Synthesis and characterization of deoxynivalenol glucuronide: its comparative immunotoxicity with deoxynivalenol

Xianai Wu

Iowa State University

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Synthesis and characterization of deoxynivalenol glucuronide: its comparative immunotoxicity with deoxynivalenol

by

Xianai Wu

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Toxicology

Program of Study Committee:
Suzanne Hendrich, Major Professor
Patricia Murphy
Joan Cunnick

Iowa State University
Ames, Iowa
2006
This is to certify that the master’s thesis of

Xianai Wu

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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Xianai Wu, Patricia Murphy, Joan Cunnick and Suzanne Hendrich

Deoxynivalenol (DON) is a mycotoxin commonly contaminating corn, wheat and barley. DON glucuronide (DONGLU) was synthesized with rat liver microsomes, uridine-5’-dephosphoglucuronic acid (UDPGA) and DON. DONGLU was purified with a Sephadex LH-20 column and HPLC (revere phase column, UV at 220 nm). β-glucuronidase hydrolysis formed a product with retention time and UV spectrum identical with DON. HPLC chromatography of DONGLU reaction mix with/without UDPGA or with/without microsomes showed that only in the complete reaction mix was there a peak more hydrophilic than DON but with similar UV spectrum, putatively DONGLU. DONGLU was further identified by mass spectrometry in negative ionization mode and NMR. The molecular mass (M-1) was 471 g/mol, in agreement with DONGLU’s expected molecular weight of 472 g/mol. β-glucuronidase hydrolysis, MS and NMR indicated that the glucuronide moiety was conjugated with the carbon-3-hydroxyl group of DON. The cytotoxicity of DON and DONGLU were compared in cell culture using human erythroleukemia cell line K562. 50% inhibition of cell proliferation was observed with a DON concentration of 1.31 µM using CellTitre96™ detection, whereas no significant cytotoxicity was observed for DONGLU at up to 270 µM. DONGLU did not influence DON toxicity at low, medium and high concentration combinations (0.5 µM, 1.3 µM and 8.4 µM) of each compound. These data show that DONGLU is a detoxification product of DON, and that measuring DON without DONGLU in human urine or plasma would be a suitable biomarker of DON exposure and toxicity.
INTRODUCTION

Deoxynivalenol (DON) is a trichothecene mycotoxin produced by *Fusarium* fungi, specifically *F. graminearum* and *F. culmorum*, which are common contaminants of various cereal crops such as wheat, maize, barley, and oats. The formation of DON in growing crops is dependent on climate and will thus vary between geographical regions. Moreover, DON can also be produced during storage when moisture content of stored grains is less rigorously controlled (Lombaert et al. 2003; Lori et al. 2003; review by WHO, 1990 and Pacin et al. 1997). A survey done in 1993 of DON in U.S. showed DON contamination in wheat samples averaging 2.0 µg/g and ranging from < 0.5 to 18 µg/g. DON contamination in barley samples averaged 4.2 µg/g and ranged from < 0.5 to 26 µg/g. About 40% of the wheat samples and 57% of the barley samples contained DON levels that were greater than the U.S. Food and Drug Administration 1982 advisory level of 2 µg/g for DON in wheat designated for milling (human consumption) (Trucksess et al. 1995). DON exposure investigated in Canada in 2003 found that DON was the most frequently detected mycotoxin and detected in 63% sample analyzed in cereal based infant food (Lombaert et al. 2003).

