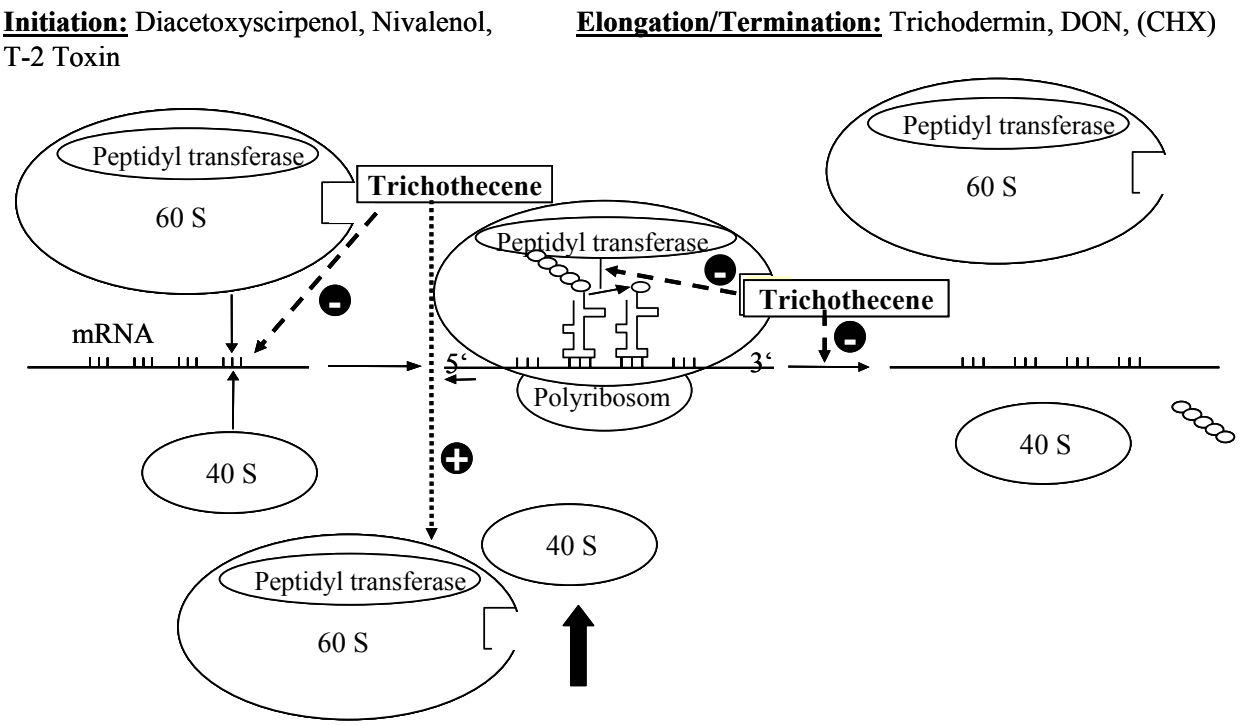


Figure 3 Mechanism of protein synthesis inhibition by trichothecenes according to Dänicke et al. (2000, modified). Inhibitors of initiation (I-Type) will accumulate free ribosomes (40S + 60S) as these are not able to bind to mRNA (initiation complex). Elongation and termination inhibitors (E-Type) will increase the amount of polyribosomes (80S) as the uncoupling from mRNA and release of peptide chain is inhibited (further details in the text, ⊖ inhibitory or ⊕ activating effects)

Plasma amino acid concentrations of guinea pigs were significantly elevated 30 min to 12 h after intramuscular (im) injection of 1 mg/kg LW T-2, indicating an increased tissue destruction and reduced utilization for protein synthesis (Wannemacher and Dinterman 1983). However, *in vivo* the effects of trichothecenes on protein synthesis have to date been rarely examined using [^{14}C]leucine incorporation only in rodents. Intraperitoneal (ip) injection of a lethal dose of T-2 toxin (2 mg/kg LW) or CHX (10 mg/kg LW) to rats decreased [^{14}C]leucine incorporation significantly in muscle, heart, kidney, liver, spleen and intestine by more than 70 % (Thompson and Wannemacher 1990). In addition, a strong overall inhibition of DNA synthesis ([^{3}H]thymidine incorporation) was observed in all tissues after T-2 and CHX treatment, but showed a higher variability than inhibition of protein synthesis (Thompson and Wannemacher 1990). At DON doses of 80 and 20 mg/kg LW ip to mice, [^{14}C]leucine incorporation was inhibited in the heart, kidney, liver and spleen by more than 50 % (Robbana-Barnat et al. 1987). However, 10 mg DON/kg LW ip only reduced [^{14}C]leucine incorporation into the heart, whereas 4 mg DON/kg showed no effect on protein synthesis *in vivo* (Robbana-Barnat et al. 1987). Furthermore, acute oral exposure of mice to 25 mg/kg LW pure DON inhibited *in vivo* protein synthesis in all organs examined (spleen, Peyer´s Patches (PP), kidney, liver, small intestine and plasma) 3, 6 and 9 h postdosing, whereas oral gavage of 5 mg DON/kg LW reduced [^{14}C]leucine incorporation only in the kidney, liver, small intestine and plasma after 3 h and tended to recover thereafter (Azcona-Olivera et al. 1995a).

Several studies have demonstrated that protein synthesis inhibitors “superinduce” cytokine gene expression and secretion (Efrat et al. 1984, Holt et al. 1988b, Zinck et al. 1995), whereby different mechanisms are feasible to transiently induce expression of specific gene transcripts. First of all, it could be considered that primarily high turnover proteins, which downregulate transcription, are affected by impaired synthesis (Zinck et al. 1995). Second, a decreased synthesis of labile selective nucleases may result in increased mRNA levels for cytokine genes (Azcona-Olivera et al. 1995a). Finally, a direct stimulation of intracellular signalling pathways, not involving protein synthesis inhibition, could be suggested to cause “superinduction” (Edwards and Mahadevan 1992, Mahadevan and Edwards 1991).

2.1.2 Other cellular and molecular mechanism

Besides the inhibition of protein synthesis, trichothecenes are considered to have multiple inhibitory effects on eukaryote cells. An inhibition of RNA and DNA synthesis, as well as adverse effects on mitochondrial function were observed (Charoenpornsook et al. 1998, Mekhancha-Dahel et al. 1990, Minervini et al. 2004, Thompson and Wannemacher 1986,

1990, Ueno 1977, 1985). However, these effects seemed to be secondary to the inhibition of protein synthesis (Thompson and Wannemacher 1990).

Further suggested toxic mechanisms of trichothecenes included impaired membrane function (Bunner and Morris 1988), altered intercellular communication (Jone et al. 1987), and deregulation of calcium homeostasis (Yoshino et al. 1996). Moreover, apoptosis was linked to alterations in cell signalling at the level of mitogen-activated protein kinases (MAPKs, Shifrin and Anderson 1999) and the "ribotoxic stress response" induced by translational inhibitors, such as trichothecenes (Moon and Pestka 2002, Iordanov et al. 1997, Yang et al. 2000). In addition, trichothecenes were reported to cause lipid peroxidation (Rizzo et al. 1994, Suneja et al. 1989) possibly due to an increased respiratory burst of macrophages (Cooray and Jonsson 1990) and a consequent release of reactive oxygen species.

2.2 Acute/subacute toxicity

In animals, the effect of DON is dependent on the dose and duration of exposure, as well as the species involved. Although DON is less toxic than other trichothecenes such as T-2 toxin, acute exposure to extremely high DON doses can produce shock-like death (Pestka and Smolinski 2005; Table 6).

Table 6 Acute toxicity of deoxynivalenol (DON) and metabolites on lethality (LD_{50})

Species, strain	Route	Toxin	Parameter	Dose (mg/kg LW)	References
Mouse, $B6C3F_1$ -female, weanling	oral	DON	LD_{50}	78	Forsell et al. 1987
		15-ADON		34	
	ip	DON	LD_{50}	49	
		15-ADON		113	
Mouse, DDY - male (6 weeks)	oral	DON	LD_{50}	46	Yoshizawa and Morooka 1974
		3-ADON		34	
	ip	DON	LD_{50}	70	
		3-ADON		49	
- female (4 weeks)	ip	DON	LD_{50}	77	
		3-ADON		47	
Mouse	ip	DON	LD_{50}	43	Thompson and Wannemacher 1986
	sc	DON	LD_{50}	45	
Duck, Peking (10 days)	sc	DON	LD_{50}	27	Yoshizawa and Morooka 1974
Chicken, Cobb, female broiler	oral	DON	LD_{50}	140	Huff et al. 1981
Cat	oral	DON	LD_{50}	200	Ueno et al. 1974

ip, intraperitoneal; LD_{50}, lethal dose of 50 %; sc, subcutaneous

In B6C3F$_1$ female mice, the lethal dose of 50 % (LD_{50}) was 78 mg/kg LW and 49 mg/kg LW given orally and ip, respectively (Forsell et al. 1987). In contrast, male DDY mice showed a LD_{50} of 70 mg/kg LW ip and 46 mg/kg LW orally (Yoshizawa and Morooka 1974). Subcutaneous exposure of 27 mg DON/kg LW was estimated as LD_{50} in 10-days old Peking ducklings (Yoshizawa and Morooka 1974), indicating that species, strain, sex and age differences may vary DON absorption and distribution and therefore its toxicity.

Table 7 Acute toxicities of deoxynivalenol (DON) on emesis

Species	Route	Source	Parameter	Dose (mg/kg LW)	References
Pig, 9 -10 kg	oral	purified	LOAEL	0.1	Forsyth et al. 1977
			NOAEL	0.075	
	ip	purified	LOAEL	0.05	
			NOAEL	0.025	
Pig, 28-51 kg	oral	purified	ED_{50}	0.088	Young et al. 1983
			LOAEL	0.07	
			NOAEL	0.035	
Pig, 7.5 kg	diet	contaminated corn	LOAEL	0.8 (20 ppm)	Young et al. 1983
			NOAEL	0.6 (11.9 ppm)	
Pig, 10-15 kg	oral	purified	LOAEL	0.05 (4/15)	Pestka et al. 1987
			NOAEL	0.025	
	ip	purified	LOAEL	0.05 (9/15)	
			NOAEL	0.025	
Pig, 12 – 25 kg, fasted	oral	purified	ED_{50}	0.085	Prelusky and Trenholm 1993
			LOAEL	0.05	
			NOAEL	0.025	
	ig	purified	ED_{50}	0.075 (2/6)	
			LOAEL	0.05 (1/5)	
			NOAEL	0.025	
	iv	purified	ED_{50}	0.02	
			LOAEL	0.02 (2/4)	
			NOAEL	0.015	
Dog, 2-3 kg	sc	purified	LOAEL	0.1	Yoshizawa and Morooka 1974
Dog	diet	contaminated wheat	LOAEL	0.45	Hughes et al. 1999
			NOAEL	0.3	
Cat	diet	contaminated wheat	LOAEL	0.4	Hughes et al. 1999
			NOAEL	0.3	

ED_{50}, emetic dose of 50 %; ig, intragastric; ip, intraperitoneal; iv, intravenous; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level

Acute/subacute DON intoxications are characterized by emesis (especially in pigs; Table 7), feed refusal, weight loss and diarrhoea (Forsell et al. 1987, Prelusky and Trenholm 1993, Yoshizawa and Morooka 1974, Young et al. 1983). Intraperitoneal application of 10 - 1000 mg/kg LW of pure DON to mice resulted in necrosis of the gastrointestinal tract, bone marrow and lymphoid tissues such as spleen and thymus (Forsell et al. 1987). Accordingly, Arnold et al. (1986b) described atrophy of thymus, spleen, Peyer´s Patches (PP) und lymph nodes in mice given 7.5 mg DON/kg LW, indicating that lymphoid tissues are particularly sensitive to DON. Furthermore, heart lesions were observed in mice after exposure to high DON doses (Forsell et al. 1987, Robbana-Barnat et al. 1987).

However, it has to be taken into account that acute effects of DON such as mortality and necrosis of tissues could be detected only at extremely high DON doses (≥ 27 mg/kg LW), which were unlikely to occur in food and feed. On the other hand, relatively low oral doses of about 50 µg/kg LW were shown to induce emesis in pigs (Forsyth et al. 1977, Pestka et al. 1987, Prelusky and Trenholm 1993, Young et al. 1983), whereby the DON contaminated feed has to be presented as one single bolus rather than as a total-day-exposure to cause an emetic effect. Therefore, it still has to be clarified whether a practical level of DON will functions differently when administered as a single dose (acute exposure) or as a chronic DON exposure (≥ 4 weeks).

2.3 Subchronic/chronic toxicity

2.3.1 Effects on performance and feed intake

Prolonged dietary DON exposure of animals was described to cause anorexia, decreased live weight gain, and altered nutritional efficiency (Pestka and Smolinski 2005). Regarding livestock production these adverse effects of DON on performance resulted in great economic losses, especially in pig production, since the tolerance of farm animals decreased in the order of ruminants > poultry > pig (Table 5). This is underscored by findings that up to 8 ppm DON did not affect the productivity of poultry (Hamilton et al. 1985a,b), and DON feeding of 66 ppm over 5 days (Cote et al. 1986a), and 6.4 ppm for 6 weeks (Trenholm et al. 1985), caused no detrimental effects on the performance or health of dairy cows. Furthermore, it has to be taken into account that although rodents were used in most studies, they were more tolerant to DON than pigs. Consequently, feed intake was reduced in mice at doses of 2.5 - 10 ppm depending on strain and sex (Forsell et al. 1986, Iverson et al. 1995, Robbana-Barnat et al. 1987, Rotter et al. 1992a), whereas rats seemed to require a DON contamination of 12 - 20

ppm before overt effects could be observed (Arnold et al. 1986a, b, Morrissey and Vesonder 1985, Morrissey et al. 1985). However, in pigs DON exposure of 1 – 2 ppm already reduced feed intake and live weight gain (Friend et al. 1982, Rotter et al. 1994, Trenholm et al. 1984), whereas 12 ppm produced complete feed refusal (Young et al. 1983). Besides the adverse effects on feed intake and live weight gain, Bergsjø et al. (1992, 1993) described a decreased feed efficiency in pigs fed naturally DON contaminated diets of 2 - 4 ppm. In contrast, Øvernes et al. (1997) observed no effect on feed intake and live weight gain, but an enhanced feed efficiency in pigs fed up to 4 ppm DON. However, it has to be noted that the control diet already contained 0.6 ppm DON.

The anorectic and emetic effects of DON are supposed to be mediated by the serotinergic system (as reviewed by Rotter et al. 1996), because DON treatment increased concentrations of serotonin (5-HT, 5-hdroxytryptamine) and its metabolites in the cerebral spinal fluids of rats and pigs (Fitzpatrick et al. 1988, Prelusky 1993, 1996), and DON-induced emesis in pigs was avoided by application of serotonin receptor antagonists (Prelusky and Trenholm 1993). In addition, peripheral serotonin receptors in the gastrointestinal tract were also supposed to be involved in the DON-induced effects (Fioramonti et al. 1993, Prelusky and Trenholm 1993).

Furthermore, DON was shown to produce a conditioned taste aversion in rats and pigs (Clark et al. 1987, Ossenkopp et al. 1994, Osweiler et al. 1990) when it was administered concomitant with a novel taste such as saccharin. Ossenkopp et al. (1994) related this conditioned (learned process) taste aversion to the area postrema, which is characterized by a reduced blood-brain barrier and may therefore be accessible and a sensor for toxins in the blood.

2.3.2 Effects on the gastrointestinal tract and other organ manifestation

Rotter et al. (1994) suggested that DON may alter the stomach epithelial cell layer, as the oesophageal region of the stomach appeared thicker and the degree of folding higher. This might have caused a change in the passage rate of the intestine and have contributed to a higher feed efficiency. The relative weight of stomach increased in pigs with increasing DON concentrations in the diet (Trenholm et al. 1984). Furthermore, at higher DON concentrations of 5 ppm swelling and keratinisation of the mucosa of oesophageal area of the stomach were detected (Trenholm et al. 1984). Accordingly, Arnold et al. (1986b) found local irritations of the gastrointestinal tract in mice and rats. Fioramonti et al. (1993) observed delayed gastric emptying and reduced small-intestine motility in mice and rats. Moreover, intestinal uptakes

of glucose and other nutrients were impaired in broilers and mice after dietary exposure to 10 ppm DON (Awad et al. 2004, Hunder et al. 1991). These findings were approved *in vitro* by Maresca et al. (2002), who found an inhibition of intestinal glucose transporter activities in human epithelial intestinal cell line HT-29-D4 by DON.

Although possible effects of DON on parenchymatous organs are discussed controversially, it was supposed that liver and kidney were affected by long-term toxin exposure. In accordance, liver weights were increased in mice and pigs after chronic DON feeding (Iverson et al. 1995, Bergsjø et al. 1993). In contrast, relative liver and kidney weights of starter pigs were lower after dietary DON exposure to 4.6 ppm for 21 days (Swamy et al. 2002). Furthermore, kidney mesangial IgA accumulation, electron dense mesangial deposits and haematurie of DON-exposed mice were frequently described and were linked to the human IgA nephropathy (Dong et al. 1991, Pestka 2003).

While intragastrical gavage of up to 7.5 mg/kg LW caused atrophy of thymus, spleen, PP, and lymph nodes (Arnold et al. 1986b), histopathological changes were not found in mice fed contaminated diets up to 1 mg DON/kg LW daily for 5-9 weeks (Arnold et al. 1986a, Tryphonas et al. 1986).

2.3.3 Effects on blood parameters

Serum albumin concentrations were increased, whereas α_1- and α_2-globulin levels were reduced in mice given up to 1 mg DON/kg LW for 5 weeks (Tryphonas et al. 1986). Accordingly, Rotter et al. (1994) reported an increased albumin/globulin ratio, resulting from increased albumin and decreased α-globulin serum concentrations, when feeding increasing DON concentrations (0.75 – 3 ppm) to pigs, indicating that DON may affect the protein profile of blood. In contrast, Döll et al. (2003) observed a decrease in serum protein and GLDH after feeding a DON contaminated maize diet up to 3.9 ppm to piglets. Decreased serum protein and albumin concentrations were also found in pigs exposed to diets contaminated with 3.5 ppm DON (Bergsjø et al. 1993). Additionally, a temporary fall in packed blood cell volume, serum calcium, and serum phoshorus was observed (Bergsjø et al. 1993). Furthermore, Arnold et al. (1986a, b) described a decrease in haematocrit, haemoglobin and erythrocyte counts with a concomitant increase of leukocyte counts after feeding mice 6.25 ppm DON for 91 days. Trenholm et al. (1994) observed a decreased concentration of total protein, anorganic phosphate, as well as a reduced activity of the alkaline phosphatase (AP) in the serum of pigs after 14 days exposure to 3.4 ppm pure DON. On the other hand, in a subsequent long-term study with DON exposure of up to 8.7 ppm to

pigs, no disturbance of these blood parameters could be reproduced (Trenholm et al. 1994). However, it has to be noted that a two-year feeding study with $B6C3F_1$ mice receiving up to 10 ppm DON resulted in no biologically relevant haematological or clinical-chemical effects (Iverson et al. 1995). Accordingly, Cote et al. (1985) were not able to observe significant alterations of creatinine, total protein, anorganic phosphate, AP, ASAT, ALAT, LDH, cholesterin, bilirubin and further parameters when pigs were fed a naturally contaminated diet up to 5.8 mg DON/kg.

Nevertheless, it has to be emphasized that most studies were not able to distinguish between indirect effects due to a DON-induced decrease in feed intake and the direct toxic effects. For determination of the direct DON effect, it is therefore necessary that all animals be fed the same amount of diet, which is voluntarily consumed by DON exposed animals (pair-feeding).

2.4 Reproduction toxicity, teratogenicity and carcinogenicity

The International Agency for Research in Cancer (IARC 1993) graded DON "not classifiable" for carcinogenicity to humans. However, mice showed no preneoplastic or neoplastic changes after daily DON consumption of 0, 1, 5, 10 ppm during two years (Iverson et al. 1995). Accordingly, DON did not show a mutagenic activity in the Ames test with *Salmonella typhimurium* (IARC 1993). However, in interpreting the literature results, the IARC (1993) concluded that DON induced cell transformation, chromosomal aberrations and inhibits gap-junctional intercellular communication in cultured mammalian cells. Furthermore, DON is capable of inducing reproductive/teratogenic effects in mice and rabbits with NOAELs of 0.5 and 0.6 mg/kg LW/d, respectively (as reviewed in Eriksen and Alexander 1998, Pestka and Smolinski 2005).

2.5 Modulation of immune function

Leukocytes as the functional cells of the immune system were regarded as a primary target for deoxynivalenol and other trichothecenes (Pestka et al. 2004). Studies investigating the immunotoxicity of DON, principally in laboratory animals and cell cultures, indicated that DON and other trichothecenes may have stimulatory as well as suppressive effects on immune function (Rotter et al. 1996). Exposure to high doses of trichothecenes resulted in necrosis and atrophy of actively dividing tissues such as bone marrow, lymph nodes, spleen, thymus and intestinal mucosa (Arnold et al. 1986b, Forsell et al. 1987). Accordingly, mitogen-induced proliferation of human and murine lymphocytes was impaired after DON exposure *in vitro* (Atkinson and Miller 1984, Forsell et al. 1985, Miller and Atkinson 1986, 1987, Tomar et al. 1986, 1987). In addition, dietary DON exposure (2 - 5 ppm) reduced the

proliferation of murine lymphocytes *ex vivo* (Robbana-Barnat et al. 1988, Tryphonas et al. 1984, 1986). However, it has to be stressed that mitogens, as potent external stimuli, were used in these *in vitro/ex vivo* studies, suggesting that the DON effect on lymphocyte proliferation *in vivo* under normal metabolic conditions can not sufficiently be concluded.

Furthermore, trichothecene-induced immunosuppression was deduced from a reduced number of circulating blood leukocytes (Ueno 1983), increased susceptibility to pathogens (reviewed by Bondy and Pestka 2000), and inhibition of antibody responses or impaired delayed-type hypersensitivity (Rotter et al. 1994, 1996, Øvernes et al. 1997). However, it has to be noted that exposure to T-2 before inoculation enhanced resistance to *Listeria*, whereas post-inoculation exposure to T-2 caused an increased susceptibility (Corrier et al. 1987).

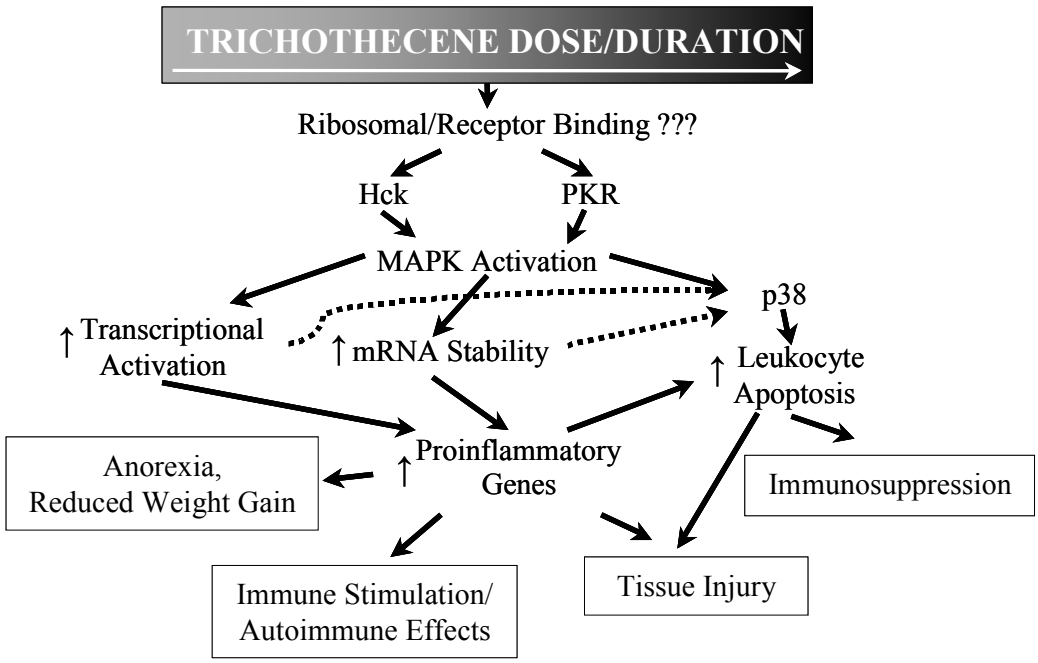


Figure 4 Interaction of molecular and cell-signaling mechanisms involved in the trichothecene-induced toxicity, PKR (double-stranded RNA-activated protein kinase), Hck (haematopoetic cell kinase), and MAPKs (mitogen-activated protein kinase) such as p38 function as molecular rheostats and define whether an immunostimulatory or immunosuppressive response will result (Reference: Pestka et al. 2004)

On the other hand, at low doses, DON was shown to elevate serum IgA levels, as well as cytokines, chemokines and other immune related proteins by stimulation of immune-associated genes (Pestka et al. 2004). While the suppressive effects were linked with the induction of apoptosis, the stimulatory effects were related to the induction of immune- and

inflammation-associated genes by protein synthesis inhibitors ("superinduction") (reviewed in Pestka et al. 2004; Figure 4).

It has been suggested that DON, like other translational inhibitors, up-regulates the expression of pro-inflammatory cytokines by inhibition of especially labile repressor proteins which results in activation of mitogen-activated protein kinases (MAPKs), possibly mediated by PKR (double-stranded RNA-activated protein kinase) and Hck (haematopoetic cell kinase) as upstream transducers (Pestka et al. 2004; Figure 4). This was assessed *in vitro* or in mice for the "extracellular signal regulated protein kinase" 1 and 2 (ERK1/2), the "c-Jun N-terminal kinase" 1 and 2 (JNK1/2) and p38 MAPK (Moon and Pestka 2002, Shifrin and Anderson 1999, Yang et al. 2000, Zhou et al. 2003). Furthermore, an increase of the binding activities of transcription factors, which are specific for regulatory units of cytokine promoters, such as "activating protein 1" (AP-1), "nuclear factor κB" (NFκB) and "CCAAT enhancer-binding protein" (C/EBP) was shown in the spleen of those mice (Zhou et al. 2003), indicating that DON works not only on the level of translation, but also on the transcriptional level. In addition, an enhanced stability of cytokine-mRNA as determined for COX-2, IL-6, and TNF-α (Chung et al. 2003b, Moon and Pestka 2003, Wong et al. 2001) may further increase cytokine-mRNA levels and therefore pro-inflammatory cytokine levels as reported for IL-1β, 2, 4, 5, 6, TNF-α, TGF-β, IFN-γ (Azcona-Olivera et al. 1995a, b, Chung et al. 2003a, Dong et al. 1994, Moon and Pestka 2002, Ouyang et al. 1995).

The DON-induced IgA production by B-lymphocytes observed in mice has been linked to the human IgA nephropathy (as reviewed by Pestka 2003). It must be noted that also T-lymphocytes and macrophages were involved in this process, as DON could not induce IgA secretion in primary B-cells (Warner et al. 1994). Therefore, it was suggested that the DON-related up-regulation of pro-inflammatory cytokines (e.g. IL-6) produced by T-lymphocytes and macrophages is essential for the differentiation of B-cells to IgA secreting plasma cells (Pestka 2003; Figure 5).

Cytotoxic and apoptotic capacities of trichothecenes were determined in macrophages, T- and B-lymphocytes *in vitro* and *in vivo* and have been related to MAPK phosphorylation (Islam et al. 1998a, b, 2003, Pestka et al. 1994, Yang et al. 2000). Furthermore, Pestka et al. (2004) hypothesized that the downstream transducers p38 and ERK act as molecular rheostats: 1) favoring immune gene expression (stimulatory) when both are activated and 2) resulting in apoptosis when only p38 is activated.

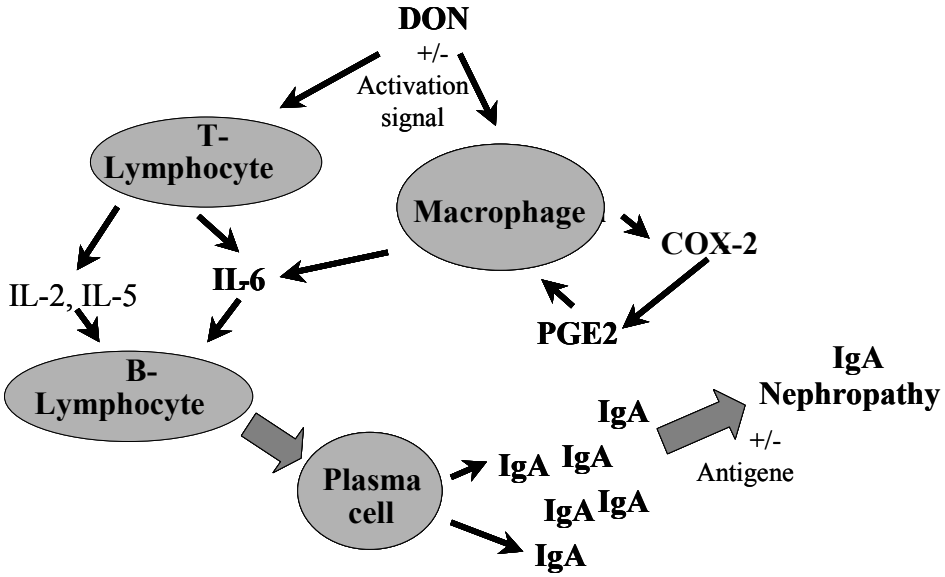


Figure 5 Cellular mechanism involved in DON-induced IgA production and IgA nephropathy (Reference: Pestka 2003)

3 Toxicokinetics of deoxynivalenol

All animal species tested have been shown to be susceptible to DON. However, the degree of susceptibility varies according to the following rank order (from most to least susceptibility): pigs > mice > rats > poultry ≈ ruminants (Prelusky et al. 1994). The difference in sensitivity may be explained by differences in absorption, distribution, metabolism, and elimination of DON (Pestka and Smolinski 2005).

In pigs, absorption of DON from the gastrointestinal tract was found to be very rapid, as DON could be detected in plasma of pigs within 15-30 min after oral dosing (Prelusky et al. 1988). The systemic absorption was estimated to be up to 82 % based on the urinary recovery (Prelusky et al. 1988). However, Coppock et al. (1985) were able to recover only 28 – 57 % of the dose given intravenously with the urine and assumed that metabolism of DON may explain the unaccounted toxin.

In comparison to pigs, the systemic absorption of DON appeared to be lower in rats (25 %, Lake et al. 1987), sheep (6-10 %, Prelusky et al. 1985, 1986b, 1987), dairy cows (< 1 % as parent toxin, Prelusky et al. 1984) and laying hens (< 1 %, Prelusky et al. 1986a). This could be explained by several reasons.

For example, Prelusky et al. (1988) reported of an eight-times lower distribution for sheep compared to pigs, which resulted in faster plasma elimination since the systemic clearance was the same for both species. Furthermore, in ruminants, ingested DON was detected mainly as its metabolite de-epoxy-DON and/or as glucuronide conjugate (Cote et al. 1986a, Prelusky

et al. 1984, 1985, 1986b, 1987, Seeling et al. 2006), whereas DON is only poorly metabolized in pigs (Prelusky et al. 1988). Additionally, it appeared to be an acquired ability since not all pigs were able to form de-epoxy-DON (Eriksen et al. 2002). However, de-epoxidation occured mainly through microbes in the large intestine of pigs and therefore probably did not contribute to a significant detoxification (Eriksen et al. 2002).

A rapid plasma clearance and excretion due to an efficient hepatic or renal first-pass effect (Rotter et al. 1996) as well as a rapid intestinal transit time (Prelusky et al. 1986a) might explain the relative tolerance of poultry.

In pigs, the main route of excretion was the urine whith only a very low proportion in the faeces (Dänicke et al. 2004a), whereas in rats 2/3 of the ingested DON, which was mainly detoxified to the metabolite de-epoxy-DON, was excreted with the faeces (Lake et al. 1987).

Although the tissue distribution of DON was extensive in pigs, no significant accumulation was found in tissues of swine 24 h after oral or iv exposure (Coppock et al. 1985, Prelusky et al. 1988, 1990, Prelusky and Trenholm 1991, 1992). Accordingly, a significant accumulation of DON was observed in neither the tissues of rats (Lake et al. 1987) or mice (Azcona-Olivera et al. 1995a), nor in the tissues and eggs of poultry (El Banna et al. 1983, Kubena et al. 1985, 1987). Furthermore, only negligible amounts of DON and de-epoxy-DON were transmitted into the milk of dairy cows (Seeling et al. 2006).

However, it has to be emphasized that the bioavailability of DON from naturally contaminated feedstuffs has not been investigated so far, since mainly radio-labeled, pure DON was used. Furthermore, it can be suggested that different feedstuffs might result in different critical concentrations for DON. Therefore, it is necessary to use one naturally contaminated source to compare the various effects of DON.

SCOPE OF THE THESIS

The aim of the present thesis was to elucidate the impact of the *Fusarium* toxin deoxynivalenol on the protein synthesis and immunological function as well as on its toxicokinetics in the pig in the view of chronic (≥ 4 weeks) or acute (one time) DON intoxication (Figure 6). Because of the principally sensitive response to DON pigs were chosen as a model for potential hazard in humans. As wheat is the main dietary DON exposure route in northern temperate regions, one batch of wheat naturally contaminated with *Fusarium* was used in the present experiments.

Since a contamination of animal feed with DON can not completely be prevented, the chronic DON exposure is a worldwide problem in animal production. Therefore, the effect of a chronic DON intoxication from naturally *Fusarium* contaminated wheat on the regulation of feed intake and performance was examined under *ad libitum* conditions. In addition, control and DON contaminated diets were fed at the same level (restrictive feeding regimen) to differentiate the specific DON effects on performance, haematological and serum parameters as well as on nutrient digestibility from the effects of feed intake (**Paper I**).

Furthermore, the bioavailability of DON from a naturally *Fusarium* contaminated source was an issue of **Paper II**, since thus far just little information on DON kinetics in the pig is available using only pure toxin exposure. For that reason, the kinetic parameters, including the bioavailability, were determined following chronic and acute feeding of a naturally DON contaminated diet with wheat grain as the source of DON to pigs in comparison to an intravenous DON application (**Paper II**).

The proliferation of peripheral blood lymphocytes, as a primary target of DON, and alterations in immunoglobulin (IgA, IgG, IgM) levels were measured after *in vitro* exposure of pure DON and following acute or chronic feeding of a DON contaminated diet (*ex vivo*) (**Paper III**).

Inhibition of protein synthesis is regarded as the main mode of action of the trichothecenes. Therefore, it was investigated whether DON modulated the lymphocyte and plasma protein synthesis *in vivo* in pigs determining the incorporation of the stable isotope L-[$^2H_5$]phenylalanine (**Paper IV**).

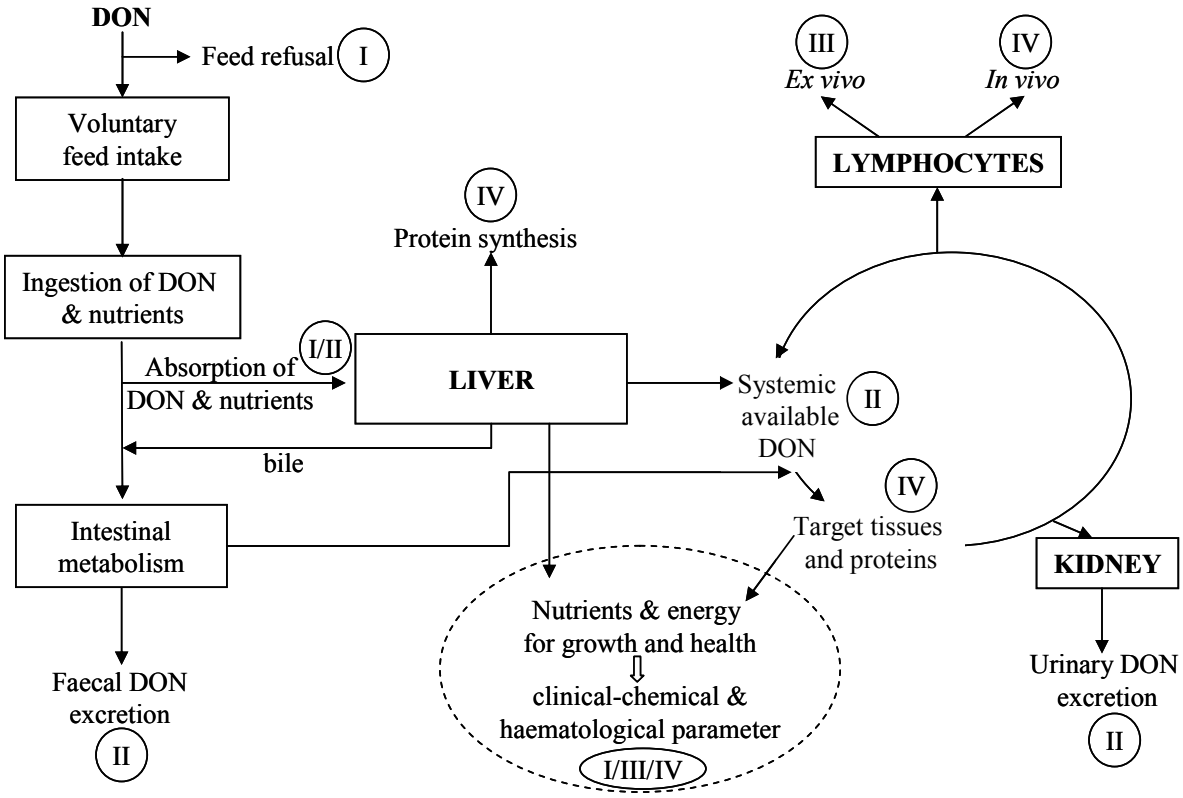


Figure 6 Scheme of the investigations carried out in the present thesis (I, II, III, IV Paper of the related investigation). As DON intake is varied by a reduction of feed intake, it was investigated to what extent a diet contaminated naturally with *Fusarium* at about 6 mg DON/kg inhibited the voluntary feed intake. The absorption of DON and the digestibility of nutrients were tested in pigs at an identical feed intake level (restrictive feeding regimen) to exclude the indirect effect of feed intake. In a balance study faecal, including the biliary reflux, and urinary DON excretion was examined together with the metabolite de-epoxy-DON as an indicator of intestinal metabolism. Systemic availability of DON after exposure to a naturally contaminated diet was assessed in order to estimate the DON dose which may act on the target tissues. The inhibition of protein synthesis, as a primary mode of action of DON, was determined *in vivo* in exported liver proteins. As lymphocytes are regarded to be particularly susceptible, the effects of DON on *ex vivo* lymphocyte proliferation were compared with *in vivo* lymphocyte protein synthesis.

PAPER I

ON THE EFFECTS OF A CHRONIC DEOXYNIVALENOL INTOXICATION ON PERFORMANCE, HAEMATOLOGICAL AND SERUM PARAMETERS OF PIGS WHEN DIETS ARE OFFERED EITHER FOR *AD LIBITUM* CONSUMPTION OR FED RESTRICTIVELY

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SUMMARY

Wheat infected naturally with *Fusarium*, contaminated mainly with DON (16.6 mg DON/kg), was added to a total constant wheat content of 400 g/kg diet. To distinguish between differences in feed intake and specific effects the DON contaminated diet, control and DON contaminated feed was administered for 11 weeks under *ad libitum* and restrictive feeding conditions to 48 pigs of both sexes, which were randomly divided into 4 groups (n = 12 per group).

Feed intake was 2.91 kg/d, live weight gain 978 g/d and feed to gain ratio 2.79 kg/kg for the *ad libitum* fed control group. The group fed DON contaminated wheat *ad libitum* significantly consumed 15 % less feed and gained 13 % less live weight, while the feed to gain ratio was unaffected. Moreover, it was concluded that the lower growth performance by DON contaminated feed resulted mainly from the lower voluntary feed intake, because there were no differences in live weight gain between the groups with the restrictive feeding regimen. On the other hand, metabolizability of energy, digestibility of organic matter, crude protein, crude fat and crude fibre were significantly increased by 3, 3, 6, 9 and 20 % in the DON group, respectively. Animals fed DON contaminated diets needed more time to consume the restrictive ration than the control group. For example in the first hour after feeding 85 % of the control pigs had consumed all feed, but only 39 % of the DON group had. There were only few differences in haematological and serum parameters which were characterized by a high variation between individuals. DON and IgA concentrations in serum were significantly influenced by DON exposure.

Keywords: deoxynivalenol, feeding regimen, restrictive feeding, fattening pigs

1 INTRODUCTION

Deoxynivalenol (DON) is one of the most prevalent *Fusarium* mycotoxin under the climatic and production conditions in Germany in terms of frequency of occurrence in relation to its toxicological relevance for farm animals (Oldenburg et al. 2000).

Studies with pigs, which are regarded to be particularly susceptible to DON, showed that reduced feed consumption and lower weight gains are the principal clinical effects following ingestion of DON in naturally contaminated feedstuffs (for reviews see Rotter et al. 1996, Trenholm et al. 1984). The effects of *Fusarium* mycotoxins on animals could be differentiated into direct and indirect effects (Chavez and Rheaume 1986, Swamy et al. 2003). Reduced voluntary feed intake is considered as an indirect effect of DON and could result in a decreased availability of nutrients, a subsequent growth depression and an increased susceptibility to diseases. The direct effects of *Fusarium* mycotoxins on tissues and organs are metabolic disturbances like altered nutrient absorption, inhibition of protein synthesis and cytotoxic effects on various cell types (Swamy et al. 2003).

To differentiate the effect of feed intake from the direct action of DON on growth, immunologic and clinical-chemical parameters several investigators (Rotter et al. 1994, 1995, Chavez and Rheaume 1986) introduced a pair-fed control group, which obtained the same feed level as the DON fed *ad libitum* group. But it has to be taken into account that this is just an average feed intake of all DON fed animals and that some of them consume less feed. Additionally, Chavez and Rheaume (1986) pointed out that even if the pigs receive the same amount of feed per day their feeding behaviour is different. As animals fed DON contaminated diets consumed small amounts continuously throughout the day, the pair-fed control pigs finished their feed intake long before end of the day and exhibited a noisy and hyperactive behaviour as a result of hunger. To exclude such effects on feed intake, in the present study the restrictively fed pigs received such an amount of feed which was completely consumed both by the pigs fed the control or DON contaminated diet and these two groups were compared to their *ad libitum* fed counterparts. The objective of this study was to determine the significance of the reduction in feed consumption on growth, haematological and clinical-chemical parameters when pigs were fed DON contaminated diets. Moreover, feed consumption behaviour, nutrient digestibility and N-balance of the restrictively fed pigs were recorded.

2 MATERIAL AND METHODS

2.1 Experimental diets

The control diet was formulated to meet or exceed all nutritional requirements of fattening pigs as recommended by the German Society of Nutrition Physiology (GfE 1987) and was split into an initial and finisher feed with a total wheat content of 400 g/kg (Table 1).

Table 1 Composition of the experimental diets (g/kg, based on a dry matter content of 88 %)

	Control diet		DON contaminated diet	
	Starter	Finisher	Starter	Finisher
Ingredients				
Barley	348	446	348	446
Wheat	400	400	0	0
Contaminated wheat	0	0	400	400
Soybean meal	200	110	200	110
Soybean oil	20	15	20	15
Dicalcium-phosphate	3.5	1	3.5	1
Sodium chloride	1	0.5	1	0.5
L-lysine-HCl	2.5	2.5	2.5	2.5
Premix[1]	25	25	25	25
Calculated composition				
Crude ash	61.9	57.6	63.2	58.9
Crude protein	178.2	149.3	180.1	150.9
Crude fat	38.7	35.0	37.7	33.9
Crude fibre	35.2	34.8	38.4	37.9
N-free extractives	573.4	612.6	568.3	607.7
Starch	430.7	477.0	424.2	470.8
ME (MJ/kg)	13.4	13.2	13.3	13.2
Lysine	10.1	8.0	10.1	8.1
Methionine + Cystine	5.8	5.0	5.9	5.1
Threonine	6.2	5.1	6.3	5.1
Tryptophan	2.3	1.9	2.3	1.9
Calcium	7.0	6.8	7.1	6.8
Total phosphorus	5.9	5.9	5.9	5.7
Sodium	2.0	1.8	2.0	1.9
Deoxynivalenol (mg/kg)	0.14	0.14	6.51	6.51
Analysed composition				
Crude protein	173.7	140.3	176.3	142.4
Deoxynivalenol (mg/kg)	0.23	0.24	6.15	6.80

[1] Provided per kg of diet: Ca 6.1 g; P 1.5 g; Na 1.4 g; Mg 0.3 g; Fe 100 mg; Cu 25 mg; Mn 50 mg; Zn 100 mg; I 1.3 mg; Se 0.4 mg; Co 0.5 mg; vitamin A 10000 IU; vitamin D_3 1000 IU; vitamin E 30 mg; vitamin B_1 18.8 µg; vitamin K_3 1.3 mg; nicotinic acid 12.5 mg; pantothenic acid 8.4 mg; choline chloride 125 mg.