DON exposure possibly inhibits immune system function through suppressing lymphocyte proliferation and decreasing non-specific host defenses resulting in an increased susceptibility for infectious diseases. In vitro exposure of human lymphocytes to DON showed that DON inhibited mitogen-induced lymphocyte proliferation with IC50 216 ng/ml (Thuvander et al. 1999). Exposure to DON induces immature and mature B cells apoptosis and cytotoxicity after exposure at 100-1000 ng/ml for 18 h, with EC50 at 500 ng/ml for apoptosis and 300 ng/ml for cytotoxicity, respectively (Uzarski et al. 2003). DON
exposure exerted an inhibitory effect on resident macrophages and natural killer (NK) cells. Mouse peritoneal macrophages pre-incubated with 1 ng DON/mL for 4 h showed inhibited phagocytosis and microbicidal killing against a model microbial, opsonized Saccharomyces cerevisiae (Ayral et al., 1992). By investigating antibody-dependent cellular cytotoxicity and natural killer (NK) cell activity, Berek et al. (2001) observed DON inhibited NK cells activity at 50 ng/ml. Male Balb/c mice (4–6 weeks of age) were fed DON daily for 1 or 2 weeks at doses of 2.5 to 100 ppm in the diet. Liver and kidney weights were not changed whereas thymus weight was reduced significantly at concentration >10 ppm (Robbana-Barnat et al., 1988). These results suggest leukocytes and the immune system are primary targets for DON.

DON is found in foods such as corn, wheat and barley. Processing, milling and baking showed little reduction in DON levels (Castells et al. 2005). After consuming DON contaminated food, DON was quickly absorbed in the stomach and the proximal small intestine in swine (Danicke et al. 2004). Following administration of a single oral 10 mg/kg dose in male PVG rats, radioactivity excreted in the urine and feces accounted, respectively, for 25 and 64% of the administered dose within 96 h (Lake et al. 1987). After oral administration the major metabolic routes are de-epoxidation to DOM-1 due to gut microbes and glucuronide conjugation due to biotransformation by UDP-glucuronosyl-transferases. DOM-1 has been found in urine of rats and in urine and plasma of cow (Worrell et al., 1989; Cote et al., 1986). De-epoxidation is a detoxification reaction. In vitro, DOM-1 was 55 times less toxic than DON in Swiss mouse 3T3 fibroblasts (3T3 cells) (Sundstol Eriksen et al., 2004). The biological activity of DON glucuronide has not been reported yet. We hypothesized that DON glucuronide is a detoxification product of DON.
that does not interact with DON toxicity. Meky et al. (2002) found that DON glucuronide is present in human urine. After treating human urine with β-glucuronidase, the increase in DON ranged from 1.2-2.8 fold. Generally, biotransformation serves to enhance parent compound detoxification and elimination. Zhang et al. (1999) found that isoflavone glucuronide was much less immunotoxic to natural killer cells in vitro than the parent compounds. However, in some cases, after conjugation, the biological activities of the conjugates were enhanced. For example, Ghaoui et al. (2003) demonstrated that clofibric acid undergoes glucuronidation-dependent bioactivation to DNA damaging species in cultured mouse hepatocytes. The formation of aromatic amine glucuronides causes bladder cancer (Thorgeirsson et al. 1983). Therefore, the current study is to synthesize and purify DON glucuronide to compare immunotoxicity of DON glucuronide and DON in an in vitro model. Human K562 erythroleukemia cell line was used in our research to compare immunotoxicity of DON and its glucuronide. K562 cells were selected as a model because they are very early erythroid precursors and like lymphoblasts in morphology (Koeffler and Golde 1980). At the same time, K562 cells are easily grown in vitro without mitogen, so they may be a convenient model of human lymphocyte precursors.
LITERATURE REVIEW

General information

Deoxynivalenol (DON, vomitoxin) is a type B trichothecene. About 150 related compounds are known as trichothecenes, which are formed by a numbers of species of Fusarium and some other fungi. The occurrence of DON is associated primarily with Fusarium graminearum (Gibberella zeae) and F. culmorum, both of which are important plant pathogens which cause Fusarium head blight in wheat and Gibberella ear rot in maize. F. graminearum grows optimally at a temperature of 25 °C and at a water activity above 0.88. F. culmorum grows optimally at 21 °C and at a water activity above 0.87. The formation of DON in growing crops is dependent on climate and will thus vary between geographical regions. Moreover, DON can also be produced during storage where moisture content of stored grains is less rigorously controlled (Christian Larsen et al., 2004; review by WHO, 1990; Isebaert et al., 2004; Lori et al., 2003).