The mycotoxin contaminated diets were formulated by replacing control wheat with wheat contaminated naturally with *Fusarium*. Based on the analysed DON concentration of the wheat, the DON concentrations of the diets were calculated at 140 and 6510 µg/kg diet (Table 1). The wheat proportions were kept constant over the course of the experiment, whereas the barley proportion was elevated and the soybean meal proportion reduced in the finisher diets to adjust to the lower protein needs of the pigs during this fattening period. Starter diets were fed for the first 7 weeks (average live weight range between 26 and 65 kg) of the experiment, and finisher diets thereafter until finishing of the experiment after 11 weeks when the heaviest group reached an average weight of approximately 100 kg.

Treatments and experiments were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany.

2.2 Growth experiment

The study was carried out with 48 pigs, crossbred German Landrace x Pietrain with an average body weight of about 26 kg at the beginning of the experiment. Similar numbers of females and castrated males (except the *ad libitum* control group with 5 castrated males and 7 females) were assigned to the control as well as the DON contaminated diet, which were fed both *ad libitum* and restrictively (for design see Table 3). The restrictive feeding regimen was introduced to assure the same amount of feed intake for all animals of both the control group and of the DON group. For this reason it was geared to the animal consuming the lowest amount of feed. This was necessary to compare the effects on the measured parameters between the control and DON group without impact of feed intake level. The pigs were subjected to one week of acclimatization with gradual adaptation to the experimental diets. Each animal was housed in a separate pen with free access to water from drinking nipples and was fed individually. Feed consumption and live weight were recorded weekly.

Additionally, duration of feed intake of the restrictively fed pigs was recorded in Weeks 6, 11 and before slaughter (Week 12).

Blood samples were drawn before the morning meal by jugular venopuncture at the beginning and after 5 weeks of the feeding experiment and prepared for differential blood counts, haematocrit and serum for determination of clinical-chemical parameters and IgA. The serum of 6 pigs per group was tested for DON concentration. Sera were prepared, frozen and stored at -20°C until analysis.

After termination of the experiment the pigs were fed the experimental diets until slaughter in the ensuing week. Six pigs from each control group and 12 animals from each group fed the DON contaminated diet were slaughtered and liver, kidney and spleen were excised, weighed and subjected a macroscopic examination. Absolute organ weights were expressed as percentage of body weight (relative weight).

2.3 Balance study

A balance study was carried out to examine the effects of the two experimental starter diets on nutrient digestibility and on N-balance according to the total collection method as described by Schiemann (1981). Each diet was tested on 5 barrows and was restricted to an amount consumed by all animals which was equivalent to the maximum level voluntarily consumed by the pigs fed the DON contaminated diet. After a four-week period of feeding the experimental diets the pigs were adjusted to the balance cages during 2 days as described by Farries and Oslage (1961). The cages allowed a quantitative collection of urine and faeces during a 5-day-collection period. At the beginning of the balance study the mean live weight was 36.3 kg ± 1.3 kg and 35.6 ± 1.3 kg for pigs fed DON contaminated and control diet, respectively. The daily feed amount of 1.0 kg was given in two equal portions at 7 a.m. and 2 p.m. after collecting the faeces. Aliquot samples of acidified urine were pooled for each individual pig and were kept frozen until analysis. Pooled faecal samples were prepared from aliquots of the daily faeces, homogenized, freeze dried and ground to pass through a 1 mm screen before being analysed.

2.4 Analyses

Representative wheat samples were taken at the beginning of the experiment and were analysed for *Fusarium* mycotoxins and nutrients contents.

Samples from the wheat batches, each diet and freeze dried faeces were analysed for the content of crude nutrients, and wheat samples were analysed additionally for amino acids, according to the methods of the "Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA)" (Naumann and Bassler 1993).

DON in wheat and diets was determined by high performance liquid chromatography (HPLC) with diode array detection (DAD) after clean-up with immuno-affinity columns (IAC, VICAM) according to a modified VDLUFA-method described by Valenta et al. (2002). The detection limit was 0.03 mg/kg. The recovery was approximately 90 % for these matrices. Further *Fusarium* mycotoxins were analysed in wheat samples by the Institute of Animal

Nutrition of the University of Hohenheim using a gas chromatography mass spectrometry (GC/MS) method (Schollenberger et al. 1998).

The determination of DON and de-epoxy-DON in serum is described by Valenta et al. (2003). Serum samples were extracted with ethyl acetate on a ChemElut cartridge (Varian), cleaned up by IAC (VICAM) and measured by HPLC-DAD. The detection limit for serum was approximately 4 ng/ml. The mean recovery in serum of DON and de-epoxy-DON was 89 and 85 %, respectively. The results of mycotoxin analysis were not corrected for recovery.

Leukocytes were counted using an improved Neubauer cell counting chamber. Haematocrit was read off after centrifugation with a haematocrit centrifuge. Differential leukocyte counts were acquired after staining the smear with Papenheim and counting 200 cells.

Activities of alkaline phosphatase (AP), aspartat aminotranferase (ASAT), glutamate dehydrogenase (GLDH), γ-glutamyltransferase (γ-GT), and creatinine kinase (CK) in serum were measured by enzymatic UV-standard procedures (Clinic for Swine and Small Ruminants, Veterinary School, Hanover, Germany). Serum protein and albumin concentrations were determined using the biurette method and a colorimetric test with bromcresol green, respectively.

Serum IgA concentrations were analysed by ELISA (Research Unit Reproductive Biology, Research Institute for the Biology of Farm Animals (FBN), Dummerstorf, Germany)**.**

2.5 Calculations and statistics

Apparent digestibility of nutrients and balance of nitrogen were calculated from the respective quantitative intake (feed intake multiplied by analysed concentration) and excretion (urine or faeces excretion multiplied by concentration). ME concentration of the diets was estimated using the prediction equation based on apparent digestible nutrients as proposed by the GfE (1987).

All statistics were carried out using the SAS-software package (Version 8.2, procedure mixed, SAS Institute, Cary, NC, U.S.A.). In our investigation we consider a feeding regimen dependent remainder variance because the homogeneity of variances due to the restricted feeding was not approved.

Performance and serum clinical-chemical parameters were subjected to analysis of variance (ANOVA) according to a three-factorial design:

$$y_{ijkl} = \mu + a_i + b_j + c_k + a \cdot b_{(ij)} + a \cdot c_{(ik)} + b \cdot c_{(jk)} + a \cdot b \cdot c_{(ijk)} + e_{ijkl}$$

where y_{ijkl} = l^{th} observation related to the wheat (DON or control) i, feeding regimen j and sex k; μ = overall mean; a_i = effect of wheat (DON or control); b_j = effect of feeding regimen (*ad libitum* or restrictive); c_k = effect of sex; $a \cdot b_{(ij)}$ = interactions between wheat and feeding regimen; $a \cdot c_{(ik)}$ = interactions between wheat and sex; $b \cdot c_{(ik)}$ = interactions between feeding regimen and sex; $a \cdot b \cdot c_{(ijk)}$ = interactions between wheat, feeding regimen and sex; e_{ijkl} = error term.

Haematological parameters and IgA concentration in serum were subjected to ANOVA according to a four-factorial design:

$$y_{ijklmn} = \mu + a_i + b_j + c_k + d_l + a \cdot b_{(ij)} + a \cdot c_{(ik)} + a \cdot d_{(il)} + b \cdot c_{(jk)} + b \cdot d_{(jl)} + c \cdot d_{(kl)} + a \cdot b \cdot c_{(ijk)} + a \cdot b \cdot d_{(ijl)} + a \cdot c \cdot d_{(ikl)} + b \cdot c \cdot d_{(jkl)} + a \cdot b \cdot c \cdot d_{(ijkl)} + animal_{(m)} + e_{ijklmn}$$

where y_{ijklmn} = n^{th} observation of the m^{th} animal related to the wheat i, feeding regimen j, sex k and week l; μ = overall mean; a_i = effect of wheat; b_j = effect of feeding regimen; c_k = effect of sex; d_l = effect of week of sample taking; $a \cdot b_{(ij)}$ = interactions between wheat and feeding regimen; $a \cdot c_{(ik)}$ = interactions between wheat and sex; $a \cdot d_{(il)}$ = interaction between wheat and week; $b \cdot c$ = interactions between feeding regimen and sex; $b \cdot d_{(jl)}$ = interaction between feeding regimen and week; $c \cdot d_{(kl)}$ = interaction between sex and week; $a \cdot b \cdot c_{(ijk)}$ = interactions between wheat, feeding regimen and sex; $a \cdot b \cdot d_{(ijl)}$ = interactions between wheat, feeding regimen and week; $a \cdot c \cdot d_{(ikl)}$ = interaction between wheat, sex and week; $b \cdot c \cdot d_{(jkl)}$ = interaction between feeding regimen, sex and week; $a \cdot b \cdot c \cdot d_{(ijkl)}$ = interaction between wheat, feeding regimen, sex and week; $animal_{(m)}$ = random animal effect to take into account repeated measurements of the same individual; e_{ijklmn} = error term.

In both models described above we used the REML-method (restricted maximum likelihood) for random effect variances and the Kenward-Roger-method for calculation of standard errors and degrees of freedom (Kenward and Roger 1997).

3 RESULTS

3.1 Composition of wheat batches

DON was the predominating mycotoxin in the contaminated wheat (16.6 mg/kg) whereas zearalenone (114 µg ZON/kg) and further *Fusarium* toxins were present in much lower concentrations (Table 2). In the control group the DON and ZON concentrations were 395 µg/kg and 45 µg/kg, respectively. Crude protein and crude ash content were slightly higher in the DON contaminated wheat, while starch concentration was lower.

Table 2 Selected nutrients and *Fusarium* toxin content of wheat (based on a dry matter content of 88 %)

	Control wheat	DON wheat
Crude protein (g/kg)	113	122
Amino acids (g/kg)		
Cystine	2.2	2.3
Methionine	1.7	1.9
Aspartic acid	5.7	7.0
Threonine	3.1	3.3
Serine	5.0	5.0
Glutamic acid	20.7	21.3
Prolin	10.4	9.5
Glycine	4.4	4.6
Alanine	4.0	4.4
Valin	4.5	4.8
Isoleucine	3.6	3.8
Leucine	7.1	7.1
Thyrosine	2.0	1.0
Phenylalanine	5.3	5.1
Histidin	2.6	2.9
Lysine	4.1	4.6
Arginine	5.1	5.5
Crude ash (g/kg)	16.7	19.5
Starch (g/kg)	719.9	667.8
Total NSP	9.0	11.0
Insoluble NSP	7.5	9.6
Soluble NSP	1.5	1.4
Deoxynivalenol (µg/kg)	395	16643
Zearalenone (µg/kg)	45	114
Nivalenol (µg/kg)	71	41
Scirpentriol (µg/kg)	<8	20
T-2-Tetraol (µg/kg)	<7	<7
Fusarenon-X (µg/kg)	<19	<19
Monoacetoxyscirpenol (µg/kg)	<3	<3
15-Acetyldeoxynivalenol (µg/kg)	20	101
3-Acetyldeoxynivalenol (µg/kg)	20	41
T-2-Triol (µg/kg)	<5	<5
Neosolaniol (µg/kg)	<6	<6
Diacetoxyscirpenol (µg/kg)	<14	<14
HT-2 Toxin (µg/kg)	<3	11
T-2 Toxin (µg/kg)	<4	<4

Table 3 Growth performance of fattening pigs (n=12) fed a basal diet with 40 % control wheat or deoxynivalenol (DON) contaminated wheat either *ad libitum* or restrictively (LSmeans ± SEM)

Feeding regime	Wheat	n	Feed intake (kg/day)			Live weight gain (g/d)			Feed to gain ratio (kg/kg)		
			Starter	Finisher	Total	Starter	Finisher	Total	Starter	Finisher	Total
ad libitum	Control	12	2.25^{a}±0.08	3.56^{a}±0.12	2.90^{a}±0.09	930.5^{a} ±25.7	1084.8^{a}±40.5	986.5^{a} ± 28.1	2.42^{a} ±0.05	3.30^{a}±0.13	2.77^{a}±0.07
	DON	12	1.92^{b}±0.08	3.02^{b}±0.12	2.47^{b}±0.09	813.3^{b} ±25.3	917.8^{b} ±39.9	851.3^{b} ± 27.7	2.36^{a} ±0.05	3.33^{a}±0.13	2.73^{a}±0.07
restrictive	Control	12	1.58^{c}±0.001	2.31^{c}±0.007	1.94^{c}±0.003	728.6^{c} ± 9.0	784.0^{c} ±20.2	746.8^{c} ± 7.4	2.19^{b} ±0.03	2.97^{b}±0.08	2.48^{b}±0.03
	DON	12	1.58^{c}±0.001	2.30^{c}±0.007	1.93^{c}±0.003	725.9^{c} ± 9.0	776.8^{c} ±20.2	744.3^{c} ± 7.4	2.18^{b} ±0.03	2.98^{b}±0.08	2.48^{b}±0.03
Probability											
Feeding regime			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001
Wheat			0.007	0.003	0.004	0.004	0.010	0.003	0.418	0.874	0.713
Sex			0.006	0.001	0.002	0.001	0.010	0.001	0.913	0.829	0.846
Feeding regime x wheat			0.006	0.007	0.004	0.005	0.018	0.004	0.486	0.959	0.689
Feeding regime x sex			0.006	0.001	0.002	0.001	0.001	<0.001	0.890	0.547	0.719
Wheat x sex			0.649	0.312	0.430	0.050	0.789	0.194	0.057	0.665	0.517
Feeding regime x wheat x sex			0.102	0.627	0.371	0.452	0.978	0.305	0.104	0.467	0.761

[abc] Values with no common superscript are significantly different within columns (P<0.05)

Starter period: 26 to 65 kg live weight (7 weeks), Finisher period: 65 to 100 kg (4 weeks), Total period: 26 to 100 kg (11 weeks)

3.2 Growth experiment

The growth experiment covered the live weight range between approximately 26 and 100 kg and lasted 11 weeks in total. No acute pathologies such as death, external lesions, or other toxic effects were seen in any of the experimental animals. The feeding system had a significant effect on all of the acquired performance parameters over the course of the study (Table 3). Pigs fed the restrictive DON diet did not show significant differences in performance compared to the restrictive control group.

However, feed intake, live weight gain and feed to gain ratio of both restrictively fed groups (DON and control) were 34 %, 24 % and 11 % lower than the control group fed *ad libitum* (Table 3). Performance of pigs fed *ad libitum* was significantly influenced by dietary DON: the *ad libitum* fed DON group consumed 15 % less feed and gained 13 % less live weight, whereas the feed to gain ratio remained unaffected over the course of the study.

The sex of the pigs significantly affected live weight gain and feed intake, as castrated males of the *ad libitum* fed groups had a significantly higher feed consumption and live weight gain than the females, while under restrictive feeding conditions no marked differences between barrows and gilts occurred. In the *ad libitum* fed control group, feed intake was 3.1 and 2.6 kg diet per day and live weight gain 1060 and 880 g/d for castrated males and females, respectively. Barrows offered the DON contaminated diet *ad libitum* consumed 2.7 kg feed/d and gained of 918 g live weight/d in comparison to 2.3 kg/d and 794 g/d for gilts.

Restrictively DON fed animals needed more time to consume the restrictive rations than the control group. 85 % of the control pigs consumed their feed within the first hour after feeding, while only 39 % of the DON group did (Figure 1).

Table 4 Results of the balance study of barrows (n = 5) fed control and deoxynivalenol (DON) contaminated starter diets restrictively (LSmeans ± SEM)

	Control		**DON**		**Probability**
Digestibility (%)					
Organic matter	83.0	± 0.6	85.3	± 0.6	0.034
Crude protein	82.2	± 1.1	87.2	± 1.1	0.025
Crude fat	63.7	± 1.7	69.7	± 1.7	0.042
N-free extractives	88.6	± 0.5	89.7	± 0.5	0.149
Crude fibre	31.6	± 2.2	39.6	± 2.2	0.042
ME (MJ/kg dry matter)	13.0	± 0.1	13.4	± 0.1	0.017
N-balance (mg/d/W(kg)$^{0.75}$)	851	± 24	943	± 24	0.037

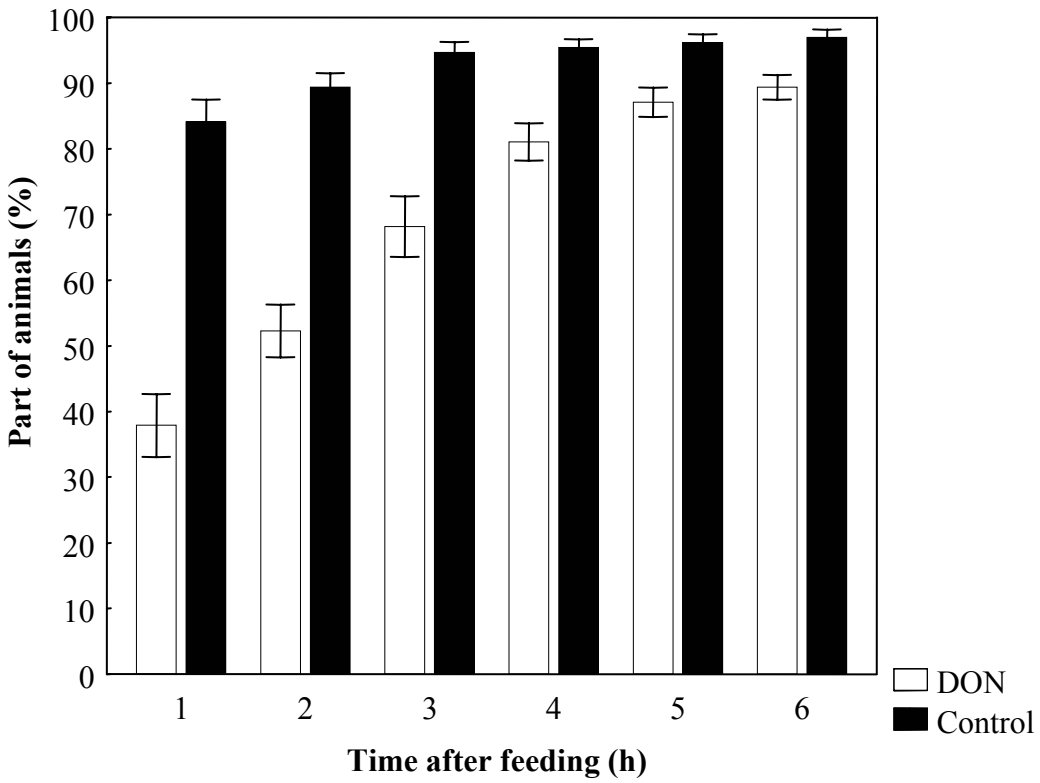

Figure 1 Time for intake the total meal of pigs (n = 12) fed restrictively control and deoxynivalenol (DON) contaminated diets (means ± SEM)

3.3 Balance study

The effect of DON on feed intake was eliminated in the balance study by adjusting all animals to the feed intake level consumed voluntarily, i.e., to the level of DON fed pigs. Metabolizability of energy, nitrogen retention, digestibility of organic matter, crude protein, crude fat and crude fibre were significantly increased by 3, 10, 3, 6, 9 and 20 %, respectively, in the DON group (Table 4). Digestibility of N-free extractives remained unaffected.

3.4 Haematology and serum examination

Only few significant differences in haematological parameters were found, accompanied by a great variation between the individuals. The leukocyte count increased significantly during the 5 week test period ($p<0.001$) and was significantly affected by the feeding regimen ($p=0.022$), while DON contamination of wheat had no significant influence (Table 5). In pigs fed DON contaminated wheat *ad libitum* over 5 weeks, serum IgA concentration increased significantly (Table 5). Male pigs fed the DON contaminated diet *ad libitum* for 5 weeks showed a significantly higher serum IgA level than the corresponding females (1.71 *versus* 0.99 mg IgA/ml serum).

The kind of feeding influenced the albumin concentration in serum. Table 6 indicates that albumin concentration of restrictively fed control pigs was significantly lower as in their *ad libitum* fed counterparts, whereas the concentration of alkaline phosphatase was significantly decreased in the *ad libitum* fed DON group. Total protein, GLDH, γ-GT, ASAT, CK concentrations were not affected by dietary treatments (Table 6).

DON was not detectable in serum of pigs which received the control diets. Pigs fed the DON contaminated diet showed a great variation of serum DON concentration, although variation appeared to be lower in the restrictively fed pigs (Figure 2).

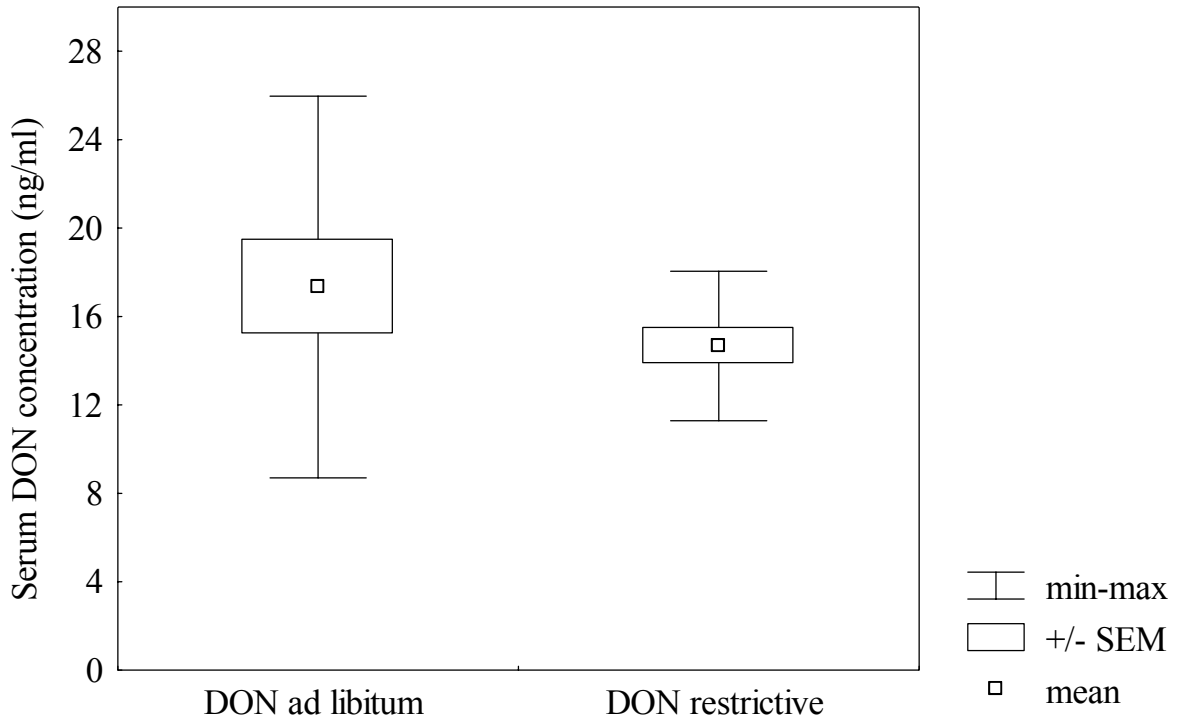

Figure 2 DON concentrations in serum (ng/ml) of pigs fed a deoxynivalenol (DON) contaminated diet either *ad libitum* or restrictively (n = 6)

Table 5 Haematologic parameters and IgA serum concentrations of pigs (n=12) fed control or deoxynivalenol (DON) contaminated diets either *ad libitum* or restrictively after 5 weeks of exposure (LSmeans ± SEM)

Feeding regime	Wheat	**IgA (mg/ml)**	**Leukocytes (G/l)**	**Lymphocytes (%)**	**Granulocytes (%)**	**Haematocrit (%)**
ad libitum	Control	0.79^{b} ±0.13	15.4^{b} ±0.66	63.7^{ab} ± 2.67	33.4^{a} ±2.76	38.0^{a} ±0.63
	DON	1.35^{a} ±0.13	16.9^{ab} ±0.65	59.5^{b} ± 2.64	36.1^{a} ±2.72	35.9^{b} ±0.62
restrictive	Control	0.93^{b} ±0.10	18.3^{a} ±1.07	65.6^{a} ± 2.63	32.5^{a} ±2.65	36.6^{ab} ±1.01
	DON	1.11^{ab} ±0.10	17.8^{ab} ±1.07	66.4^{a} ± 2.62	31.1^{a} ±2.64	38.1^{a} ±1.01
Probability						
Feeding regime		0.873	0.022	0.069	0.277	0.557
Wheat		0.020	0.076	0.162	0.251	0.443
Week		0.002	<0.001	0.199	0.171	0.074
Sex		0.039	0.704	0.268	0.254	0.975
Feeding regime x wheat		0.394	0.342	0.768	0.859	0.004
Feeding regime x week		0.631	0.495	0.722	0.691	0.201
Wheat x week		0.045	0.330	0.497	0.362	0.183
Feeding regime x wheat x week		0.115	0.573	0.086	0.197	0.937

[abc] Values with no common superscript are significantly different within columns (P<0.05)

Table 6 Serum clinical-chemical parameters of pigs (n=12) fed control or deoxynivalenol (DON) contaminated diets either *ad libitum* or restrictively for 5 weeks covering the live weight range between approximately 26 kg and 65 kg (LSmeans ± SEM)

Feeding regime	Wheat	**Total protein (g/l)** (≤86)*	**Albumin (g/l)** (22.1-27.5)*	**Alkaline phosphatase (U/l)** (≤170)*†	**Glutamate dehydrogenase (U/l)** (≤4)*	**γ-Glutamyl-transferase (U/l)** (≤26)*	**Aspartate aminotransferase (U/l)** (≤35)*	**Creatinine kinase (U/l)** (≤2000)*
ad libitum	Control	60.9 ±1.7	40.6[a] ± 1.7	220[a] ±10	1.0 ±0.4	20.1 ±1.1	25.6 ±3.5	1341 ±399
	DON	59.9 ±1.6	35.9[ab] ± 1.7	191[b] ±10	0.7 ±0.4	17.8 ±1.1	19.2 ±3.4	1681 ±393
restrictive	Control	59.4 ±1.0	36.3[b] ± 0.9	217[ab] ±13	1.5 ±0.5	19.5 ±1.2	19.5 ±1.5	1186 ±281
	DON	60.8 ±1.0	38.7[ab] ± 0.9	207[ab] ±13	1.2 ±0.5	18.1 ±1.2	20.1 ±1.5	1689 ±281
Probability								
Feeding regime		0.842	0.607	0.563	0.203	0.918	0.336	0.833
Wheat		0.885	0.403	0.108	0.451	0.098	0.285	0.228
Sex		0.414	0.256	0.776	0.833	0.915	0.133	0.185
Feeding regime x wheat		0.367	0.014	0.391	0.936	0.688	0.193	0.814
Feeding regime x sex		0.766	0.286	0.606	0.701	0.895	0.483	0.853
Wheat x sex		0.517	0.840	0.889	0.497	0.752	0.295	0.682
Feeding regime x wheat x sex		0.861	0.393	0.678	0.777	0.033	0.077	0.051

[ab] Values with no common superscript are significantly different within columns ($P<0.05$)

* Reference value (Clinic for Swine and Small Ruminants, Veterinary School, Hanover, Germany)

† Highly age-related; sows: ≤ 170 (U/l); young pigs: ≤ 700 (U/l)

3.5 Organ weights and gross pathological findings

Table 7 shows that the absolute weights of liver, kidney and spleen were significantly lower in the control and DON treated pigs fed restrictively. DON contamination of wheat had a significant effect on relative kidney weight (Table 7). Here, the relative kidney weight was significantly elevated, while the relative weights of liver or spleen were not significantly changed (Table 7). Examination of liver, kidney and spleen offered only non-relevant macroscopic lesions in kidneys, like cysts (8 pigs), scars (4 pigs) and anaemic infarcts (4 pigs) which were independent of dietary treatments. In addition, the wall of bile ducts in two control animals appeared thickened.

Table 7 Absolute and relative (g per kg live weight) organ weights of pigs fed control (n=6) or deoxynivalenol (DON) (n=12) contaminated diets over 12 weeks (LSmeans ± SEM)

		Absolute weight (g)			**Relative weight (g/kg LW)**		
Feeding regime	Wheat	Liver	Kidney	Spleen	Liver	Kidney	Spleen
ad libitum	Control	1775.2[a] *± 113.2*	349.7[a] *± 16.7*	173.2[a] *± 12.3*	16.35 *± 1.35*	3.18[ab] *± 0.13*	1.57 *± 0.11*
	DON	1610.4[a] *± 80.0*	332.1[a] *± 11.8*	151.3[ab] *± 8.7*	16.45 *± 0.96*	3.38[a] *± 0.09*	1.54 *± 0.08*
restrictive	Control	1415.0[b] *± 50.1*	273.3[c] *± 7.5*	135.0[b] *± 8.2*	15.33 *± 0.53*	2.97[b] *± 0.09*	1.47 *± 0.09*
	DON	1343.9[b] *± 35.4*	293.8[b] *± 5.3*	136.5[b] *± 5.8*	15.14 *± 0.38*	3.31[a] *± 0.06*	1.54 *± 0.06*
Probability							
Feeding regime		< 0.001	< 0.001	0.007	0.207	0.158	0.576
Wheat		0.136	0.898	0.269	0.959	0.012	0.818
Sex		0.705	0.968	0.037	0.128	0.032	0.361
Feeding regime x wheat		0.544	0.105	0.207	0.871	0.471	0.577
Feeding regime x sex		0.656	0.081	0.067	0.207	0.370	0.880
Wheat x sex		0.540	0.046	0.064	0.895	0.124	0.192
Feeding regime x wheat x sex		0.479	0.060	0.098	0.964	0.325	0.413

[ab] Values with no common superscript are significantly different within columns (P<0.05)

4 DISCUSSION

4.1 Experimental feed

The wheat grains contaminated naturally with *Fusarium* contained not only DON, but many other *Fusarium* toxins (Table 2), which could interact with DON and thereby enhance the net toxicity (Rotter et al. 1992).

The higher crude protein and crude ash, and the lower starch concentrations of the infected wheat can not solely be attributed to the effects of fungal invasion. But results from inoculation experiments with a direct comparison of nutrient composition of non-infected and infected wheat of the same variety produced under identical conditions (Matthäus et al. 2004) would strongly support this assumption. Similarly, Friend et al. (1986) could even show an increase of dietary protein and ash content with increasing DON contaminated wheat content in diets at a total wheat content of 70 %.

4.2 Growth experiment

Pigs fed the DON contaminated diet *ad libitum* consumed 15 % less feed and gained 13 % less live weight compared to the *ad libitum* control group over the course of the study (Table 3). But DON had no influence on live weight gain or feed to gain ratio when pigs were fed the same amount of diet (restrictively). This finding supports the hypothesis that the adverse effects of DON contaminated diets on the growth performance of growing pigs is primarily caused by depressing the voluntary feed intake (Rotter et al. 1994). This is in agreement with Swamy et al. (2002), who also determined a significant decrease of growth performance and feed intake of 34.6 and 32.6 %, respectively, but no significant effect on gain to feed ratio when feeding feed contaminated naturally with *Fusarium* (DON 5.6 ± 0.6 ppm) *ad libitum* to starter pigs over 21 d. On the other hand, Bergsjø et al. (1993) assessed a significant reduction of feed utilisation in fattening pigs (21.3 – 100 kg) after feeding a diet containing 3.5 mg DON/kg *ad libitum*. Smith et al. (1997) reported a linear, and Young et al. (1983) a linear and quadratic decrease of feed to gain ratio due to the inclusion of *Fusarium* mycotoxin contaminated grains. Other studies (Swamy et al. 2003, Rotter et al. 1994, Øvernes et al. 1997) even revealed an increase in feed efficiency, and supposed that pigs adjusted to reduced feed intake by improving feed utilization. On the other hand, Dänicke et al. (2004a) inferred no significant effects on performance when fattening pigs were fed with increasing DON concentrations (0.2, 0.7, 1.2, 2.5, 3.7 mg/kg) from wheat inoculated artificially with *Fusarium*

culmorum. Discrepancies among experiments with dietary DON concentration and the effect on animals could be due to different weights and ages of animals at the beginning of the study, sex, source of contamination (grain type or pure DON), presence of other known and unknown fungal metabolites, the duration of the study (adaptation - recovery), statistical design of experiment and number of animals used (Rotter et al. 1995).

As a result of the restricted amount of feed and therefore lower availability of nutrients the restrictively fed groups without distinction of control or DON contaminated diet gained less than *ad libitum* fed pigs. Our suggestion is supported by the results reported by Øvernes et al. (1997) who showed that the *ad libitum* fed groups had higher feed uptake, higher average weight gain, lower feed utilization and lower meat percentage than the groups fed restricted rations, which were fed according to the Norwegian C-standard and not as in the present study where all pigs in the restricted groups had to accept the whole ration. While restrictively fed pigs consumed the meal relatively quickly after feeding, the *ad libitum* fed pigs were observed to rummage in the diet when offered in the through in the present study. Dänicke et al. (2004a) observed this behaviour as well and concluded that the feed wastage was probably higher in *ad libitum* fed groups which resulted not only in a higher recorded feed intake but also in an increased feed to gain ratio.

In the present study, *ad libitum* fed castrated males showed a significantly higher feed intake and live weight gain than the corresponding females (data not shown) over the course of the experiment, while under restrictive feeding conditions there was no influence of sex due to the equal amount of feed. In contrast to these effects, Cote et al. (1985) reported lower weight gain in castrated males compared with the females during the first 4 weeks on DON contaminated feeds at levels of 3.1 and 5.8 mg/kg, while Bergsjø et al. (1992) observed no differences between sexes for any level of DON.

Pigs did not only consume less DON contaminated feed when fed *ad libitum*, they actually needed more time to consume an equal amount of feed when compared to control fed animals. Moreover, pigs fed the DON contaminated diet restrictively lost interest and laid down shortly after feeding, while most of the restricted controls kept eating. This is in accordance with Øvernes et al. (1997), who observed a visibly reduced appetite when pigs were fed 1.8 and 4.7 mg DON/kg compared to the control.

4.3 Balance study

In the balance study DON contamination did significantly increase metabolizability of energy, nitrogen retention, digestibility of organic matter, crude protein, crude fat and crude fibre by 3, 10, 3, 6, 9 and 20 %, respectively (Table 4). The digestibility of N-free extractives remained unaffected. The positive effect of the DON contaminated diet on nutrient digestibility could neither compensate the negative effects on feed intake in *ad libitum* fed animals nor exceed performance data of restrictively fed control pigs. Dänicke et al. (2004c) reported a DON related significant increase in nutrient digestibility with the exception of the crude fibre digestibility. Friend et al. (1986) observed a greater N-retention in pigs fed the DON contaminated diet, but could not differentiate between a direct effect of DON or an indirect due to a higher N-intake, because of the higher crude protein content of the DON contaminated wheat. On the other hand, Dänicke et al. (2004a,d) found no or inconsistent effects of graded levels of DON in pigs diets on nutrient digestibility. The feeding value of the contaminated feedstuff could be modified by the *Fusarium* colonisation. Matthäus et al. (2004) observed marked changes in nutrient composition and other characteristics when wheat was highly infected with *Fusarium*. The authors found that the activities of non-starch-polysaccharide hydrolyzing enzymes, of amylase and protease were markedly increased in *Fusarium* inoculated wheat and Dänicke et al. (2004a) suggested that this could be regarded as a partial "pre-digestion."

4.4 Haematology and serum examination

In the present study, we observed high variation between the individuals in the investigation in the haematological and clinical-chemical parameters. An increase of leukocyte count (Table 5) from the beginning of the experiment to the 5th week was independent of DON feeding and could be explained by antigen stimulation as adaptation to a new environment (Davis 1980). It is possible that the elevated leukocyte count is a stress response due to insufficient feed intake at the restrictive feeding regimen. However, stress is known to cause an excessive release of corticosteroids which can stimulate erythropoesis resulting, for example, in an increase in numbers of erythrocytes and neutrophils and haemoglobin concentration (Jain 1986). We did not find any elevation of haematological parameters in pigs after 5 weeks exposure to DON in the diet. Our finding is supported by the results reported by Swamy et al. (2003) who observed a linear increase of absolute numbers of lymphocytes in the peripheral blood of pigs with increasing DON concentrations (0.4, 3.9 and 5.8 mg/kg) on day 7, but not on day 21, which was supposed to be an adaptation to feeding DON

contaminated grains. In contrast, Rotter et al. (1994) found a marked decrease in leukocyte count after the initial 7 days of exposure to *Fusarium* toxins (0, 0.75, 1.5, and 3 mg DON/kg diet), while after 4 weeks the total leukocyte count ascended with increasing DON concentration because of the elevation of segmented and band neutrophils.

DON and other trichothecenes were shown to stimulate or suppress immune responses, sometimes even at identical dosages (Rotter et al. 1996). Numerous studies observed a pronounced elevation in serum IgA and concurrent depression in IgM and IgG in mice exposed to DON > 2 mg /kg feed (as reviewed by Rotter et al. 1996). But effects on serum IgA in swine are rather inconsistent. In the present investigation serum IgA concentration increased significantly when pigs were fed the DON contaminated wheat *ad libitum* over 5 weeks (Table 5). Drochner et al. (2004) demonstrated an IgA elevation in pigs fed a diet without grain components with 0.6 mg pure DON/kg diet under highly standardized conditions. Similarly, Swamy et al. (2002) observed such an effect at 4.6 mg DON/kg in a feeding experiment with contaminated corn and wheat, but some investigations failed to show interactions between DON (concentrations up to 5.8 mg/kg) and IgA serum concentrations (Swamy et al. 2003, Bergsjø et al. 1992, 1993, Dänicke et al. 2004a,d, Döll et al. 2003). In the current study, serum IgA concentrations of barrows were higher than their female counterparts, reaching only significance ($P = 0.007$) in the DON *ad libitum* group after 5 weeks. This could be a result of the higher feed intake levels and therefore a higher DON intake of the castrated males, because DON had no sex effect in the restricted fed group. A predilection of males to an elevation of serum IgA was observed after feeding DON contaminated diets to mice of different strains (Greene et al. 1994a,b), whereby castrated male mice showed only a moderate increase of serum IgA (Greene et al. 1995). The authors suggested a higher susceptibility of male mice to DON induced IgA nephropathy and related this to androgenic sex hormones (e. g. 5α-dihydrotestosterone).

Young et al. (1983) reported alterations in various serum parameters in piglets fed DON contaminated diets (9 to 43 mg DON/kg), but the effects could not be separated from the impact of a low feed intake. Therefore it has to be emphasised that feeding the same amount of feed is very useful to differentiate the effects between different diets. In the present study, there were no significant effects on serum clinical-chemical parameters between the groups fed the restrictively control and the DON contaminated diet while in the DON *ad libitum* fed group the alkaline phosphatase, which is not specific to particular tissues and highly affected by age, was significantly lower compared to the controls (Table 6). Similarly, the significantly

lower albumin concentration in the restrictively fed control group could not be explained by the restricted feeding, but probably due to variation of the individuals. Additionally, it has to be noticed that the serum albumin concentration is increased in all groups compared to reference values, which could be due to dehydration. A couple of studies observed a significant decrease in total serum protein and albumin (Bergsjø et al. 1993, Rotter et al. 1995) or elevated albumin levels and a decreased α-globulin fraction and an overall increase in albumin:globulin ratio (Rotter et al. 1994) when pigs were fed DON contaminated diets. However, in the present study there was no evidence of DON altering protein synthesis or serum activities of liver enzymes.

DON was not detectable in serum of pigs fed the control diet containing very small DON concentrations and consequently only small amounts of 0.47 to 1.06 mg DON/d were consumed by these pigs. Pigs fed the DON contaminated diet showed a greater variation of serum DON concentration (Figure 2). This could be elucidated by the time of feed intake, especially in the *ad libitum* fed group, because Dänicke et al. (2004b) found that the serum DON concentration was maximal after 4 hours of feeding a DON contaminated meal (4.2 mg DON/kg). This could explain, at least in part, why the variation in serum DON concentration of the restrictively fed pigs was smaller in the present study. The variation of serum DON concentration in restrictively DON fed pigs was neither related to the termination of feeding nor to the DON intake per kg live weight. So it is likely that variation between individuals and analysis should not be unconsidered.

4.5 Organ weights and gross pathological findings

Pathological examination of liver, kidney and spleen showed no marked lesions for any of the groups in the present study. This is in accordance with results reported by Øvernes et al. (1997), who could not show any relation of gross pathological findings, but only small changes like 8 visible kidney cysts, 2 enlarged renal pelvis and a few small, white cortical scars not-related to feeding method or DON level. The authors observed histopathological changes in liver samples, such as eosinophilic interstitial hepatitis found in 13 cases in relation to DON level (Øvernes et al. 1997). In the current experiment, the absolute weight of liver, kidney and spleen was significantly lower in restrictively fed groups. DON only significantly affected the relative weight of kidneys. It has to be taken into account that the absolute organ weights are directly related to the nutritional status of animals and organ mass changes depend directly on changes in body weight (Forsell et al. 1986). Therefore, organ weights related to live weight seem to be more appropriate for interpreting the DON effects.

Swamy et al. (2002) pointed out that on the one hand absolute weights of liver, kidney and spleen were significantly lower in pigs fed 5.6 ppm DON, but the relative weight just of liver and kidney were significantly decreased. In contrast, in other experiments the weights of liver, kidney, and spleen expressed as a percentage of live weight were not altered (Swamy et al. 2003, Rotter et al. 1995). Trenholm et al. (1994) observed a significant increase in relative liver and kidney weights in pigs fed 3.9, 5.0, and 8.7 mg DON/kg from contaminated wheat for seven weeks. The effect of DON on relative organ weights seems to be dependent on the age of pigs, duration of exposure of pigs to mycotoxins, and the dose of DON (Swamy et al. 2002).

In conclusion, it could be shown by the present results that DON affects performance of pigs mainly by a decrease in feed intake. The significantly improved digestibility of nutrients of the *Fusarium* contaminated wheat could neither compensate the lower feed intake in the pigs offered the DON containing diet for *ad libitum* consumption nor result in a superimposed performance fed this diet restrictively. It needs to be clarified if this apparent contradiction indicates alterations in post-absorptive metabolism of nutrients and energy. The serum clinical-chemical and haematological parameters which were determined in the present study seem not to be specific enough to answer this question.

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PAPER II

BIOAVAILABILITY OF THE *FUSARIUM* TOXIN DEOXYNIVALENOL (DON) FROM NATURALLY CONTAMINATED WHEAT FOR THE PIG

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ABSTRACT

Experiments were carried out with 16 castrated male pigs (41.5 ± 2.0 kg) to examine the toxicokinetics of deoxynivalenol (DON) from naturally contaminated wheat (16.6 mg DON/kg) after chronic exposure or one single oral dose (acute). The systemic absorption (bioavailability) of DON was estimated based on the area under the curves (AUC) after oral (chronic or acute) and intravenous application of pure DON (53 µg/kg live weight). Additionally, a balance study was conducted to quantitatively trace the DON metabolism.

After intravenous (IV) DON application (n = 5), serum DON concentrations decreased biphasically with terminal elimination half-lives ($t_{1/2}$ β) of between 4.2 and 33.6 h. DON was rapidly absorbed following oral exposure and reached maximal plasma concentrations (C_{max}) of 21.79 and 15.21 ng DON/ml serum after (t_{max}) 88.4 and 99.1 min in the chronic (n = 5) and acute (n = 6) fed group, respectively. Thereafter serum DON levels declined slowly with an elimination half-life ($t_{1/2}$ β) of 6.28 and 5.32 h for both oral groups. The mean bioavailability (F) of DON was 89 % for the chronic group and 54 % for the acute oral group. DON was highly distributed in all groups, with an apparent volume of distribution (V_d) higher than the total body water. Glucuronide conjugation of deoxynivalenol was found in serum samples after oral exposure, but not after intravenous application.

Dietary DON caused a significant increase in DON concentrations of urine and faeces, whereby the metabolite de-epoxy-DON was found only in the trials with a pre-period of longer than 4 weeks. The total recovery was 66.6 ± 39.0 and 54.0 ± 9.7 % for the control and the chronic DON groups, respectively, with urine being the main excretory route.

In conclusion, orally administered DON was quickly absorbed to an extent of over 50 %, highly distributed and only poorly metabolized. Twenty-four hours following oral dosing, DON could not be detected in the serum, except in one chronically fed pig at the level of the detection limit.

Keywords: balance study, deoxynivalenol, wheat, toxicokinetics, bioavailability, pig

1 INTRODUCTION

Moulds of the *Fusarium* species often infect wheat, especially under temperate weather conditions, and do not only cause profit cuts through decreases in grain yield related to *Fusarium* head blight (scab), but they can also produce secondary metabolites (mycotoxins). The trichothecene deoxynivalenol (DON) plays an important role worldwide for farm animal and human health due to its frequent occurrence in toxicologically relevant concentrations (Placinta et al. 1999). Knowledge of the kinetic parameters of a toxic agent like DON is essential to understand its mode of action, and to evaluate animal and human health risks. Swine are known to be especially susceptible to DON (Rotter et al. 1996) and could therefore serve as a model for human sensitivity to this mycotoxin. Varying toxic effects of DON for pigs were reported in the literature even when similar dietary concentrations were fed (for reviews see Dänicke et al. 2001). Hence, it would be of interest whether these varying effects depend on bioavailability of DON from different sources.