DON is one of the more polar trichothecenes with molecular weight, 296.3 g mol⁻¹ and the molecular formula C₁₅H₂₀O₆. It has a 12, 13 epoxy group, OH group, and an α, β-unsaturated keto group, with the chemical name is 12, 13-epoxy-3α, 7α, 15-trihydroxy-trichothec-9-en-8-one (Fig 1). It is soluble in water and polar solvents such as methanol and acetonitrile. DON has a maximum absorption at 218nm. For UV-determination and spectrophotometric determination; acetonitrile is a better solvent than methanol. DON is heat stable at 120°C and is not decomposed under mildly acidic conditions (Krska et al., 2001).

DON occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale. Because it is a stable compound it has also been
detected in a range of processed cereal products including breakfast cereals, bread, noodles, infant foods, malt and beer. Concentrations have been reported at up to 26 mg/kg in barley and 18 mg/kg in wheat (Trucksess et al. 1995, 1996; Lombaert et al. 2003; MacDonald et al. 2004; Castells et al. 2005).

The cause of head blight (scab) of wheat and barley has emerged as the plant disease with the greatest impact on U.S. agriculture and society during the past decade. Scab has occurred frequently in the Midwest because of recurrent cool rainy weather during wheat and barley flowering (McMullen et al., 1997; Windels, 2000). In contaminated cereals 3- and 15-acetyl DON can co-occur in significant amounts with DON. 3-acetyl DON is prevalent in Europe; 15-acetyl DON is prevalent in North America. These toxins are often present at the levels of 10-20% that of DON (Christian Larsen et al., 2004). It is possible that these toxins can be degraded to DON due to microbial in the alimentary tracts of ruminants and non-ruminants (Binder et al. 1998; Eriksen et al. 2003).

Austria, Canada, US and the European Union (EU) have guideline limits for DON. US Food and Drug Administration voluntary advisory levels for DON stipulate no more than 1 mg/kg for bran, flour, and germ for human consumption (FDA/CFSAN, Chapter 7). Several European guidelines recommend maximum levels of DON of 500 ng/g for cereal products as consumed and 750 ng/g for flour used as raw materials in food products and raw cereals (Codex Alimentarius Commission FAO/WHO, 2003).

**Detoxification strategies**

DON is thermally stable so it is difficult to eliminate from grain once formed. Because DON is water-soluble a significant proportion can be removed by washing grain but commercially this represents an additional stage and an effluent problem. Other
detoxification strategies include enzyme reactions, food processing or other alternative methods.

**Fig 1: The chemical structure of DON (MW=296)**

Biological detoxification strategies include the use of exogenous enzymes or bacteria to hydrolyze the 12-13 epoxide ring, which is responsible for the toxicological properties of DON. It is determined that de-epoxy DON has 55 times less toxicity than DON in Swiss mouse 3T3 fibroblasts (Sundstol Eriksen et al. 2004). For example, the incubation of DON-contaminated corn with the cecal microflora from chickens reduced the anorectic potential of the corn and improved the performance of swine. He et al. (1992) found that microorganisms in large intestines of chickens (MLIC) completely transformed pure DON. A single metabolite was isolated and identified as de-epoxy DON, DOM-1 (Fig 2). The DON transformation rate was not affected by either the ratio of MLIC to DON (5 to 0.2 g of MLIC per mg of DON) or the initial concentration of DON (14 to 1,400 ppm) in the medium. Sodium azide at a 0.1% (wt/vol) concentration in the medium blocked the transformation of DON, indicating that the de-epoxidation of DON is energy dependent and microbial. However, DON in the moldy corn was not transformed when MLIC were added to corn without culture medium. Biotransformation of DON was completely inhibited when
the pH in the medium was lowered to 5.2. After incubation of MLIC and moldy corn, approximately 79 and 47% of the initial DON were recovery after 48 and 96 h of incubation. However, the pH in the medium decreased from 6.5 to 3.5 in the 96 h incubation period, presumably because of organic acids produced during corn fermentation. At the same time, the buffer failed to neutralize the acid produced from corn fermentation. These results indicate that control of the pH in the incubation medium is critical for further transformation. As a result, incubation of MLIC and moldy corn for 96 h can partial biotransform DON to de-epoxy-DON. Depending on the concentration of DON, this moldy corn could be used feed animals such as cows and sheep.

DON can also be transformed to DOM-1 by microorganisms in rumen fluid and soil. Binder et al. (1998) found DON and 3-acetyl DON were transformed by ruminal microorganisms to a single metabolite, which was identified by GC-MS as DOM-1. DOM-1 from either trichothecene showed a significant reduction in toxicity against Saccharomyces cerevisiae. Aerobic biotransformation of DON and 3-acetyl DON by microorganisms in soil, respectively, gave five new metabolites as indicated by HPLC and TLC determination. These five new metabolites were unknown. 3-acetyl DON was de-acetylated to DON within 24h; the following reactions also produced five new metabolites by soil microorganisms just like DON as original mycotoxin. The toxicity of these metabolites against S. cerevisiae was reduced.

DON can be transformed to 3-keto-4-deoxynivalenol by some bacteria in soil (Fig 3). A soil bacteria, strain E3-39, Agrobacterium-Rhizobium group, was isolated by Shima et al. (1997). This bacterium completely eliminated exogenously supplied DON from culture medium after incubation for 1 day. Thin-layer chromatographic analysis indicated the
presence of one major and two minor metabolites of DON in ethyl acetate extracts of the E3-39 culture filtrates. The main metabolite was identified as 3-keto-4-deoxynivalenol by mass spectroscopy and $^1$H and $^{13}$C nuclear magnetic resonance analysis. This compound exhibited a remarkably decreased (to less than one tenth) immunosuppressive effect evaluated by means of a bioassay based on the mitogen-induced and mitogen-free proliferations of mouse spleen lymphocytes.

![Image of chemical structure of de-epoxy-deoxynivalenol (DOM-1)](image)

**Fig 2:** The chemical structure of de-epoxy-deoxynivalenol (DOM-1)

Under aerobic conditions, Volkl et al. (2004) found that DON was biotransformed to 3-keto-4-deoxynivalenol by a microorganism after screening 1285 microbial cultures from farmland soil, cereal grains, insects and other sources. DON was isolated both from ears that were visibly infected with *Fusarium* and from symptomless grain grown on test fields. One mixed culture transformed DON into two chromatographically separable products. The main product of the transformation was purified and its structure was elucidated by mass spectroscopy, and NMR spectroscopy. The structure of this product was determined to be 3-keto-4-deoxynivalenol. The DON-transforming mixed culture survived and retained its transforming activity during a starvation period of six months at $20^\circ$ C. Transformation of DON was suppressed by low concentrations of glucose and high concentrations of tryptone.
and yeast extract. Trichothecenes 15-acetyl DON, 3-acetyl DON and fusarenon-X were also transformed.