Only few studies investigated the toxicokinetic parameters of DON in pigs (Coppock et al. 1985, Prelusky et al. 1988, 1990), but these experiments were performed with very high doses of pure DON (300 - 1000 µg DON per kg live weight) given intravenously or intragastrically. Therefore, the bioavailability of DON was estimated by studies using standards, but the systemic absorption and kinetic behaviour of DON contaminated feedstuffs remained unclear.

Additionally, the excretion and possible metabolism of DON should not be excluded in the risk assessment of DON in pigs. Prelusky et al. (1988) examined the 24 h recovery of radio-labeled DON in urine and bile of pigs following intravenous and intragastric administration. However, inaccuracy of the DON excretion in a 24 h sampling could be result of varying amounts of faeces and urine from day to day.

The purpose of the present study was to examine the effects of DON on the toxicokinetic parameters of pigs under the terms of a native DON exposure and to distinguish the effects of an acute and chronic DON intoxication as both situations might occur under practical feeding conditions. For this reason, fattening pigs of approximately 40 kg LW were fed a wheat infected naturally with *Fusarium* and contaminated mainly with DON either chronically (≥ 4 weeks) or as one single dose (acutely). The oral kinetics were then related to those after intravenous application of pure DON in order to estimate the systemic absorption (bioavailability) of DON.

Furthermore, a balance study was conducted to collect further metabolic data, to determine a quantitative DON balance over a longer period of time (5 days), after chronic exposure and to estimate systemic DON absorption based on its urinary excretion.

2 MATERIAL AND METHODS

2.1 Experimental diets

The control diet was formulated to meet or exceed all nutritional requirements for fattening pigs as recommended by the 'German Society of Nutrition Physiology' (GfE 1987) with a total wheat content of 400 g/kg (Table 1). The mycotoxin contaminated diets were prepared by replacing 'uncontaminated' control wheat with DON contaminated wheat. The DON contaminated diet comprised 5.7 ± 0.3 mg DON per kg diet, whereas the control diet contained 0.14 ± 0.06 mg DON per kg (Table 1).

Table 1 Composition of the experimental diets (g/kg, based on a dry matter content of 88 %)

	Control diet		**DON contaminated diet**	
Ingredients				
Barley	348		348	
Wheat	400		0	
Contaminated wheat	0		400	
Soybean meal	200		200	
Soybean oil	20		20	
Dicalcium-phosphate	3.5		3.5	
Sodium chloride	1		1	
L-lysine-HCl	2.5		2.5	
Premix[1]	25		25	
Analysed Composition				
Crude protein	173.7		176.3	
Deoxynivalenol [mg/kg]	0.14	± 0.06	5.7	± 0.3

[1] Provided per kg of diet: Ca 6.1 g; P 1.5 g; Na 1.4 g; Mg 0.3 g; Fe 100 mg; Cu 25 mg; Mn 50 mg; Zn 100 mg; I 1.3 mg; Se 0.4 mg; Co 0.5 mg; vitamin A 10000 IU; vitamin D_3 1000IU; vitamin E 30 mg; vitamin B_1 18.8 µg; vitamin K_3 1.3 mg; nicotinic acid 12.5 mg; pantothenic acid 8.4 mg; choline chloride 125 mg.

2.2 Surgery and DON kinetic studies

16 castrated male pigs, crossbred German Landrace x Pietrain, were housed individually and fed control (0.14 mg/kg; n = 11) or DON (5.7 mg/kg; n = 5) contaminated diets for at least four weeks until weighing more than 30 kg. Following an adaptation to the balance cages, the surgery was performed under hospital conditions. After anaesthesia with azaperone, ketamine (intramuscular) and thiopental (IV) sterile human *vena subclavia* catheters (1.5 x 2 mm, 50

cm, Vygon, No. 9153.8220) were used for cannulation of the external *vena jugularis* to facilitate the frequent blood collection over a period of 24 h. The catheter was slid forward into the heart lumen via the jugular vein and fixed with ligatures around the cannulated vein. After subcutaneous cannulation and the addition of a three-way valve, the catheter was fixed in the mid-dorsal area of the pig's thorax. The cranial portion of the jugular vein was tied off to control haemorrhage. Animals were allowed to recover for one day before the kinetic study was initiated.

DON kinetics following chronic (≥ 4 weeks, Table 2) dietary DON exposure of 5.7 mg DON per kg diet was examined in five pigs weighing 42.4 ± 2.3 kg. On the day of the kinetic study, 11 control pigs were divided in two groups. The acute oral DON group (41.6 ± 1.4 kg; n = 6) was once fed the same DON contaminated meal as for chronic exposure (5.7 mg DON/kg). The other five pigs (39.7 ± 0.8 kg) were fed the control diet (0.14 mg DON/kg) and were once dosed intravenously with 53 µg of pure DON/kg LW (Sigma D-0156, Deisenhofen, Germany, 0.2 mg DON was dissolved in one ml 0.9 % NaCl-solution). This DON concentration was calculated on the basis of the DON ingestion of the first two orally tested pigs.

Serial blood samples (10 ml) were drawn prior to feeding and after 5, 10, 15, 20, 30, 45, 60, 90, and 120 min, then every hour until 12 h, and after 24 h. Blood samples were centrifuged to separate the serum. Sodium heparin (2 ml/500ml normal saline) was used to keep the catheters in functioning.

Treatments and experiments were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany (File Number 509.42502/09-02.02).

Table 2 Design and number of pigs of the kinetic and balance studies

Study	Group	DON application		n	Mean dose [µg/kg LW/d]	
		Duration	Form			
Kinetic	DON chronic	5-8 weeks	Wheat	5	68.5	± 4.9
	DON acute oral	Single bolus	Wheat	6	77.3	± 2.4
	DON acute iv	Single bolus	crystalline	5	53.0	± 0.0
Balance	Control	-	-	11	4.2	± 0.4
	DON chronic	4-6 weeks	Wheat	11	162.9	± 15.9

2.3 Balance study

A balance study was carried out to investigate the effects of the two experimental diets (control and DON contaminated, Table 2) on DON concentrations in urine and faeces as a precondition for the estimation of the quantitative DON balance according to the total collection technique (Schiemann 1981). In order to obtain sufficient observations for both groups the experiment was repeated four times (Trial 1 to 4). Each diet was tested on 11 barrows, crossbred German Landrace x Pietrain, and was restricted to an amount consumed by all animals, which was equivalent to the maximum level voluntarily consumed by the pigs fed the DON contaminated diet. The pigs were fed control or DON contaminated diets for at least four weeks (chronic DON exposure; Trial 1 & 3 = 4 weeks; Trial 2 & 4 = 6 weeks) in order to obtain quantitative data on DON balance, until they weighed more than 30 kg each. After this pre-period, the pigs were adjusted for two days to the balance cages, which allowed a quantitative collection of urine and faeces during a 5-day collection period. At the beginning of the balance study, the mean live weight (LW) was 35.4 ± 2.3 kg and 34.1 ± 2.1 kg for pigs fed DON contaminated and the control diet, respectively. The daily feed amount of 1.0 kg (Trials 1 & 2) and 1.1 kg (Trials 3 & 4) was given in two equal portions at 7 a.m. and 2 p.m. after collecting the faeces. Aliquot samples of acidified urine were pooled for each individual pig and were kept frozen until analysis. Pooled faeces samples were prepared from aliquots of the daily faeces, homogenized, freeze dried and ground to pass through a 1 mm screen before being analysed.

2.4 Analyses

Samples from the experimental diets were analysed for DON by high performance liquid chromatography (HPLC) with diode array detection (DAD) after clean-up with immuno-affinity columns (IAC, VICAM, Watertown, MA, USA) according to a modified VDLUFA-method (Valenta et al. 2002). The detection limit was 0.03 mg/kg and the recovery was approximately 90 %.

The determination of DON and de-epoxy-DON in serum, urine and freeze dried faeces is described by Valenta et al. (2003). Freeze dried faeces were analysed in principle as described for diets. In addition to the so-analysed free fraction of DON in serum (without incubation), selected serum samples (after 0.5, 2, 4, 8 and 24 h) were analysed after incubation with β-glucuronidase to yield the total DON concentration (free plus conjugated). In brief, after addition of one ml of sodium acetate buffer (pH 5.5) to 1.5 ml of serum, the mixture was incubated for 16 hours with 6,000 U β-glucuronidase (Sigma, G 0876). For determination of

DON in urine, 2.5 ml phosphate buffer (pH 6.8) and 8,000 U β-glucuronidase were added to 2.0 ml urine and incubated 16 h at room temperature. Urine and serum samples were extracted with ethyl acetate on a ChemElut cartridge (Varian, Middelburg, Netherland), cleaned up by IAC (VICAM) and measured by HPLC-DAD. The detection limit for DON and its metabolite de-epoxy-DON was approximately 4 ng/ml, 8 ng/ml and 20 ng/g for serum, urine and freeze dried faeces, respectively. The mean recovery of DON and de-epoxy-DON was 89/85% in serum, 82/81% in urine and 83/64% in faeces. The results of mycotoxin analysis were not corrected for recovery.

2.5 Calculations and statistics

Individual animal serum data after oral DON exposure were fitted to the Bateman function according to Dost (1963) (1):

$$c_t = \frac{(D \cdot k_i)}{(V \cdot (k_i - k_e))} \cdot (e^{(-k_{el} \cdot t)} - e^{(-k_i \cdot t)}) \qquad (1)$$

where c_t = DON concentration at time t, D = dose of DON [µg/kg body weight], V = apparent volume of distribution [l/kg], k_i = invasion constant, k_{el} = elimination constant, t = time [h].

DON serum concentrations of IV dosed pigs were fitted into a two-compartment model (2, Prelusky et al. 1985, 1990):

$$c_t = A \cdot e^{(-\alpha \cdot t)} + B \cdot e^{(-\beta \cdot t)} \qquad (2)$$

where c_t = DON concentration at time t, A, B = initial concentration at t = 0, α, β = rate constant of distribution or elimination phase, t = time [h]. The non-linear curve fitting module of the Statistica for the Windows^{TM} operating system (StatSoft Inc. 1994) was used to fit the data to equation (1 & 2).

The half-life was calculated as $t_{1/2}\,\alpha = \frac{\ln(2)}{k_i \text{ or } \alpha}$ for invasion or distribution phase and $t_{1/2}\,\beta = \frac{\ln(2)}{k_{el} \text{ or } \beta}$ for the elimination phase. The total area under the serum-time curve (AUC) for DON was determined by using the trapezoidal rule method. The apparent volume of distribution (V_d) following IV administration was determined by $V_d = \frac{D}{AUC} \cdot \frac{t_{1/2}\beta}{\ln(2)}$.

The serum clearance (Cl) of DON was estimated by $Cl = \frac{(\ln(2) \cdot V_d)}{t_{1/2}\,\beta}$.

After extrapolation of the elimination curve to t = 0 the DON concentration ($C_0 = \frac{D}{V}$) was determined. The maximal serum concentration (C_{max}) and the times at peak concentrations (t_{max}) were estimated numerically based on the individually fitted curves. The systemic availability of DON (F) following oral administration was determined as the ratio of the dose-normalized AUC value for the oral route to that of the IV route $F(\%) = \frac{AUC_{oral} \cdot D_{iv}}{AUC_{iv} \cdot D_{oral}} \cdot 100$.

The different parameters were determined according to Prelusky et al. (1985, 1990). Toxicokinetic parameters, performance and DON balance data were subjected to the non-parametric Mann-Whitney U-test.

All statistics were carried out using the Statistica for Windows^{TM} operating system (StatSoft Inc. 1994).

3 RESULTS

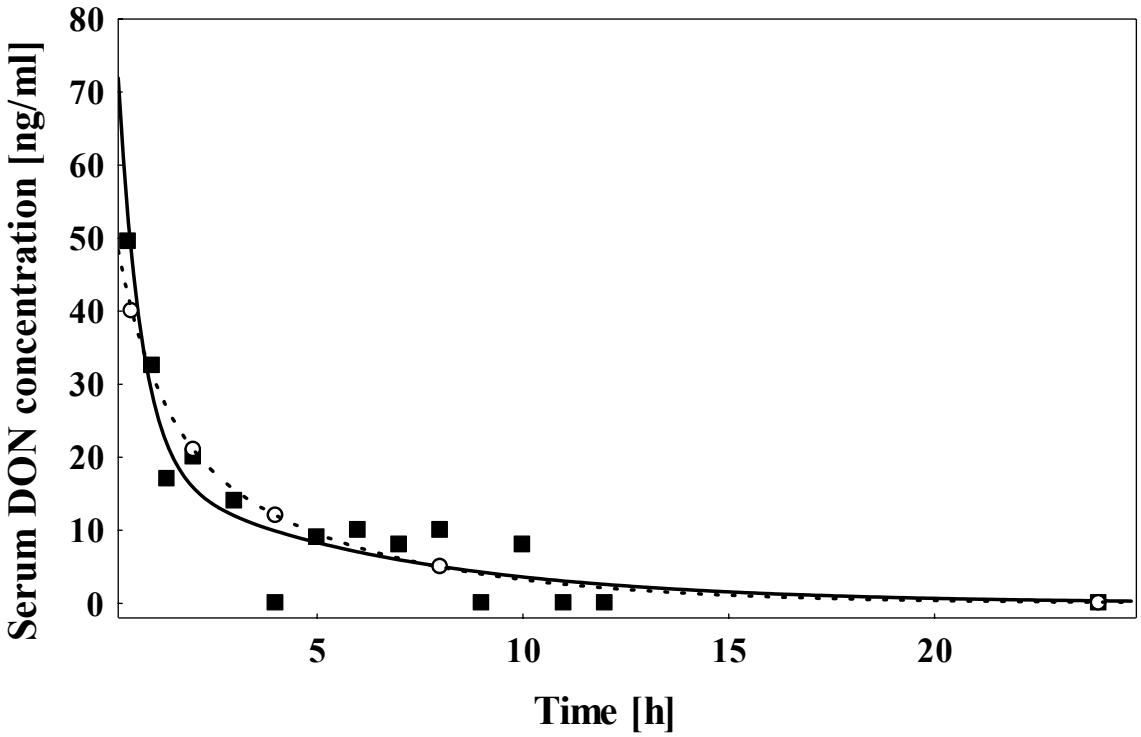

Figure 1 DON concentrations in serum [ng/ml] of one exemplary pig (IV-1) administered a DON dose of 53 µg/kg LW intravenously with (···O···; total DON) or without (—■—; free DON) β-glucuronidase incubation.

3.1 Intravenous (IV) administration of DON

All pigs administered 53 µg DON/kg LW intravenously showed signs of acute DON toxicosis, like retching and emesis (4 - 9 times), within a few minutes (7 - 13 min). No further emesis appeared after 30 min, indicating that DON has no long-lasting emetic effect. Between 13.8 - 56.7 % of the dry matter intake was regurgitated with the vomit.

Following intravenous dosing, the disappearance of DON is described by a two compartment model (Table 3). By the way of example the curve progression is shown for one pig (IV-1) in Figure 1. Initial distribution was rapid with a mean half-life ($t_{1/2}$ α) of 0.72 ± 0.48 h, followed by a slower terminal elimination phase ($t_{1/2}$ β) of 15.24 ± 12.93 h (Table 3).

Table 3 Parameter values of pigs dosed intravenously (IV) with pure DON (53 µg/kg LW) dissolved in 0.9 % NaCl

Animal	IV-1	IV-2	IV-3	IV-4	IV-5	mean	±SD
Live weight [kg]	39.0	41.0	40.0	39.4	39.0	**39.7**	**0.8**
DON [µg/kg LW]	53.0	53.0	53.0	53.0	53.0		
A [ng/ml]	73.6	42.0	87.8	40.1	51.5	**58.6**	**20.5**
α [1/h]	1.77	0.84	6.10	0.52	0.73	**1.99**	**2.35**
B [ng/ml]	19.1	9.5	52.1	6.1	9.6	**19.3**	**19.0**
β [1/h]	0.17	0.03	0.34	0.02	0.05	**0.12**	**0.14**
$t_{1/2}$ α [h]	0.39	0.82	0.11	1.32	0.95	**0.72**	**0.48**
$t_{1/2}$ β [h]	4.16	21.06	2.03	33.61	15.34	**15.24**	**12.93**
V_d [l/kg]	2.08	4.74	0.93	6.91	4.16	**3.76**	**2.34**
Cl [ml/kg*min]	5.28	2.60	5.28	2.34	3.13	**3.81**	**1.56**
AUC [ng*h/ml]	155.1	208.0	167.2	186. 9	210.3	**185.1**	**25.0**
RSD [ng/ml]	3.71	2.66	2.01	2.45	3.46	**2.86**	**0.71**
R^2	0.96	0.95	0.99	0.95	0.95	**0.96**	**0.02**
β-glucuronidase							
Total DON [AUC]	152.1	159.8	180.5	198.2	198.2	**177. 8**	**21.4**
Not-conjugated [%]	100.6	130.1	92.6	94.3	106.1	**104.8**	**15.2**
conjugated [%]	-0.6	-30.1	7.4	5.7	-6.1	**-4.8**	

A, B = initial concentration at t = 0; α = distribution constant; β = elimination constant; $t_{1/2}$ = biological half-life of α = distribution or β = elimination; V_d = apparent volume of distribution; Cl = clearance; AUC = area under the curve; RSD = residual standard deviation; R^2 = stability index.

The apparent volume of distribution of free DON was higher than the total body water (V_d = 3.76 ± 2.34 l/kg LW) and the serum clearance (Cl) of DON was 3.81 ± 1.56 ml/kg*min

(Table 3). Comparing the AUC´s of free (unconjugated) DON and total DON (after incubation with β-glucuronidase) following IV administration, DON in serum seemed not to be conjugated, except in two pigs, where only 5.7 and 7.4 % of the administered dose was in the form of the glucuronide conjugate of DON (Table 3 & Figure 1).

3.2 Chronic oral DON exposure

No signs of toxicity were observed in pigs fed the DON contaminated diet. The serum profile for oral DON dosing was characterized as a two-compartment model with first-order absorption and elimination (Figures 2 & 3). In three out of five chronically fed pigs (C-1, C-2 and C-5), low serum DON concentrations of 10, 11 and 5 ng/ml, respectively, were found in samples taken before feeding after a 17 h feed withdrawal. DON was rapidly absorbed with a mean invasion half-life ($t_{1/2}$ α) of 0.38 ± 0.26 h from the gastrointestinal tract of swine fed DON chronically, and was first detected in serum samples after 5 min (C-3) and 15 min (C-4).

Table 4 Parameter values of pigs chronically fed a DON contaminated diet (C)

Animal	C-1	C-2	C-3	C-4	C-5	mean	±SD
Live weight [kg]	39.0	41.0	43.8	43.5	44.6	**42.4**	**2.3**
DON [µg/kg LW]	69.2	65.8	61.6	73.9	72.1	**68.5**	**4.9**
k_i [1/h]	5.422	2.296	2.097	2.111	0.846	**2.555**	**1.704**
k_{el} [1/h]	0.071	0.149	0.101	0.107	0.191	**0.124**	**0.047**
C_0 [ng/ml]	27.8	28.3	23.8	20.0	33.5	**26.7**	**5.1**
C_{max} [ng/ml]	26.3	23.4	20.4	17.1	21.7	**21.8**	**3.4**
t_{max} [h]	0.81	1.27	1.52	1.49	2.27	**1.47**	**0.53**
$t_{1/2}$ α [h]	0.13	0.30	0.33	0.33	0.82	**0.38**	**0.26**
$t_{1/2}$ β [h]	9.75	4.65	6.85	6.50	3.63	**6.28**	**2.35**
V [l/kg]	2.49	2.52	2.59	3.71	2.19	**2.65**	**0.61**
Cl [ml/kg*min]	2.95	5.77	4.37	6.59	6.85	**5.30**	**1.64**
AUC [ng*h/ml]	319.6	184.4	213.3	171.8	173.1	**212.4**	**62.2**
F [%]	132.2	80.3	99.1	66.6	68.7	**89.4**	**27.2**
RSD [ng/ml]	2.07	2.99	1.67	2.18	1.85	**2.15**	**0.51**
R^2	0.86	0.81	0.90	0.81	0.91	**0.86**	**0.05**
β-glucuronidase							
Total DON [AUC]	222.0	281.3	299.8	268.4	205.2	**255.3**	**40.2**
Not-conjugated [%]	144.0	65.6	71.1	64.0	84.4	**85.8**	**33.5**
Conjugated [%]	-44.0	34.4	28.9	36.0	15.6	**14.2**	**33.5**

k_i = invasion constant; k_{el} = elimination constant; C_0 = extrapolated serum concentration at time zero; C_{max} = maximum serum DON level; t_{max} = time of maximum serum DON concentration; $t_{1/2}$ = biological half-life of α= absorbtion or β= elimination; V = apparent volume of distribution; Cl = clearance; AUC = area under the curve; F = bioavailability; RSD = residual standard deviation; R^2 = stability index.

Peak concentrations (C_{max}) of 17.1 - 26.3 ng free DON per ml serum were reached (t_{max}) between 0.81 to 2.27 h (Table 4). The mean elimination half-life ($t_{1/2}$ β) was 6.28 ± 2.35 h for free DON. Free DON was widely distributed (V = 2.65 ± 0.61 l/kg LW) and the clearance (Cl) of serum covered a range from 2.95 to 6.85 ml/kg*min. Apart from one pig (C-1) fed chronically with DON, 15.6 to 36.0 % of the serum DON was conjugated, based on area under the curve (AUC) comparison (Table 4).

3.3 Acute oral DON exposure

DON was found in serum of pigs as early as 15 min after feeding a DON contaminated meal as a single dose. This quick systemic absorption exhibited a mean invasion half-life ($t_{1/2}$ α) of 0.73 ± 0.61 h (Table 5).

Table 5 Parameter values of pigs acutely fed a DON contaminated diet (O)

Animal	O-1	O-2	O-3	O-4	O-5	O-6	mean	±SD
Live weight [kg]	41.2	41.2	41.8	40.2	44.2	41.2	**41.6**	**1.4**
DON [µg/kg LW]	78.1	78.1	76.9	80.0	72.8	78.1	**77.3**	**2.4**
k_i [1/h]	2.49	0.70	3.72	0.73	3.12	0.40	**1.86**	**1.43**
k_{el} [1/h]	0.16	0.21	0.08	0.13	0.10	0.24	**0.15**	**0.07**
C_0 [ng/ml]	12.4	25.9	13.0	26.7	21.4	35.5	**22.5**	**8.9**
C_{max} [ng/ml]	10.3	15.4	12.0	18.3	19.1	16.4	**15.2**	**3.3**
t_{max} [h]	1.19	2.45	1.07	2.87	1.11	1.23	**1.65**	**0.79**
$t_{1/2}$ α [h]	0.28	0.99	0.19	0.95	0.22	1.73	**0.73**	**0.61**
$t_{1/2}$ β [h]	4.42	3.26	9.04	5.26	7.11	2.84	**5.32**	**2.38**
V [l/kg]	6.30	3.02	5.93	2.99	3.41	2.20	**3.98**	**1.71**
Cl [ml/kg*min]	16.48	10.69	7.58	6.57	5.54	8.96	**9.30**	**3.95**
AUC [ng*h/ml]	77.0	120.7	141.8	192.5	197.3	144.2	**145.6**	**45.2**
F [%]	28.2	44.3	52.8	68.9	77.6	52.9	**54.1**	**17.6**
RSD [ng/ml]	1.68	1.87	1.81	1.67	1.11	2.63	**1.80**	**0.49**
R^2	0.76	0.85	0.70	0.89	0.95	0.73	**0.81**	**0.10**
β-glucuronidase								
Total DON [AUC]	111.6	302.0	274.2	211.8	230.3	292.7	**237.1**	**70.9**
Not-conjugated [%]	69.0	40.0	51.7	90.9	85.7	49.3	**64.4**	**20.8**
conjugated [%]	31.0	60.0	48.3	9.1	14.3	50.7	**35.6**	

k_i = invasion constant; k_{el} = elimination constant; C_0 = extrapolated serum concentration at time zero; C_{max} = maximum serum DON level; t_{max} = time of maximum serum DON concentration; $t_{1/2}$ = biological half-life of α= absorbtion or β= elimination; V = apparent volume of distribution; Cl = clearance; AUC = area under the curve; F = bioavailability; RSD = residual standard deviation; R^2 = stability index;

Although the mean DON dose per kg LW was higher than in the chronic group, the mean peak serum concentration (C_{max}) of 15.2 ± 3.5 ng/ml for free DON was significantly (P = 0.018) lower. Peak concentrations were reached within 1.65 ± 0.79 h and were followed by monoexponential decay with a half-life ($t_{1/2}$ β) of 5.32 ± 2.38 h. The apparent volume of distribution (V) of 3.98 ± 1.71 l/kg LW for free DON was in the same order of magnitude as after intravenous DON application.

The mean serum clearance of 9.30 ± 3.95 ml/kg*min was nearly twice as great as in the chronic DON fed group and significantly (P = 0.011) higher than in the intravenous treated group (Table 5). Based on AUC calculations, between 9.1 and 60.0 % of the total DON in serum was in the form of the glucuronide conjugate when pigs were fed the DON contaminated diet acutely (Table 5 & Figure 3), indicating a high variability of glucuronide conjugation ability between the individuals. Comparison of AUC from acute and chronic DON fed pigs revealed a higher DON level in the chronic group about 46 %, but this was predominantly the effect of pig C-1.

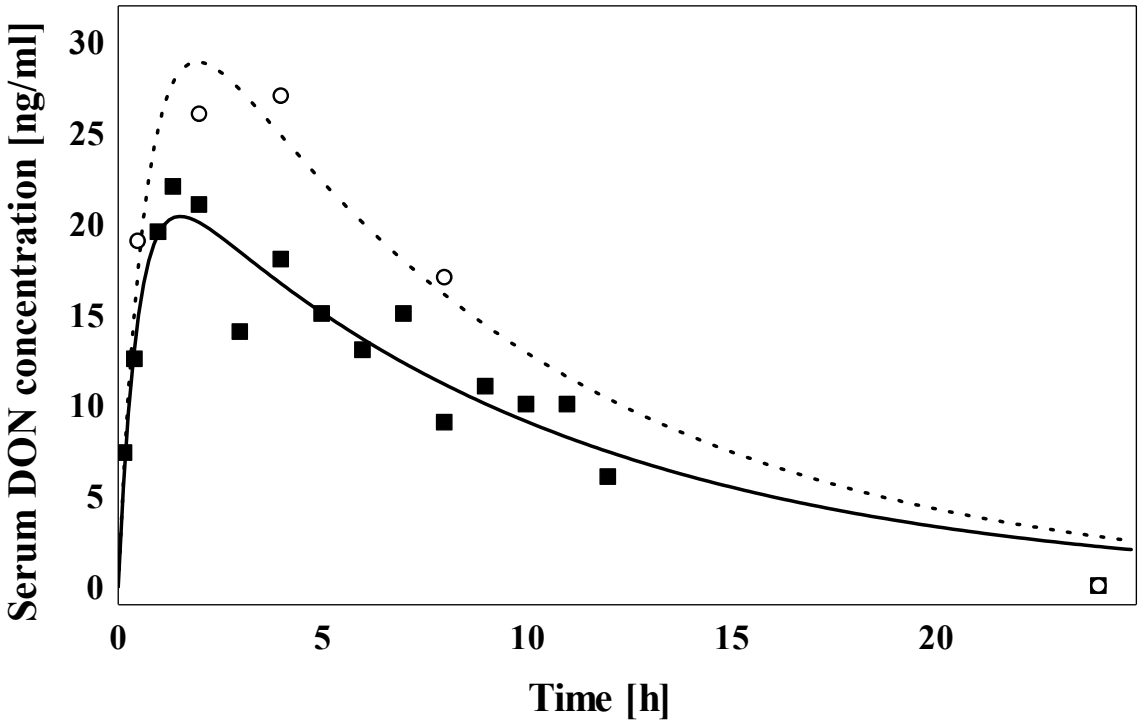

Figure 2 DON concentrations in serum [ng/ml] of one exemplary pig (C-2) fed a DON contaminated diet [5.7 mg/kg] chronically with (⋯○⋯; total DON) or without (—■—; free DON) β-glucuronidase incubation.

3.4 Bioavailability

The mean bioavailability (F) calculated from AUC was 89.4 ± 27.2 % for free DON and 112.3 ± 24.2 % for total DON (after incubation with β-glucuronidase) in pigs fed the DON contaminated chronically, whereas the bioavailability of 54.1 ± 17.5 % for free DON in the acute DON fed group was significantly (P = 0.045) lower. Systemic absorption of total DON after a single DON meal was 91.5 ± 27.4 %.

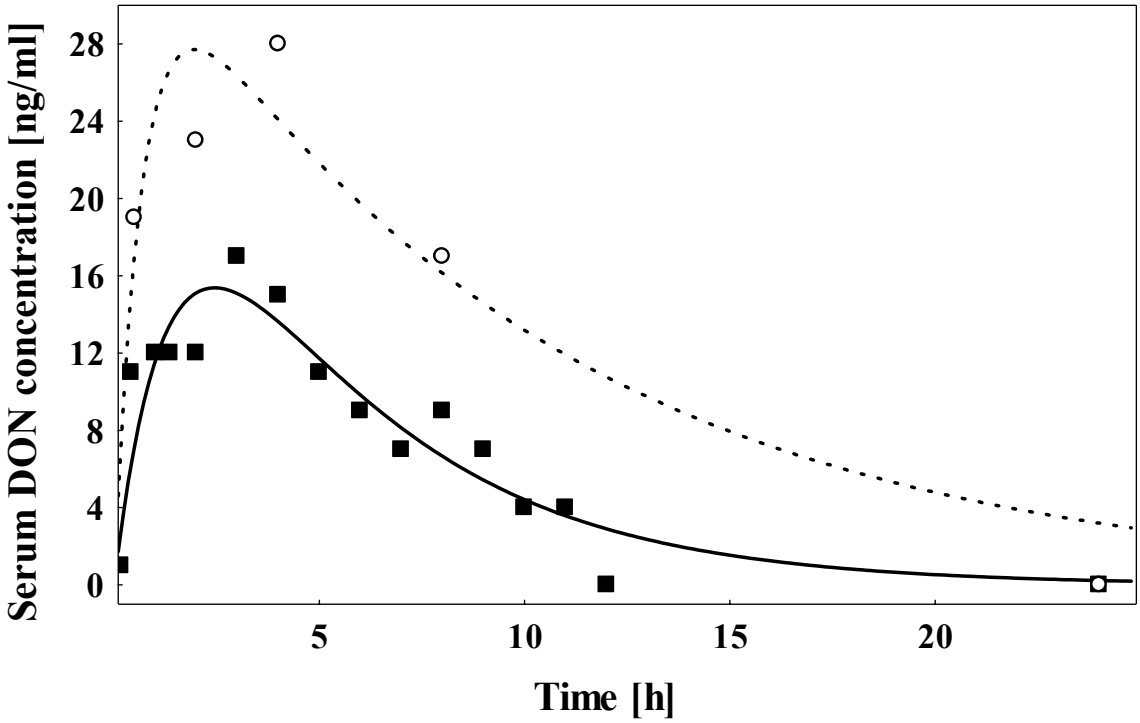

Figure 3 DON concentrations in serum [ng/ml] of one exemplary pig (O-2) fed a DON contaminated diet [5.7 mg/kg] acutely with (···o···; total DON) or without (—■—; free DON) β-glucuronidase incubation.

3.5 Balance study

In the balance study, live weight gain was not affected by dietary DON exposure with 436 ± 152 g/d and 429 ± 172 g/d for the control and DON group, respectively.

DON excretion was significantly influenced by dietary treatment: Concentrations of DON, but not its metabolite de-epoxy-DON, in urine and faeces were significantly increased after feeding the DON contaminated diet. In the control and DON contaminated group 61.8 and 49.7 % of the ingested DON was eliminated with the urine (P = 0.533), which was the main way of excretion. Whereas just 4.8 and 2.5 % of the DON intake of control and DON pigs was excreted by faeces (P = 0.718).

Table 6 DON excretion of pigs fed control or DON contaminated diets (n = 11 per group)

	Control			DON			Probability*
	mean	SD	Range	mean	SD	Range	Diet
DON-intake (mg/d)	0.15	± 0.01	(0.14-0.16)	5.96	± 0.30	(5.68-6.25)	<0.001
Concentration							
DON in urine (mg/l)	0.06	± 0.04	(0.01-0.13)	1.99	± 0.35	(1.40-2.77)	<0.001
De-epoxy-DON in urine (mg/l)	n.d.	± 0.0	n.d.	0.05	± 0.07	(0-0.19)	0.071
DON in faeces (mg/kg)	0.04	± 0.05	(0-0.14)	1.08	± 1.08	(0.04-2.99)	0.005
De-epoxy-DON in faeces (mg/kg)	n.d.	± 0	n.d.	0.20	± 0.26	(0-0.67)	0.071
Excretion in urine as % of DON-intake							
DON	61.8	± 34.7	(14.4-120.6)	49.7	± 9.3	(31.8-60.2)	0.533
De-epoxy-DON	0	± 0	0	1.3	± 1.9	(0-5.3)	0.071
Excretion in faeces as % of DON-intake							
DON	4.8	± 5.6	(0-16.1)	2.5	± 2.4	(0.1-6.2)	0.718
De-epoxy-DON	0	± 0	0	0.4	± 0.6	(0-1.5)	0.072
De-epoxy-DON-excretion as % of the excretion of DON+de-epoxy-DON							
In urine	0	± 0	0	3.9	± 5.4	(0-15.2)	0.071
In faeces	0	± 0	0	40.1	± 46.2	(0-92.2)	0.071
Total recovery (sum of DON and de-epoxy-DON in urine and faeces) as % of DON-intake	66.6	± 39.0	(14.8-129.5)	54.0	± 9.7	(34.3-64.7)	0.718

* Significantly different at $P < 0.05$ Mann-Whitney-U-test

De-epoxy-DON was only detected in excrements of pigs fed the DON contaminated diets over 6 weeks (Trial 2 & 4, data not shown), whereas it was not detected after 4 weeks DON exposure (Trial 1 & 3) or in the control group. Occurrence of de-epoxy-DON was accompanied by a superproportional reduced excretion of the parent toxin. The mean excretion of de-epoxy-DON in pigs fed a DON contaminated diet was approximately 10 times higher in the faeces than with the urine (Table 6), whereby de-epoxy-DON was the predominant compound in faeces (81 - 92 % of total metabolites) in Trial 2 & 4 (data not shown). Total recovery of the ingested DON as parent toxin and its metabolite de-epoxy-DON in faeces and urine was 66.6 and 54.0 % for the control and DON contaminated group, respectively (Table 6).

4 DISCUSSION

4.1 Clinical signs

DON is known to induce a dose-related feed refusal (anorexia) at lower concentrations and emesis at higher levels. Both effects have been linked to increased central (CNS) serotoninergic activity and also peripheral $5HT_3$ receptors in the gastrointestinal tract seemed to be involved (as reviewed by Rotter et al. 1996). Following an intravenous injection, DON reaches primarily the most perfused organs, which are the lungs, myocardial muscle, kidneys and brain. Prelusky et al. (1990) found that DON quickly crosses the blood-brain barrier, because the DON kinetic parameters in the cerebral spinal fluid generally reflected the plasma profile. Peak DON concentrations (C_{max}) of 582 ± 175 ng/ml cerebral spinal fluid were found 30-60 min after IV administration of 1 mg DON/kg LW to pigs weighing 20 - 26 kg, whereas the peak concentration in cerebral spinal fluid was delayed (t_{max} = 270 ± 64.8 min) and five times lower (C_{max} = 113 ± 28 ng/ml) when the same dose was given intragastrically (orally) (Prelusky et al. 1990). In the present study, vomiting was observed in swine 7 to 13 min after the injection and persisted for 12 to 24 min after IV administration of 53 µg DON/kg LW. Prelusky et al. (1988) reported emesis in pigs at 300 µg DON/kg LW intravenous and 600 µg DON/kg LW intragastric dosing, whereas sheep did not show obvious signs of toxicity up to 4000 µg DON/kg LW IV (Prelusky et al. 1987). After intravenous application of 500 µg DON/kg LW to two female pigs (30 kg LW), Coppock et al. (1985) observed severe signs of acute DON intoxication, like vomiting after 6 to 7 minutes for 4 h, mahogany flushing of the skin, diarrhoea after 1.5 h, and progressive muscular weakness and tremors. The fewer clinical signs in the present experiment are probably due to the 10 times lower DON dose

given intravenously. Prelusky and Trenholm (1993) reported that 50 % of the pigs vomited at an effective dose (ED_{50}) of 20 µg/kg LW when DON was administered intravenously. However, it has to be mentioned that both other groups in the current study (oral DON exposure) showed no signs of acute intoxication with DON, although it was reported that intragastric gavage of 50 µg pure DON/kg LW was the minimum effective dose (MED) that evoked emesis (Pestka et al. 1987), and 50 % of the pigs vomited at oral DON doses of 75 - 85 µg DON/kg LW (Prelusky and Trenholm 1993, Young et al. 1987). In studies with pigs weighing 9 - 10 kg, Forsyth et al. (1977) noticed that the oral route was about one-quarter to one-half as effective as the intraperitoneal (ip) route in inducing vomiting, and the response was generally delayed.

4.2 Toxicokinetics

4.2.1 Intravenous (IV) administration of DON

The course of the serum DON concentrations after IV application in the current study fitted best into the two-compartment model (Figure 1), whereas Coppock et al. (1985) described a one-compartment model for IV administration of DON in pigs. Prelusky et al. (1988, 1990) adapted a two- and a three-compartment model and suggested that the fitting model depends on the frequency of blood sampling during the initial minutes after dosing as well as the sensitivity of the analytical methods. On the other hand, Prelusky et al. (1988) analysed not the toxin DON *per se*, but only the disappearance of radioactivity in plasma after application of ^{14}C-labeled DON and revised this method in a later study because of the inaccuracy at low doses (Prelusky et al. 1990). In the present experiment, the absorption rate and time has reduced the maximal DON serum concentrations after oral ingestion of 61.6 - 80 µg DON/kg LW (C_{max} = 10.3 - 26.3 ng/ml, acute and chronic group, Tables 4 & 5) compared to IV administration of 53 µg DON/kg LW (A+B = 46.2 – 139.9 ng/ml, Table 3). Besides the difference between the kinetic after oral or IV administration *per se*, Coppock et al. (1985) suggested that IV administration of DON probably resulted in higher plasma concentrations than would be achieved by oral ingestion of DON contaminated feedstuffs. This could be explained by the hepatic 'first pass' effect and additionally due to decreased feed consumption, which was observed by linear or exponential relationships with increasing dietary DON concentrations (Dänicke et al. 2001, Friend et al. 1982, Forsyth et al. 1977). However, the effect of DON on feed consumption was excluded in the current experiments due to the restricted feeding regimen, where all pigs received the same amount of feed.

Total serum clearance of DON after IV administration was significantly lower than after oral DON exposure, which could be a result of the lower hepatic clearance or the metabolism of DON.

4.2.2 Oral DON exposure

In the current experiment, the serum DON concentrations after oral exposure comply with the Bateman function with a first-order absorption and elimination (Figures 2 & 3).

Fifteen minutes after oral exposure, DON was found in all serum samples. This early detection of DON in serum showed that the absorption of DON was rapid and indicated that the absorption could start in the stomach or in the upper part of the duodenum as is assumed by the findings of Eriksen et al. (2003) and Dänicke et al. (2004b).

The wheat used in the present study was contaminated mainly with DON (16643 µg/ kg) and to a small extent with other trichothecenes (Goyarts et al. 2005), whereby 15-acetyl-DON (101 µg/ kg) and 3-acetyl-DON (41 µg/ kg) probably were metabolized rapidly into DON (Eriksen et al. 2003). This has to be taken into account in interpreting the present results, because it could enhance the availability of oral DON. However, in the view that the proportions of 15-acetyl-DON and 3-acetyl-DON amounted just 0.6 and 0.2 % of the total DON intake, respectively, their contribution to overall DON metabolism should not be overestimated.

The increased serum DON levels (AUC & C_{max}) after chronic, compared to acute, dietary DON exposure could be due to a higher baseline level of 5.2 ± 5.3 ng DON/ml before feeding. Moreover, a higher baseline level could be an explanation for the significantly increased bioavailability in the chronic group. Subtraction of a baseline-AUC of 62.3 for pigs fed DON chronically, assumed by the linear decrease of initial DON-concentration over time (y = 5.2-0.217*x), would result in an AUC of 150.12 and a bioavailability of 62.8 %, which are both in the same range with that of pigs fed the DON contaminated diet acutely (AUC = 145.58, F = 54.1 %). On the other hand, the bioavailability of a toxic agent depends on the 'first pass' effect and consequently on the liver function. Hence, a disturbance or disease of the liver, which might be due to the chronic intoxication with DON, could result in an increase of the bioavailability. However, the bioavailability of DON in pigs fed acutely (54.1 ± 17.5 %) corresponds with results from Prelusky et al. (1988), who found a bioavailability of 54.8 ± 8.6 % after intragastric dosing of 600 µg radio-labeled DON/kg LW to three barrows weighing 14 - 17 kg. The rapid decline of DON in the gastric and proximal duodenum (Dänicke et al,

2004b) associated with the findings of the present study (a bioavailability of over 50 %, a fast appearance of the maximal plasma concentrations (t_{max}) and a major role of the urinary excretion of DON) indicate a significant gastrointestinal absorption of DON from naturally contaminated feedstuffs in pigs, which are probably representative for monogastric animals and for humans.

Serum DON concentration was maximal after 4.1 h and declined thereafter to a baseline 15 h after feeding a DON contaminated diet (4.2 mg DON/kg) (Dänicke et al. 2004b). Following an intragastric application of 1000 µg/kg pure DON to four pigs weighing 20 - 26 kg, Prelusky et al. (1990) measured maximal DON concentrations (C_{max}) of 367 ± 37 ng/ml after 3.75 ± 1.26 h (t_{max}). In the present study, the maximal serum concentration in pigs fed DON acutely were achieved rapidly after 1.65 ± 0.79 h, and the mean C_{max} was 24 times lower (15.2 ± 3.3 ng/ml), although the dose was only 13 times lower (77.3 ± 2.4 µg/kg). This difference between dose and C_{max} could not be solely attributed to the intragastric application, because Eriksen et al. (2003) fed five castrated male pigs a diet with 2.5 mg pure 3-acetyl-DON (50 µg/kg LW) and observed a maximal plasma concentration of 52 ± 7 ng DON/ml after 2.82 ± 0.33 h. The higher DON levels in relation to exposure could be explained by the fact that pure DON is possibly more readily available for absorption than in naturally contaminated feed. Furthermore, it could be assumed that the high DON doses caused a saturation of the DON transport from the gut into the blood (higher t_{max}) and/or a saturation of the excretion mechanisms, e.g., a conjugation reaction (higher DON plasma levels in relation to the DON dose). A delay of the elimination after application of pure DON would not only explain the later t_{max}, but also the higher C_{max} in comparison to the dose as discussed above, because DON could longer accumulate to a higher but later maximal concentration (C_{max}). Additionally, the high variation between the individuals has to be taken into account, because in another study (Prelusky et al. 1988), the maximum concentrations varied between 135 – 322 ng/ml and occurred 0.75 – 9.0 h after intragastric application of 600 µg radio-labeled DON/kg LW to three pigs.

The elimination half-life ($t_{1/2}\beta$) of 6.3 ± 2.4 h and 5.3 ± 2.4 h in chronic and acute DON fed pigs in the current study was in the same range as reported by other experiments, indicating a slow release from a deep tissue compartment (i.e., fat) (Prelusky et al. 1988, 1990). Research by Eriksen et al. (2003) supported this hypothesis, because DON could still be detected in the urine 48 h after the last feeding, whereas no DON was present in plasma at this time, but the

authors found a faster elimination half-life of 2.39 ± 0.71 h, possibly because the authors used a one-compartment open model neglecting the absorption phase.

As in the present experiment, Prelusky et al. (1988) observed a higher apparent volume of distribution of DON than the total body water and assumed a DON uptake by tissues because the high volume could not be attributed to plasma protein binding. In contrast to pigs, sheep exhibited a distribution of DON in the dimension of extracellular water and a several times lower terminal elimination phase (Prelusky et al. 1985, 1987, 1990). These findings, in combination with a low bioavailability of 7.5 % (Prelusky et al. 1985), and an extensive metabolism of DON in the rumen, might explain the relative tolerance of ruminants. The nearly twofold lower total serum clearance after chronic compared to acute dietary DON exposure in the present study possibly results from an inhibition of the elimination mechanisms, but both oral DON exposed groups exhibited higher clearances as described by other experiments (Coppock et al. 1985, Prelusky et al. 1988). A total of 97 % of DON would be eliminated after five times of the elimination half-life ($t_{1/2}\beta$), that means 31.4 and 26.6 h after feeding a DON contaminated diet chronically and as one single dose, respectively. In any case, DON was not detected in serum after 24 h in either oral group, except at the level of detection limit in one pig (C-1) fed chronically.

4.2.3 Glucuronide conjugation

In contrast to ruminants, where the major metabolite in plasma was the glucuronide conjugate of DON (Prelusky et al. 1985, 1987, 1990), Prelusky et al. (1988) reported only small increases in the DON level after incubation with β-glucuronidase and concluded that just small amounts of DON are present as the glucuronide conjugate metabolite in swine. This is in accordance with our findings after intravenous DON application, but following oral DON exposure, 9.1 to 60.0 % of total DON appeared to be conjugated. On the other hand, Eriksen et al. (2003) observed that 42 ± 7 % of the total plasma DON was in a glucuronide conjugated form after a dietary exposure of 3-acetyl-DON. In accordance with the present study, Prelusky et al. (1985) reported a difference of DON glucuronide conjugation between the routes of administration, because the plasma proportion of conjugated DON was 73 % after oral gavage of 5 mg DON/kg LW and only 20 % after IV application of 0.5 mg DON/kg LW to sheep.

Conjugation with activated glucuronic acid (UDPGA = uridine diphospho-glucuronic acid) proceeds in the endoplasmatic reticulum (ER) and is the major Phase II reaction to enhance water solubility and to facilitate the excretion with the urine or bile (depending on molecular size and varying between species) (Fichtl et al. 1992). Glucuronide conjugation of DON was

assumed to occur particularly in the liver (Galtier and Alvinerie 1981, Prelusky et al. 1986, Seeling et al. 2005), but a 'first pass' effect after oral exposure of DON could not solely explain the lack of glucuronide conjugation after intravenous DON administration. This is in accordance with Cote et al. (1987) who were not able to find a DON glucuronidation in rat and pig hepatic microsomes *in vitro*. Phase II enzymes are also widespread in extrahepatic tissues, especially in lungs, kidneys, skin and in the gastrointestinal tract (Fichtl et al. 1992). Therefore, it is likely that DON was conjugated in the intestine before absorption. The results of *in vitro* incubation of zearalenone with intestinal mucosa of pigs would contribute to this assumption (Olsen et al. 1987). It could be hypothesized that glucuronide conjugated DON is not as toxic as free DON, possibly because it does not fit into the activation centre of the 60S subunit of eukaryotic ribosomes and is eliminated faster with the urine. On the other hand, Prelusky et al. (1985, 1986, 1987) observed a longer elimination half-life ($t_{1/2}\beta$) for glucuronide conjugated DON in comparison to non-conjugated (free) DON in sheep.

4.3 DON balance

Steady state conditions (with a quantitative balance of constant DON intake and DON excretion with urine and faeces over five days) were preferred for the DON metabolism study because the effect of variation in the daily excretion should be excluded.

In the present study, the majority of the ingested DON was excreted via the urine (61.8 % (control) and 49.7 % (chronic DON), Table 6). Other balance studies agreed with this finding, but the urinary recovery of ingested DON varied between 50 % and 63 % (Friend et al. 1986), 42 % and 71 % (Dänicke et al. 2004a), 39 % and 90 % (Dänicke et al. 2004c), and 45 % and 55 % (Dänicke et al. 2004d). A large urinary excretion of a toxin or its metabolites after oral administration indicated a high gastrointestinal absorption, while faecal elimination could result from a lack of systemic absorption or from an efficient biliary excretion (Galtier 1998). In the present experiment, the urinary recovery of ingested DON as DON and de-epoxy-DON of 51.1 % (33.2 – 60.2 %) for the DON group in the balance study was in the same range as the systemic absorption of pigs fed DON acutely (54.1 ± 17.6 %), whereas the bioavailability after chronic DON exposure was visibly higher (89.4 %).

Considering the ratio of de-epoxy-DON excretion of the sum of DON and de-epoxy-DON excretion, Dänicke et al. (2004a) observed that dietary DON in the urine was principally excreted in its unmetabolised form (95.2 %), whereas in faeces the majority was excreted as the metabolite de-epoxy-DON (97.4 %). This is in accordance with our findings in the two trials (Trial 2 & 4), where we were able to detect de-epoxy-DON in urine and faeces. The

detection of de-epoxy-DON was only possible in pigs receiving the DON contaminated diet for a period longer than 4 weeks, whereas control pigs showed no de-epoxidation ability, indicated by urinary or in faecal de-epoxy-DON excretion. Accordingly, Hedman and Pettersson (1997) hypothesised that the ability of de-epoxidation is acquired and may be a result of the presence of the trichothecene. It is possible, that bacteria with this ability proliferate because they were favoured by the presence of the trichothecenes in the feed. However, Eriksen et al. (2002) was unable to detect a difference between bacterial DNA profiles. *In vitro* studies indicated that the gastrointestinal microflora in some pigs had the ability to de-epoxidate trichothecenes (Kollarczik et al. 1994) while other pigs lacked this ability (He et al. 1992). Bacteria from human faeces were not able to de-epoxidate trichothecenes (Eriksen and Pettersson 2003). Furthermore, it was noticed that pigs from commercial pig farms had the ability, whereas specific pathogen-free (SPF) pigs or pigs from research stations were not able to de-epoxidate trichothecenes, but the ability to transform trichothecenes appeared to be transferred between pigs in a stock (Eriksen et al. 2002). It is also noticeable that in the trials of the present study, where de-epoxy-DON was found in the excrements (Trial 2 & 4), the total recovery of DON is on a lower level, so Eriksen et al. (2003) suggested that there are further decompositions to unknown substances in the gut after degradation to de-epoxy-DON.

Eriksen et al. (2003) were unable to identify de-epoxy-DON in the urine and plasma of pigs with known faecal de-epoxidation ability and concluded that this ability could not protect the pig against the toxic effect of DON, because the de-epoxidation activity is located too far down in the gastrointestinal tract. This is in accordance with Dänicke et al. (2004b), who examined the passage of DON in consecutive segments of the digestive tract of pigs (88.1 ± 3.9 kg LW) after feeding a diet containing DON (4.2 mg/kg) at different times with a comparative slaughter technique. DON was almost completely absorbed in the proximal small intestine, where only traces of de-epoxy-DON were found, whereas the proportion of de-epoxy-DON of the sum of DON plus de-epoxy-DON increased from small intestine to the rectum by up to approximately 80 %. These findings make clear why only low proportions of de-epoxy-DON could be found in the urine, and could explain the low recovery of total DON plus de-epoxy-DON in the faeces of the present experiment.

In summary, the results of the DON balance study do confirm the assumption that not all animals are able to detoxify DON to the metabolite de-epoxy-DON and that this metabolism

occurs primarily in the large intestine, where unlikely absorption proceeds. Therefore, the ability to de-epoxidate does not seem to contribute to a substantial detoxification of DON. Furthermore, it was shown that quantitative urinary recovery of DON can be considered as an indicator for its systemic absorption as it approximates the bioavailability as estimated by the kinetic study.

It has to be taken into account that for real risk assessment, the oral exposure of naturally contaminated material is essential. Oral exposure of a diet contaminated naturally with DON in the present study results in a rapid absorption, a high distribution and low metabolism. More than 50 % of DON was absorbed from naturally contaminated feedstuffs and could be recovered in the serum of pigs by the AUC-method and in urine employing the quantitative balance technique. This could explain, at least in part, the high susceptibility of pigs to DON in contrast to other species, e. g., ruminants. However, the effects of glucuronide conjugation of DON on toxicity and excretion behaviour in pigs have to be clarified. Assuming a high comparability of digestion and excretion in humans and swine, it could be concluded that although DON is poorly detoxified, it is rapidly excreted and is not found in remarkable concentrations in serum after 24 h.

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PAPER III

EFFECT OF THE *FUSARIUM* TOXIN DEOXYNIVALENOL (DON) ON IGA, IGM AND IGG CONCENTRATIONS AND PROLIFERATION OF PORCINE BLOOD LYMPHOCYTES

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ABSTRACT

An important effect of the trichothecene mycotoxins is the impairment of the immune function, but immunotoxicity studies have mainly been conducted on the mouse model. In the present study, the effect of deoxynivalenol (DON) on the proliferation of ConA stimulated porcine peripheral blood lymphocytes (PBL) was assessed *in vitro* after adding of 70 to 560 ng DON per ml medium, and *in vivo* after chronic and acute (one single dose) dietary DON exposure (5.7 mg/kg). Immunoglobulin (IgA, IgG, IgM) concentrations were measured by ELISA in supernatants and serum of pigs. The proliferation rate was estimated with two different assays (BrdU incorporation and MTT cleavage). *In vitro* the ConA stimulated proliferation was inhibited to 50 % (IC_{50}) at 200 and 309 ng DON/ml for the BrdU and MTT assay, respectively, indicating a higher sensitivity of DNA synthesis to DON. Immunoglobulin concentrations in the supernatant after *in vitro* proliferation of PBL with increasing DON concentrations for 72 h were significantly decreased, with IC_{50} values of 120.6, 84.1 and 71.7 ng DON/ml for IgA, IgM and IgG, respectively.

In vivo significant inhibition of lymphocyte proliferation was observed only in the DON acute group using the MTT assay, but values tended to be decreased in the BrdU assay and after chronic DON exposure. Immunoglobulins (IgA, IgM and IgG) in the supernatant of cultured lymphocytes were not significantly affected after dietary DON exposure. Serum IgA of pigs showed no significant differences between the groups, whereas IgM and IgG were significant increased in the DON acute group.

Keywords: deoxynivalenol; *ex vivo*; immunoglobulin; *in vitro*; *in vivo*; lymphocytes; pig; proliferation

1 INTRODUCTION

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium* fungi, is frequently found as a contaminant in cereal grains (Rotter et al. 1996, Placinta et al. 1999). Among farm animals swine are regarded to be most susceptible to DON because of reduced feed intake and decreased weight gain already at lower dose levels (Rotter et al. 1996). However, exposure to DON and other trichothecenes results not only in economical losses due to worse performance, but also in an alteration of immune cell function, dysregulation of the humoral immune response and impairment of host resistance to pathogens (Rotter et al. 1996). Mitogen-induced proliferation, a common technique to assess immunotoxicity, was observed to be impaired or enhanced (at low concentrations) after *in vitro* exposure of human and murine lymphocytes to DON and other trichothecenes (Bondy and Pestka 2000, Rotter et al. 1996). DON was also shown to affect the humoral immunity (Bondy and Pestka 2000). IgA, IgM and IgG secretion was significantly impaired in murine lymphocyte cultures exposed to DON *in vitro* (Warner et al. 1994). Pestka (2003) reported that chronic exposure to DON up-regulates serum immunoglobulin A (IgA) in mice, whereas IgM and IgG levels were decreased. Immunoglobulin disturbances as well as the effects on proliferative response of lymphocytes caused by DON were mainly studied in mice, with comparatively few investigations on possible effects on humans or domestic animals. Understanding the basis for the immuno-dysfunction attributable to DON in pigs is also important in understanding whether human exposure to DON may have unfavourable effects, because swine are physiologically quite similar to humans (Tumbleson and Schook 1996) and are widely used as models for human disease.

The purpose of the present study was to examine the alteration of immune function in pigs by DON using two different *in vitro* proliferation assays (BrdU and MTT assay) as well as the measurement of immunoglobulins (IgA, IgM and IgG). For this reason, different concentrations of pure DON were tested *in vitro* on mitogen stimulated porcine peripheral blood lymphocytes (PBL) derived from untreated pigs. To take into account that the effects of trichothecenes on lymphocyte proliferation were mainly attributed to an inhibition of protein synthesis (Rotter et al. 1996), in the present study the influence of the known protein synthesis inhibitor cycloheximide (CHX) was compared to the effect of pure DON *in vitro*. Furthermore, it was tested whether the lymphocyte proliferation of pigs was affected by a chronic (≥ 4 weeks) or acute (one single dose) dietary DON exposure from naturally with *Fusarium* contaminated wheat (*in vivo* study), indicating a hazard of DON on the porcine immune

system. Finally, IgA, IgM and IgG levels were determined in supernatants of DON exposed cultured lymphocytes and in serum of pigs fed a DON contaminated diet.

2 MATERIAL AND METHODS

If not otherwise stated, all chemicals were purchased from Sigma (Deisenhofen, Germany).

2.1 Sample preparation

Blood samples were drawn from the pigs by jugular venopuncture. The heparinized blood was diluted 1:1 with RPMI 1640 medium (R-8758). Peripheral blood lymphocytes (PBL) were separated from the diluted blood samples using a Ficoll (F-4375) density gradient (centrifugation 400 x g for 15 min). This procedure was described to enhance the recovery of mononuclear cells that were mostly lymphocytes, with a small amount of contamination by other cells (Bøyum 1968). The opaque interface was washed two times (centrifugation 250 x g for 8 min) in RPMI 1640 medium supplemented with 1 M HEPES buffer (H-3784), 2 mM L-glutamine (G-6392), 5 mM mercaptoethanol (M-7522), 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 0.25 µg/ml amphotericin B (ABAM, A-7292) and 5 % heat inactivated foetal calf serum (FCS, Biochrom AG seromed®, Berlin, Germany). Cell viability was evaluated by the trypan blue exclusion technique and was always greater than 95 %. Isolated lymphocytes were adjusted to a final concentration of 1 x 10^6 viable cells/ml and 100 µl of cell suspension was pipetted in quadruplicate into 96-well microtitre plates (MTP, Nunc A/S, Roskilde, Denmark, Cat.No. 167008). After addition of 50 µl mitogen (2.5 µg ConA/ ml) and 50 µl toxin, suspension medium was appended to an amount that a final solution of 200 µl per well was obtained. Cell cultures were incubated at 37°C in a humidified incubator at 5 % CO_2 for 72 h, after centrifugation and collection of 100 µl supernatant, 10 µl of BrdU or MTT (5 mg/ml PBS) was added and incubated for another 4 h. A BrdU proliferation kit (Roche Diagnostic GmbH, Mannheim, Germany, Cat. No. 1647229) was performed according to the manufacturer's instructions and read by microplate photometer (Powerwave, Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany) at a test wavelength of 450 nm and a reference wavelength of 690 nm. The optical density of MTT assay was measured by an ELISA reader at 570 nm after dissolving the crystalline formazan product with 100 µl of 0.01 N HCl/SDS-solution.

2.2 In vitro experiments

The applicability and sensitivity of two different assays to measure effects of DON on porcine lymphocytes were compared: 1) DNA synthesis with BrdU (5-Bromo-2`-deoxyuridine), which

is incorporated into the DNA of proliferating cells instead of thymidine and 2) metabolic activity with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay, in which the yellow tetrazolium salt is metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product (Mosmann 1983, Widestrand et al. 1999).

In preliminary tests, it was found that 1 x 10^5 cells stimulated with 2.5 µg ConA/ml were optimal to examine the effects of DON on porcine PBL.

The effect of different DON concentrations (70, 140, 280, 560 ng/ml, 76 h incubation) were tested on ConA stimulated peripheral blood lymphocytes of one pig fed an uncontaminated diet. In order to compare these effects of DON with the effect of protein synthesis inhibition the known protein synthesis inhibitor cycloheximide (CHX) was added as a positive control at 50, 100, 200, 500 and 750 ng/ml [0.18, 0.36, 0.71, 1.78, 2.67 µM] to lymphocyte cultures (76 h). The proliferation rate was measured by BrdU and MTT assays.

Furthermore, immunoglobulin concentrations were evaluated in the supernatants of ConA stimulated lymphocytes exposed to DON (70, 140, 280, 560 ng/ml) for 72 h. IgA, IgM and IgG concentrations were analysed by an indirect enzyme-linked immunosorbent assay (ELISA). By way of example the determination of IgA is described. The antibody and reference serum were purchased from Natu Tec-Bethyl (Frankfurt a.M., Germany). Microtitre plates (Nunc C bottom Immunoplate 96 well, Cat. No. 4466612; Nunc, Wiesbaden, Germany) were coated for 60 min at RT, with goat anti-pig IgA (E 100-102) diluted 1:100 in 50 mM carbonate buffer, pH 9.6. Coating plates were washed three times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05 % Tween 20, pH 8.0) to remove excess capture antibodies (Ab). Wells were incubated with 200 µl of blocking (postcoat) solution (50 mM Tris, 0.14 M NaCl, 1 % BSA, pH 8.0) for 30 min to block nonspecific protein binding and then washed three times with washing solution. For determination of IgA, supernatants from the cell culture plate were used undiluted and serum was diluted to 1:15,000 (50 mM Tris, 0.14 M NaCl, 1 % BSA, 0.05 % Tween 20, pH 8.0). From both, 100 µl were added to appropriate wells. Standard curve was diluted from the reference serum, covering a range from 7.8 to 1000 ng/ml. The concentrations of IgA in the test serum samples were determined using this standard curve. Plates were incubated at RT for 60 min, washed five times in washing solution, and then 100 µl peroxidase-conjugated goat anti-pig IgA (A100-102P; diluted 1:100,000 in solution for samples) was added to each well. After incubation (60 min, RT), unbound peroxidase-conjugate was removed and each well washed five times with washing solution. Bound peroxidase was determined with

tetramethylbenzidine (TMB, 0.1 mg/ml) and H_2O_2 (0.006 %) in 0.1 M acetate, pH 6.0 and 100 µl of this substrate was transferred to each well. After 15-60 min incubation, the TMB reaction was stopped by the addition of 100 µl of 2 M H_2SO_4 to each well, and the optical density was measured at 450 nm with a microplate reader (Anthos Instruments, Salzburg, Austria).

2.3 In vivo experiment

Wheat infected naturally with *Fusarium* and contaminated mainly with DON was included in a pig diet at a total wheat content of 400 g/kg diet. Analysed toxin concentrations of the contaminated wheat (in µg/kg) were: deoxynivalenol (16643), zearalenon (114), nivalenol (41), 15-acetyl-DON (101), 3-acetyl-DON (41), scirpentriol (20), HT-2 toxin (11), whereas the trichothecenes fusarenon X, monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol, T-2 toxin, T-2 tetraol and T-2 triol were not detectable (Goyarts et al., 2005). The DON concentration of the contaminated diet was 5.7 ± 0.3 mg/kg diet, whereas the control diet contained 0.14 ± 0.06 mg DON per kg (Table 1).

Table 1 Composition of the experimental diets (g/kg, based on a dry matter content of 88 %)

	Control diet	**DON contaminated diet**
Ingredients		
Barley	348	348
Wheat	400	0
Contaminated wheat	0	400
Soybean meal	200	200
Soybean oil	20	20
Dicalcium-phosphate	3.5	3.5
Sodium chloride	1	1
L-lysine-HCl	2.5	2.5
Premix[1]	25	25
Analysed Composition		
Deoxynivalenol (mg/kg)	0.14 ± 0.06	5.7 ± 0.3

[1] Provided per kg of diet: Ca 6.1 g; P 1.5 g; Na 1.4 g; Mg 0.3 g; Fe 100 mg; Cu 25 mg; Mn 50 mg; Zn 100 mg; I 1.3 mg; Se 0.4 mg; Co 0.5 mg; vitamin A 10000 IU; vitamin D_3 1000 IU; vitamin E 30 mg; vitamin B_1 18.8 µg; vitamin K_3 1.3 mg; nicotinic acid 12.5 mg; pantothenic acid 8.4 mg; choline chloride 125 mg.

The *in vivo* study was carried out with 36 castrated male, crossbred German landrace x Pietrain pigs with an initial average body weight of 16.5 ± 3.0 kg. Each animal was housed in a separate pen with free access to water from drinking nipples and was fed individually. After one week of acclimatization with gradual adaptation to the experimental diets, pigs were fed restrictively (the same amount of feed for each pig) a control (n = 23) or a DON contaminated

diet (chronic DON group; n = 13) diet over 4 weeks. Haematological parameters were determined from all pigs (control and DON chronic) at the beginning (Week 0) and the end (Week 5) of the study (Table 2).

In the 4th week, 23 control pigs were divided in two groups. One group (acute DON group; n = 12) was fed the DON contaminated meal (5.7 mg DON/kg) once. The other pigs were fed a control diet (control group; n = 11). One hour after feeding (550 g diet, entirely consumed by all pigs) venous blood (20 ml) from mycotoxin-treated and control pigs was collected in sterile syringes, containing sodium heparin, by puncture of the *vena jugularis* and was treated as mentioned above. Lymphocytes (1 x 10^5 per well, 100 µl) of each pig were tested on proliferative response and immunoglobulin (IgA, IgM and IgG) secretion in the supernatant (*ex vivo*) without (nonstimulated) and with 2.5 µg/ml ConA in 5 wells, respectively. Additionally, serum of each pig was analysed on DON and IgA, IgM and IgG concentrations. The determination of DON in serum was performed according to Valenta et al. (2003) with small modifications. In brief, one ml of sodium acetate buffer (pH 5.5) was added to 1.5 ml of serum and was thereafter incubated for 16 hours with 6,000 U β-glucuronidase (G-0876) and measured by HPLC-DAD. The detection limit for serum was approximately 4 ng DON/ml. Supernatant and serum IgA, IgG and IgM concentrations were determined as described above.

Treatments and experiments were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany (file number 509.42502/09-02.02).

2.4 Calculations and statistics

Optical density of blank wells (medium without cells) was subtracted from the measured extinction values of the cell cultures and mean values and standard deviation of quadruplicates were compared with the corresponding controls. The stimulation index (SI) was calculated: SI = absorbance of mitogen-stimulated lymphocytes / absorbance of nonstimulated lymphocytes.

The dose-response curves were fitted to a non-linear regression equation was calculated according to Mercer et al. (1987, modified):

$$\% \text{ of inhibition} = \frac{R_{max} \cdot \text{toxin}^x}{(K_{0.5})^x + \text{toxin}^x} \quad (1)$$

where toxin = concentration of the toxin (DON or CHX), R_{max} = maximum theoretical inhibition, $K_{0.5}$ = time at $0.5 \cdot R_{max}$, x = apparent kinetic order. Inhibiting concentration of 50 %

(IC_{50}) was derived from (1). The non-linear curve fitting module of the Statistica for the Windows™ operating system (Statsoft Inc. 1994) was used to fit the data to equation (1).

DON analyses, haematological and performance data were subjected to analysis of variance (ANOVA) according to a one-factorial design:

$$y_{ij} = \mu + a_i + e_{ij}$$

where y_{ij} = j^{th} observation related to the DON exposure, μ = overall mean, a_i = effect of DON exposure (control, acute, chronic), e_{ij} = error term. Significant mean values differences were evaluated by the Tukey test. Immunoglobulin analyses (serum and supernatant of *in vivo* study) were subjected to the non-parametric Kruskal-Wallis test, because of the not normally distribution. All statistics were carried out using the Statistica for Windows™ operating system (Statsoft Inc. 1994).

3 RESULTS

3.1 In vitro experiments

A dose-response-dependent decrease of MTT and BrdU values in ConA stimulated PBL was observed when pure DON (70, 140, 280 and 560 ng DON/ml) was added to lymphocyte culture, reaching significance at 280 ng DON/ml (Figure 1). Absorbance for ConA stimulated lymphocytes were 106, 97, 58, 31 % and 87, 85, 16, 0 % of the stimulated control (= 100 %) in the MTT and BrdU assay, respectively. Stimulation index (SI) after addition of 2.5 µg ConA/ml was 2.2 in the MTT and 212 in the BrdU assay. DON concentrations of 309 and 200 ng/ml resulted in a 50 % reduction of optical density (IC_{50}) in the MTT and BrdU assay, respectively (Figure 1).

Figure 2 shows a significantly decreased proliferation of stimulated lymphocytes of 88, 57, 28, 25, 26 % and 52, 27, 10, 9, 7 % in MTT and BrdU assay compared to the control, respectively, when 50, 100, 200, 500 and 750 ng/ml of the protein synthesis inhibitor CHX was added. IC_{50} values were 52.7 and 111.5 ng CHX/ml in the BrdU and MTT assay, respectively.

In vitro supernatant immunoglobulin concentrations were significantly dose-dependently declined to 80, 43, 24 and 23 % (IgA), 62, 23, 9 and 9 % (IgM) and 59, 39, 34 and 46 % (IgG) compared to the stimulated control (= 100 %) when 70, 140, 280 and 560 ng DON/ml were added to cultured lymphocytes. IC_{50} values of stimulated porcine PBL were 120.6, 84.1 and 71.7 ng DON/ml for IgA, IgM and IgG, respectively (Figure 3).

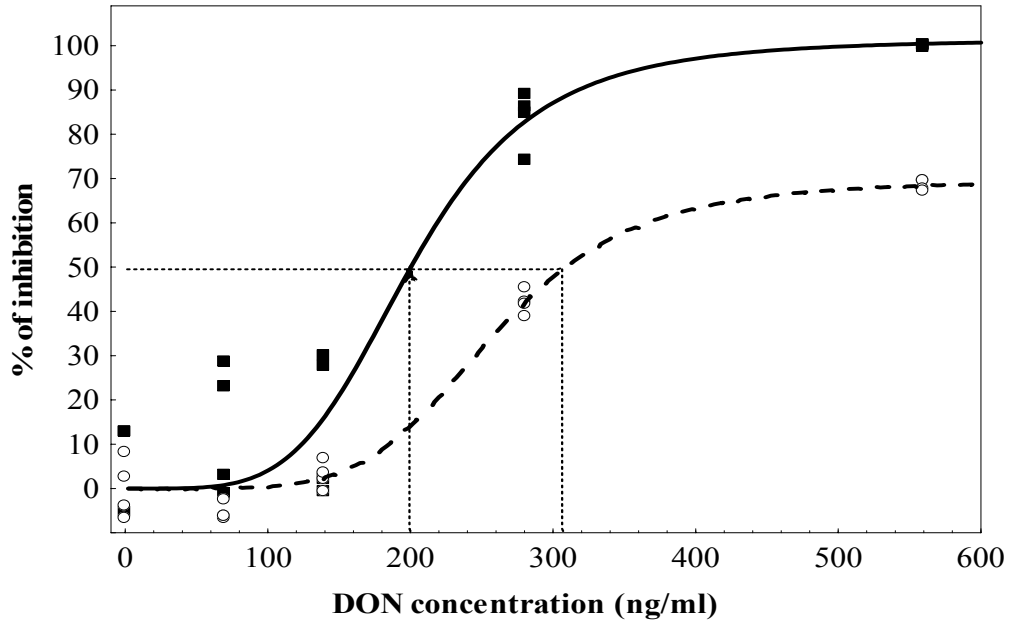

Figure 1 Inhibiting effect of DON (70, 140, 280, 560 ng/ml) after incubation of 76 h on *in vitro* proliferation of ConA stimulated porcine blood lymphocytes in the MTT (–⊖–, 570 nm; $r^2 = 0.98$) and BrdU (—■—, 450-690 nm, $r^2 = 0.92$) assay; ..... IC_{50} (inhibiting concentration of 50 %); n = 4 for each DON concentration.

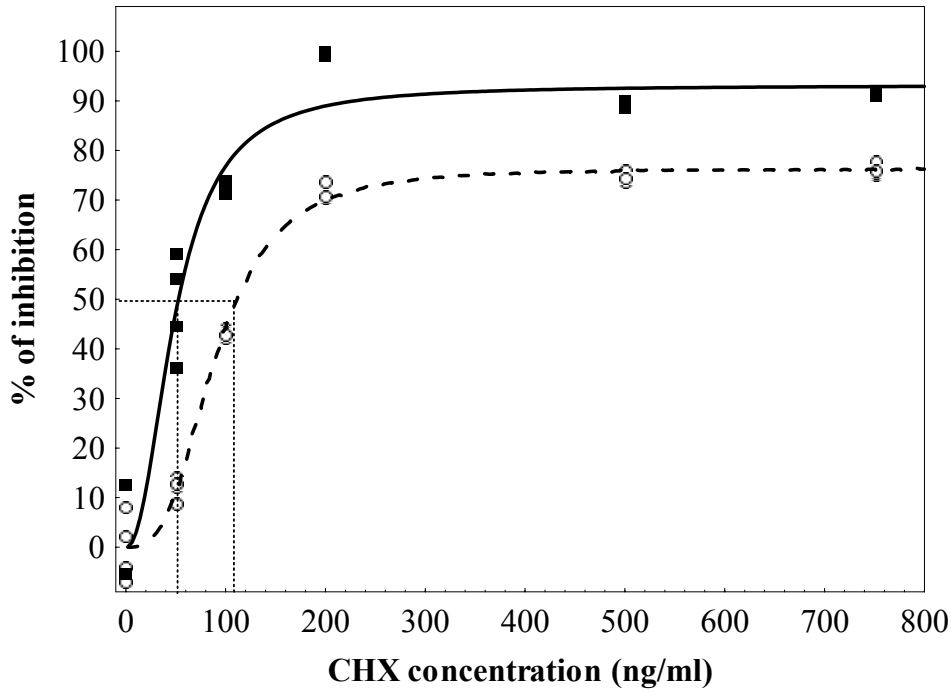

Figure 2 Inhibiting effect of increasing CHX concentrations (0, 50, 100, 200, 500, 750 ng/ml) after 76 h incubation on *in vitro* proliferation of porcine blood lymphocytes (fresh) in the MTT (–⊖– 570 nm, $r^2 = 0.99$) and BrdU (—■— 450-690 nm, $r^2 = 0.95$) assay; ..... IC_{50} (inhibiting concentration of 50 %); n = 4 for each CHX concentration.

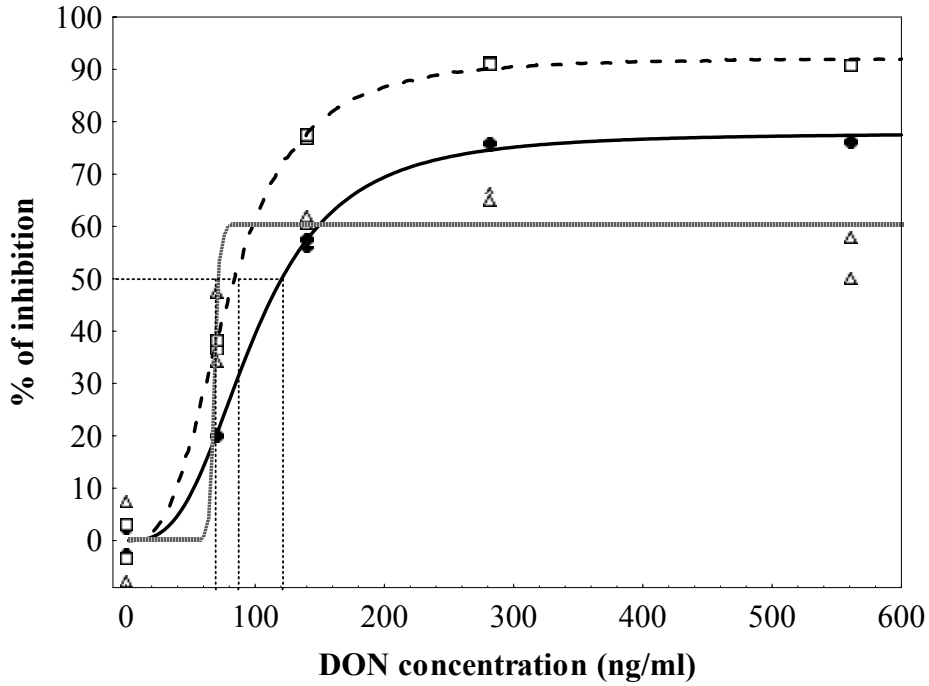

Figure 3 Inhibiting effects of DON (0, 70, 140, 280, 560 ng/ml) on immunoglobulin concentrations (—●— IgA [r^2 = 0.99]; —□— IgM [r^2 = 0.99]; ···△··· IgG [r^2 = 0.98]) in the supernatant after 72 h *in vitro* proliferation of stimulated porcine blood lymphocytes (fresh); ..... IC_{50} (inhibiting concentration of 50%); n = 2 for each DON concentration.

3.2 In vivo experiments

Between the chronic DON exposed (n = 13) and the control group (n = 23) differences could be observed neither in feed intake (1.1 ± 0.04 kg/d) and live weight gain (364 ± 39 g/d) over the course of the study (5 weeks) nor in haemotological parameters (Table 2) at the beginning or the end of the study.

Table 2 Haematological data of pigs fed a control or DON contaminated diet at the begin and after 5 weeks of exposure (means ± SD)

	Week	Control		DON chronic		Probability*
N		23		13		
Leukocytes (G/l)	0	17.4	± 6.3	17.6	± 3.2	0.941
	5	16.1	± 3.2	14.9	± 1.8	0.237
Lymphocytes (%)	0	57.0	± 10.9	60.9	± 9.3	0.288
	5	59.4	± 10.5	63.2	± 8.2	0.265
Granulocytes (%)	0	41.9	± 10.9	38.1	± 9.4	0.299
	5	40.0	± 10.4	35.2	± 8.1	0.155
Haematocrit (l/l)	0	0.32	± 0.07	0.31	± 0.05	0.542
	5	0.30	± 0.05	0.29	± 0.05	0.569

*not significant with P > 0.05

On the day of blood collection for lymphocyte proliferation the live weight was 29.4 ± 3.8, 30.7 ± 3.4 and 29.6 ± 3.0 kg for pigs of control, DON chronic and DON acute groups, respectively (Table 3). After feeding 550 g diet per meal the DON intake was 2.6 µg/kg LW for control pigs and 102 or 106 µg/kg LW for pigs fed DON chronically or acutely. Serum DON concentrations one hour after feeding were significantly different in the control, DON chronic and DON acute groups with 0 ± 0, 43.7 ± 13.3 and 22.7 ± 12.9 ng DON/ml for, respectively (Table 3).

Ex vivo, spontaneous proliferation by nonstimulated lymphocytes was not significantly affected by treatment (Table 3). Pigs fed the DON diet acutely exhibited a significantly lower optical density when lymphocytes were stimulated with ConA than the control group in the MTT assay, but not in the BrdU assay. Although there seemed to be a reduction of absorbance and stimulation index in both the MTT and the BrdU assay, when pigs were fed DON contaminated diet acutely or chronically, the decrease did not reach significance except in the mentioned case (Table 3).

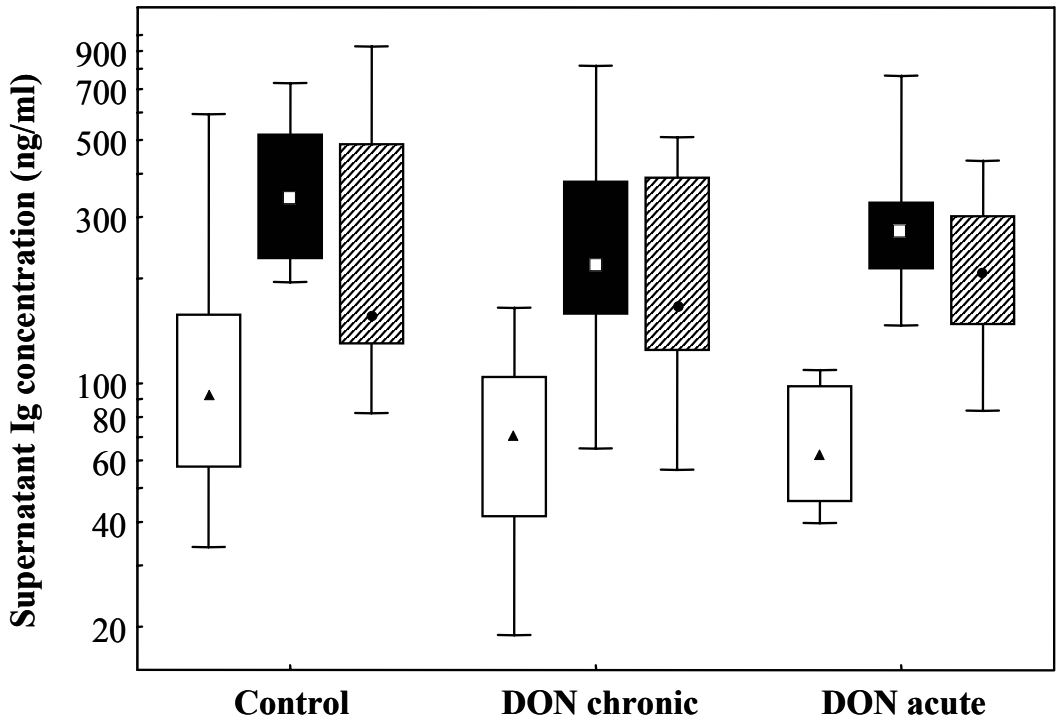

Figure 4 Semilogarithmic plot of IgA (▭), IgM (▬) and IgG (▨) concentrations (ng/ml) in the supernatant after 72 h *in vitro* proliferation of ConA stimulated blood lymphocytes from pigs fed control (n = 11) or DON contaminated diet chronically (n = 13) and acutely (n = 12) (* not significant different from control with P > 0.05; median; box: 25 %, 75 %, whisker: min, max).

Table 3 Absorbance of nonstimulated and ConA-stimulated blood lymphocytes in MTT (570 nm) and BrdU (450-690 nm) assay after feeding 550 g of a control or DON contaminated diet (acute and chronic) to pigs (means ± SD)

				MTT (570 nm)					BrdU (450-690 nm)				
	Live weight	**Serum DON**	**nonstimulated**		**+ 2.5 µg/ml ConA**		**SI**	**nonstimulated**		**+ 2.5 µg/ml ConA**		**SI**
	n	(kg)	(ng/ml)	Absorbance	%	Absorbance	%		Absorbance	%	Absorbance	%	
Control	11	29.4 ±3.8	0.0[c] ± 0.0	0.128 ±0.067	**100**	0.318[a] ±0.060	**100**	3.00 ±1.25	0.031 ±0.026	**100**	0.972 ±0.233	**100**	57.9 ±50.7
DON chronic[1]	13	30.7 ±3.4	43.7[a] ±13.3	0.113 ±0.050	**88**	0.290[ab] ±0.060	**91**	2.78 ±0.73	0.026 ±0.019	**84**	0.854 ±0.321	**88**	51.2 ±40.4
DON acute[2]	12	29.6 ±3.0	22.7[b] ±12.9	0.100 ±0.025	**78**	0.252[b] ±0.040	**79**	2.65 ±0.77	0.027 ±0.015	**90**	0.898 ±0.302	**92**	56.3 ±75.3
Probability		0.598	<0.001	0.424		0.020		0.655	0.833		0.613		0.954

[abc] means with various letters are significantly different within the column ($P < 0.05$)

[1] oral DON exposure for 4 weeks, [2] one single oral dose of DON

Supernatant immunoglobulin concentrations of unstimulated and ConA-stimulated lymphocyte cultures were not significantly influenced by dietary DON exposure (chronically or acutely), but displayed a high inter-individual variation (Figure 4). Stimulation indices after ConA stimulation varied between 1.1 – 4.6, 1.1 – 8.9 and 0.8 – 2.3 for IgA, IgM and IgG secretion, respectively, with no difference between the groups.

Serum IgA concentrations of pigs showed no significant differences between control and the DON (acute and chronic) fed groups, whereas mean IgM and IgG serum concentrations of the DON acute group was significantly increased about 33 and 77 %, respectively (Figure 5). In pigs fed the DON contaminated diet chronically mean serum IgM and IgG values were 23 and 18 % higher, respectively, than in the control group, but did not reach significance.

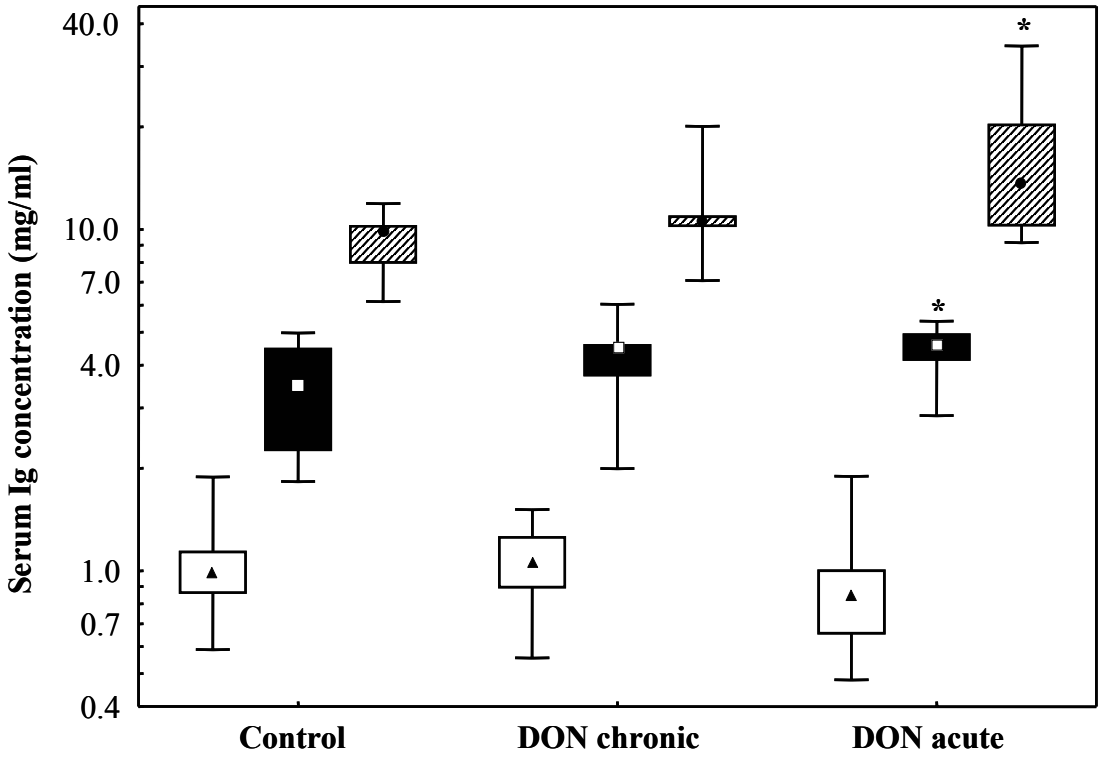

Figure 5 Semilogarithmic plot of serum IgA (▲), IgM (■) and IgG (●) concentrations (mg/ml) of pigs fed control (n = 11) or DON contaminated diet chronically (n = 13) and acutely (n = 12) on the day of lymphocyte proliferation (* significantly [P < 0.05] different from control; median; box: 25 %, 75 %, whisker: min, max).

4 DISCUSSION

In the present study, beside determination of immunoglobulin concentrations in lymphocyte culture supernatants and sera of DON fed pigs, the mitogen-stimulated proliferation response was investigated *in vitro* in porcine lymphocytes pre-exposed to DON for 76 h, and *ex vivo* in pigs fed a DON contaminated diet (5.7 mg/kg) acutely (one single dose) or chronically (exposure for 4 weeks) compared to pigs fed a control diet.

For evaluation of proliferation rate we applied the immunochemical BrdU method which is faster and easier to employ and not as time consuming as the traditionally used radioactive [^{3}H]-thymidine incorporation assay. On the other hand, the MTT assay is widely used for determination of cell viability and proliferation because of its convenience and rapidity (Mosmann 1983).

In the current *in vitro* study, DON concentrations of 309 and 200 ng/ml inhibited proliferation of ConA stimulated porcine PBL by 50 % in the MTT and BrdU assay, respectively (Figure 1). Bovine ConA stimulated PBL exhibited IC_{50} values of 70 ng DON/ml in [^{3}H]thymidine incorporation and 700 ng DON/ml in the MTT cleavage assay (Charoenpornsook et al. 1998), indicating that metabolic disturbances of DON were more obvious in porcine lymphocytes. This is also supported by the findings of Hanelt et al. (1994) who observed a higher sensitivity of porcine kidney cells to DON compared to cell lines of other species. IC_{50} values for the trichothecenes T-2 toxin and DON vary depending on species, mitogen, assay, input of cell counts and protocol, with lymphoid cells and fibroblasts as the most sensitive cell types (Charoenpornsook et al. 1998, Gutleb et al. 2002, Holt et al. 1987, 1988, Reubel et al.1989, Rotter et al. 1993). The influence of trichothecenes on proliferation rate is dependent on the type of trichthecene. Potency of *in vitro* inhibition of rodent and human lymphocyte proliferation decreased in the order of macrocyclic > type A group > type B group trichothecenes (Bondy and Pestka 2000, Visconti et al. 1991). In Swiss mouse 3T3 fibroblasts, the concentration inhibiting 50 % of the DNA synthesis (IC_{50}, BrdU assay) was 444, 4890, 510 and 23300 ng/ml for DON, 3-acetyl-DON, 15-acetyl-DON and de-epoxy-DON, respectively (Eriksen et al. 2004), representing that acetylation decreased immunotoxicity and deepoxidation leads practically to a detoxification of DON. Examination of proliferation of mitogen-stimulated human lymphocytes with various type B trichothecenes showed a rank order of fusarenon X > NIV > DON > 15-acetyl-DON (Forsell and Pestka 1985, Visconti et al. 1991). These structure-activity responses are similar to those observed for protein synthesis inhibition and Rotter et al. (1996) suggested that the translational arrest

is the underlying mechanism for impairment of proliferation. This assumption was underlined by other researchers (Holt et al. 1987, 1988, Minervini et al. 1993) as inhibitory effects of DON and T-2 toxin on cell metabolism (MTT cleavage), DNA ([^{3}H]thymidine) and protein synthesis ([^{3}H]leucin incorporation) were comparable in different cell lines. Inhibition of the DNA synthesis could be explained by the fact that eukaryotic cells depend on newly synthesized protein to enter the S phase of the cell cycle (Feinberg and McLaughlin 1989).