Fig 3: The chemical structure of 3-keto-4-deoxynivalenol

Alternative approaches for the removal of DON from grains include the use of washing or soaking procedures and physical methods, such as dehulling. With respect to the latter, House et al. (2003) researched the efficiency of DON removal from barley samples through the use of an abrasive pearling procedure at varying levels of contamination. The results showed that pearling was an effective means of reducing the DON content of barley, with improvements in nutrient levels of barley because DON could affect nutrient absorption in the intestine through modulating the activities of intestinal transporters (Maresca et al., 2002). Following 15 s of pearling, the grain contained 34% of the initial DON content, irrespective of the initial level of contamination. However, the need to reduce the DON content of contaminated barley to less than 1 ppm for swine will necessitate the removal of a significant amount of the grain mass for heavily contaminated samples. Additional pearling resulted in a linear decline of grain mass. Further pearling resulted in continued significant (p < 0.05) reductions in the percent of DON remaining to a level of 7.9% after 120 s but with significant losses in grain mass.
Many spices and herbs and their extracts possess antimicrobial activity because of their essential oil fraction. Therefore, these essential oils could be used as alternatives to food preservatives currently in use. Velluti et al. (2004) investigated the efficacy of cinnamon, clove, lemongrass, palmarose and oregano oils on growth rate and DON production by *F. graminearum* in irradiated maize grain at different water activities and temperature conditions. The influence of the essential oils was tested on irradiated maize at two concentrations (500 and 1000 mg/kg), at different water activity (aw) (0.950 and 0.995) and temperature (20 and 30 °C) levels. At 0.995 aw, all essential oils tested had an inhibitory effect on growth rate of *F. graminearum* at both temperatures studied. At this aw, DON production in general was significantly inhibited by all essential oils at 30°C compared to control. Therefore, essential oils should be considered as alternative post-harvest natural fungicides. These results also indicate that antifungal and antimycotoxigenic activity of the essential oils depended on environmental conditions.

**Detection methods**

DON can be extracted from cereals using aqueous solvent mixtures such as methanol and water or acetonitrile and water. Mechanical shaking or blending with aqueous acetonitrile (acetonitrile: water 84:16, v/v) usually results in extraction of the relatively polar DON. The main procedures employed for clean up in trichothecene analysis are liquid-liquid partitioning, solid-phase extraction (SPE), column chromatography, and use of immunoaffinity column (IAC) and multifunctional clean-up columns. Liquid-liquid partitioning can be employed by shaking the sample extract with an immiscible solvent in a tube, such as ethyl acetate (Gareis et al., 1987). Column chromatography can be performed
on a variety of stationary phases. Sep-pak cartridge (C18) has been successfully used to clean up DON in our laboratory. The most frequently employed column packing material for DON is a mixture of charcoal, alumina, and celite with ratio of alumina: celite: charcoal (0.35 + 0.25 + 0.4 g) (Omurtag and Beyoglu 2003) or alumina: charcoal: celite (7 + 5 + 3) (Fernandez, 1994). Use of an immunoaffinity column to extract DON was based on DON binding to DON-specific antibodies linked to an organic carrier material, and then DON could be eluted by use of pure organic solvents. Commercial multifunctional Mycosep® clean-up columns are available to clean several trichothecenes simultaneously. Combined HPLC and GC Mycosep® columns are frequently employed.

Because DON has some UV absorbance, it is possible to detect it using TLC or HPLC with UV detection methods. C_{18} reversed phase columns with methanol-water mixture or acetonitrile-water mixture as mobile phase were used for HPLC. Recently, LC-MS instruments, particularly using APCI (atmospheric pressure chemical ionization) interfaces have recently been employed for the determination and identification of trichothecenes, including DON, at trace levels. GC-ECD (Electron Capture Detector) and GC-MS are available to detect DON with detection limit 20 ng/g. The advantage of this is that other related compounds can be determined simultaneously with high sensitivity. The disadvantages are that DON is non-volatile and must be derivatized to form a stable compound suitable for analysis. Expensive equipment and skilled operators are required for GC-MS. ELISA assays and cell proliferation assays have been developed for quick and simple screening. The limit of ELISA is that it cannot distinguish between DON, 3-acetyl DON, 15-acetyl DON, and 3, 15, di-acetyl DON because the antibodies used are designed against 3, 7, 15-tri-acetyl DON (Krska et al. 2001). The elimination of cytotoxic matrix
substances is crucial when screening food samples for toxic substances using cell proliferation assays. Therefore, thorough purification of the sample is required. At the same time, the sensitivity of the cell line is critical when applying an in vitro cytotoxicity assay for screening of DON. As a result, the method of choice depends on the instrumentation available, the required detection limit, matrix composition and the properties of the analyte. ELISA assay has the lowest detection limit with 0.2 ng/ml in buffer without clean up after extraction (Ngundi et al. 2006), however, ELISA has crossreactivity between DON and other trichothecenes (Krska et al. 2001). Immunoaffinity column enrichment and HPLC-MS/UV detector has a very low detection limit at 12-20 ng/ml and can detect individual compounds specifically (Janes and Schuster 2001; Meky et al. 2003). The cheapest method is clean up using alumina and charcoal SPE column combined with cell proliferation assay. At the same time, it was reported that this method has a good correlation with an HPLC method (Landgren 2005). Table 1 shows the current methods to use for detection of DON in different matrices.