In the present *in vitro* experiment, CHX was applied as an inhibitor of protein synthesis in order to relate the DON effects. IC_{50} values in the present study were at 111.5 and 52.7 ng CHX/ml [0.40 and 0.19 µM] for the MTT and BrdU assay, respectively. This is in accordance with data reported by Minervini et al. (2004), who found a 50 % inhibition in a human erythroleukemia cell line after 48 h of incubation at 0.88 and 0.14 µM [248 and 39 ng/ml] by the MTT and BrdU tests, respectively, suggesting a higher sensitivity of the BrdU assay.

As in the current study saturation at approximately 60 and 100 % were observed at high toxin concentrations (> 200 ng CHX/ml, 560 ng DON/ml) for the MTT and the BrdU assay, respectively, it could be assumed that metabolism is not completely inhibited at concentrations where no further cell division occurred, indicating likewise a higher sensitivity of the DNA synthesis measurement. This is in accordance with Charoenpornsook et al. (1998), who tested the mycotoxin damage (T-2, DON, FB_1 and OTA) on bovine PBL and observed a sensitivity in decreasing order [H^3]-thymidine > LDH > MTT > Trypan blue uptake. Furthermore, the results were confirmed by data reported by Widestrand et al. (1999) who found a higher sensitivity of the BrdU assay compared to the MTT and LDH assay in 3T3 mouse fibroblasts exposed to the mycotoxins T-2, HT-2, DON and NIV. The authors assumed that cytostatic rather than cytotoxic effects of the type B trichothecenes (DON and nivalenol) occur.

Immunoglobulin secretion in porcine lymphocytes was significantly inhibited by DON in a dose-dependent fashion in the present *in vitro* study (Figure 3), with IC_{50} values of 120.6, 84.1 and 71.7 ng DON/ml for IgA, IgM and IgG, respectively. Cytotoxic or cytostatic effects and inhibition of proliferation after DON application appear to be responsible for the decrease of immunoglobulins in the supernatant. IgA secretion of murine lymphocyte cultures (unstimulated or stimulated with LPS or ConA) from Peyer´s Patches (PP) or spleen as well as purified splenic B cells were significantly inhibited after 7 d incubation with 1000 ng DON/ml, whereas IgM and IgG levels were already decreased at 100 ng DON/ml (Warner et al. 1994). This was also observed by Minervini et al. (1993), who reported a 50 % inhibition

(IC_{50}) of IgA and IgM production in LPS-stimulated CH12LX cells, a B cell lymphoma line, at DON concentrations of 115 and 120 ng/ml, respectively. Analogous IC_{50} values were 170, 130, 120 and 110 ng DON/ml for the trypan blue exclusion, MTT assay, [^{3}H]thymidine and [^{3}H]leucine incorporation assay, respectively, indicating that cell proliferation and DNA synthesis which are dependent on protein synthesis, were inhibited at similar dose levels of DON (Minervini et al. 1993). Human PBL were inhibited in cell proliferating and producing IgA, IgM and IgG by four trichothecenes (T-2, DAS, NIV, DON), but at low toxin concentrations the immunoglobulin production was stimulated at levels which inhibited mitogen-induced proliferation by approximately 25 % (Thuvander et al. 1999).

Due to the restricted feeding regimen (where all pigs consumed the same amount of feed) in the present *in vivo* study variability in feed intake (1.1 ± 0.04 kg/d) or live weight gain (364 ± 39 g/d) over a period of 5 weeks could not be observed. Furthermore, chronic dietary DON exposure (5 weeks) did not result in disturbance of haematological parameters in pigs. This is in contrast to Forsell and Pestka (1985) who observed a dose-dependent decrease in total leukocyte count with a concomitant decrease of lymphocytes and monocytes and increase of neutrophil granulocytes after feeding up to 25 ppm DON to mice over 8 weeks. However, haematological parameters appeared not to be a good indicator of immunologic disturbances as many situations such as stress under blood collection might influence the results.

Therefore, it was proved in the current experiment whether feeding a DON contaminated diet (5.7 mg/kg) had an effect on *ex vivo* lymphocyte proliferation. Dietary DON decreased the absorbance and stimulation index of porcine lymphocytes in both the BrdU and MTT assay *ex vivo*, reaching only significance in the MTT cleavage of the DON acute group. Rotter et al. (1994) found a lower incorporation of [^{3}H]thymidine in ConA stimulated PBL of 36 weaned pigs fed 3 mg DON/kg diet, but the DON effect already existed at the beginning of the experiment prior to DON exposure. Dietary exposure of pigs to other trichothecenes such as to 5 mg/kg nivalenol over three weeks (n = 6; Hedman et al. 1997) or 8 mg/kg T-2 toxin for 30 days (n = 9) did not affect lymphocyte blastogenesis (Harvey et al. 1994), whereas Rafai et al. (1995) found a decreased proliferation of stimulated porcine PBL (n = 10) using spectrofluorometric measurement at dietary T-2 toxin concentrations of 2 and 3 mg/kg. T-2 toxin decreased responses of porcine lymphocytes even following topical or inhalative exposure (Pang et al. 1987, 1988).

Immunoglobulin (IgA, IgM and IgG) secretion of porcine PBL cultures in the present *ex vivo* study showed no significant effect after feeding a DON contaminated diet (5.7 mg/kg) to pigs

either chronically or acutely. On the contrary, Pestka et al. (1990) observed increased IgA and IgG supernatant concentrations in cultures of Peyer´s patches lymphocytes after 4, 8 and 12 weeks dietary DON exposure (25 ppm) of mice, whereby the IgA and IgG secretion was highest in ConA stimulated compared to LPS stimulated or unstimulated cultures. However, in spleen lymphocyte cultures, as a surrogate for systemic immunoglobulin production, supernatant concentrations of IgA and IgG were decreased after 4 weeks of dietary DON exposure (Pestka et al. 1990), indicating that immunoglobulin secretion is depending on exposure time and on type of lymphocytes used. Although ConA is described to preferentially stimulate T lymphocytes and might not be an optimal stimulus for B lymphocytes, it could be suggested that ConA-stimulated T cells enhance immunoglobulin secretion by B cells by production of cytokines such as IL-4, 5 and 6 (Pestka et al. 1990).

Dietary DON caused dose-dependent decreases in serum IgM and IgG, in circulating lymphocyte and monocyte numbers, in spleen and thymus weight, but dose-dependent increases in serum IgA in mice (Forsell et al. 1986). This is in contradiction to the present findings, as there was no difference in serum IgA concentrations between the groups (Figure 5), but even a significant elevation of IgM (33 %) and IgG (77 %) in serum of pigs fed the DON contaminated diet once (acute). As reviewed by Pestka (2003), DON exposure to mice was repeatedly linked to the frequently occurring human IgA nephropathie, with elevated IgA concentrations in serum, mesangial deposits and hematuria. With regard to pigs, the effects on serum IgA are rather inconsistent. An IgA elevation in pigs after dietary DON exposure was demonstrated by Drochner et al. (2004), Goyarts et al. (2005) and Swamy et al. (2002), whereas other investigations failed to show interactions between DON and IgA serum concentrations (Bergsjø et al. 1992, 1993, Dänicke et al. 2004a, b, Döll et al. 2003, Swamy et al. 2003). Swamy et al. (2002) observed significantly elevated IgA (103 %) and IgM (39 %) values, but no effect on IgG concentration after feeding starter pigs a DON (5.5 mg/kg) contaminated diet for 21 days. The increased IgM and IgG serum concentrations in the present study are in accordance with Atroshi et al. (1994), who found significantly increased IgA, IgG and IgM values in mice fed a DON contaminated diet (6.25 mg/kg body weight) for one week. Otherwise, the authors observed no significant increase of IgG and IgM values in serum of mice after a single dose of 12.5 mg DON/kg body weight (Atroshi et al. 1994). However, in the current investigation the elevation of IgM and IgG occurred in the DON acute group (serum DON concentration 22.7 ± 12.9 ng/ml), whereas in the chronic DON group (43.7 ± 13.3 ng DON/ml serum) the immunoglobulin values were not significantly affected.

The effects of dietary DON *in vivo/ex vivo* are not in contradiction to the dose-dependent effects of DON on porcine PBL cultures in the present *in vitro* study. First of all, the significant effect of acute dietary DON in the MTT assay, although the BrdU assay was found to be more sensitive in the *in vitro* study, might be a result of a lower variance within the MTT assay because of fewer working steps. Furthermore, it has not to be neglected that inter-individual variation was excluded in the *in vitro* study as lymphocytes from only one pig were used for all cultures.

The difference in the immunoglobulin secretion *in vitro* (dose-dependent inhibition) and the increased serum IgM and IgG concentrations after dietary DON exposure might be due to the lower serum concentration of between 10 and 63 ng DON/ml in contrast to 70 ng/ml as the lowest DON concentration of the *in vitro* study. This suggestion would be underlined by Minervini et al. (1993) who observed *in vitro* a slightly stimulated IgA secretion between 5 – 50 ng DON/ml, whereas higher DON concentrations caused an inhibition. To the author's knowledge this is the first report of DON affecting the IgM and IgG secretion in porcine lymphocyte cultures (inhibition, 70 – 560 ng DON/ml) and serum of pigs (stimulation, 10 – 63 ng/ml) more than IgA production which is in contrast to other studies with rodents (Pestka et al. 1990, Pestka 2003). However, the question why DON caused in pigs diverse immunoglobulin disturbances than in mice could not be answered and should be part of further research.

In conclusion, the proliferation of porcine peripheral blood lymphocytes is an appropriate method to measure the effects of DON *in vitro*, whereby the BrdU assay (DNA synthesis) showed a higher sensitivity than the MTT cleavage (metabolic activity) in the present study. The assumption that inhibition of proliferation could be mainly attributed to the inhibition of protein synthesis was supported by the strong inhibiting effects of the known protein synthesis inhibitor CHX on proliferation of porcine PBL. The results of the present *in vivo* study indicated a more pronounced disturbance in the immunologic parameters examined (MTT assay, IgG and IgM) for the pigs fed a DON contaminated meal once (acute group) than the DON chronic group, although the serum DON concentrations one hour after feeding were lower. Further research is necessary to examine the effect of exposure time (acute and chronic) and a possibly adaptation to DON.

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PAPER IV

EFFECTS OF THE *FUSARIUM* TOXIN DEOXYNIVALENOL (DON) FROM NATURALLY CONTAMINATED WHEAT ON THE *IN VIVO* PROTEIN SYNTHESIS OF PERIPHERAL BLOOD LYMPHOCYTES AND PLASMA PROTEINS IN THE PIG

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ABSTRACT

Trichothecenes, such as deoxynivalenol (DON), are known to inhibit the protein synthesis *in vitro* by binding at the 60S subunit of eukaryotic ribosomes. Therefore, cells and tissues with high protein turnover, such as lymphocytes and the liver (albumin & fibrinogen synthesis), were suggested to react most sensitively to DON. However, to the author's knowledge this observation was not proven *in vivo* in pigs, which were regarded as the farm animals most susceptible to DON.

A total of 31 castrated male, crossbred German Landrace x Pietrain pigs weighing approx. 40 kg were fed a DON contaminated diet (5.7 mg/kg) either acutely (one single dose) or chronically (≥ 4 weeks) or a control diet (0.1 mg/kg). In addition, one group received an intravenous injection of 53 µg DON/kg LW. One hour after feeding, a "flooding dose" of the stable isotope L-[$^2H_5$]-phenylalanine (125 mg/kg LW) was given and frequent blood samples (permanent catheter) were collected over a 60 min period. The molar percent excess (MPE) of plasma free and protein-bound phenylalanine were measured by GC/MS.

No differences could be observed in the plasma concentrations of total protein, albumin, fibrinogen and serum enzymes between the groups. On the other hand, fractional synthesis rates (FSR, %/d) of albumin were significantly decreased by 32, 45 and 26 % and FSR of lymphocytes declined by 27, 19 and 24 %, whereas fibrinogen was not significantly affected after chronic or acute oral and intravenous DON exposure, respectively. Additionally, the absolute synthesis rate (ASR, g/d) of albumin and the proportion of albumin to total body protein synthesis were reduced in the same manner, whereas the albumin secretion time ranged between 6.8 – 34.4 min and was not affected by treatment. In conclusion, the flooding dose technique appeared to be suitable for distinguishing DON-related effects on the protein synthesis, while determination of plasma protein concentrations seemed not to be an appropriate parameter.

Keywords: deoxynivalenol, L-[$^2H_5$]-phenylalanine, intravenous application, *in vivo*, oral exposure, pig, protein synthesis

1 INTRODUCTION

In northern temperate regions, the *Fusarium* toxin deoxynivalenol (DON) is one of the most frequent contaminant occurring in toxicologically relevant concentrations in wheat and other cereal grains (Bottalico and Perrone 2002, Logrieco et al. 2002, Oldenburg et al. 2000). Previous investigations considered pigs as the most susceptible farm animals, because of emesis after ingestion of high DON doses (Young et al. 1983). Besides these rarely occurring acute intoxications, the chronic DON exposure causes enormous economical losses in animal livestock due to reduced feed intakes and live weight gains, as well as disturbances in immunological function (Bondy and Pestka 2000, Rotter et al. 1996). The primary mode of action of the trichothecenes, such as deoxynivalenol, is the inhibition of the protein synthesis at the level of translation. Here, the epoxide-group of the trichothecenes binds at the 60S subunit of eukaryotic ribosomes and impairs the initiation or the elongation of the peptide chain by interfering with the peptidyl transferase (Feinberg and McLaughlin 1989). Therefore it is likely that rapidly dividing cells, e.g., lymphocytes, as well as tissues with a high protein turnover rate, such as the liver, respond particularly sensitively to the action of trichothecenes.

Up to now, only few studies have investigated the effect of DON on protein synthesis *in vivo* just measuring the radioactivity of rodent tissues after [^{14}C]leucine gavage (Azcona-Olivera et al. 1995a, Robbana-Barnat et al. 1987).

Growth, aging, nutrition, hormones, injury and diseases can affect protein synthesis in individual tissues, while having little effect on other tissues. Therefore, it is important to measure the effect on specific proteins rather than on the body as a whole to understand the mechanisms by which protein metabolism is altered during such physiopathological situations (Guillet et al. 2004). The liver synthesizes both exported and constitutive proteins. As blood is the most easily accessible tissue, many investigations dealt with the exported plasma proteins, such as albumin and fibrinogen. Since albumin is the most abundant blood protein, and exclusively synthesized by the liver, albumin concentrations are used as indicator of nutritional status and/or liver disturbances (Ballmer et al. 1995a). Albumin represents approximately 50 % of the liver proteins produced and its synthesis amounts to 11 - 18 % of total liver protein synthesis in well-fed animals (Guillet et al. 2004). However, it has to be emphasized that the measurement of the plasma concentration only represents the status of one moment and is unlikely to reflect the dynamic processes, because the vascular albumin concentration results not only from synthesis, but also from degradation, distribution (plasma

volume) and elimination (Ballmer et al. 1990, Dänicke et al. 2005b). Furthermore, it has to be noticed that albumin has a long biological half-life of ~ 3 weeks and that the transcapillary escape rate may be varied (increased) by different clinical conditions, such as acute and chronic diseases, sepsis, surgical trauma, diabetes mellitus or high blood pressure (Ballmer et al. 1995a). Besides a direct effect of DON on plasma protein synthesis, an indirect effect could be suggested as a DON-induced deregulation of the cytokine secretion of T-lymphocytes. For instance, an increase of interleukin 6 (IL-6) would mediate an acute-phase response. This is characterized by an enhanced concentration of positive acute-phase proteins such as fibrinogen and a decrease of negative acute-phase reactants, mainly albumin (Baumann and Gauldie 1994, Heinrich et al. 1990, Jia and Pestka 2005, Kinser et al. 2005, Mackenzie et al. 2003).

In the same way, the immunological status is usually estimated indirectly, either on the basis of blood cell counts, as well as cell populations and subpopulations, or on the basis of *in vitro* proliferative responses (Walrand et al. 2001, 2004). However, a large inter-individual variation in response to mitogenic stimulation and poor reproducibility are well-known drawbacks of *in vitro* methods (Januszkiewicz et al. 2001b, 2002, Smith et al., 1988). Accordingly, it was shown that dietary DON decreased the proliferation of ConA-stimulated porcine blood lymphocytes *ex vivo* (Goyarts et al. 2005b), but whether these observations reflect *in vivo* changes in lymphocyte protein metabolism remains unclear.

Alternatively to the measurement of the concentration and/or cell counts, the *in vivo* protein synthesis of plasma proteins and lymphocytes can be measured by the application of stable isotopes, such as L-[$^2H_5$]-phenylalanine (Ballmer et al. 1996, Dänicke et al. 2005b, Imoberdorf et al. 2001, Park et al. 1994, Walrand et al. 2001). The tracer may be given either alone in a constant infusion or with a significant amount of tracee as a bolus ('flooding dose') (Garlick et al. 1994). The aim of a flooding dose of an amino acid is to produce a constant isotopic enrichment of the free amino acid in plasma compared to that in the tissue. Under that condition, the measurements of the precursor enrichment can conveniently be made on plasma (Ballmer et al. 1990). In contrast to muscle, the turnover rate of liver proteins is rapid and 30 – 50 % of protein synthesized in the liver appears in the blood circulation within 30 min (Guillet et al. 2004). On this account, the flooding dose is the method of choice when exported proteins and high turnover proteins are issues, since the recycling of labeled amino acids from intracellular protein breakdown could be neglected because of the short labeling

period (Papet et al. 2002, Simon 1989). Furthermore, recent data has shown that albumin synthesis measured in plasma is a good indicator of liver albumin synthesis (Ruot et al. 2000).

The objective of the present study was to investigate the effect of the mycotoxin deoxynivalenol on *in vivo* protein synthesis in pigs. Moreover, it was studied if the type of DON exposure affects the protein synthesis *in vivo*. Therefore, pigs were exposed to a DON contaminated diet either chronically (≥ 4 weeks) or once (acute oral exposure) to simulate two distinct DON exposure scenarios which might occur under practical feeding conditions. In addition, these scenarios were compared to an intravenous application of pure DON which has been recently used for studying DON-related effects. Since protein synthesis rates are influenced by feed and protein intake level (de Feo et al. 1992), the feed intake of the present study was standardized on an equal level for all pigs by a restrictive feeding regimen.

2 MATERIAL AND METHODS

2.1 Chemicals and reagents

Deoxynivalenol (DON), diethyl ether, acetonitrile, sodium citrate, ammonium sulphate, L-tyrosin decarboxylase, pyridoxal 5-phosphate, cycloheximide (CHX), RPMI-1640 medium (Roswell Park Memorial Institute medium), histopaque 1077, 5-sulfosalicylic acid dehydrate (SSA), N-(tert-butyl-dimethyl-silyl)-N-methyl-trifluoro-acetamide (MTBSTFA), and L-phenylalanine were obtained from Sigma-Chemie, Deisenhofen, Germany.

Ammonia solution, ammonium chloride, sodium chloride, sodium hydroxide (NaOH), perchloric acid (PCA), trichloroacetic acid (TCA), absolute ethanol (99.5 %) and phenol crystals were purchased by Merck, Darmstadt, Germany. Additionally, the cation exchange resin AG 50W-X8 (H^+ form, 100-200 mesh, Bio-Rad Laboratories GmbH, München, Germany), TRIS (Roth, Karlsruhe, Germany) and hydrochloric acid (HCl, AppliChem GmbH, Darmstadt, Germany) were used. Sodium heparin and isotonic sodium chloride solution (0.9 %) were purchased by B. Braun, Melsungen, Germany. L-[$^2H_5$]-phenylalanine (98 %) was supplied by Euriso-top (Bât Homère, Saint Aubin, France) and Chemotrade (Leipzig, Germany).

Crystalline DON was dissolved in isotonic sodium chloride solution (0.2 mg/ml) for intravenous application. 3 g L-[$^2H_5$]-phenylalanine and 7 g L-phenylalanine were dissolved

under steady agitation and slight warming in 400 ml isotonic sodium chloride (150 mmol/l, 29 molar percent excess (MPE)) and sterilised using syringe filters before intravenous injection.

For lyses of red blood cells in the lymphocyte preparation, 9 parts ammonium chloride (8.3 g/L) and 1 part TRIS buffer (20.594 g/l double distilled water, pH 7.65) were used.

2.2 Experimental diets

The control diet was formulated to meet or exceed all nutritional requirements for fattening pigs as recommended by the German Society of Nutrition Physiology (GfE 1987), with a total wheat content of 400 g/kg (Table 1). The composition of both diets was the same, with exception of the wheat, which was replaced by a naturally *Fusarium* infected and mainly DON contaminated wheat. The wheat batches did not differ substantially in nutrient composition, but only in mycotoxin concentrations (Goyarts et al. 2005). Therefore, the experimental diets differed only in mycotoxin concentrations. The DON concentration of the contaminated diet was 5.7 ± 0.3 mg/kg, whereas the control diet contained 0.14 ± 0.06 mg DON per kg (Table 1).

Table 1 Composition of the experimental diets (g/kg, based on a dry matter content of 88 %)

	Control diet	**DON contaminated diet**
Ingredients		
Barley	348	348
Wheat	400	0
Contaminated wheat*	0	400
Soybean meal	200	200
Soybean oil	20	20
Dicalcium-phosphate	3.5	3.5
Sodium chloride	1	1
L-lysine-HCl	2.5	2.5
Premix[1]	25	25
Analysed Composition		
Deoxynivalenol (mg/kg)	0.14 ± 0.06	5.7 ± 0.3

[1] Provided per kg of diet: Ca 6.1 g; P 1.5 g; Na 1.4 g; Mg 0.3 g; Fe 100 mg; Cu 25 mg; Mn 50 mg; Zn 100 mg; I 1.3 mg; Se 0.4 mg; Co 0.5 mg; vitamin A 10000 IU; vitamin D_3 1000 IU; vitamin E 30 mg; vitamin B_1 18.8 µg; vitamin K_3 1.3 mg; nicotinic acid 12.5 mg; pantothenic acid 8.4 mg; choline chloride 125 mg.

* Mycotoxin composition (µg/kg wheat): deoxynivalenol 16643, zearalenone 114, 15-acetyl-deoxynivalenol 101, 3-acetyl-deoxynivalenol 41, nivalenol 41, scirpentriol 20, HT-2 toxin 11.

2.3 Surgery

31 castrated male pigs (crossbred German Landrace x Pietrain) with an initial live weight (LW) of 23.6 ± 2.4 kg were individually housed and fed control or DON contaminated diets for at least four weeks until weighing more than 30 kg. Following an adaptation to metabolism cages (for facilitated blood sampling), the surgery was performed under sterile conditions. After anaesthesia with azaperon, ketamin (im) and thiopental (iv) sterile human *vena subclavia* catheters (1.5 x 2 mm, 50 cm, Vygon, No. 9153.8220) were used for cannulation of the external *vena jugularis* to facilitate the frequent blood collection over 60-90 min. The catheter was tunnelled subcutaneously and exteriorized dorsally between the shoulders. The cranial portion of the jugular vein was tied off to control haemorrhage. Sodium heparin dissolved in isotonic sodium chloride solution (1 ml/l) was used to maintain patency. The return to normal feed intake and activity levels were used as indices for recovery from surgery, so that the measurement of protein synthesis could be conducted on the second day.

2.4 Protein synthesis studies

The experimental design and number of pigs per treatment are described in Table 2 and Figure 1.

Table 2 Design and number of pigs of the in vivo protein synthesis studies

GROUP	DON application		N	Live weight [kg]	
	Duration	Form			
Control	-	-	7	38.4	± 3.0
DON chronic	4-6 weeks	Wheat	10	39.3	± 3.1
DON acute oral	Single bolus	Wheat	7	37.4	± 1.8
DON acute iv	Single bolus	crystalline	7	38.5	± 1.5

Following chronic (≥ 4 weeks) dietary DON exposure to 5.7 mg DON/kg feed *in vivo* protein synthesis was examined on 10 pigs weighing 39.3 ± 3.1 kg. On the day of sampling, 21 control pigs were divided in three groups: 1) Seven pigs with a mean weight of 37.4 ± 1.8 kg were once fed the DON contaminated diet (DON acute oral), 2) Seven pigs (38.5 ± 1.5 kg LW) were fed the control diet (0.14 mg DON/kg) and were intravenously administered 53 µg pure DON/kg LW one hour thereafter (DON acute iv), and 3) the remaining seven pigs (38.4 ± 3.0 kg) served as unexposed control group. The intravenous DON dose was based on the DON ingestion of the first chronically fed pigs.

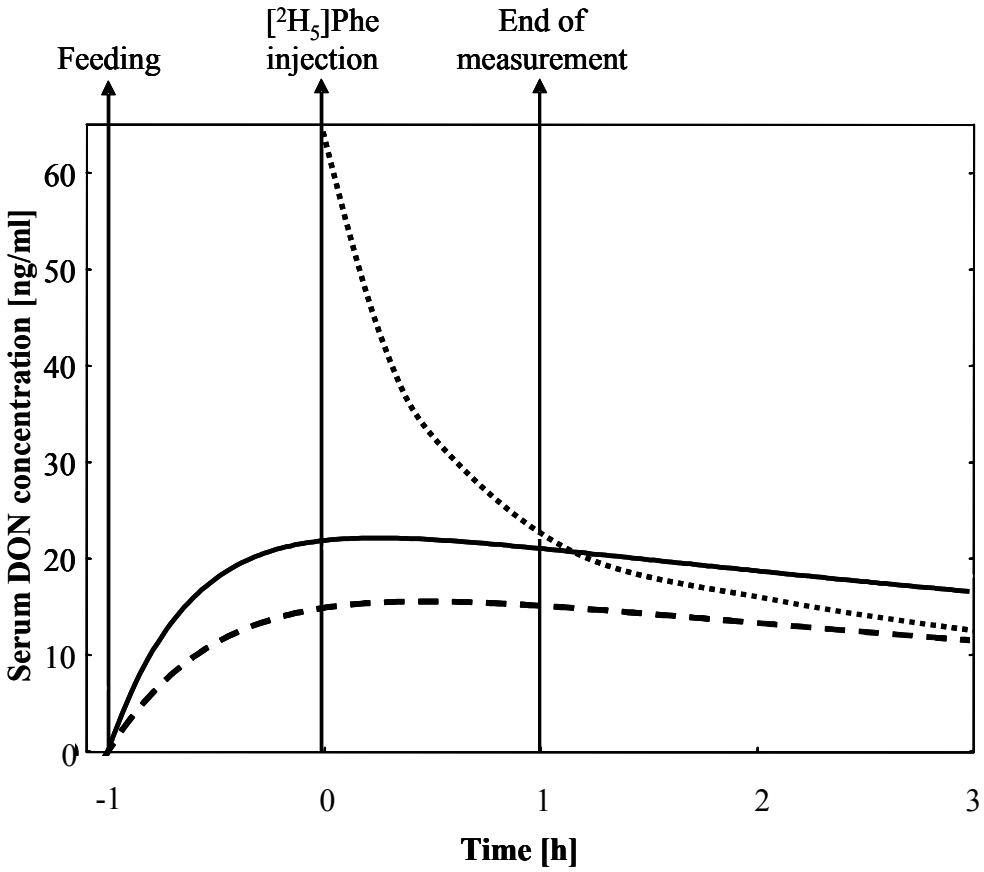

Figure 1 Study design in relation to the time-mean serum DON concentration course of pigs after intravenous (.....; 53 µg DON/kg LW) or oral DON exposure (5.7 mg/kg diet) either chronically (——) or acutely (– – –) according to Goyarts and Dänicke (2006).

One hour after feeding, and directly followed by the intravenous DON administration, 5 ml/kg (= 125 mg/kg LW) of the L-[$^2H_5$]-phenylalanine solution (150 mmol/l, 29 MPE) was injected intravenously (< 3 min). This dose (125 mg/kg LW) was proven to give an adequate enrichment of the plasma precursor pool (Dänicke et al. 2005).

For albumin and fibrinogen synthesis determination, heparinized and citrated blood samples were drawn prior to feeding and after 30, 45, and 60 min. Blood samples were centrifuged (3000 rpm for 15 min at 15 °C) to separate the plasma, and stored until analyses at -70 °C. Additionally, after 60 min, blood was sampled in a heparinized tube containing cycloheximide to prevent further phenylalanine incorporation during cell separation (Park et al. 1994), for analyses of the lymphocyte protein synthesis.

Treatments and experiments were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the

Regional Council of Braunschweig, Lower Saxony, Germany (File number 509.42502/09-02.02).

2.5 Analyses

2.5.1 Determination of DON

The experimental diets were analysed for DON by high performance liquid chromatography (HPLC) with diode array detection (DAD) after clean-up with immuno-affinity columns (IAC, VICAM, Watertown, MA, USA) according to a modified VDLUFA-method (Valenta et al. 2002). The determination of plasma DON is described by Valenta et al. (2003). In brief, after addition of one ml of sodium acetate buffer (pH 5.5) to 1.5 ml of plasma, the mixture was incubated for 16 hours with 6,000 U β-glucuronidase (Sigma, G 0876). Plasma samples were extracted with ethyl acetate on a ChemElut cartridge (Varian, Middelburg, Netherlands), cleaned up by IAC (VICAM) and measured by HPLC-DAD. The detection limits of DON in diets and plasma were 0.03 mg/kg diet and 4 ng/ml plasma, respectively, and the recovery was approximately 90 %. The results of mycotoxin analysis were not corrected for recovery.

2.5.2 Determination of protein synthesis

a) Standards

For determination of free phenylalanine (high enrichment of phenylalanine), a series of nine standards, spanning the range of enrichment from 0 to 24 MPE, were prepared from labeled (103 mg L-[$^2H_5$]-phenylalanine, 98 molar percent, in 10 ml 0.1 n HCl) and unlabeled (10 g L-phenylalanine, 0.015 molar percent, in 1000 ml 0.1 n HCl) stock solutions. Nine standards for GC/MS analysis of albumin, fibrinogen and protein of lymphocytes (low enrichment of L-[$^2H_5$]-phenylalanine) ranging between 0 – 0.1 MPE were obtained by mixing diluted L-[$^2H_5$]-phenylalanine solution (1.1 ml labeled phenylalanine solution in 100 ml unlabeled stock solution (1.0811 molar percent) and unlabeled stock solution. Fifteen µl of each standard was dried and derivatisated as described below.

b) Plasma sample preparation: free phenylalanine and albumin

The sample preparation for plasma free and albumin-bound phenylalanine was described previously (Dänicke et al. 2005). One ml heparinized plasma was mixed with 10 ml ice-cold TCA (12 %), left on ice for 10 min and centrifuged for 15 min at 3500 rpm and 4 °C. The supernatant was used to analyze the free phenylalanine enrichment in plasma as an indicator for the albumin and fibrinogen synthesis precursor pool. Amino acids were isolated from the

supernatant by using a cation exchange resin (AG 50W-X8). The loaded amino acids were washed with 4 ml distilled water, and then eluted by 2 ml 6 n ammonia solution followed by 1 ml of doubly distilled water. The eluate was evaporated to dryness under a stream of nitrogen at 40 °C. Thereafter free phenylalanine was dervatisated as described below.

For determination of the albumin synthesis, the pellet was re-dissolved in ice-cold TCA (12 %) and centrifuged again for 20 min at 4000 rpm and 4 °C. Thereafter, the pellet was stirred to a creamy mass and 10 ml non-denatured absolute ethanol (99.5 %) was added, mixed and incubated for 15 min at room temperature (RT). Following centrifugation (20 min, 4000 rpm, RT), three ml of the supernatant were used to determine the purity of albumin via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 10 %) as described by Dänicke et al. (2005), while the remaining 7 ml supernatant were dried under nitrogen stream (40 °C). After addition of 6 ml 0.3 n NaOH and incubation for 20 min in a water bath (37 °C), 2 ml ice-cold PCA (20 %) were added, mixed and left on ice for 5 min. The sample was washed with 6 ml PCA (2 %) two times (centrifugation 20 min, 3500 rpm, RT). To the pellet 500 µl 0.3 n NaOH was added and it was transferred with 6 ml 4 n HCl in a Pyrex vial containing 2-3 phenol crystals. Then it was hydrolyzed in the drying oven for 18 h at 110 °C. Three ml of the hydrolysate were dried in the drying oven with air circulation (60 °C) and washed with 5 ml doubly distilled water and dried again. The further preparation is described below.

c) Plasma sample preparation: fibrinogen

Fibrinogen was isolated from plasma according to the method of Takeda (1966) with some modifications. For the isolation of plasma fibrinogen, 3 ml citrate plasma was mixed with 3 ml sodium citrate (0.09 n) and centrifuged for 10 min at 3000 rpm and RT. The supernatant was precipitated with 4 ml 4 n ammonium sulphate ($(NH_4)_2SO_4$) and centrifuged (10 min, 4000 rpm, RT). Ten ml of 1 n $(NH_4)_2SO_4$ were mixed with the pellet (ultrasonic bath) and centrifuged as described above. The pellet was dissolved in 3 ml 0.005 n sodium citrate (warming in water bath 38 °C) and again precipitated with 5 ml 4 n $(NH_4)_2SO_4$ (leaving 10 min at RT) and centrifugation (10 min, 4000 rpm, RT). The pellet was then washed with 6 ml 1 n $(NH_4)_2SO_4$ (centrifugation) and thereafter dissolved in 3 ml 0.005 n sodium citrate (water bath, 38 °C). Fifty µl were removed to determine the purity of the isolated fibrinogen by SDS-PAGE. Four ml of ice-cold PCA (2 %) were added to the remaining sample and were left on ice for 10 min before centrifugation (15 min, 4000 rpm, 4 °C). The pellet was hydrolyzed as described above.

d) Plasma sample preparation: lymphocytes

For determination of the lymphocyte protein synthesis, the blood sample (tube with heparin and CHX) was diluted 1:1 with RPMI medium (or isotonic saline). 5 ml of this diluted sample was layered on 5 ml Histopaque (1.083 kg/l) in each of the 8 tubes before centrifugation (15 min, 2100 rpm, RT, without break and acceleration). The 'buffy coats' (PBMC, mononuclear cells consisting of more than 95 % lymphocytes) from each pig were transferred in one new spun tube and ice-cold saline was added up to 10 ml. After centrifugation (15 min, 3100 rpm, 4 °C) the pellet was washed three times in 4 ml ice-cold lysis buffer (9 parts ammonium chloride (8.3 g/l) and 1 part TRIS buffer (20.594 g/l)). The pellet was resuspended in 5 ml doubly distilled water and could be frozen at -20 °C.

To the defrosted sample 0.8 ml of 48 % (w/v) SSA was added, kept on ice for 10 min and then centrifuged (20 min, 3100 rpm, 4 °C). The protein pellet was washed with 8 ml SSA (8 %) two times and was thereafter hydrolyzed as described above.

e) Decarboxylation and derivatisation of standards and hydrolysates for GC/MS analysis

For low enrichment of protein-bound phenylalanine (albumin, fibrinogen, lymphocytes, standards) a conversion to β-phenylethylamine was conducted to improve chromatographic separation (Calder et al. 1992), whereas decarboxylation was not required for plasma free phenylalanine because of its relatively high enrichment. In brief, after drying of the samples or 15 µl of the standards, the residues were dissolved in 1 ml 0.5 n citrate buffer (pH 6.3), and 200 µl enzyme solution (4 ml sodium citrate buffer (0.5 n, pH 6.3) + 40 mg (= 14 U) L-tyrosin decarboxylase + 5 mg pyridoxal 5-phosphate) was added, vortex-mixed and incubated for 15 h in a water bath of 50 °C. Following addition of 100 µl 6 n NaOH, the vial was centrifuged for 10 min at 6000 rpm. The supernatant was extracted with 400 µl diethyl ether and the ether fraction was mixed with 100 µl 0.1n HCl and taken to dryness under a stream of nitrogen (40 °C). Samples were converted to the tertiary-butyldimethylsilyl (t-BDMS) derivates according to Slater et al. (1995) by addition of 200 µl of a 1:1 mixture of MTBSTFA and acetonitrile and incubation for 1 h at 60 °C in a heating block.

f) GC/MS analysis of L-[$^2H_5$]-phenylalanine and β-phenylethylamine

L-[$^2H_5$]-phenylalanine (plasma free phenylalanine) and β-phenylethylamine (protein-bound phenylalanine from albumin, fibrinogen and lymphocytes; standards) were measured as their t-BDMS derivates in a GC/MS system consisting of the autosampler A200S (CE Instruments), the GC 8000 (Fisons Instruments) and the quadrupole mass spectrometer MD

800 (Fisons Instruments) as described by Dänicke et al. (2001). In brief, ratios between the peak areas at m/z 239 and m/z 234 were used for calculation of MPE of plasma free phenylalanine, whereas the peak areas at m/z 183 and m/z 180 of samples and standards were recorded in the selected ion recording mode under electron ionization conditions (Dänicke et al. 2001, 2005).

2.5.3 Other analytical procedures

Total plasma protein and albumin concentrations were analysed using the biurette method and a colorimetric test with bromcresol green, respectively. Plasma fibrinogen was determined by a gravimetric method according to de Maat et al. (1999). In brief, 1 ml citrated plasma was diluted with 2 ml phosphate buffer (pH 6.35) and rapidly mixed with 50 µl thrombin solution (= 25 IU) and incubated at RT for 2 h. The clot was blotted to dryness with filter paper and was washed twice with 0.15 mol/l NaCl solution and thereafter in doubly distilled water for 30 min, blotting dry between each washing. The gravimetric determination was carried out following drying for 2 h at 100 °C in the drying oven.

Plasma glucose concentrations and activities of alkaline phosphatase (AP), aspartate aminotranferase (ASAT), glutamate dehydrogenase (GLDH), γ-glutamyltransferase (γ-GT), and creatinine kinase (CK) were measured by enzymatic UV-standard procedures (Clinic for Swine and Small Ruminants, Veterinary School, Hanover, Germany).

2.6 Calculations and statistics

The fractional synthesis rate (FSR) of albumin and fibrinogen is defined as the percentage of the intravascular albumin/fibrinogen pool synthesized per day (%/d) and was calculated from the ratio between the time-related linear increase in albumin/fibrinogen L-[$^2H_5$]-phenylalanine enrichment and the corresponding area under the MPE-time curve of the precursor (free plasma phenylalanine) times 100 (Ballmer et al. 1990):

$$\text{FSR} = \frac{(P_2 - P_1)}{\text{AUC}} \cdot 100 \quad (1)$$

where FSR = fractional synthesis rate (%/d), P_1, P_2 = enrichment at t_1 and t_2, respectively and AUC = area under the MPE-time curve of the precursor (plasma free phenylalanine).

The absolute synthesis rate (ASR) is the amount of protein synthesized each day expressed as g/d or as mg/kg LW/d and was calculated from the FSR multiplied by the intravascular

albumin/fibrinogen mass, which was estimated by the mean plasma albumin/fibrinogen concentration and the estimated plasma volume.

(2) $$ASR = \frac{PV \cdot C \cdot FSR}{100}$$

where ASR = absolute synthesis rate (g/d), PV = plasma volume (PV (l) = 1.06 + 0.037 * LW (kg) according to Yang and Lin 1997), C = plasma concentration (g/l) and FSR = fractional synthesis rate (%/d).

The secretion time (t_s) is estimated from the extrapolated intercept on the x-axis.

Data are expressed as means ± standard deviation (SD) or range. Statistical comparisons between the groups were performed by analysis of variance (ANOVA) and Fisher LSD test. Data which were not normally distributed were subjected to the nonparametric Kruskal-Wallis-test.

3 RESULTS

3.1 Clinical signs

Emesis (4 – 9 times) was observed in all of the seven pigs dosed intravenously with 53 µg DON/kg LW within 7 to 10 min after application. No further emesis occurred after 30 min, indicating that DON has no long-lasting emetic effect. Between 5.5 – 31.0 % of the dry matter intake was regurgitated with the vomit. In orally dosed pigs no signs of acute toxicity, like retching and vomiting, were observed.

3.2 Clinical-chemical parameters and DON concentration

Plasma DON concentrations were significantly affected by the treatment ($P < 0.001$, Table 3): 90 min after feeding, which corresponds to 30 min after the flooding dose (Figure 1), oral exposure to a DON contaminated diet (5.7 mg/kg) given chronically or acutely resulted in a significantly increased serum DON concentration (1.9 versus 20.5 and 18.7 ng/ml). Pigs dosed intravenously with 53 µg DON/kg LW showed the highest mean serum DON concentration of 44.6 ± 4.0 ng/ml.

Table 3 Clinical-chemical parameters and deoxynivalenol (DON) concentration in plasma of pigs after oral (5.7 mg/kg diet, chronically and acutely) or intravenous (53 µg/kg LW) DON exposure (mean, range)

GROUP	N	DON	Total Protein	CK	ASAT	GLDH	γ-GT	AP	Glucose
		ng/ml	g/l	U/l	U/l	U/l	U/l	U/l	mmol/l
Control	7	1.9[c]	57.6	448.7	15.3	0.53	24.9	167.3	3.65
		(0-7)	*(50.6-64.4)*	*(165-1371)*	*(5.9-28.1)*	*(0-2.2)*	*(15.1-33.5)*	*(74-221)*	*(2.53-4.53)*
DON chronic	10	20.5[b]	55.7	503.4	17.1	2.32	26.9	154.8	3.61
		(12-28)	*(48.0-59.1)*	*(117-950)*	*(3.5-42.7)*	*(0-5.9)*	*(13.7-42.1)*	*(58-209)*	*(2.47-5.35)*
DON acute oral	7	18.7[b]	59.7	620.4	30.0	1.96	27.7	164.6	3.82
		(13-26)	*(57.8-61.8)*	*(224-2528)*	*(10.1-94.0)*	*(0-5.9)*	*(15.9-49.9)*	*(134-208)*	*(1.88-5.08)*
DON acute iv	7	44.6[a]	59.2	436.7	14.3	1.47	28.2	196.1	3.37
		(39-51)	*(49.7-64.4)*	*(82-1097)*	*(9.6-20.9)*	*(0-3.6)*	*(21.5-38.2)*	*(165-254)*	*(2.34-4.63)*
Probability									
Group		<0.001	0.187	0.905	0.388	0.248	0.837	0.281	0.997

[abc] data with distinct superscript are significantly different within a column ($p<0.05$, Kruskal-Wallis-test), CK = creatinine kinase (≤ 2000), ASAT = aspartate aminotransferase (≤ 35), GLDH = glutamate dehydrogenase (≤ 4), γ-GT = γ-glutamyl transferase (≤ 26), AP = alkaline phosphatase (≤ 170), Glucose (4 – 6.4)

Clinical-chemical parameters were not significantly affected by the treatment (Table 3), but one clinically inconspicuous pig of the DON acute oral group showed elevated values of creatinine kinase (CK, 2528 U/l) and aspartate aminotransferase (ASAT, 94 U/l), indicating a muscle trauma possibly due to the surgical treatment. Omitting this animal, the activity of ASAT was positively correlated with GLDH (r = 0.57) and γ-GT (r = 0.57), but inversely correlated with plasma albumin and fibrinogen concentrations (r = -0.52 and -0.49) as well as their mass (r = -0.55 and -0.50).

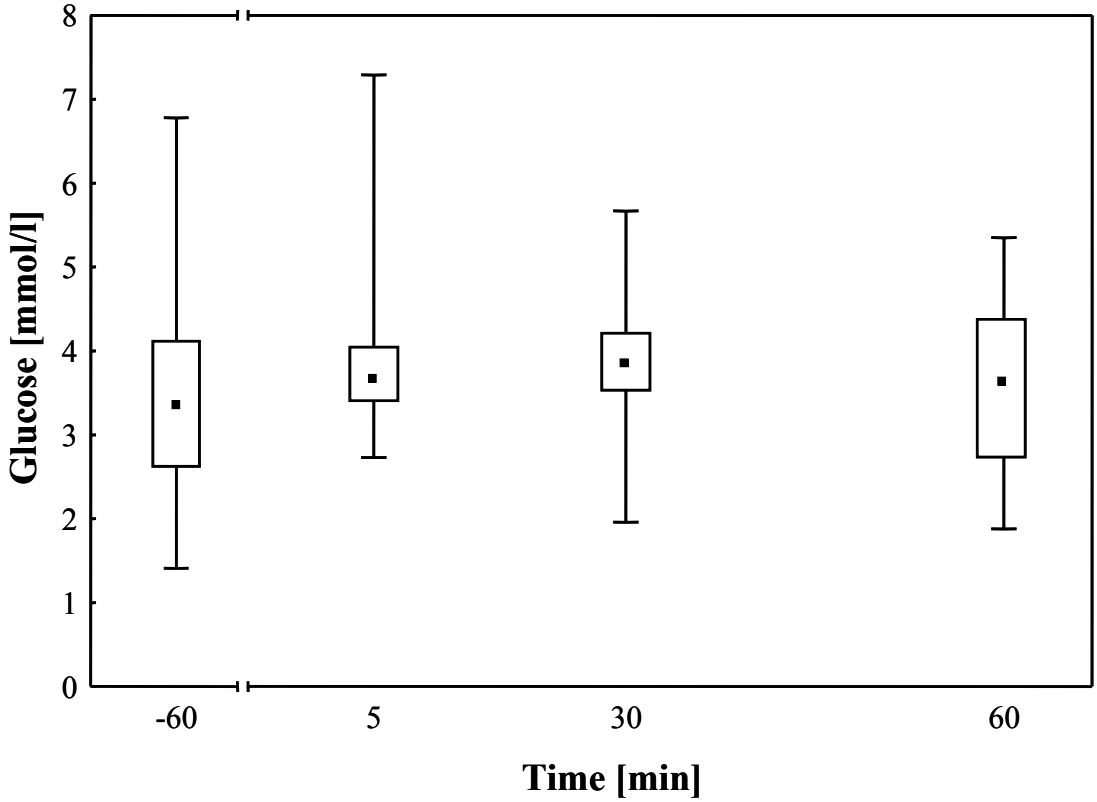

Figure 2 Plasma glucose concentrations [mmol/l; median, box: 25%, 75%, whisker: min, max] of pigs before feeding (-60 min) and 5, 30 and 60 min after receiving a flooding dose of L-[$^2H_5$]-phenylalanine.

In all groups of the present study the AP plasma activities exceed the reference value of 170 U/l. Furthermore, the sensitive, liver-specific and mitochondrially-bound glutamate dehydrogenase (GLDH) was higher than the reference value of 4 U/l in four chronically and one acutely DON fed pigs with a concomitant increase of the liver specific, cytosolic located, but slower reacting γ-glutamyl transferase (γ-GT), whereas the control and intravenously dosed groups showed only some negligible elevation of the γ-GT. The plasma glucose

concentration showed no significant group- or time-dependent differences (Figure 2) and was inversely correlated with the liver enzymes (ASAT, GLDH and γ-GT), but positively correlated with the albumin and fibrinogen plasma concentration.

3.3 Electrophoresis

In Figure 3, the SDS-PAGE (10 %) revealed that albumin and fibrinogen fractions were purely isolated from the other protein fractions. The porcine fibrinogen marker (Lane 1, 2.5 µg) was divided into three proportions: an α, β and γ-chain with a molecular weights (MW) of 64,000, 57,000 and 48,000 Da, respectively, which were reflected by the purified samples (Lanes 2 & 3). The porcine albumin standard (Lane 5, 1 µg) with a MW of 68,000 Da, and the albumin samples (Lanes 6 & 7), were close to the bovine serum albumin (66,000 Da) of the wide molecular weight marker.

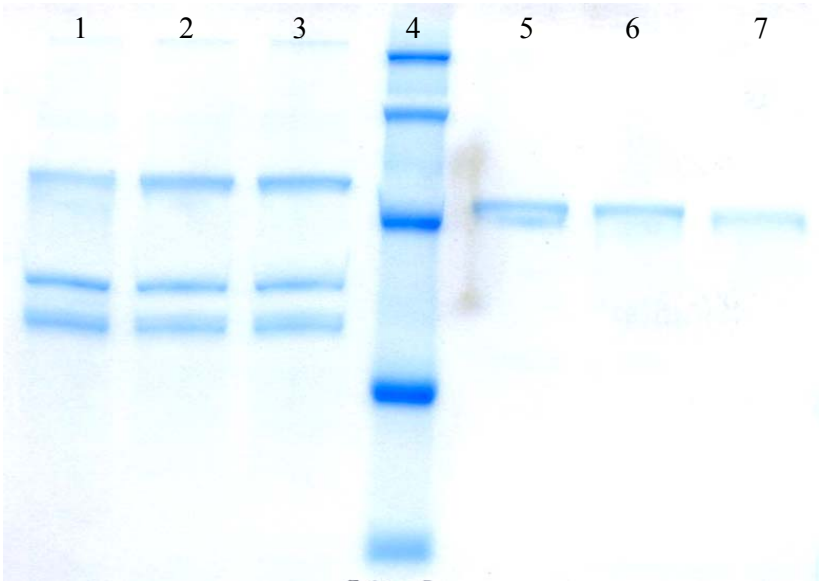

Figure 3 Determination of the purity of albumin and fibrinogen isolates by SDS-PAGE (10 %). 1 - porcine fibrinogen standard (MW: α = 64,000 Da, β = 57,000 Da, γ = 48,000 Da, 2.5 µg); 2 - Pig 1, 0 min fibrinogen; 3 - Pig 1, 60 min fibrinogen; 4 - molecular weight standard (Roti®-Mark STANDARD, Roth, Germany: bovine myosin = 200,000 Da, β-galactosidase = 119,000 Da, bovine serum albumin = 66,000 Da, ovalbumin = 43,000 Da, carboanhydrase = 29,000 Da, soya trypsin inhibitor = 20,000 kDa, and chicken lysozyme = 14,500 Da); 5 – porcine albumin standard (MW 68,000 Da, 1 µg); 6 – Pig 13, 0 min albumin; 7 – Pig 13, 60 min albumin.

Table 4 In vivo albumin synthesis in pigs after oral (5.7 mg/kg diet, chronically and acutely) or intravenous (53 µg/kg LW) deoxynivalenol (DON) exposure measured by a flooding dose of L-[$^2H_5$]-phenylalanine (mean, range)

GROUP	N	Conc.[1]	Albumin: Plasma protein[2]	Intravasal mass[3]	FSR[4]	ASR[5]		t_s[6]	Albumin: body protein synthesis[7]
		g/l	%	g	%/d	g/d	mg/kg/d	min	%
Control	7	34.7 *(27.3-42.5)*	62.2 *(55.0-75.4)*	86.2 *(67.1-103.7)*	18.8[a] *(14.9-25.9)*	16.1[a] *(10.6-23.4)*	420.4[a] *(280-624)*	25.9 *(17.7-34.4)*	3.5[a] *(2.36-5.25)*
DON chronic	10	35.1 *(27.0-43.3)*	66.5 *(46.8-89.9)*	91.0 *(71.1-128.4)*	12.8[b] *(5.5-20.5)*	11.3[b] *(5.9-17.7)*	283.4[b] *(156-478)*	23.1 *(17.7-30.8)*	2.4[b] *(1.26-4.02)*
DON acute oral	7	33.5 *(27.0-36.6)*	57.1 *(46.8-57.7)*	79.9 *(65.1-85.8)*	10.4[b] *(5.8-15.2)*	8.2[b] *(5.1-11.1)*	214.5[b] *(140-284)*	23.6 *(13.7-29.1)*	1.8[b] *(1.18-2.38)*
DON acute iv	7	33.7 *(27.6-42.6)*	60.3 *(43.5-68.1)*	86.2 *(67.9-100.6)*	13.9[ab] *(7.7-25.9)*	12.2[ab] *(5.9-22.3)*	322.0[ab] *(155-557)*	19.9 *(6.8-29.2)*	2.7[ab] *(1.31-4.68)*
Probability									
Group		0.961	0.362	0.830	0.029	0.032	0.028	0.252	0.028

[ab] data with distinct superscript are significantly different within a column ($p<0.05$, Fisher LSD test), [1] plasma albumin concentration, [2] percentage of albumin concentration of total plasma protein concentration, [3] intravascular albumin mass = plasma concentration x plasma volume, [4] FSR = fractional synthesis rate, [5] ASR = absolute synthesis rate, [6] t_s = secretion time, [7] whole body protein synthesis = LW (kg) x 0.17 x 0.07 (17 % protein, 7 % FSR (Simon, 1989)

3.4 Protein synthesis

3.4.1 Albumin

The mean plasma albumin concentrations (g/l) did not differ between the groups and amounted to 44 - 90 % of the plasma total protein concentration (Table 4). A positive correlation between the plasma albumin concentration and fibrinogen concentration (r = 0.67), but not with the plasma protein (r = 0.05), was observed. However, albumin plasma concentration was neither correlated with the albumin secretion time (r = 0.17) nor with the FSR of albumin (r = -0.09).

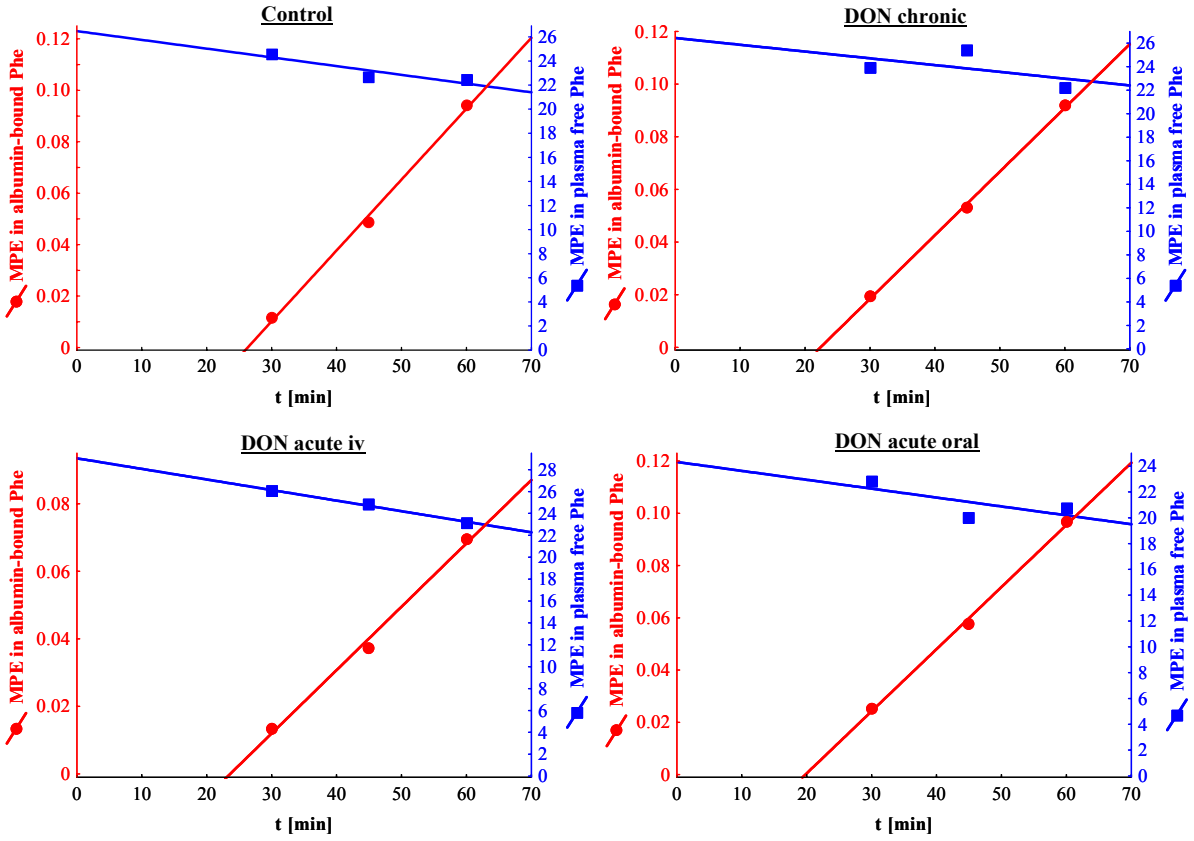

Figure 4 Time course of molar percent excess (MPE) in albumin-bound phenylalanine (Phe) (●) and in plasma free Phe (■) of one exemplary pig per treatment after receiving a flooding dose of L-[$^2H_5$]-phenylalanine (150 mmol/l, 29 MPE).

The intravascular albumin mass, as a product of the concentration and the estimated plasma volume, ranged between 65.1 and 128.4 g, with no significant (P = 0.830, Table 4) differences between the groups. On the other hand, chronic or acute dietary DON significantly decreased the fractional and absolute synthesis rate of albumin by 32 and 33 %, or 45 and 49 %,

respectively, whereas the intravenous DON application resulted in 26 and 24 % lower, but not significantly different values (Table 4). The proportion of albumin synthesis of the whole body protein synthesis ranged between 1.18 and 5.25 % and reflected the results of the lower synthesis rates after oral DON application (Table 4).

Figure 4 proved the assumption that after a flooding dose of L-[$^2H_5$]-phenylalanine (125 mg/kg LW, 150 mmol/l, 29 MPE) the plasma-free phenylalanine remained nearly constant, whereas following a lag period, the incorporation of labeled phenylalanine in plasma albumin increased linearly. The interception on the x-axis is regarded as the so-called secretion time (t_s) of albumin, which means the time required until newly synthesized albumin (from the liver) is detectable in the plasma (Figure 4). However, DON had no significant effect on the t_s of albumin, which ranged between 6.8 and 34.4 min (Table 4).

3.4.2 Fibrinogen

DON exposure neither significantly affected plasma concentration nor altered the fractional and absolute synthesis rates of fibrinogen (Table 5). Nevertheless, mean FSR and ASR of fibrinogen were increased by 27 and 61 % after chronic, and by 49 and 23 % after acute oral DON exposure. In contrast, intravenous DON exposure resulted in a decrease of fibrinogen FSR and ASR by 18 and 40 % compared to the control. However, fibrinogen synthesis parameters showed a very high inter-individual variation (FSR = 6.8 – 60.9 %/d; ASR = 0.40 – 8.87 g/d or 7.0 – 212.3 µg/kg LW/d).

Mean plasma fibrinogen concentrations (g/l) accounted for 2.2 – 18.9 % of the plasma total protein concentration, whereas the absolute fibrinogen synthesis (g/d) contributes to approximately to 0.06 – 1.78 % of the calculated total body protein synthesis. The fibrinogen secretion time ranged between 4.5 – 28.2 min and was also not affected by treatment (Table 5).

3.4.3 Lymphocytes

Fractional synthesis rates of the isolated blood lymphocytes were 16.4 ± 4.1, 12.3 ± 3.4, 13.6 ± 3.9, and 12.9 ± 3.4 %/d for the control, DON chronic, DON acute oral and DON acute iv group, respectively (Figure 5). However, the decrease of the lymphocyte FSR by 27, 19 and 24 % compared to the control only reached significance after chronic dietary and intravenous DON exposure.

Table 5 *In vivo* fibrinogen synthesis of pigs after oral (5.7 mg/kg diet, chronically and acutely) or intravenous (53 µg/kg LW) deoxynivalenol (DON) exposure measured by a flooding dose of L-[$^2H_5$]-phenylalanine (mean, range)

GROUP	N	Conc.[1]	Fibrinogen : Plasma protein[2]	Intravasal mass[3]	FSR[4]	ASR[5]		t_s[6]	Fibrinogen : body protein synthesis[7]
		g/l	%	g	%/d	g/d	mg/kg/d	min	%
Control	7	4.7	7.8	11.9	14.0	1.88	46.9	16.1	0.39
		(3.4-7.7)	*(5.7-12.0)*	*(8.4-20.2)*	*(7.2-24.6)*	*(0.60-4.98)*	*(20.1-117.4)*	*(8.7-23.8)*	*(0.17-0.99)*
DON chronic	10	4.5	8.0	11.7	17.8	3.03	72.1	15.6	0.61
		(2.5-10.5)	*(4.3-18.9)*	*(5.9-28.1)*	*(6.8-53.2)*	*(0.40-8.87)*	*(11.6-212.3)*	*(4.5-27.9)*	*(0.10-1.78)*
DON acute oral	7	4.4	7.4	11.0	20.9	2.31	60.2	19.0	0.51
		(2.5-6.2)	*(4.3-10.1)*	*(6.0-15.4)*	*(8.0-60.9)*	*(0.70-7.47)*	*(19.1-191.6)*	*(7.8-28.2)*	*(0.16-1.61)*
DON acute iv	7	3.5	5.7	8.5	11.5	1.12	30.5	16.9	0.26
		(1.2-6.1)	*(2.2-9.6)*	*(3.0-14.4)*	*(9.0-18.8)*	*(0.27-2.71)*	*(7.0-77.1)*	*(14.1-19.6)*	*(0.06-0.65)*
Probability									
Group		0.699	0.724	0.683	0.630	0.681	0.565	0.812	0.565

ns = $p > 0.05$ (Kruskal-Wallis-test), [1] plasma fibrinogen concentration at t = 60 min, [2] percentage of fibrinogen concentration of total plasma protein concentration, [3] intravascular fibrinogen mass, [4] FSR = fractional synthesis rate, [5] ASR = absolute synthesis rate, [6] t_s = secretion time, [7]whole body protein synthesis = LW (kg) x 0.17 x 0.07 (17 % protein, 7 % FSR (Simon, 1989)

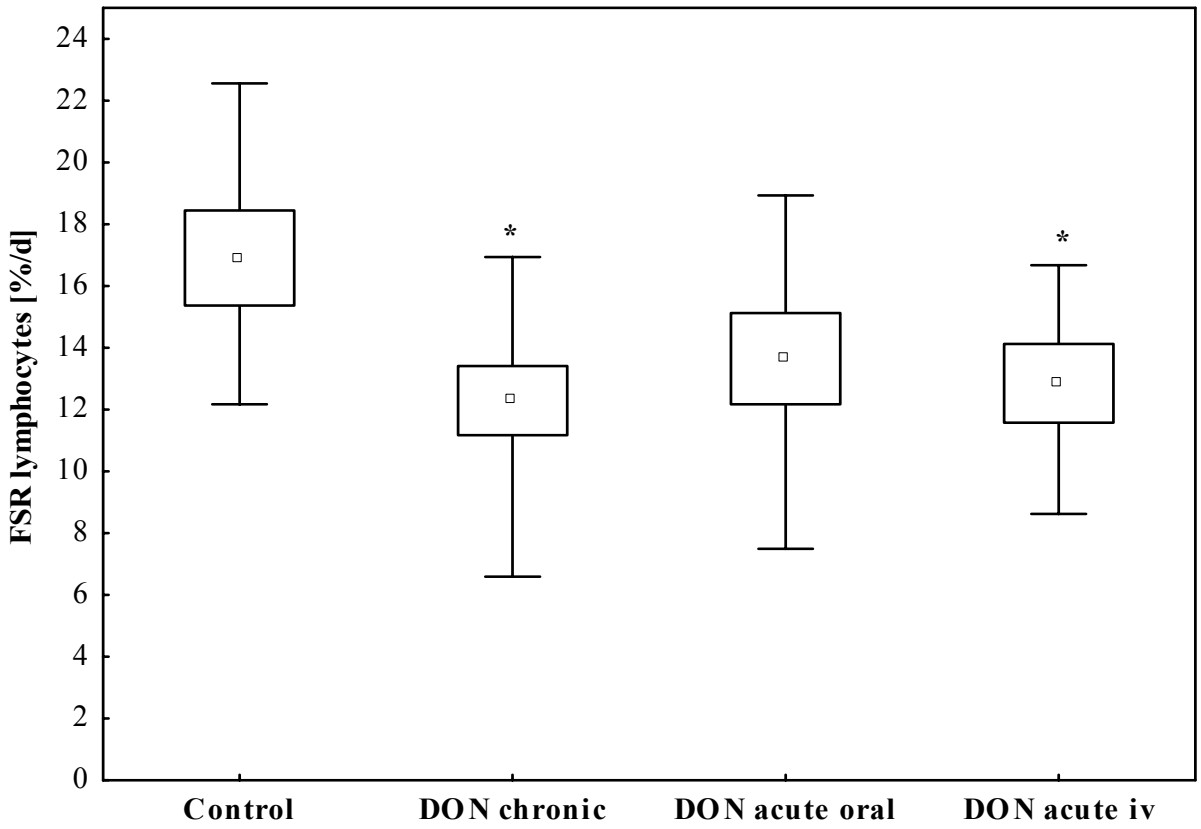

Figure 5 Fractional synthesis rates (FSR, %/d) of porcine blood lymphocytes following oral (chronically and acutely) or intravenous deoxynivalenol (DON) exposure (mean, box = standard deviation, whisker = min-max), * significantly different from control ($P < 0.05$, Fisher LSD test).

4 DISCUSSION

The present study aimed to relate the measurement of plasma protein concentrations and activity of liver enzymes to *in vivo* protein synthesis of albumin, fibrinogen and lymphocytes following an oral (acute or chronic) or intravenous DON exposure to pigs as it was suggested that DON primarily affects the protein synthesis of rapidly dividing cells or tissues with a high metabolic turnover.

It was previously shown by Goyarts and Dänicke (2006), that serum DON concentrations are maximal at between 0.81 to 2.87 h after feeding pigs with a DON contaminated diet (5.7 mg/kg), either chronically or acutely. For this reason, the measurements in the present study were made in the time period 60 – 120 minutes after feeding (Figure 1). As the serum DON concentration decreased rapidly after intravenous application ($t_{1/2}$ α = 0.11 – 1.32 h, Figure 1, Goyarts and Dänicke, 2006), the intravenous DON dose was given directly following the flooding dose of L-[$^2H_5$]-phenylalanine (125 mg/kg LW). Thirty minutes after the flooding

dose, which is equivalent to 90 minutes after feeding, the plasma concentration of DON was significantly increased in pigs following acute and chronic oral DON exposure (12 – 28 ng DON/ml) and was even significantly higher in pigs dosed 53 µg DON/kg LW intravenously (39 – 51 ng DON/ml, Table 3).

4.1 Clinical-chemical parameters

In the present study, AP plasma activities were higher than the reference value in all groups, which may be explained by the state of adolescence of pigs. Since AP is highly active in osteoblasts, it correlates closely to age, which results in reference value up to 700 U/l for weaned piglets in contrast to only 170 U/l for sows (Kraft and Dürr 1999).

The correlation between higher activities of liver enzymes with decreasing plasma concentrations of albumin and fibrinogen, as well as the surpassing of the GLDH reference values, possibly indicated a slight disturbance in the liver function, in particular for the chronic DON group. On the other hand, it has to be taken into account that neither the activity of the measured liver enzymes, nor the plasma protein concentrations, showed a correlation to the fractional synthesis rate of albumin or fibrinogen (FSR, %/d) corroborating the assumption that these plasma parameters are inadequate for estimating the protein synthesis capacity of the liver. Furthermore, it has to be emphasized that we were not able to notice a significant effect of dietary or intravenous DON exposure on liver enzymes or plasma protein concentrations (total protein, albumin, and fibrinogen) in the present study. Similarly, feeding a DON contaminated diet of approximately 6 mg DON/kg, either *ad libitum* or restrictively, over five weeks to growing pigs (~ 65 kg LW) did not result in differences for these parameters (Goyarts et al. 2005). Effects of DON on plasma protein concentrations were rather inconsistent. Bergsjø et al. (1993) and Rotter et al. (1995) found a significant decrease in total protein and albumin, whereas Rotter et al. (1994) described elevated albumin, decreased α-globulin levels and a general increase in albumin:globulin ratio following feeding DON contaminated diets to pigs. Alterations in various serum parameters in piglets fed DON contaminated diets up to 43 mg DON/kg were reported by Young et al. (1983). However, the authors were not able to separate these effects from the impact of a low feed intake which reconfirmed the importance of feeding the same amount of diet to differentiate the DON effect.

In the present study, plasma glucose concentrations were neither affected by DON treatment and feeding nor by intravenous injection of a large dose phenylalanine. Bregendahl et al. (2004) were also not able to detect time-dependent changes in plasma insulin levels or in

plasma glucose concentration (with an exception after 15 min) following an intraperitoneal (ip) injection of a flooding dose of [$^2H_5$]phenylalanine to piglets weighing 5.7 ± 0.2 kg. This could possibly undermine a previous assumption of Smith et al. (1998) that insulin and/or glucose plasma concentrations would be influenced by a large dose of amino acids as used by the flooding dose method. Furthermore, plasma glucose levels and many other biochemical and haematological parameters in pigs were found to be changed by acute human-pig interactions, such as a snare restraint to enable the puncturing of the *vena jugularis* (Dubreuil et al. 1993). This effect could be excluded in the present study as permanent catheters were used and pigs were inconspicuous (relaxed, lying down or sleeping) during blood sampling.

4.2 Plasma concentration and synthesis of albumin and fibrinogen

Although the albumin plasma pool (intravascular mass = albumin concentration x PV) was not different between the groups of the current investigation, the FSR of albumin was significantly lower after dietary DON exposure (Table 4). Thus, despite a lower fractional synthesis rate, the pool size of albumin was maintained, indicating that the decrease in the amount of protein synthesized was balanced by an equal reduction in the rate of catabolism.

However, it has to be emphasized that plasma albumin level is influenced by several mechanisms, including changes in circulating plasma volume, albumin synthesis and catabolism, distribution of the protein in the extravascular space, lymphatic return, and urinary or intestinal losses (Ruot et al. 2003). As albumin escapes from the intravascular space across the capillary membrane and returns to it via the lymphatic system, there is a continuous circulation of albumin between intra- and extravascular spaces. Since the transcapillary escape rate is 8-10 times higher than the rate of synthesis or degradation it was suggested that an altered transcapillary escape rate affects the plasma albumin concentration more rapidly than any modification in albumin synthesis or catabolism (Ballmer 1990, Fleck 1989, Ruot et al. 2003). However, the transcapillary escape was not determined in the present study but these findings make clear that the plasma concentration might not be an appropriate parameter to determine protein synthesis, as it is influenced by many other factors.

In the present study, FSR of albumin was decreased by 32, 45 or 26 % after chronic and acute dietary exposure (~80 µg/kg LW) or intravenous injection of DON (53 µg/kg LW) to pigs, respectively. In mice, plasma protein synthesis ([^{14}C]leucine incorporation) was reduced by more than 50 and 80 % after 3 h oral exposure of 5 and 25 mg DON/kg LW. Protein synthesis appeared to recover to normal levels 6 and 9 h after exposure to 5 mg DON/kg LW (inhibition of about 20 and 0 %), while it remained inhibited to the same extent at the high DON dose

(Azcona-Olivera et al. 1995a). *In vivo* liver protein synthesis was affected to a lower extent, as the [^{14}C]leucine incorporation decreased by only about 20 and 50 % 3 h after receiving the oral DON dose of 5 and 25 mg/kg LW, respectively (Azcona-Olivera et al. 1995a). Accordingly, Robbana-Barnat et al. (1987) observed an inhibition of [^{14}C]leucine incorporation into the liver of mice by about 60 and 90 % after ip injection of 20 and 80 mg DON/kg LW, but no difference compared to the control values after receiving 4 and 10 mg pure DON ip. Moreover, it has to be taken into account that these studies compared only the radioactivity levels as indicators of protein synthesis of tissues after exposure to very high doses of pure toxin to that of unexposed mice. Furthermore, it could be assumed that plasma albumin synthesis is a more sensitive indicator than the whole liver protein synthesis, because many proteins could superpose the effect of DON. This may be an explanation for the unaffected liver protein synthesis of pigs treated in the same manner as in the present study (Dänicke et al. 2006).

The fractional synthesis rates of albumin in the control group of the present study (14.9 – 25.9 %/d) are in accordance with values obtained by other investigations with pigs (10 – 40 %/d: Dänicke et al. 2005, Jahoor et al. 1994, 1999b, Mackenzie et al. 2003) whereas the FSR declined after dietary DON exposure to 5.1 – 17.7 %/d. Anyway, protein turnover appeared to be higher in the growing pig compared to that in humans (FSR albumin 3 – 10 %/d: Ballmer et al. 1990, 1995a, c, 1996, Hunter et al. 1995, 2001, McNurlan et al. 1996, Slater et al. 1995).

Furthermore, feeding has been shown to significantly stimulate albumin synthesis in humans by ~ 20 – 30 % using the flooding dose technique and oral feeding (Barber et al. 2000, Hunter et al. 1995) and by ~ 90 % using the primed constant infusion method and enteral feeding (de Feo et al. 1992). Therefore, in the present study all pigs were fed the same amount of feed (1100 g/d) with the same composition of both diets, except of the wheat (control and *Fusarium* contaminated), in which crude nutrients and amino acid content were not substantially different (Goyarts et al. 2005). In healthy humans albumin synthesis accounts for 5 - 7 % of whole body protein synthesis in a fasting state, whereas this might increase to > 10 % in the fed state (Cayol et al. 1997, de Feo et al. 1992). In the present study, 90 min after feeding a meal of 6.2 – 6.8 MJ ME to pigs, the calculated proportion of albumin of whole body protein synthesis varied between 1.2 - 5.2 %. The lower proportion of albumin of the whole body protein synthesis in the growing pig compared to adult human is probably a result of the higher impact of muscle protein synthesis. As fractional synthesis rates of skeletal muscle in growing pigs (5.0-5.6 %/d, Dänicke et al. 2006) are considerably higher than in

adult humans (1.5-2.0 %/d, Garlick et al. 1994), the ASR will be even higher because of the very large mass of muscle protein.

Additionally, an adequate protein supply has to be assured as chronic protein deficiency resulted in a suppression of total protein synthesis which may not include all plasma proteins. For example, fibrinogen synthesis was not reduced while muscle protein and albumin synthesis were decreased in pigs after 4 weeks of protein deficient feeding (Jahoor et al. 1999b).

Since DON was observed to upregulate pro-inflammatory cytokine production, for example IL-6, TNF-α, and IFN-γ, *in vitro* and *in vivo* in mice (Azcona-Olivera et al. 1995a,b, Chung et al. 2003, Jia et al. 2004, Jia and Pestka 2005, Meky et al. 2001, Moon and Pestka 2003, Sugita-Konishi and Pestka 2001), it was suggested that DON may induce an acute-phase response. The up-regulation of cytokines might be due to a so-called superinduction of immediate-early genes (such as c-*fos*) by protein synthesis inhibitors which is possibly caused by inhibition of a labile repressor and is manifested in at least three ways: (1) mRNA stabilization, (2) activation of intracellular signalling cascades, and (3) interference with transcriptional down-regulation (Zinck et al. 1995).

Consequences of the acute-phase response for protein metabolism following injury or infection appeared to be an attempt to adjust to the higher requirements due to increased lymphocyte proliferation and synthesis of cytokines, immunoglobulins, and positive acute-phase proteins (APP, for instance fibrinogen, C-reactive protein and haptoglobin) by a redistribution of amino acid precursors with a drawback from synthesis of skeletal muscle and the negative APP, such as albumin, transthyretin and retinol-binding protein (Baumann and Gauldie 1994, Fleck 1989, Jahoor et al. 1999a). The assumption of reduced synthesis rates of negative reactants during the acute-phase response is underlined by findings of Ballmer et al. (1995b) who observed a decrease in hepatocyte albumin mRNA in the liver of animals given either IL-1 or turpentine (a potent stimulus for pro-inflammatory cytokine production). In contrast, albumin synthesis was found to be increased in rats and children after infection or trauma (Jahoor et al. 1999a, Mansoor et al. 1997, Morlese et al. 1996). It has to be emphasized that the synthesis rate of acute-phase proteins is known to be influenced not only by pro-inflammatory cytokines, for example IL-6 (Castell et al. 1990), but also by factors such as insulin and the counterregulatory hormones (O'Riordain et al. 1995). Conversely, the present study could not entirely prove the assumption of a DON-caused acute-phase response since plasma concentrations of fibrinogen and albumin were not influenced by DON as

expected. Anyway, albumin synthesis was inhibited by DON in the current investigation, whereas dietary DON increased fibrinogen synthesis. However, the increase of fibrinogen synthesis after dietary DON, as well as the decrease after iv DON exposure, did not reach significance because of the extremely high inter-individual variation, indicating that fibrinogen is a highly variable parameter which is suitable for detection of DON-caused effects only to a limited extent. Possibly a DON-caused inhibition of the protein synthesis (direct effect) is intensified by a DON-related acute-phase response and cytokine disturbance (indirect DON effect) in the case of albumin, but attenuated to some extent in the case of fibrinogen. Anyway, the difference between the fibrinogen FSR of pigs (6.8 - 60.9 %/d) in the present study and the results of Jahoor et al. (1999b) (pigs: 40 – 90 %/d) and Barber et al. (2000) (humans: 11.6 – 24.6 %/d) could not be solely explained by a different method used (flooding dose *versus* constant infusion).

The protein nutritional status of the individual has an important bearing on the ability of the liver to mount an adequate acute-phase response (Reeds et al. 1994). As most of the negative acute-phase proteins play major roles in the transport of nutrients, hormones, metabolites, and drugs, a reduction might further impede nutrient utilization (Jahoor et al. 1999a).

Albumin secretion times of the present study (6.8 – 34.4 min) were in the same range as reported by Dänicke et al. (2005) in pigs (6 – 28 min). Furthermore, secretion time of fibrinogen (4.5 – 28.2 min) resembled that of albumin. A biological reason for such a great variation between the individuals in the present study could not be identified, as all pigs were crossbred barrows of similar live weight. It is worth noting that albumin secretion time is neither significantly correlated with plasma DON and albumin concentration (r = -0.35 and 0.164) nor with fractional and absolute synthesis rates of albumin (r = -0.06), suggesting that other mechanisms are responsible for the variation of t_s. Human albumin seemed to occur slightly later in the plasma as secretion times varied between 24 and 37 min (Ballmer et al. 1990, Essen et al. 1998, Hunter et al. 1995, Slater et al. 1995).

4.3 Lymphocyte protein synthesis

In contrast to *in vitro* assays that determine the capacity of lymphocytes to respond to strong external stimuli such as mitogens, the *in vivo* estimate of protein synthesis is a measure of the metabolic state of lymphocytes within the body (Caso et al. 2001, McNurlan et al. 1996). In the present study, the fractional synthesis rates of porcine blood lymphocytes were decreased by 27, 19 and 24 % after chronic or acute oral DON exposure (5.7 mg/kg diet) and intravenous (53 µg DON/kg LW) application, respectively, compared to the control group.

This is in agreement with the results presented by Goyarts et al. (2006) who determined the ConA-stimulated proliferation of blood lymphocyte with two different assays (MTT reduction *versus* BrdU incorporation) *ex vivo* from pigs (~ 30 kg LW) exposed either chronically or acutely to a similar dietary DON concentration as in the present experiment. Accordingly, Park et al. (1994) found a positive correlation between *in vivo* lymphocyte protein synthesis and *ex vivo* lymphocyte proliferation. Furthermore, Park et al. (1994) reported greater variability in the *ex vivo* lymphocyte proliferation using [^{3}H]thymidine uptake compared with the *in vivo* protein synthesis by a flooding dose of [^{13}C]leucine. On the other hand, Januszkiewicz et al. (2001a) were not able to replicate the decrease of *in vivo* lymphocyte protein synthesis after infusion of stress hormones (epinephrine, cortisol and glucagon) in the *ex vivo* [^{3}H]thymidine incorporation assay using the same human PBMC (peripheral blood mononuclear cells). But it has to be considered that in contrast to the proliferation assays, the measurement of the lymphocyte protein synthesis *in vivo* probably will not only detect the lymphocyte proliferation, but also the synthesis of cell receptors, cytokines, prostaglandins, immunoglobulins and other secretory proteins (Januszkiewicz et al. 2001a, Park et al. 1994).

In the present study, FSR of blood lymphocytes varied between 6.6 and 22.6 %/d. This is in accordance with findings in healthy humans in the basal state who exhibited fractional synthesis rates of PBMC (mainly consisting of lymphocytes) between 4.2 and 14.6 %/d using the flooding dose technique (Caso et al. 2001, Essen et al. 1996, Januszkiewicz et al. 2001a, McNurlan et al. 1996, Park et al. 1994). Furthermore, Essen et al. (1996) observed a high inter-individual variation of the protein synthesis rate and concluded that the variability might be due to an unbalanced distribution of lymphocytes between circulation and lymphoid tissue.

In well-nourished rats, the FSR decreased in the order bone marrow (place of stem cells, 117 %/d), thymus (site of maturation, 73 %/d) and circulating mature blood lymphocytes (29 %/d) (Papet et al. 2002), which is consistent with their replicative and metabolic activities. Moreover, it has to be noted that the fractional lymphocyte protein synthesis rates in rats (29.2 ± 3.8 %/d; Papet et al., 2002) seemed to be markedly higher than in human PBMC. Accordingly, Rennie et al. (1994) reported higher FSR for muscle, intestine and albumin in rats compared to humans.

Nevertheless, it has to be emphasized that there are fundamental differences between protein synthesis rates of leukocytes. Januszkiewicz et al. (2000) described the measurement of *in vivo* protein synthesis of T-lymphocytes isolated by the rosette method. In doing so, it could be shown that the FSR of normal human T-lymphocytes (10.4 – 14.7 %/d) were in the range

of human PBMC (9.6-14.6 %/d), but were higher than FSR of total leukocytes (3.2 ± 1.2 %/d) (Januszkiewicz et al. 2000, 2001a, 2002). Using a constant infusion with [^{13}C]leucine, Walrand et al. (2004) reported of two-fold lower *in vivo* protein synthesis rates in PMN (polymorphonuclear neutrophil, FSR = 3.0 ± 0.7 %/d) compared to FSR of the corresponding PBMC of 6.0 ± 2.1 %/d (3.3 – 8.6 %/d). However, Park et al. (1994) observed higher FSR of PBMC (7.2 – 11.4 %/d) in healthy subjects using the flooding dose technique. This difference might be due to the different methods used. As a large part of the synthesized proteins of immune cells like lymphocytes will be exported, these newly synthesized export proteins probably remain in the cell during the short flooding dose measurements, whereas they were released in the course of a constant infusion (Walrand et al. 2004).

Furthermore, *in vivo* lymphocyte protein synthesis appeared to be influenced by stress as a 60 % decrease at the end of the 6-hour stress hormone infusion was shown in humans by McNurlan et al. (1996). However, 18 h after cessation of the infusion, the FSR in lymphocytes had returned to pre-infusion levels indicating that the inhibitory effect of stress hormones was transient (McNurlan et al. 1996).

In summary, deoxynivalenol was shown to inhibit the protein synthesis of lymphocytes and albumin *in vivo* after dietary exposure of 5.7 mg DON/kg diet or intravenous (53 µg/kg LW) DON application to pigs weighing ~ 40 kg. Moreover, this study provided evidence that plasma concentrations of proteins are not appropriate for deducing DON effects on protein synthesis.

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GENERAL DISCUSSION

As infection of small grains and maize with *Fusarium* species and subsequent toxin production occurs predominantly at the pre-harvest stage and can not be completely prevented under the conditions of current agricultural practice, deoxynivalenol is a critical issue in animal and human health.

Since deoxynivalenol was isolated from a maize batch which caused emesis in pigs (vomitoxin) in the 1970´s (Vesonder et al. 1973), pigs are considered to be particularly susceptible to this mycotoxin (Rotter et al. 1996). Because of their receptiveness, pigs may be a good indicator for human health risk according to this mycotoxin. Besides economic losses in agricultural production caused by losses in crop yield and a decreased animal performance, a *Fusarium* contamination may have an impact on animal health.

Therefore, the present thesis investigated the effects of the *Fusarium* mycotoxin deoxynivalenol from naturally contaminated wheat on animal health and performance, toxicokinetic parameters (including the bioavailability of DON from naturally contaminated feedstuffs) and immunological parameters. Furthermore, *in vivo* lymphocyte and plasma protein synthesis were determined because protein synthesis inhibition is considered to be the mode of action of trichothecenes, whereas most other effects of DON are regarded as secondary effects. To assess the risk to human and animal health it is essential to relate these toxic effects to each other.

1 Feed intake and performance

Decreased feed intake and feed refusal were often described for pigs exposed to deoxynivalenol (Rotter et al. 1996, Young et al. 1983). While few field studies reported adverse effects on feed intake at as low DON doses as 1 mg/kg (Schuh 1981, 1983), it is generally suggested that DON affects the performance of pigs at diet concentrations exceeding 1 mg/kg (Dänicke et al. 2001; Figure 1).

Feedstuffs contaminated naturally with *Fusarium* were observed to reduce feed intake to a greater extent than an oral gavage of pure DON, indicating that mycotoxins not detected in the contaminated diet contribute to the higher toxic effects (Foster et al. 1986, Trenholm et al. 1994). Therefore, wheat contaminated naturally with *Fusarium* (Paper I, Table 2) was used in the present experiments to mimic DON exposure under practical conditions. Although further *Fusarium* toxins were present in the wheat (Paper I, Table 2) and 15-acetyl-DON (101 µg/kg)

and 3-acetyl-DON (41 µg/kg) may be metabolized rapidly into DON (Eriksen et al. 2003), the observed effects can mainly be attributed to DON (16643 µg/kg) as the proportion of the other toxins amounted to just about 2 %.

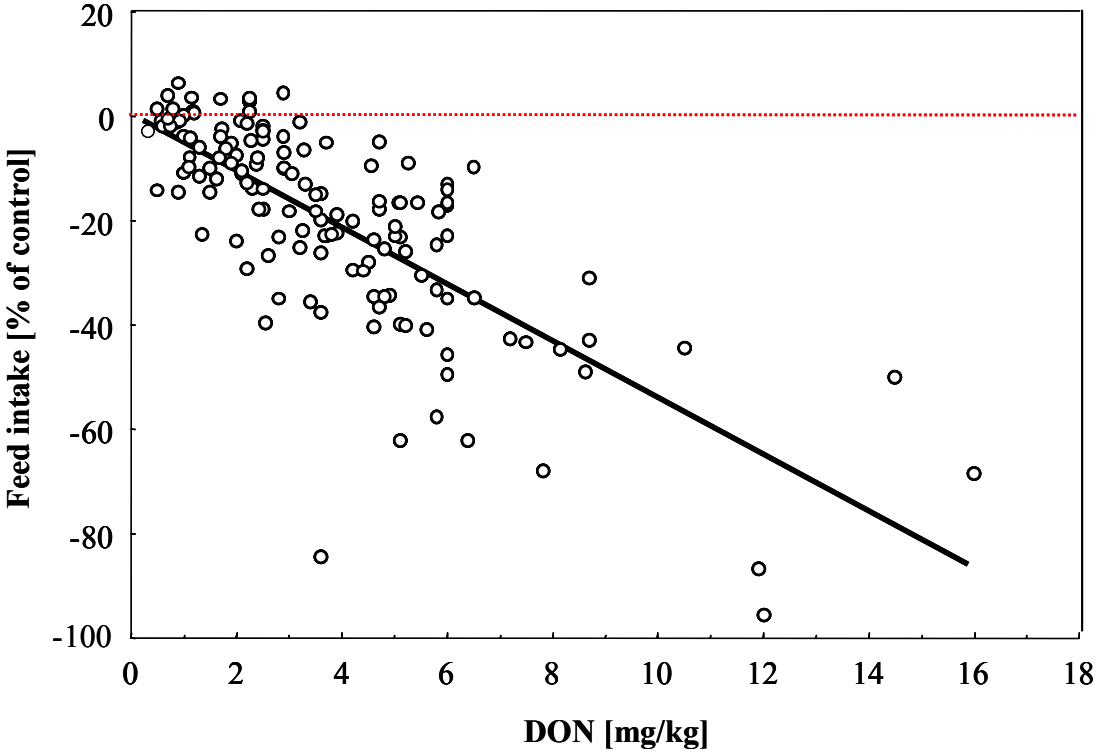

Figure 1 Effect of DON concentration of the diet on the feed intake of pigs (y = 0.7 - 5.5 * x, r^2= 0.570) (update according to Dänicke et al. 2001; References: Bergsjø et al. 1992, 1993; Chavez 1984; Chavez and Rheaume 1986; Dänicke et al. 2004a, c, d; Döll et al. 2003, 2004; Forsyth et al. 1977; Foster et al. 1986, 1987; Friend et al. 1982, 1983, 1984, 1986b,c, 1992; Harvey et al. 1996; He et al. 1993; House et al. 2002; Lun et al. 1985; Øvernes et al. 1997; Pollmann et al. 1985; Prelusky et al. 1994; Richter et al. 1996; Rotter et al. 1992b, 1994, 1995; Schätz 1982; Smith et al. 1997; Swamy et al. 2002, 2003; Weiß et al. 1999; Wetscherek et al. 1998; Young et al. 1981, 1983)

A reduction of feed consumption by 15 % was observed in pigs fed a DON contaminated diet of about 6 mg/kg *ad libitum* from wheat infected naturally with *Fusarium* compared to the *ad libitum* fed control group (Paper I, Table 3). After compiling literature data on the depressant effect of DON in pig diets on feed intake in comparison to the control, a mean feed intake reduction of 5.5 % can be predicted after an increase of the dietary DON concentration by 1 mg/kg (Figure 1; according to Dänicke et al. 2001). The predicted reduction of feed intake by more than 30 % for the DON concentration of the present experimental diet exceeds the observed reduction approximately twice, but the observed depression falls within the overall variation of this regression. Thus, further experimental conditions such as the age, sex and

breed of pigs, duration of experiment, source of DON and presence of additional mycotoxins contribute to the variation in feed intake due to DON concentration of pig diets (Dänicke et al. 2001).