Table 1: Quantitative and screening detection method for DON

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Matrix</th>
<th>Extraction, clean-up and special considerations</th>
<th>Recovery and detection limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>grain</td>
<td>Hazardous waste production, time consuming</td>
<td>300 ng/g</td>
<td>Scott (1995)</td>
</tr>
<tr>
<td>GC</td>
<td>Corn, milk, urine, feces</td>
<td>acetonitrile: water (84:16), Mycochar column, and C_{18} cartridge</td>
<td>82%, 50 ng/g</td>
<td>Cote et al. (1986)</td>
</tr>
<tr>
<td>Detection method</td>
<td>Matrix</td>
<td>Extraction, clean-up and special considerations</td>
<td>Recovery and detection limit</td>
<td>Reference</td>
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<tr>
<td>HPLC-APCI-MS</td>
<td>Pig urine, maize</td>
<td>Extract using acetonitrile: water (84:16). Clean-up using MycoSep® column, simultaneous determination of B-trichotheccenes and DOM-1. Dexamethasone as internal standard</td>
<td>63.7-102.3% 25-150 ng/g</td>
<td>Razzazi-Fazeli et al. (2003)</td>
</tr>
<tr>
<td>Polar-RP C18 column</td>
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<tr>
<td>HPLC-UV (218 nm)</td>
<td>Cereal and flour</td>
<td>Extract using water and polyethylene glycol. Immunoaffinity column-HPLC MycoSep 225-GC/ECD</td>
<td>80-96% 20 ng/g</td>
<td>Kotal and Radova (2002)</td>
</tr>
<tr>
<td>Supelco® - C18 GC-ECD</td>
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<tr>
<td>LC-APCI-MS</td>
<td>Wheat</td>
<td>Extract using acetonitrile: water (84:16). MycoSep column, Detect both of DON glucoside and DON. Silica gel normal phase MPLC(medium pressure) and semipreparative reversed phase HPLC (Jupiter C18, 250 mm x 15 mm i.d., 10 μM) used to purify DON glucoside.</td>
<td>No information</td>
<td>Berthiller et al. (2005)</td>
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<td>(230 nm)</td>
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<tr>
<td>RP C18 Aquasil® column,</td>
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<td></td>
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<tr>
<td>100mmx 4.6mm, 3μm</td>
<td></td>
<td></td>
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<tr>
<td>HPLC-MS</td>
<td>Rat plasma, urine and</td>
<td>Immunoaffinity column enrichment First paper reported measurement of a urinary biomarker for DON in human</td>
<td>&lt;12 ng/ml</td>
<td>Meky et al. (2003)</td>
</tr>
<tr>
<td>Supelcosil® RP C18</td>
<td>Human urine</td>
<td></td>
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<tr>
<td>column. 25 cm x 4.6 mm,</td>
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<tr>
<td>5 μm</td>
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<tr>
<td>HPLC-UV</td>
<td>Yolk and albumen of</td>
<td>Extract using acetonitrile: water and a mixture of charcoal, alumina and celite, and pass through an immunoaffinity column. Detected DON and DOM-1</td>
<td>72-80% 1-2.5 ng/g</td>
<td>Valenta and Danicke (2005)</td>
</tr>
<tr>
<td>eggs</td>
<td></td>
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<tr>
<td>Detection method</td>
<td>Matrix</td>
<td>Extraction, clean-up and special considerations</td>
<td>Recovery and detection limit</td>
<td>Reference</td>
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<tr>
<td>HPLC-MS Aquasil® RP C18 100 mm x 4.6 mm, 3 µm</td>
<td>Maize</td>
<td>Multifunctional MycoSep column. Simultaneous detection of major trichothecenes</td>
<td>0.3-3.8 ng/g</td>
<td>Berthiller et al. (2005)</td>
</tr>
<tr>
<td>HPLC-UV (220 nm) Lichrosorb® RP C18 5µm, 4 x 250mm</td>
<td>Swine Blood, bile, Urine and excrement</td>
<td>Fresh blood stirred 2h. Extract using acetonitrile-water (84:16) and clean up using immunoaffinity column. Additional purification step required for bile and excreta.</td>
<td>75-90% 0.01-0.02 µg/ml</td>
<td>Janes and Schuster (2001)</td>
</tr>
<tr>
<td>HPLC-UV</td>
<td>Wheat</td>
<td>Extract using acetonitrile: water (84:16), clean using alumina and charcoal SPE column. First use of caffeine as internal standard</td>
<td>100.5%</td>
<td>Landgren (2005)</td>
</tr>
<tr>
<td>Enzyme linked immunosorbent assay GC-MS</td>
<td>Cereals and foods</td>
<td>Components derived from the wheat extract did not interfere with the ELISA kits. Rapid (&lt;2 h). Good correlation with GS-MS</td>
<td>80 ng/g 93.8-112%</td>
<td>Yoshizawa et al. (2004)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Food matrixes and indoor air samples</td>
<td>Extracted with methanol-water (3:1) and assayed without cleanup or preconcentration</td>
<td>0.2 ng/mL in buffer to 50 ng/g in oats, air sampler was 4 ng/mL</td>
<td>Ngundi et al. (2006)</td>
</tr>
<tr>
<td>Bioassay K562 cells</td>
<td>Corn and wheat</td>
<td>Extract using acetonitrile: water (84:16), alumina and charcoal SPE column. MTS assay</td>
<td>140 ng/ml</td>
<td>Landgren (2005)</td>
</tr>
</tbody>
</table>
Absorption, distribution, and excretion