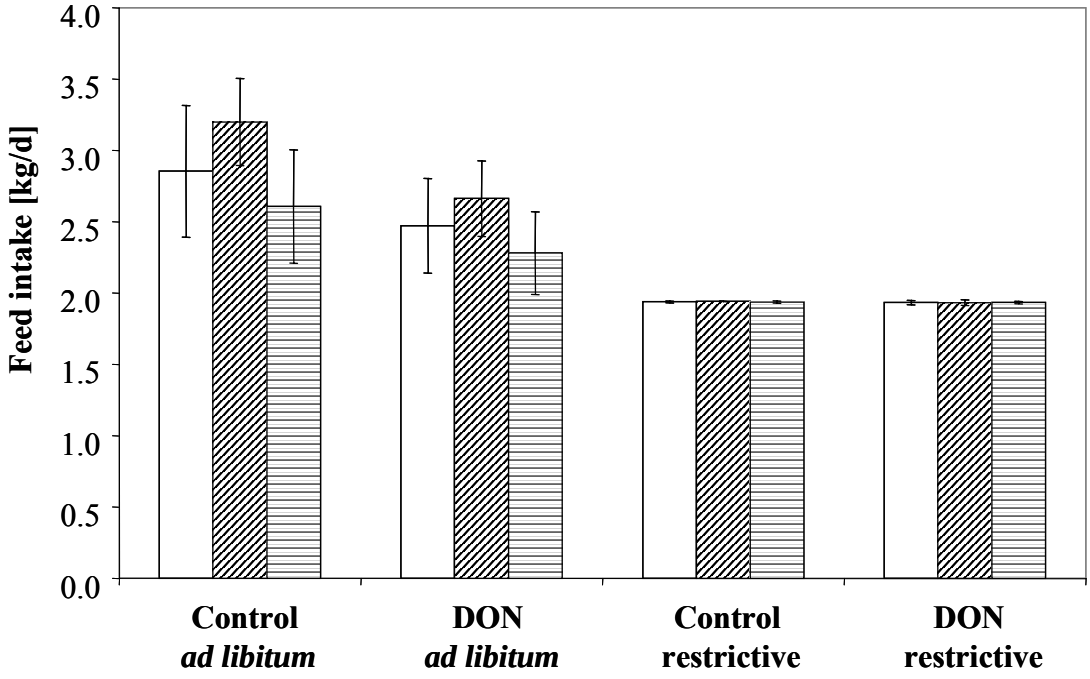

Figure 2 Feed intake [kg/d] of pigs fed a control or DON contaminated diet either *ad libitum* or restrictively over a period of 11 weeks (▭ all pigs, n = 12; ▨ barrows, n = 6; ▤ females, n = 6; means ± SD)

Furthermore, it has to be emphasized that a DON-caused reduction of feed intake may indirectly affect further parameters such as live weight gain, feed efficiency, disturbances in serum/plasma parameters (protein and albumin concentration, activity of liver enzymes) and immunological response. Thus, the results of Paper I strongly indicate that the impaired live weight gain can mainly be attributed to the reduced feed intake, as there were no differences in performance when feeding the pigs the same level of either the control or DON contaminated diet (Paper I, Table 3). Consequently, to distinguish the indirect effects of the direct action of DON, a restrictive feeding regimen characterized by identical daily feed consumption is required.

Castrated male pigs fed a control or DON contaminated diet *ad libitum* consumed 18.5 and 14.3 % more than their female counterparts, respectively (Figure 2), whereas the restrictive feeding regimen prevented sex differences between the both diets. Accordingly, barrows gained 19.5 and 12.7 % more live weight when the control or DON contaminated diet was offered *ad libitum*. However, it has to be noted that no significant interaction was observed

between DON and sex on performance data (Paper I, Table 3). Nevertheless, in further studies (Papers II-IV), only barrows were exposed to the test diets with a restrictive feeding regimen in order to exclude both sex differences and the level of feed intake.

Although DON is considered as less acutely toxic, it is regarded as one of the most potent anorectic and emetic trichothecenes with a minimum oral emetic dose of 50 µg DON/kg LW (Pestka et al. 1987, Prelusky and Trenholm 1993, Rotter et al. 1996).

Moreover, feed refusal and vomiting have both been linked to an interaction with serotinergic and dopaminergic receptors in the area postrema as well as with gastrointestinal $5HT_3$ receptors (Fioramonti et al. 1993, Prelusky and Trenholm 1993, Rotter et al. 1996). However, it has to be noted that in the present experiments no emetic effects were observed in pigs fed the *Fusarium* contaminated diet with a DON content of about 6 mg/kg, which resulted in a daily DON dose of 140-250 µg/kg LW/d. Even if this dose is divided in half for the DON dose per meal in the case of restrictive feeding regimen, the dose will be much higher than the above mentioned minimum emetic dose of 50 µg/kg LW using pure toxin (Pestka et al. 1987, Prelusky and Trenholm 1993). Therefore, it can be assumed that pure DON is possibly more readily available for absorption compared to DON from a naturally contaminated source, as this toxin possibly has to be released from organic bindings. The lack of emesis despite the higher dose due to slower DON release after oral exposure to naturally contaminated feedstuff does not necessarily result in a lower systemic availability of DON, but in a longer period of systemic distribution. On the other hand, all pigs dosed intravenously with 53 µg/kg LW vomited within a few minutes, but after 30 min no further emesis occurred (Paper II, Paper IV), indicating that DON has no long-lasting emetic effect.

Besides the decreased feed intake of pigs fed *ad libitum* (Figure 2) it was noticed that pigs fed the DON contaminated diet restrictively consumed the amount of diet more slowly than the restrictive control group (Paper I, Figure 1). Therefore, it can be suggested that adverse sensory effects of DON attenuated feed acceptance in pigs. This was also concluded by Prelusky (1997), who reported a lesser effect on feed intake of pigs after intraperitoneal (ip) compared to oral DON exposure, although DON is likely to be systemically available immediately after its injection. In contrast, Clark et al. (1987) were not able to observe a change in the diet preference of rats with the choice between control and DON contaminated diet (0.25 – 8 mg/kg), indicating that dietary taste *per se* is not altered by DON concentrations of 8 mg/kg in rats. But it has to be taken into account that swine are probably more sensitive

to the taste of DON than rodents, which also required higher levels of DON (20 – 40 mg/kg) to induce feed refusal (Vesonder et al. 1973, 1979). Furthermore, a concomitant treatment of a novel taste (saccharin) with DON either orally or intraperitoneally of rats and pigs was able to establish a conditioned taste aversion, whereby this effect was related to the area postrema and might therefore be postingestional (Clark et al. 1987, Ossenkopp et al. 1994, Osweiler et al. 1990). However, this conditioned taste aversion was only transitory, suggesting that part of the feed refusal syndrome may be a learned response (Clark et al. 1987).

A decrease in feed intake might further be a result of an inhibition of gastric emptying and small intestine motility as observed by Fioramonti et al. (1993) following oral DON administration (50 – 1000 µg/kg LW) in mice and rats. Additionally, one element of feed refusal could be due to an upregulation of pro-inflammatory cytokines as particularly IL-6 is known to induce anorexia (EFSA 2004, Wong and Pinkney 2004). Anyway, it has to be emphasized that the DON effect on feed intake is highly variable in pigs. While a few pigs already completely refused a DON contaminated diet at the first exposure, other pigs consumed as much as control animals. The reason for this variability has to be clarified.

2 Absorption and gastrointestinal effects

Deoxynivalenol appeared to be rapidly absorbed following ingestion of a naturally *Fusarium* contaminated diet, since (1) DON was found in serum samples within 15 min, (2) half-life of absorption ($t_{1/2}\alpha$) ranged between 8 - 104 min and (3) maximum serum concentrations (t_{max}) were reached within 49 - 172 min (Paper II). Additionally, Dänicke et al. (2004b) investigated the passage of DON in pigs using a comparative slaughter technique (0, 1, 2, 3, 4, 5, 6, 8, 15, 18, 24 h) and found that DON ($t_{1/2}$ = 1.8 h) passed the stomach more rapidly as compared to the dry matter ($t_{1/2}$ = 4.8 h) following feeding a diet contaminated naturally with *Fusarium* of 4.2 mg DON/kg. The disappearance of DON from the stomach corresponded with a rapid increase of DON in the ingesta of the first part of the small intestine (t_{max} = 3.4 h), but this fraction accounted for less than 1 % of the total DON intake. Therefore, it was suggested that absorption of DON may start in the stomach and is nearly completed in the upper part of the duodenum (Dänicke et al. 2004b, Eriksen et al. 2003).

Furthermore, the bioavailability from a diet contaminated naturally with *Fusarium* ranged between 66 - 132 % and 28 - 78 % for chronic and acute dietary exposure, respectively (Paper II, Table 4 & 5), when free (unconjugated) DON in serum was determined (without incubation of β-glucuronidase; Figure 3). However, the higher bioavailability after chronic

DON ingestion may be due to a higher baseline level of DON. In addition, a bioavailability of about 50 % would be in line with the finding that 49.7 ± 9.3 % of the ingested DON was excreted via urine (Paper II, Table 6). A rapid absorption of DON and a bioavailability of 55 % after intragastrical gavage of high doses of radio-labeled DON to pigs was also reported by Prelusky et al. (1988, 1990).

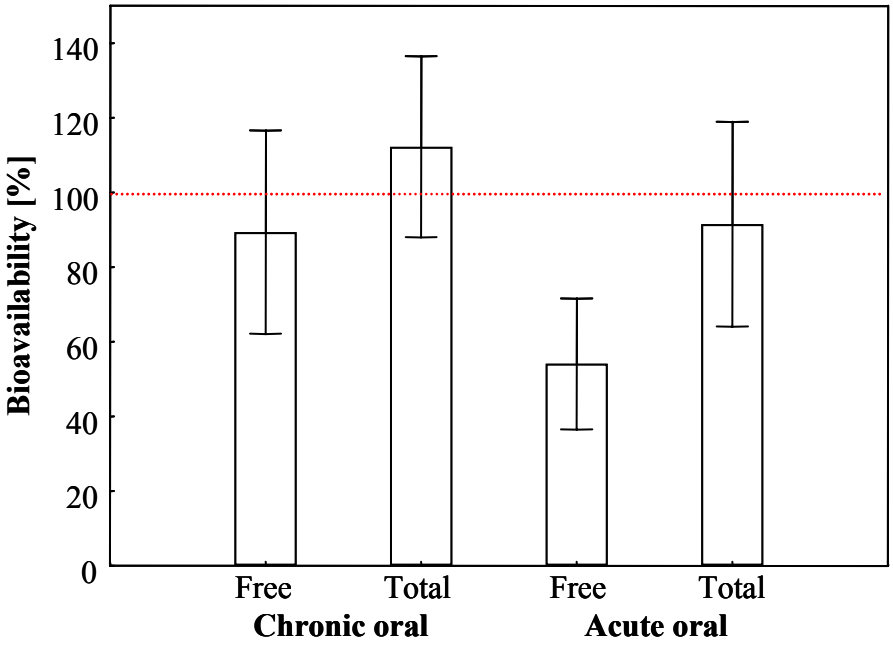

Figure 3 Bioavailability [%] of DON in pigs fed a *Fusarium* contaminated diet of 5.7 mg DON/kg either chronically (≥ 4 weeks, n = 5) or acutely (once, n = 6) when serum was treated without (free DON) or with β-glucuronidase (total DON) (means ± SD)

On the other hand, when total serum DON (free + conjugated DON, after incubation with β-glucuronidase) was considered, a bioavailability of 85 - 127 % and 43 - 115 % in the chronic and acute group was found (Figure 3), respectively. This nearly total systemic availability may explain the finding of Dänicke et al. (2004b) that less than 1 % of ingested DON can be detected in the ingesta of duodenum. Therefore, a determination of only the unconjugated (free) part of DON in serum or plasma may probably underestimate the bioavailability of DON. This has to be taken into account when interpreting the results of Prelusky et al. (1988), who only used free DON for estimating the bioavailability.

Additionally, pigs acutely exposed to a DON contaminated diet appeared to have a higher value of conjugated DON (9 – 60 %) compared to chronically DON exposed pigs (0 – 36 %), indicating that a chronic DON exposure may reduce the glucuronidation grade. Besides the

fact that conjugation with glucuronic acid is known to enhance water solubility and therefore urinary or biliary excretion, the decreased proportion of free DON in acutely exposed pigs might cause a lower elimination half-life compared to chronically exposed pigs (5.3 ± 2.4 h *versus* 6.3 ± 2.4 h). In contrast, a longer elimination half-life for conjugated DON was described in sheep (Prelusky et al. 1985, 1986b, 1987). Moreover, it could be suggested that glucuronide conjugated DON is not as toxic as free DON, as it might not fit into the activation centre of the 60S subunit of eukaryotic ribosomes. However, these results should not be overestimated, because of the high inter-individual variation of the glucuronidation grade.

On the one hand, one can assume that almost 100 % of the ingested DON is systemically available. On the other hand the question emerges of whether DON itself has an influence on structures before or at absorption.

Following the ingestion of contaminated food or feed, the gastrointestinal tract is the first barrier before entry of mycotoxins and might therefore be exposed to high toxin concentrations (Bouhet and Oswald 2005). Intercellular structures including tight junctions, adhere junctions and desmosomes are necessary for the functioning of a physical barrier, whereby the trans-epithelial electrical resistance (TEER) of cell monolayers are regarded as an appropriate indicator for epithelial integrity (as reviewed by Bouhet and Oswald 2005). Deoxynivalenol and several other toxins were found to decrease the TEER of a human epithelial cell line (Maresca et al. 2002). These disaggregating effects of mycotoxins on epithelial intestinal cells may at least in part explain the intestinal lesions observed in humans and animals.

In any case, alterations of the gastrointestinal tract, such as corrugation of the mucosa in the stomach, duodenitis, jejunitis, intestinal bleeding and necrosis, have been associated with the exposure to DON and other *Fusarium* toxins (Arnold et al. 1986b, D´Mello et al. 1999, Forsell et al. 1987, Rotter et al. 1994). However, in the present studies the macroscopic examination of the gastrointestinal tract (oesophagus, stomach, small and large intestine) as well as the other examined organs (liver, kidney, spleen) revealed no such changes (Paper I). Marked lesions of the gastrointestinal tract may possibly occur only at extremely high DON doses or be caused by other factors such as stress, high feed intake or too finely ground diets.

Moreover, DON was shown to significantly inhibit the activities of intestinal transporters in the human epithelial intestinal cell line HT-29-D4 *in vitro* at low concentration (10 µM/l = 2.96 µg DON/ml) by 50, 42, 30 and 38 % in the sodium-dependent D-glucose transporter, D-

fructose transporter, the active and passive L-serine transporters, respectively, whereas the passive transporter of D-glucose was only slightly decreased by 15 % (Maresca et al. 2002). In contrast, DON did not affect lipid uptake (palmitic acid and cholesterol) in the latter study (Maresca et al. 2002). Additionally, impaired intestinal transfer and uptake of glucose were reported in mice and broilers after dietary exposure to 10 mg/kg pure DON over 6 weeks (Awad et al. 2004, Hunder et al. 1991). On the other hand, in the jejunum of chronically exposed (5.7 mg DON/kg) pigs originating from the present investigations, Zerull et al. (2006) were not able to find a negative effect on Na^+-dependent glucose transporters.

Maresca et al. (2002) suggested that low amounts of DON (< 10 µmol/l = 2.96 µg/ml) may cause an aqueous diarrhoea because of inhibition of the intestinal sodium-dependent D-glucose transporter which results in a reduced D-glucose-associated water absorption and therefore an increased water content in the intestinal lumen. However, pigs fed a DON contaminated diet of 6 mg/kg showed no clinical signs of diarrhoea. Furthermore, dry matter content of faeces obtained by the balance studies were not different between pigs fed a DON contaminated or control diet.

In order to maintain an effective barrier function, intestinal epithelia have to rapidly regenerate throughout life (regeneration of entire intestinal tissue in approximately one week) (Bouhet and Oswald 2005). In any case, DON was reported to inhibit the proliferation of human colonic epithelial cells *in vitro* (Maresca et al. 2002) and was found to interfere with structural and functional characteristics of enterocyte differentiation *in vitro* (Kasuga et al. 1998). On the other hand, in the present investigation, DON exposure (chronic and acute oral, iv) of pigs had no effect on *in vivo* protein synthesis (incorporation of [$^2H_5$]phenylalanine) of duodenum, jejunum, jejunal mucosa cells or ileum compared to the control group (Dänicke et al. 2006).

As DON possibly interferes with nutrient uptake and structural characteristics of the gastrointestinal tract, it can be assumed that also the feed efficiency and digestibility of nutrients may be modulated by DON.

However, feed efficiency was improved in pigs after dietary treatment of 4 mg/kg pure DON over 7 days (Prelusky 1997), this could not be established after oral exposure of 3 mg/kg pure DON over a period of several weeks (Prelusky et al. 1994). However, it has to be noted that pigs fed a diet contaminated naturally with *Fusarium* of 6 mg DON/kg *ad libitum* or

restrictively over a period of 11 weeks exhibited no change in feed-to-gain ratio compared to the corresponding control group (Paper I, Table 3). Furthermore, it can not to be overlooked that the feed-to-gain ratio of pigs in the present investigation was not affected by DON although the nutrient digestibility appeared to be improved (Paper I, Table 4).

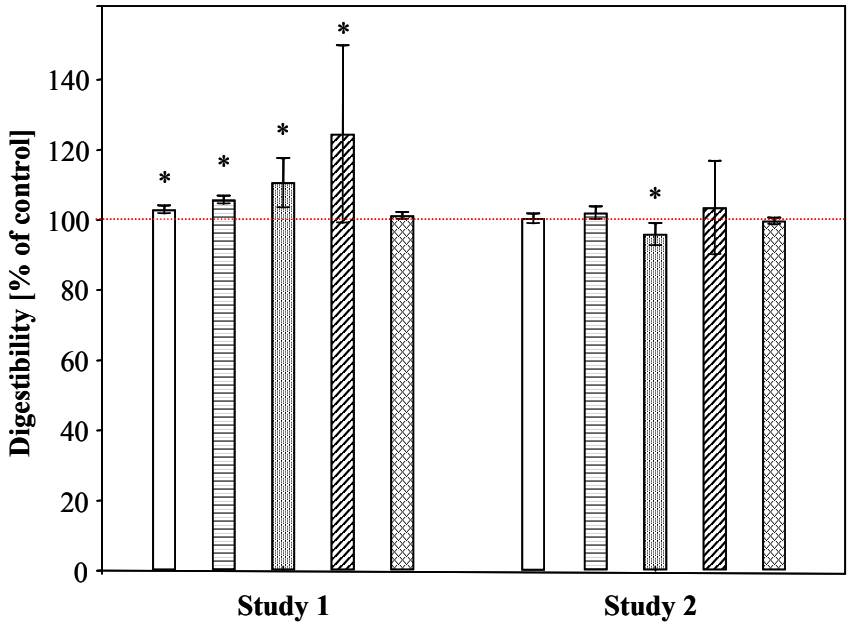

Figure 4 Digestibility in % of control of organic matter (▭), crude protein (▤), crude fat (▦), crude fibre (▨) and N-free extractives (▩) after feeding pigs (Study 1, n = 5 (Paper I); Study 2, n = 6) a DON contaminated diet over ≥ 4 weeks (* statistically significant from control with $P < 0.05$, means ± SD)

The effect of *Fusarium* contamination on nutrient digestibility appeared to be rather contradictory, while digestibility of crude nutrients with the exception of N-free extractives was improved in the *Fusarium* contaminated diet (Paper I, Table 4). These results could not be reproduced in a second trial investigating the same diet composition in 6 pigs (Figure 4). These findings are comparable with other investigations reporting an increased nutrient digestibility (Dänicke et al. 2004c) on the one hand, but no or inconsistent (reduced to improved) effects on digestibility on the other hand (Dänicke et al. 2004a, d) due to *Fusarium* contamination. Nevertheless, it could be assumed that *Fusarium* infection results in an improved nutrient availability because of a "pre-digestion" attributable to an increased activity of non-starch-polysaccharide hydrolysing enzymes (Dänicke et al. 2004a, Matthäus et al. 2004).

3 Distribution, metabolism and toxic action

Following absorption deoxynivalenol will be distributed throughout the body with the blood. The apparent volume of distribution (V_d) of DON covered a range of 0.9 – 6.9 l/kg (Paper II, Tables 3 - 5) which is considerably higher than the total body water. This is in accordance with findings of Prelusky et al. (1988) who suggested that this fact may be due to an uptake of DON by tissues.

3.1 Effects on lymphocytes

Therefore, it is likely that DON may first act on peripheral blood lymphocytes (PBL), in particular because they are regarded as highly sensitive to DON. In Paper III, lymphocyte cultures of PBL from untreated pigs exposed to increasing concentrations of pure DON (70 - 560 ng/ml) *in vitro* caused a sigmoid inhibition (Paper III, Figure 1) in both the BrdU assay (DNA synthesis) and the MTT assay (metabolic activity), suggesting that considerable inhibition does not occur below 100 ng DON per ml supernatant and is saturated at DON concentrations above 500 ng/ml (Paper III, Figure 1).

The 50 % inhibiting DON concentration (IC_{50}) were 200 ng for DNA synthesis (BrdU assay) and 309 ng/ml for metabolic activity (MTT assay) (Figure 5), suggesting a higher sensitivity of DNA synthesis to DON *in vitro*. Furthermore, it has to be noted that saturation for both assays was found at different levels, as DNA synthesis was inhibited to nearly 100 % whereas metabolic activity was reduced by only 60 - 70 % (Paper III, Figure 1). Therefore, it can be assumed that despite complete inhibition of lymphocyte proliferation, metabolism of lymphocytes is maintained at a lower level. Accordingly, Widestrand et al. (1999) supposed that the effects of Type B trichothecenes are cytostatic rather than cytotoxic.

In accordance to the IC_{50} values obtained in the lymphocyte proliferation assays (Figure 5), protein synthesis using [^{3}H]leucine incorporation was inhibited by 50 % after incubation with 280 and 250 ng DON/ml in EL-4 T-lymphocytes (Dong et al. 1994) and rat spleen lymphocytes (Thompson and Wannemacher 1986), respectively. However, Vero cells appeared to be less susceptible to *in vitro* protein synthesis inhibition indicated by an IC_{50} level of 444 ng DON/ml (Thompson and Wannemacher 1986). It should be taken into account that primary cultures, and especially lymphocytes, appeared to be more susceptible to trichothecene mycotoxins compared to other cells and cell lines (Charoenpornsook et al. 1998, Gutleb et al. 2002, Reubel et al. 1987), with porcine cells being particularly sensitive (Hanelt et al. 1994). Furthermore, it should be mentioned that protein synthesis inhibition in

Vero cells and rat lymphocytes induced by DON was below 1 % of that of T-2 toxin (Thompson and Wannemacher 1986).

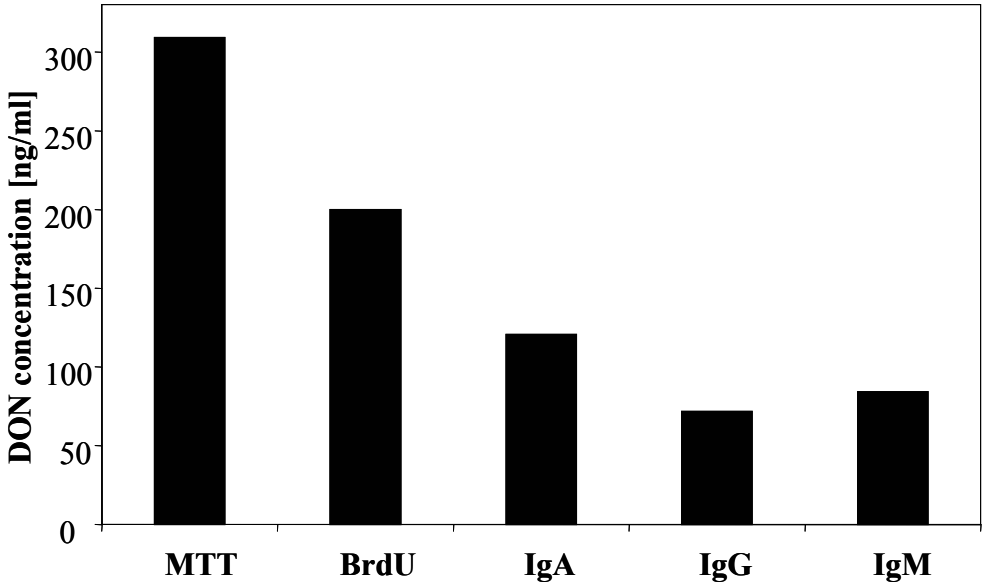

Figure 5 DON concentrations [ng/ml] inhibiting the MTT and BrdU absorbance or IgA, IgG and IgM supernatant concentrations by 50 % (IC_{50}) after *in vitro* incubation of ConA-stimulated porcine peripheral blood lymphocytes with increasing DON concentrations (0, 70, 140, 280, 560 ng/ml)

Peripheral blood lymphocytes (PBL) of pigs exposed to DON either orally (chronically and acutely) or intravenously to 53 µg/kg LW showed a decreased protein synthesis (*in vivo*, FSR), metabolic activity (*ex vivo*, MTT assay) and DNA synthesis (*ex vivo*, BrdU assay) compared to pigs fed a control diet (Figure 6). However, the reduction in absorbance of PBL of pigs fed a *Fusarium* contaminated diet of 5.7 mg DON/kg either chronically or acutely (Paper II, Table 3) was only significant in the acute DON fed group using the MTT assay, possibly because of the high inter-individual variation especially in the BrdU assay (Figure 6). Furthermore, it has to be noted that findings of *in vivo* lymphocyte protein synthesis using the incorporation of the stable isotope [$^2H_5$]phenylalanine (Paper IV) approve the results of the *ex vivo* lymphocyte proliferation assays, indicating that these assays are an appropriate method to investigate the influence of toxicants on porcine lymphocytes. This assumption may be underlined by findings of Holt et al. (1987, 1988a) and Minervini et al. (1993), who observed comparable inhibitory effects of DON and T-2 on cell metabolism (MTT cleavage), DNA ([^{3}H]thymidine incorporation) and protein synthesis ([^{3}H]leucine incorporation) in different cell lines.

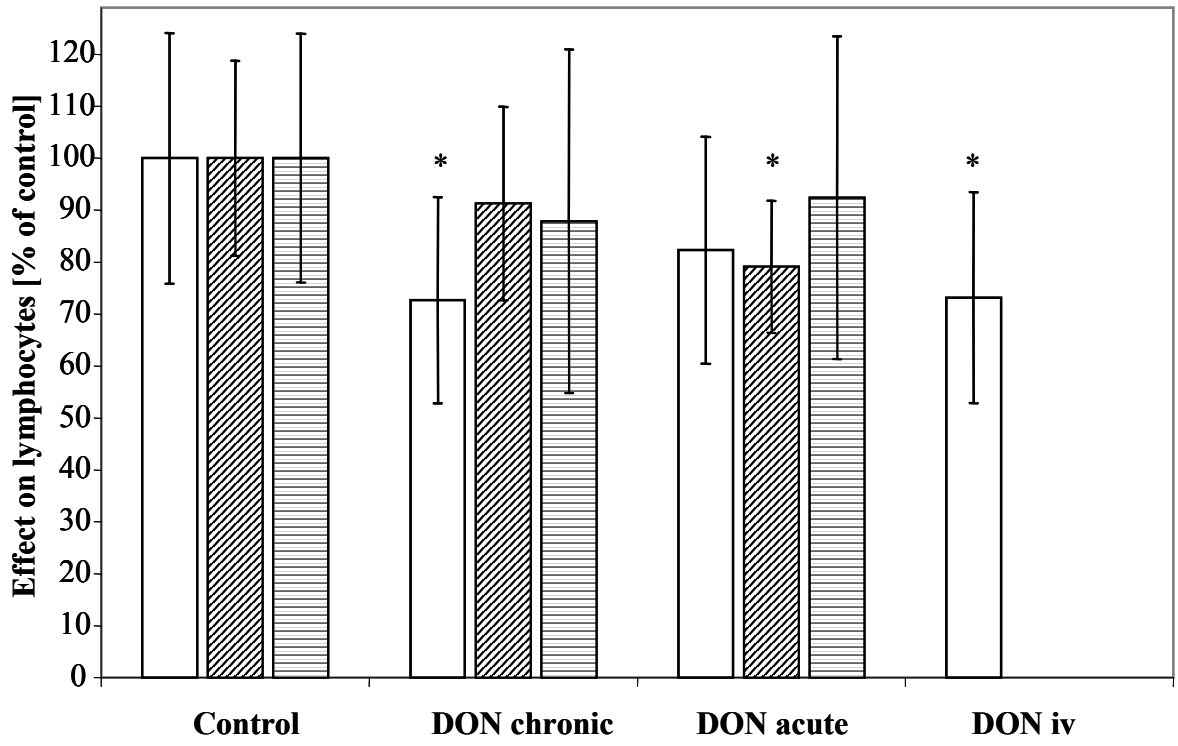

Figure 6 Effect of DON on lymphocytes [% of control] of protein synthesis (▭ FSR, *in vivo*), metabolic activity (▨ MTT, *ex vivo*) and DNA synthesis (▤ BrdU assay, *ex vivo*) after chronic and acute oral exposure to a DON contaminated diet of 5.7 mg/kg or following an intravenous (iv) application of 53 µg/kg LW to pigs compared to pigs fed a control diet (* significantly different from control with $P < 0.05$, means ± SD)

Besides the inhibition of proliferation and protein synthesis, further functions of lymphocytes may be affected by DON (Figure 7). Many *in vitro* studies in murine and human cell lines as well as *in vivo* studies in mice indicated an increase in cytokines (IL-1β, 2, 4, 5, 6, TNF-α, TGF-β, IFN-γ), chemokines (MIP-2, CINC-1, MCP-1, MCP-3, CRG-2) and other immune-relevant proteins, probably due to an inhibition of highly labile repressor proteins by trichothecenes, which results in an induction of their mRNA (Figure 7; Azcona-Olivera et al. 1995a, b, Chung et al. 2003a, Dong et al. 1994, Moon and Pestka 2002, Ouyang et al. 1995). Furthermore, it was shown that stability of mRNA for IL-6 and TNF-α was increased under the action of DON (Chung et al. 2003b, Wong et al. 2001), which may also cause an increase in mRNA levels.

Besides the regulatory effect on feed intake (Wong and Pinkney 2004), IL-6 is known to play an important role in the differentiation of B-lymphocytes into IgA secreting plasma cells (Beagley and Elson 1992). Therefore, the amplified expression of cytokines might be a reason for the frequently observed high increase in IgA serum concentrations (Figure 7), IgA-

complex deposits in the kidney mesangium and haematurie in mice exposed to DON which was linked to human IgA nephropathy (Dong et al. 1991, Dong and Pestka 1993, Forsell et al. 1986, Greene et al. 1994, Pestka 2003).

However, it has to be mentioned that a disturbance of IgA, as observed in mice, is rather inconsistent in pigs. Only few studies were able to confirm a significant IgA elevation in pigs after dietary DON (Drochner et al. 2004, Swamy et al. 2002), while others entirely failed to show an effect of DON (Bergsjø et al. 1992, 1993, Dänicke et al. 2004a, d, Döll et al. 2003, Swamy et al. 2003). IgA serum concentrations were significantly increased in pigs fed a DON contaminated diet of about 6 mg/kg over 5 weeks *ad libitum*, whereas restrictively DON fed pigs showed only a moderate increase of IgA serum levels (Paper I, Table 5), indicating that serum IgA concentrations were affected by DON only at higher doses, as the *ad libitum* fed pigs consumed more of the DON contaminated diet compared to the restrictive fed groups (Paper I, Table 3). This assumption may be further underscored by the fact that serum IgA concentrations were significantly higher in barrows with a higher feed intake than in their female counterparts with a lower feed intake, when the DON contaminated diet was offered *ad libitum* (Paper I). Furthermore, pigs exposed to dietary DON acutely (once) or chronically (≥ 4 weeks) displayed no change in IgA serum concentration (Paper III, Figure 5), but it has to be taken into account that these pigs were fed restrictively as well. On the other hand, serum IgG and IgM were significantly increased in pigs fed the DON contaminated diet once (acute DON exposure, Paper III, Figure 5). In contrast, most studies in mice observed a concomitant decrease in IgM and IgG values besides an elevation in IgA serum concentrations (Forsell et al. 1986, Pestka 2003). However, Atroshi et al. (1994) found a significant increase of IgA, IgG and IgM serum concentrations in mice fed a DON contaminated diet of 6.25 mg/kg LW for one week, but no significant effects on IgG and IgM values after a single dose of 12.5 mg/kg LW. Possibly, the minor DON effects on immunoglobulin levels of pigs compared to studies in mice may be due to the lower DON concentrations used, but further research is necessary to clarify potential differences in the mode of action between these two species.

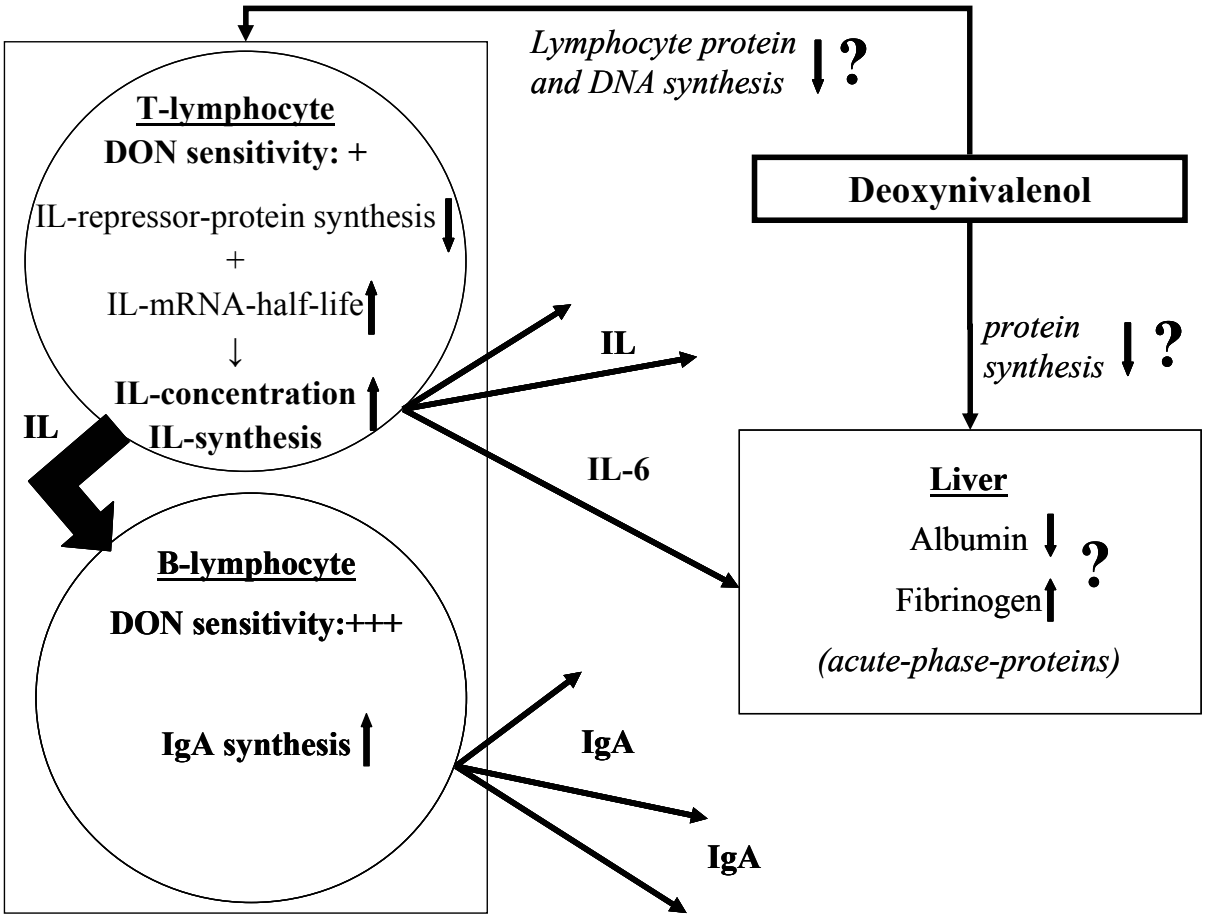

Figure 7 It is suggested that the trichothecene mycotoxin deoxynivalenol primarily inhibits highly sensitive repressor proteins. In the case of lymphocytes the synthesis of interleukin (IL) repressor protein might be reduced which will cause, in combination with a higher IL-half-life, an increase of IL concentrations. IL might further stimulate B-lymphocytes to synthesize immunoglobulins (Ig), especially IgA. For example, IL-6 mediates the acute-phase-response, which is characterized by decreased albumin, but increased fibrinogen plasma concentrations. However, plasma concentrations could hardly reflect the *in vivo* albumin and fibrinogen synthesis. Therefore, the effect of DON on *in vivo* protein synthesis of albumin, fibrinogen and lymphocytes has to be clarified (for references see text).

Furthermore, it has to be mentioned that the supernatant immunoglobulin concentrations of nonstimulated or ConA-stimulated lymphocyte cultures obtained from pigs fed a control or a DON contaminated diet of 5.7 mg/kg either acutely or chronically (*ex vivo*, Paper III) are not correlated with serum immunoglobulin levels of those pigs (Figure 8). Therefore, it seems questionable if one could predict a disturbance of parameters like immunoglobulins from such *in vitro* cultures, as this would assess only a limited view of the whole immune system.

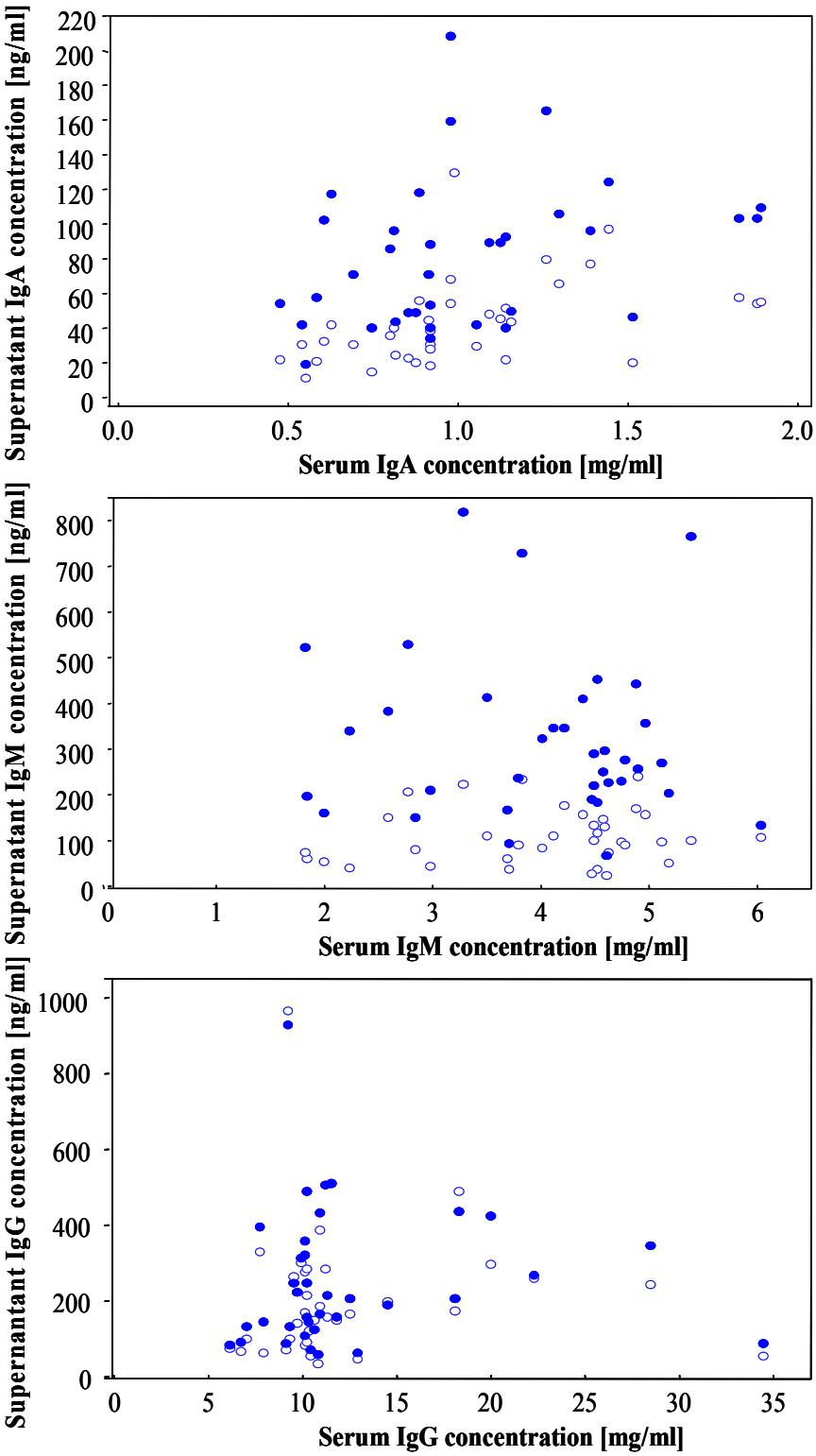

Figure 8 Supernatant immunoglobulin (IgA, IgM, IgG) concentrations [ng/ml] of nonstimulated (○) and ConA-stimulated (●) porcine peripheral blood lymphocyte cultures in relation to the serum Ig concentration [mg/ml] of pigs fed a control or DON contaminated diet (5.7 mg/kg; acute and chronic)

On the other hand, IgA, and to a lower extent IgG, concentrations of supernatants incubated with increasing DON concentrations *in vitro* (Paper III) correlated linearly with the metabolic activity (MTT assay), but not with the DNA synthesis (BrdU assay) of lymphocyte cultures (Figure 9), indicating that immunoglobulin synthesis may be more dependent on metabolic activity than on an increasing number of B-lymphocytes.

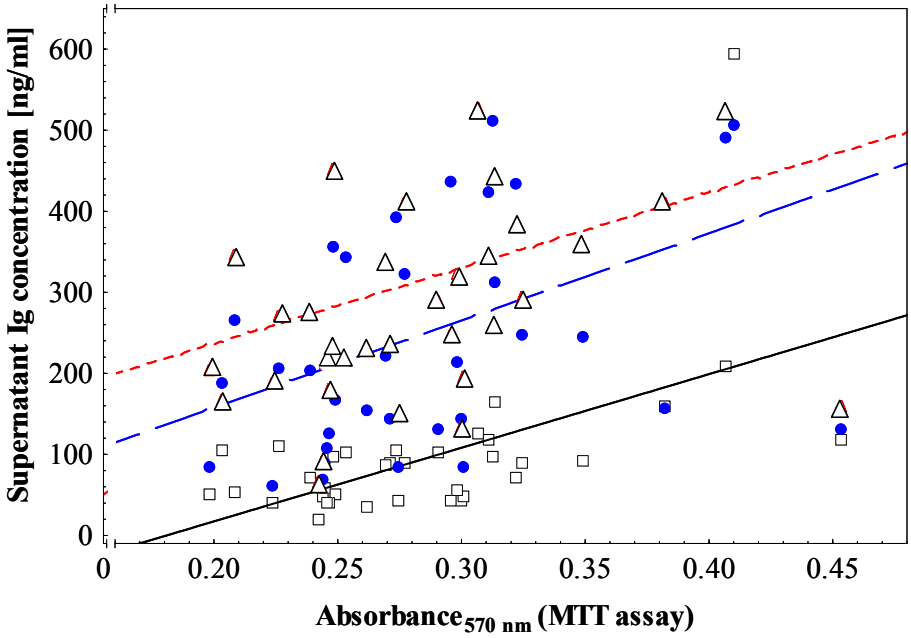

Figure 9 Supernatant immunoglobulin (Ig) concentrations [ng/ml] with increasing absorbance (570 nm; MTT assay) after feeding a control (n = 11) and DON contaminated diet chronically (n = 13) or acutely (n = 12) to pigs (IgA = -164 + 908*x; $r^2 = 0.32$; $p < 0.001$; IgG = -58 + 1076*x; $r^2 = 0.12$; $p = 0.036$; IgM = 50 + 932*x; $r^2 = 0.09$; $p = 0.068$)

Moreover, after incubation (72 h) of porcine PBL with increasing DON concentrations (0, 70, 140, 280, and 560 ng/ml) *in vitro* (Paper III), it was observed that IgA as well as IgG and IgM supernatant concentrations were dose-dependently inhibited with IC_{50} values of 120.6, 71.7 and 84.1 ng/ml, respectively (Figure 5). However, it should be noted that these inhibiting concentrations indicate a higher sensitivity of immunoglobulins to adverse effects of DON compared to the MTT (IC_{50} = 309 ng/ml) and BrdU (IC_{50} = 200 ng/ml) assay with IgG and IgM being more sensitive than IgA (Figure 5). This prompts the assumption that lymphocytes will first slope the production of secretory proteins, such as immunoglobulins, and thereafter their proliferation (measured by DNA synthesis) to preserve their maintenance requirements when exposed to toxic agents.

3.2 Effects on haematology and clinical-chemical parameters

As DON can be found rapidly in the blood and remains there for several hours, it could be suggested that it might modify the number or proportion of blood cells. For example, granulo-monocytic progenitors (CFU-GM) from human umbilical cord blood and from rat bone marrow were reported to be inhibited when cultured in the presence of DON for 14 days and measured by the clonogenic assay (Lautrait et al. 1997).

In mice, the total leukocyte count was decreased after 8 weeks of exposure to 25 mg/kg pure DON (Forsell et al. 1986). However, in the present investigations no significant effect of a chronic dietary exposure to 6 mg DON/kg could be observed on haematological parameters (Paper I, Table 5; Paper III, Table 2). Accordingly, haematological parameters were not affected by dietary DON in rats, horses and pigs (Arnold et al. 1986a, Raymond et al. 2003, Swamy et al. 2002). Therefore, it could be concluded that haematological parameters seemed not to be a sensitive marker for DON exposure, in particular because many situations such as stress and immune response to other agents might influence the results.

Piglets fed DON contaminated diets of 9 – 43 mg/kg exhibited alterations in different serum parameters (Young et al. 1983). However, it must be noted that the authors were not able to separate these effects from the feed intake level (Young et al. 1983). Furthermore, it has to be emphasized that in the present investigations (Paper I, Table 6; Paper IV, Table 3) dietary DON of ~ 6 mg/kg at a similar feed intake (restrictive feeding regimen) failed to affect clinical-chemical parameters in pigs, indicating that these parameters are not specific enough to demonstrate effects of DON.

3.3 Effects on the liver and detoxification

The liver will probably be the first organ reached by DON with venous blood after its absorption from the intestine. Furthermore, the liver is known to detoxify many toxic agents. Interestingly though, de-epoxy-DON is the primary metabolite found in urine and faeces of animals exposed to DON, but de-epoxy DON is produced via intestinal and rumen microbe activity (Cote et al. 1986b, Eriksen et al. 2002, He et al. 1992, Swanson et al. 1988) rather than by the liver or other organs (Cote et al. 1987, Gareis et al. 1987).

In any case, it was noticed in the present investigations that not all pigs have the ability to de-epoxidate DON (Paper II, Table 6). This is in accordance with Eriksen et al. (2002), who observed that specific pathogen-free pigs or pigs from research farms lacked the ability to de-epoxidate trichothecenes, while pigs from commercial pig farms had the ability. Furthermore, Eriksen et al. (2003) concluded that the de-epoxidation activity in pigs is not a protection

against the toxic effect of DON, because it occurs principally in the large intestine (microbes) when most of the toxin is already absorbed (Dänicke et al. 2004b).

Moreover, glucuronide conjugation of DON, a major Phase II reaction to enhance water solubility and urinary or biliary excretion, was assumed to occur predominantly in the liver (Galtier and Alvinerie 1981, Gareis et al. 1987, Prelusky et al. 1986b). However, the lack of glucuronide conjugation after intravenous DON application in contrast to DON-glucuronidation after oral DON exposure (Paper II, Tables 3-5) can not only be explained by a "first pass" effect, because intravenous DON probably passed the liver several 100 times within 24 h. Since glucuronidating enzymes (Phase II) also exist in extrahepatic tissues, it was suggested that DON was conjugated in the intestine before absorption. This assumption may be underlined by Olsen et al. (1987) who described how zearalenone is probably conjugated before it is reduced to zearalenol by intestinal mucosa *in vitro*. Furthermore, it can be supposed that ingesta or intestinal microbes are involved in the glucuronidation process.

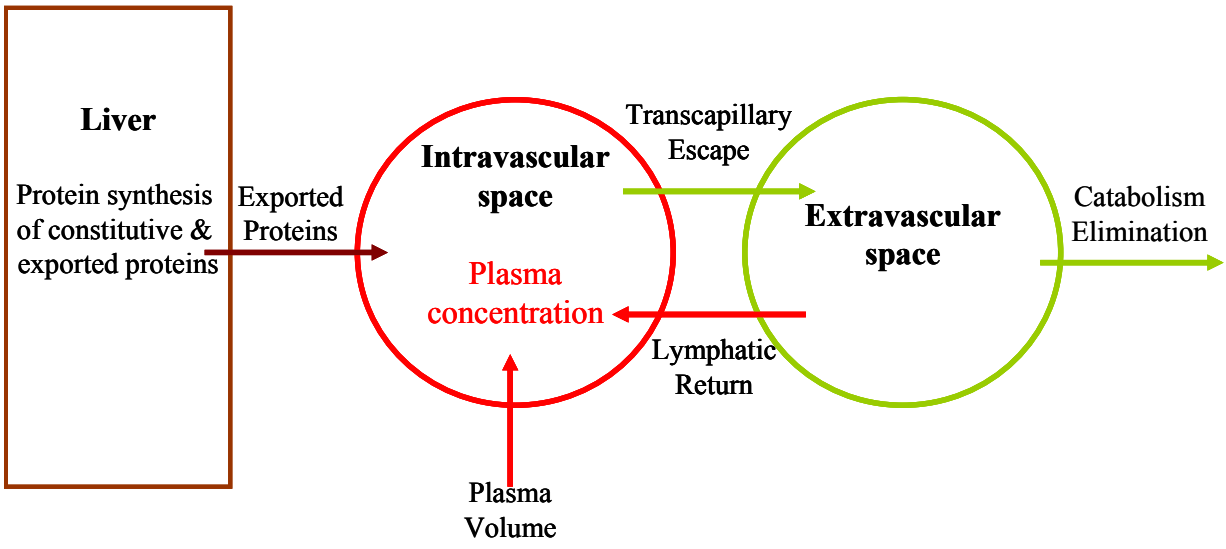

Figure 10 Protein synthesis can hardly be reflected by plasma protein concentration, since the latter will be influenced additionally by the plasma volume, transcapillary escape, lymphatic return, catabolism and elimination

Since protein synthesis inhibition is the main mode of action of trichothecenes and the liver one of the most important protein production sites, it seems likely that DON may particularly affect the liver protein synthesis. However, it has to be emphasized that plasma protein concentrations are not appropriate to ascribe protein synthesis because many other factors such as plasma volume, transcapillary escape and elimination can influence the concentration

(Figure 10). Only few studies examined the effects of trichothecenes on protein synthesis of tissues *in vivo* using radioactivity measurements by incorporation of [^{14}C]leucine in rodent models. But in any case, [^{14}C]leucine uptake in the kidney, liver and spleen of mice was reduced after ip injection of 20 and 80 mg DON/kg LW, but not at 4 and 10 mg/kg LW (Robbana-Barnat et al. 1987). Furthermore, Azcona-Olivera et al. (1995a) observed a protein synthesis inhibition in all examined tissues (spleen, Peyer´s patches, kidney, liver, small intestine and plasma) of mice 3 h after oral exposure to 5 and 25 mg DON per kg live weight, respectively, but it appeared to recover after 6 and 9 h in mice receiving the low DON dose. However, it has to be noted that both of these studies used very high doses of pure toxin, which may not apply to the exposure under practical conditions.

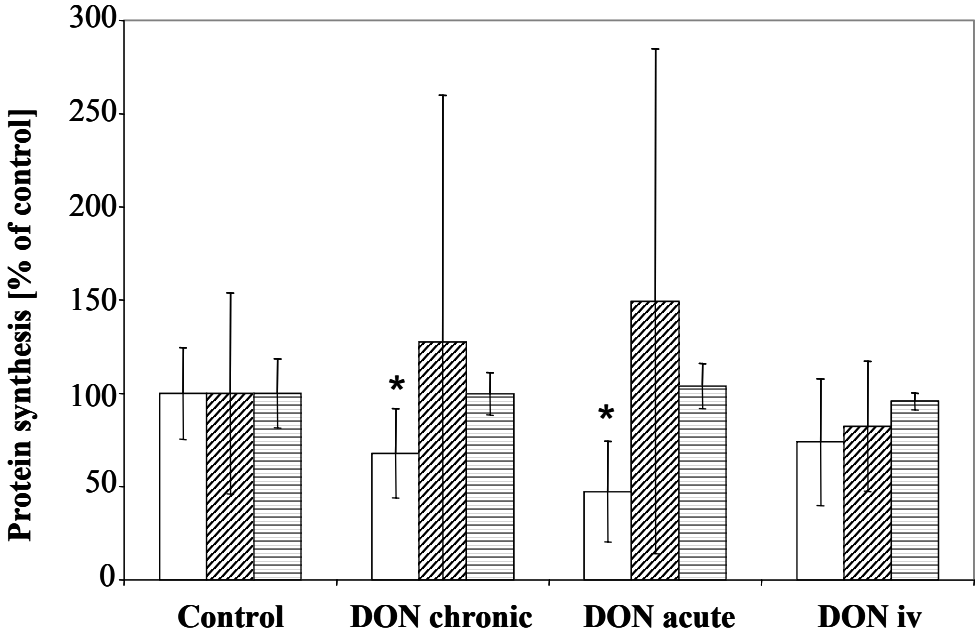

Figure 11 Effect of DON on *in vivo* protein synthesis (FSR) [% of control] of albumin (▭), fibrinogen (▨) and total liver protein (▤ according to Dänicke et al. 2006) after chronic or acute oral exposure to a DON contaminated diet of 5.7 mg/kg or following an intravenous (iv) application of 53 µg/kg LW to pigs compared to pigs fed a control diet (* significantly different from control with $P < 0.05$, means ± SD)

Therefore, we investigated two of the most abundant blood proteins (albumin and fibrinogen) in pigs after dietary exposure to a diet contaminated naturally with *Fusarium* (5.7 mg DON/kg) compared to a control and an intravenously (53 µg/kg LW) dosed group using a "flooding dose" of the stable isotope [$^{2}H_5$]phenylalanine (Paper IV). Although albumin plasma concentrations were not affected by treatment (Paper IV, Table 4), fractional albumin

synthesis rates (FSR, %/d) of pigs weighing ~ 40 kg were significantly decreased by 32 and 45 % after chronic and acute dietary DON exposure compared to the control fed group (Figure 11). Absolute synthesis rates (ASR, g/d), as the product of the intravascular albumin mass and the FSR, were affected in the same fashion as chronic and acute dietary DON exposure caused an inhibition by 30 and 49 %, respectively (Paper IV, Table 4). The decrease of FSR and ASR of pigs dosed intravenously by 26 and 24 % was not significant (Figure 11), probably because of the high inter-individual variation (Paper IV, Table 4). It should further be noted that albumin secretion time (t_s), that means the time required before newly synthesized albumin is occurring in the plasma, varied between 6.8 – 34.4 min independent of treatment. In contrast, fibrinogen synthesis was not affected by DON exposure (Paper IV, Table 5), but displayed a high inter-individual variation in all of the measured parameters (Figure 11), indicating that fibrinogen is not an appropriate parameter to assess DON effects on *in vivo* protein synthesis. Moreover, the effects of an acute-phase response, possibly due to a DON-related IL-6 up-regulation (Pestka et al. 2004), might enhance (albumin) or diminish (fibrinogen) the effects on *in vivo* protein synthesis.

Furthermore, the DON exposure of pigs, either orally (5.7 mg/kg; ~ 80 µg/kg LW) or intravenously (53 µg/kg LW) seemed not to significantly modulate total liver protein synthesis (synthesis of constitutive and exported proteins) *in vivo* (Figure 11; Dänicke et al. 2006), indicating that the inhibiting effect on albumin synthesis is overlaid by several other proteins synthesized in the liver, as for example fibrinogen. However, RNA content of the liver was significantly increased in both oral groups (chronic and acute DON) (Dänicke et al. 2006). In addition, DON appeared not to affect *in vivo* protein synthesis of *musculus longissimus thoracis*, heart, kidney, lung, pancreas, duodenum, jejunum, jejunal mucosa cells and mesenterial lymph nodes (Dänicke et al. 2006). The effect of DON on ileal protein synthesis seemed to be rather inconsistent, as FSR was decreased after chronic, but increased after acute, dietary DON exposure (Dänicke et al. 2006). On the other hand, fractional synthesis rates of spleens were significantly reduced after chronic dietary DON of 5.7 mg/kg (- 25%) and intravenous DON exposure (-19 %), whereas the decrease by 16 % in the acute oral DON group could not be statistically supported (Dänicke et al. 2006). Interestingly, the DON effects on splenic protein synthesis are in accordance with the findings of *in vivo* protein synthesis of peripheral blood lymphocytes (Paper IV, Figure 5). The splenic FSR seemed to be twice as high as the FSR of PBL, prompting the assumption that the lymphatic tissue of the spleen is more activated possibly due to maturing of T- and B-lymphocytes in the growing pig.

In summary, when feed was offered for *ad libitum* consumption, dietary DON of about 6 mg/kg decreased feed intake by 15 % (Figure 12). Within a fattening period of 11 weeks, the mean feed consumption of the DON contaminated diet was approximately 2.5 kg/d, accounting for a voluntary dietary DON intake of about 15 mg DON/d. The absorption of nutrients seemed not be affected by DON, as for example, no effect on the jejunal glucose transporter (Zerull et al. 2006) was observed. Furthermore, the digestibility of crude nutrients varied inconsistently with DON ranging from reduced to enhanced digestibility.

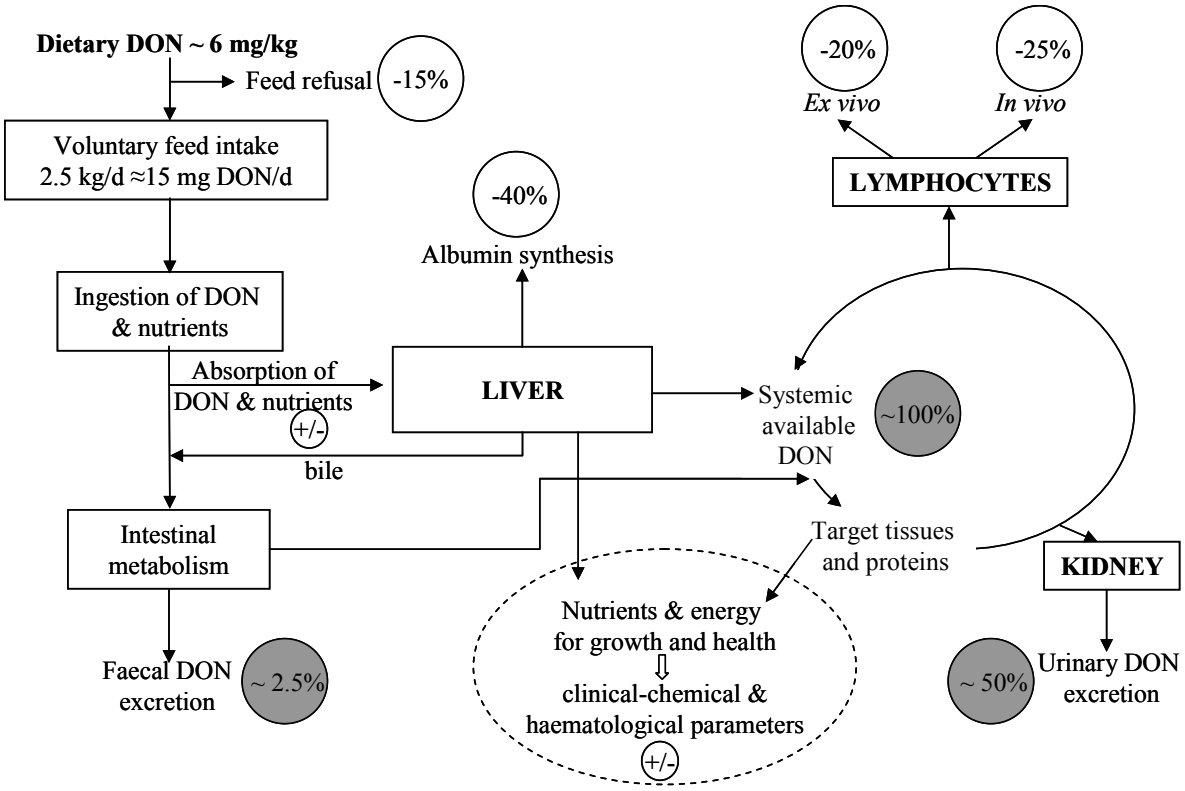

Figure 12 Scheme of the effects after dietary exposure of pigs to a DON contaminated diet of ~ 6 mg/kg (% indicated the difference from control values; % indicated the percentage of ingested DON; +/- no or inconsistent effects; see text for more information)

DON from naturally contaminated wheat appeared to be rapidly and nearly completely absorbed, resulting in a systemic availability of about 100 %. The urinary route was the main path of DON excretion (~ 50 %), whereas faecal excretion (~ 2.5 %) contributes only to a small extent to the total DON elimination. Thus, it can be assumed that DON was either metabolized to unknown substances, which might also have an influence on target tissues, or was eliminated by other pathways such as saliva, transpiration or exhalation. Protein synthesis inhibition was confirmed in the present investigation, as albumin synthesis was reduced by

about 40 % after dietary DON treatment, whereas total liver protein synthesis (Dänicke et al. 2006) was not consistently affected. Lymphocyte proliferation (*ex vivo*) and protein synthesis (*in vivo*) was decreased by 20 % and 25 %, respectively. It is suggested that the systemic toxic effects of DON seemed not to influence the nutrient and energy requirements and availability, as pigs showed no signs of growth retardation (restrictive feeding regimen) or health disturbances. Furthermore, clinical-chemical and haematological parameters appeared not to be specific enough to deduce on a DON exposure.

CONCLUSIONS

It was shown that a dietary DON concentration of about 6 mg/kg affects the performance of pigs mainly by a decrease in feed intake, since no differences could be observed following restrictive feeding.

No evidence of an altered nutrient digestibility due to a naturally *Fusarium* contamination could be concluded, because the results are rather inconsistent (ranging from reduced to enhanced effects).

Haematological and clinical-chemical parameters, such as plasma protein concentrations and activity of liver enzymes, seemed not to be specific enough to demonstrate effects of a chronic DON exposure.

In pigs, deoxynivalenol from a naturally *Fusarium* contaminated source appeared to be rapidly absorbed, since it is found in serum within 15 min after feeding.

Considering only free (unconjugated) serum DON, the bioavailability of 89.4 ± 27.2 and 54.1 ± 17.6 % after chronic and dietary exposure, respectively, may be underestimated, as the systemic availability of total (unconjugated + conjugated) serum DON is about 100 % for both type of exposure.

The apparent volume of distribution of DON in pigs exceeded the volume of total body water, indicating a DON uptake of tissues. On the other hand, DON was not close or lower than the detection limit in serum 24 h after ingestion of a meal containing 3.1 mg DON, because of the elimination half-life ($t_{1/2}\beta$) of 2.84 – 9.75 h.

Ingested DON was excreted mainly by urine (49.7 ± 9.3 %) and only to a small extent by faeces (2.5 ± 2.4 %). However, the total recovery was only 54.0 ± 9.7 %, indicating a

metabolism to still unknown DON metabolites or another way of excretion (expiratory, salivary or transpiratory losses).

The ability to de-epoxidate DON to its nearly non-toxic metabolite de-epoxy-DON was found in only few pigs, whereby this metabolite was primarily detected in the faeces, only to a limited extent in the urine, but not at all in the serum of pigs. Since this metabolism obviously occurs principally through adapted microbes in the large intestine, it seemed not to contribute to a considerable detoxification of DON for the pig.

A glucuronidation of DON was observed in serum of pigs exposed to a DON contaminated diet, whereas it did not appear after an intravenous DON application. Therefore, it was supposed that glucuronidation of DON proceeds in the intestine either by ingesta or by microbes before or during absorption rather than in the liver.

Porcine blood lymphocyte cultures exposed to pure DON *in vitro* showed a higher sensitivity of DNA synthesis (BrdU assay, IC_{50} = 200 ng DON/ml) than of their metabolic activity (MTT assay, IC_{50} = 309 ng DON/ml). Furthermore, at high DON concentrations, DNA synthesis of cultured lymphocytes was nearly completely inhibited, whereas metabolic activity was only reduced by 60 %, suggesting that DON effects on lymphocytes are cytostatic rather than cytotoxic.

Lymphocytes obtained from pigs fed a DON contaminated diet of 5.7 mg/kg either acutely or chronically exhibited a lower DNA synthesis and metabolic activity after *ex vivo* culturing. Moreover, *in vivo* protein synthesis using incorporation of [$^2H_5$]phenylalanine was also reduced after intravenous or chronic and acute oral DON exposure. Therefore, porcine lymphocytes seemed to react very sensitively to dietary DON concentrations of as low as 80 µg/kg LW/meal *ex vivo* and *in vivo*, suggesting that the *ex vivo* proliferation is a suitable technique to examine these adverse effects of DON.

Serum IgA concentrations of pigs fed a DON contaminated diet *ad libitum* were significantly increased, whereas pigs fed the same diet restrictively (lower feed intake) showed only

moderately elevated IgA serum levels, suggesting that IgA is affected only at higher DON doses. This assumption is substantiated by the fact that serum IgA concentrations of castrated males fed *ad libitum* are significantly higher than in their female counterparts, which corresponds to their higher DON intake.

Serum IgG and IgM concentrations were significantly increased in barrows once (acutely) fed a DON contaminated diet restrictively, while IgA levels were unaffected. In addition, supernatants of porcine lymphocyte cultures exposed to pure DON *in vitro* showed an inhibition of IgG and IgM at lower DON concentrations than IgA, arousing the assumption that immunoglobulins in pigs may be affected in a different way as observed in mice.

Dependent on the IC_{50} values, it could be suggested that lymphocytes *in vitro* reduced first the production of secretory proteins (immunoglobulins), and thereafter their proliferation (DNA synthesis) to prolong their maintenance requirements when exposed to toxic agents like DON.

In vivo protein synthesis of albumin was inhibited by 32, 45, and 26 % compared to the control level following chronic or acute oral and intravenous DON exposure, respectively. On the other hand, fibrinogen synthesis was not affected by DON treatment, but exhibited a very high inter-individual variation.

Pigs fed a naturally *Fusarium* contaminated diet of 5.7 mg DON/kg once (acutely) exhibited a more obvious alteration of immunological parameters (reduced metabolic activity of lymphocytes, higher IgG and IgM serum concentrations) compared to pigs after chronic DON exposure despite lower serum DON concentrations. However, inhibition of *in vivo* protein synthesis of lymphocytes and albumin was not different between the chronic and acute oral group. Thus it can be assumed that the chronically fed pigs may adapt to the effects of DON on their immune systems, but can not adjust to protein synthesis inhibition.

Further research is necessary to clarify the effects of DON on porcine Ig synthesis and lymphocyte proliferation in the view of sex and exposure (acute and chronic, restrictive and *ad libitum*) differences. However, as for albumin synthesis it can be predicted that

measurement of the immunoglobulin concentration in serum and supernatant is not able to fully describe their synthesis. *In vivo* determination of Ig synthesis using a flooding dose of stable isotope will be needed to elucidate the effect of DON on immunoglobulins.

Furthermore, it has to be emphasized that the bioavailability of DON from a naturally contaminated source was limited to wheat in the present research, but it can be supposed that the bioavailability is different in other naturally *Fusarium* contaminated materials, such as maize and straw.

SUMMARY

Tanja Goyarts

EFFECTS OF THE *FUSARIUM* TOXIN DEOXYNIVALENOL (DON) ON PROTEIN SYNTHESIS, IMMUNOLOGICAL PARAMETERS AND DON-KINETICS IN THE PIG

In northern temperate regions, the contamination of cereal grains, especially wheat, in the field with the *Fusarium* toxin deoxynivalenol (DON) is common and can not completely be prevented. Therefore, it is likely that food and feed are always contaminated to a lesser or greater extent, indicating a potential hazard to both human and farm animal health. In particular pigs react sensitively to DON, showing overt signs of toxicity such as feed refusal, emesis and diarrhoe following exposure to high DON concentrations. But the primary economical losses in animal production are due to reduced feed intake and live weight gain caused by chronic exposure to moderate amounts of DON. Furthermore, DON is described to inhibit protein synthesis via binding at 60S subunit of eukaryotic ribosomes and to modulate immune functions. These adverse effects of DON have not been studied under practical feeding conditions in pigs thus far. In addition, the toxicokinetic activity of DON may explain the susceptibility of pigs, but up to now the bioavailability of DON from a naturally contaminated source has not been examined. Moreover, it could be suggested that DON toxicokinetics and effects may be different if pigs were exposed the first time (acutely) or over a period of ≥ 4 weeks (chronically). Therefore, the present research aimed to investigate the bioavailability and the effects of DON from wheat contaminated naturally with *Fusarium* in pigs from the perspective of a chronic (≥ 4 weeks) and acute (one-time) exposure.

To evaluate the adverse effects on feed intake and performance of a naturally DON contaminated diet of 6 mg/kg, 48 crossbred (German Landrace x Pietrain) pigs of both sexes were divided into four groups (n = 12), fed a control or DON contaminated diet either *ad libitum* or restrictively over 11 weeks (26 – 100 kg live weight (LW)). The restrictive feeding regimen (the same feed intake level for all pigs) was used to separate the indirect influence of feed intake from the direct effects of DON. The voluntary feed intake and live weight gain of pigs fed the DON contaminated *ad libitum* was decreased by 15 and 13 %, respectively. Furthermore, the consumption of the DON contaminated diet appeared to be delayed, since one hour after feeding 85 % of the control, but only 39 % of the DON group had consumed all offered feed. However, the lower growth performance seemed mainly to be a result of the reduced feed intake level, as there were no differences in the pigs fed restrictively.

Accordingly, chronic dietary DON exposure was not shown to modulate haematological and clinical-chemical parameters, indicating that these parameters were not appropriate to deduce to a DON intoxication.

IgA serum concentrations of pigs were increased by 70 and 20 %, after *ad libitum* or restrictive feeding of the DON contaminated diet, respectively. It has to be noted that *ad libitum* DON fed barrows consumed more of the DON contaminated diet and showed higher serum IgA levels compared to their female counterparts, indicating that only higher DON intake may influence serum IgA concentrations in pigs.

DON affects the digestibility of nutrients rather inconsistently. In the first experiment digestibility of organic matter, crude protein, crude fat, and crude fibre, as well as the metabolizable energy and the nitrogen-balance of the *Fusarium* contaminated diet were significantly increased. On the other hand, the digestibility of crude fat was significantly decreased in a second balance study.

In castrated male pigs (41.5 ± 2.0 kg LW), dietary DON from naturally contaminated wheat appeared to be rapidly and nearly completely absorbed after chronic (≥ 4 weeks, n = 5) and acute (once, n = 6) exposure, resulting in a mean bioavailability of 89 and 54 % for free DON, and of 112 and 92 % for total (conjugated + free) DON, respectively. Following chronic or acute oral DON exposure maximal serum concentrations (C_{max}) of 21.2 and 15.2 ng/ml free DON were detected after (t_{max}) 88.4 and 99.1 min, which declined slowly thereafter with an elimination half-life ($t_{1/2}\beta$) of 6.3 and 5.3 h. While DON kinetics after oral DON exposure followed the Bateman function, serum DON concentrations decreased biphasically after intravenous application (n = 5) of 53 µg/kg LW pure DON with a $t_{1/2}\beta$ of 15.2 h. In addition, the apparent volume of distribution of DON was higher than the total body water. No remarkable DON concentrations could be detected in serum of pigs 24 hours after oral or intravenous DON exposure. Dietary DON caused a significant increase in DON excretion with urine and faeces, whereby 50 % of the ingested DON were excreted via urine and only 2.5 % via the faeces. Furthermore, it was shown that only some pigs, which received the DON diet longer than 4 weeks, had the ability to detoxify DON to its metabolite de-epoxy-DON. When pigs had the de-epoxidation ability, de-epoxy-DON was excreted only to limited extent in the urine (8.5 %), but as a main substance in faeces (88.2 %). However, it has to be noted that de-epoxy-DON contributes only to a low extent (< 2 %) to the total recovery of 54 %, based on the urinary and faecal excretion of DON and de-epoxy-DON. Therefore, it can be assumed that de-epoxidation of DON occurs predominantly through adapted microbes in the

large intestine, where unlikely systemic absorption proceeds, and will not result in a substantial detoxification for the animal.

The adverse effects of DON on the proliferation of ConA-stimulated porcine peripheral blood lymphocytes as well as immunoglobulin (IgA, IgG and IgM) concentrations was studied after adding 70 to 560 ng pure DON per ml medium *in vitro* using two different assays (BrdU incorporation and MTT cleavage). The proliferation was inhibited by 50 % (IC_{50}) at 200 and 309 ng DON/ml for the BrdU and MTT assay, respectively. IgA, IgM and IgG concentrations of lymphocyte culture supernatant were inhibited by 50 % at 121, 84 and 72 ng DON/ml, respectively, indicating that porcine lymphocytes exposed to DON first decreased the production of secretory proteins (Ig´s) and thereafter their proliferation (DNA synthesis, BrdU assay) to maintain their metabolic requirements (metabolic activity, MTT assay).

After feeding a DON contaminated diet of 5.7 mg/kg to barrows either chronically (n = 13) or acutely (n = 12) *ex vivo* lymphocyte proliferation tended to decrease compared to the control (n = 11), but the reduction was only significant in the case of acute exposure of pigs in the MTT assay. Serum IgA concentrations of pigs were not different between the groups, whereas IgM and IgG values were significantly increased in the DON acute group, suggesting that pigs exposed for the first time (acutely) to a DON contaminated diet showed a more pronounced response in the immunological parameters, although the serum DON concentration one hour after the meal were lower compared to chronically DON fed pigs.

The effect of DON on the protein synthesis of castrated male pigs of about 40 kg LW was measured using a flooding dose of the stable isotope L-[$^2H_5$]phenylalanine. The pigs were fed either a control (n = 7) or DON contaminated diet of 5.7 mg/kg chronically (n = 10) and acutely (n = 7) or were intravenously dosed with 53 µg DON/kg LW (n = 7). Plasma concentrations of total protein, albumin, fibrinogen and clinical-chemical parameters showed no difference between the groups, while protein synthesis appeared to be inhibited in lymphocytes and albumin, whereas fibrinogen synthesis was not affected. The fractional synthesis rates (FSR, %/d), meaning the percentage of protein renewed per day, were decreased by 32, 45 and 26 % for plasma albumin and by 27, 19 and 24 % for peripheral blood lymphocytes after chronic or acute oral and intravenous DON exposure, respectively.

In conclusion, DON was shown to affect the performance of growing pigs mainly by reduction of feed intake. Clinical-chemical and haematological parameters seemed not to be appropriate to deduce a DON exposure. Dietary DON of 5.7 mg/kg inhibited *in vivo* synthesis of albumin by 40 %, whereas fibrinogen synthesis was characterized by a high intra-

individual variation, but unaffected by DON. Furthermore, dietary DON appeared to disturb the immunologic function in pigs. So, lymphocyte proliferation (*ex vivo*) and lymphocyte protein synthesis (*in vivo*) was decreased by 20 and 25 %, respectively. Moreover, immunoglobulins were affected *in vitro* and *in vivo* by DON, whereby the influence of sex, feeding regimen and acute or chronic dietary DON exposure needs to be further clarified.

ZUSAMMENFASSUNG

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EFFEKTE DES *FUSARIUM* TOXINS DEOXYNIVALENOL (DON) AUF DIE PROTEINSYNTHESE, IMMUNOLOGISCHE PARAMETER UND AUF DIE DON-KINETIK BEIM SCHWEIN

Die Kontamination von Getreide, insbesondere Weizen, mit dem *Fusarium* Toxin Deoxynivalenol (DON) auf dem Feld ist in nördlich gemäßigten Regionen häufig und kann nicht vollständig verhindert werden. Daher ist es wahrscheinlich, dass Nahrung und Futter immer zu einem geringeren oder größeren Anteil kontaminiert sind, und damit von einer potentiellen Gesundheitsgefährdung von Mensch und Tier auszugehen ist. Insbesondere Schweine reagieren empfindlich gegenüber DON, mit Vergiftungserscheinungen wie Futterverweigerung, Erbrechen und Diarrhoe nach einer Belastung mit hohen DON-Konzentrationen. Die primären ökonomischen Verluste in der Tierproduktion werden jedoch durch eine chronische Exposition mit moderaten DON-Gehalten verursacht, die durch eine reduzierte Futteraufnahme und Lebendmassezunahme gekennzeichnet sind. Weiterhin wird DON die Fähigkeit zur Hemmung der Proteinsynthese durch die Bindung an die 60S Untereinheit der Ribosomen und zur Modulation von immunologischen Funktionen zugeschrieben. Bisher sind diese negativen Auswirkungen von DON jedoch noch nicht unter praktischen Fütterungsbedingungen an Schweinen erforscht worden. Zusätzlich könnte die Toxikokinetik von DON Aufschluss über die Anfälligkeit von Schweinen geben, aber bislang ist die Bioverfügbarkeit von DON aus einer natürlich kontaminierten Quelle nicht untersucht worden. Außerdem kann vermutet werden, dass die Toxikokinetik und Auswirkungen von DON unterschiedlich sind, wenn Schweine zum ersten Mal (akut) oder über einen Zeitraum von mehr als 4 Wochen (chronisch) mit DON in Kontakt kommen. Deswegen beabsichtigte die vorliegende Arbeit, die Bioverfügbarkeit und die Auswirkungen von DON aus natürlich mit *Fusarium* kontaminiertem Weizen in Schweinen im Hinblick auf eine chronische (≥ 4 Wochen) oder akute (einmalige) Exposition zu untersuchen.

Um die nachteiligen Auswirkungen auf die Futteraufnahme und Leistung einer natürlich mit DON kontaminierten Fütterung von 6 mg/kg zu evaluieren, wurden 48 Schweine einer Zweirassenkreuzung (Deutsche Landrasse x Pietrain) beiderlei Geschlechtes in vier Gruppen (n = 12) eingeteilt, die ein Kontroll- oder ein DON kontaminiertes Futter entweder zur *ad libitum* Aufnahme oder restriktiv zugeteilt über 11 Wochen (26 – 100 kg Lebendmasse (LM)) erhielten. Das restriktive Fütterungsregime (mit demselben Futteraufnahme-Niveau für alle Schweine) wurde verwendet, um die indirekten Einflüsse der Futteraufnahme von den

direkten DON-Auswirkungen differenzieren zu können. Die freiwillige Futteraufnahme und die Lebendmassezunahme der Schweine, denen das DON kontaminierte Futter *ad libitum* verabreicht wurde, war um jeweils 15 und 13 % vermindert. Außerdem erschien der Verzehr des DON kontaminierten Futters verzögert, da eine Stunde nach der Fütterung 85 % der Kontrolle, aber nur 39 % der DON-Gruppe das gesamte angebotene Futter aufgenommen hatte. Die geringere Wachstumsleistung schien jedoch vor allen Dingen eine Folge der verringerten Futteraufnahme zu sein, da es keine Unterschiede zwischen den restriktiv gefütterten Schweinen gab. Weiterhin zeigte eine chronische DON-Belastung über das Futter keine Veränderung der hämatologischen und klinisch-chemischen Parameter, so dass diese Parameter nicht geeignet zu sein scheinen, um auf eine DON-Intoxikation zurückschließen zu können.

Die IgA Serum-Konzentrationen der Schweine waren um 70 bzw. 20 % nach einer *ad libitum* oder restriktiven DON-Fütterung erhöht. Es sollte beachtet werden, dass *ad libitum* DON gefütterte Börge nicht nur mehr von dem DON kontaminiertem Futter verzehrt haben, sondern auch höhere Serum-IgA-Spiegel als die weiblichen Tiere aufwiesen, so dass vermutlich erst eine höhere DON-Aufnahme die Serum-IgA-Konzentrationen in Schweinen verändert.

Der Einfluss von DON auf die Verdaulichkeit der Nährstoffe ist relativ uneinheitlich. Während die Verdaulichkeit der organischen Masse, des Rohproteins, des Rohfettes und der Rohfaser sowie die metabolisierbare Energie und die Stickstoff-Bilanz im ersten Experiment signifikant erhöht war, war die Verdaulichkeit des Rohfettes in einer zweiten Bilanz-Studie signifikant erniedrigt.

In kastrierten, männlichen Schweinen (41,5 ± 2,0 kg LM) zeigte DON aus natürlich kontaminiertem Weizen nach chronischer (≥ 4 Wochen, n = 5) und akuter (einmalig, n = 6) Aufnahme eine schnelle und fast vollständige Absorption mit einer mittleren Bioverfügbarkeit von 89 und 54 % für freies DON sowie von 112 und 92 % für Gesamt-DON (konjugiert + frei). Nach chronischer oder akuter oraler DON-Belastung wurden maximale Serumkonzentrationen (C_{max}) von 21,2 und 15,2 ng/ml freies DON nach (t_{max}) 88,4 und 99,1 min gemessen, die danach langsamer mit einer Eliminations-Halbwertszeit ($t_{1/2}\beta$) von 6,3 und 5,3 h absanken. Während die DON-Kinetik nach oraler Exposition der Bateman-Funktion folgte, verringerte sich die DON-Konzentration nach intravenöser Verabreichung (n = 5) von 53 µg/kg LM reinem DON biphasisch mit einer $t_{1/2}\beta$ von 15,2 h. Dabei war das scheinbare Verteilungsvolumen höher als das Gesamt-Körperwasser. Vierundzwanzig Stunden nach

oraler oder intravenöser DON-Exposition konnten keine nennenswerten DON-Konzentrationen im Serum der Schweine nachgewiesen werden. Die DON-Fütterung verursachte eine signifikante Erhöhung der DON-Exkretion mit Urin und Fäzes, wobei 50 % des aufgenommen DON mit dem Urin und nur 2,5 % mit dem Kot ausgeschieden wurden. Ferner zeigte sich, dass lediglich wenige Schweine, die das DON kontaminierte Futter länger als 4 Wochen erhielten, die Fähigkeit zur Detoxifizierung von DON zu seinem Metaboliten de-epoxy-DON aufwiesen. Schweine, die die Fähigkeit zur De-epoxidierung hatten, schieden de-epoxy-DON nur zu geringen Anteilen mit dem Harn (8,5 %), sondern vornehmlich über den Kot (88,2 %) aus. Allerdings sollte beachtet werden, dass de-epoxy-DON lediglich einen geringen Teil (< 2 %) der Gesamt-Wiederfindung von 54 % ausmacht, die auf der Urin- und Kotausscheidung von DON und de-epoxy-DON basiert. Deswegen kann angenommen werden, dass die De-epoxidierung von DON durch adaptierte Mikroben insbesondere im Dickdarm vorkommt, in dem kaum eine systemische Absorption stattfindet, und somit nicht zu einer substantiellen Detoxifizierung für das Tier beiträgt.

Die nachteiligen Auswirkungen von DON auf die Proliferation von ConA-stimulierten porcinen, peripheren Blut-Lymphozyten sowie auf die Immunglobulin- (IgA, IgG und IgM) Konzentrationen wurde *in vitro* nach Zugabe von 70 bis 560 ng reinem DON pro ml Medium mit Hilfe von zwei unterschiedlichen Tests (BrdU-Einbau und MTT-Spaltung) untersucht. Die Proliferation wurde bei 200 und 309 ng DON/ml im BrdU und MTT assay zu 50 % gehemmt (IC_{50}). Die IgA-, IgM- und IgG-Konzentrationen im Überstand der Lymphozytenkultur wurde bei jeweils 121, 84 und 72 ng DON/ml zu 50 % inhibiert, so dass davon ausgegangen werden kann, dass DON-exponierte Schweine-Lymphozyten zuerst die Produktion von sekretorischen Proteinen (Ig´s) und danach ihre Vermehrung (DNA-Synthese, BrdU assay) einstellen, um ihren Erhaltungsbedarf für den Stoffwechsel aufrechterhalten zu können (metabolische Aktivität, MTT assay).

Nach chronischer (n = 13) oder akuter (n = 12) Verfütterung eines DON kontaminierten Futters von 5,7 mg/kg an Börge, zeigte sich eine Tendenz zu einer verringerten *ex vivo* Lymphozyten-Proliferation im Vergleich zur Kontrolle (n = 11), die jedoch nur in akut DON-exponierten Schweinen im MTT assay Signifikanz erreichte. Zwischen den Gruppen gab es keinen Unterschied in den Serum-IgA-Konzentrationen der Schweine, während die IgM- und IgG-Werte in der akuten DON-Gruppe signifikant erhöht waren. So kann vermutet werden, dass Schweine, die zum ersten Mal (akut) Kontakt zu einem DON kontaminiertem Futter haben, eine ausgeprägtere Reaktion der immunologischen Parameter aufweisen, obwohl die

Serum-DON-Konzentration eine Stunde nach der Mahlzeit geringer sind als bei den chronisch mit DON gefütterten Schweinen.

Der Effekt von DON auf die Proteinsynthese von kastrierten, männlichen Schweinen mit etwa 40 kg LM wurde mittels einer "flooding dose" des Stabil-Isotopes [$^2H_5$]Phenylalanin gemessen. Den Schweinen wurde entweder ein Kontroll- (n = 7) oder ein DON kontaminiertes Futter von 5,7 mg/kg chronisch (n = 10) und akut (n = 7) verabreicht oder sie erhielten eine Dosis von 53 µg DON/kg LM intravenös (n = 7). Die Plasma-Konzentrationen von Gesamtprotein, Albumin, Fibrinogen und den klinisch-chemischen Parametern wiesen keine Unterschiede zwischen den Gruppen auf, während die Proteinsynthese der Lymphozyten und des Albumins gehemmt, die Fibrinogen-Synthese jedoch unbeeinflusst erschien. Die fraktionellen Syntheseraten (FSR, %/d), d.h. der Prozentsatz an neu synthetisiertem Protein pro Tag, von Albumin waren um je 32, 45 und 26 % und von den peripheren Blut-Lymphozyten um je 27, 19 und 24 % nach einer chronisch oder akut oralen und intravenösen DON-Belastung verringert.

Somit kann geschlussfolgert werden, dass DON die Leistung von Mastschweinen hauptsächlich durch eine Reduktion der Futteraufnahme beeinflusst hat. Klinisch-chemische und hämatologische Parameter sind anscheinend nicht geeignet, um auf eine DON-Belastung zurückzuschließen. Futter mit einem DON-Gehalt von 5,7 mg/kg hemmte die *in vivo* Synthese von Albumin um etwa 40 %, während die Fibrinogen-Synthese durch eine hohe intra-individuelle Variation gekennzeichnet, aber nicht durch DON beeinflusst war. Außerdem schien aus Futter stammendes DON immunologische Funktionen in Schweinen zu beeinträchtigen. So waren die Lymphozyten-Proliferation (*ex vivo*) sowie die Lymphozyten-Proteinsynthese (*in vivo*) um 20 bzw. 25 % vermindert. Darüber hinaus waren die Immunglobuline *in vitro* und *in vivo* durch DON beeinflusst, wobei der Einfluss des Geschlechtes, des Fütterungsregimes und der akuten oder chronischen DON-Belastung noch weiter aufgeklärt werden sollte.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass die vorliegende Dissertation: "Effects of the *Fusarium* toxin deoxynivalenol (DON) on protein synthesis, immunological parameters and DON-kinetics in the pig (Einfluss des *Fusarium* Toxins Deoxynivalenol (DON) auf die Proteinsynthese, immunologische Parameter und auf die DON-Kinetik beim Schwein)" selbständig und nur unter der Verwendung der angegebenen Literatur und Hilfsmittel angefertigt wurde. Folgende Hilfe Dritter wurde in Anspruch genommen: Die Mykotoxin-Analysen sowie die GC/MS-Messungen wurden von Frau Grove aus dem Institut für Tierernährung der FAL Braunschweig vorgenommen. Weitere Trichothecene im Weizen wurden mittels GC/MS durch Frau Dr. Schollenberger aus dem Institut für Tierernährung der Universität Hohenheim analysiert. Die Bestimmung der Serum-Enzyme sowie des Proteingehaltes wurde von der Klinik für kleine Klauentiere der Tierärztlichen Hochschule durchgeführt. Die Immunglobulingehalte im Serum und in den Überständen wurden von Frau Dr. Tiemann des Forschungsbereichs Fortpflanzungsbiologie der FBN Dummerstorf bestimmt. Das statistische Modell in Paper I wurde mit Hilfe von Herrn Prof. Dr. Spilke der Landwirtschaftlichen Fakultät der Martin-Luther-Universität in Halle-Wittenberg entwickelt.

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten enthalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation am Institut für Tierernährung der Bundesforschungsanstalt für Landwirtschaft (FAL) in Braunschweig angefertigt.

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

Hannover, den ……

Tanja Goyarts

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