(a) Gastrointestinal metabolism

De-expoxidation of DON is a main metabolism of DON in alimentary tracts due to microorganism in the gastrointestinal tract. De-epoxidation is a detoxification reaction because the epoxide group seems to be essential for toxicity. Ruminants are less sensitive to the effects of trichothecenes, which may be due at least in part to differences in metabolism of trichothecenes by microorganisms present or difference in microbes in the alimentary tracts of ruminants and non-ruminants. DON was incubated in vitro for 12, 24 and 48 h with rumen microorganisms obtained from a fistulated dairy cow. Gas chromatographic and gas chromatographic-mass spectrometric analyses of extracts indicated DON was partially converted to a product identified as de-epoxy DON (DOM-1) under anaerobic condition (Swanson 1987). Similar results showing rumen fluid microbial degradation of DON to DOM-1 has been found in other studies under anaerobic conditions (He et al., 1992; Binder et al., 1998). Binder et al. (1997) tried to screen for DON detoxifying anaerobic rumen microorganisms. After several serial dilutions in optimized medium under anaerobic conditions the culture consists of two microorganisms. Both microorganisms were Gram-positive, the active biotransforming bacterium in this coculture was a nonspore-forming, strictly anaerobic irregular rod (0.2 by 1-1.5 µm) occurring singly and in long chains up to 150 µm. No growth occurred on solid media. Yeast extract and hemin were essential.

Worrell et al. (1989) found gut microorganisms in male Sprague-Dawley rats that transformed DON to de-epoxy DON (DOM-1). Oral administration of 10 mg/kg of $^{14}$C-
DON to rats resulted in the appearance of a de-epoxy metabolite in urine and feces. Incubation of DON with a strictly anaerobic preparation of gut contents resulted in the progressive appearance of de-epoxy DON during a 24 h incubation period. Incubation of DON with rat liver homogenate did not result in the appearance of the de-epoxy DON metabolite. These results indicate that the presence of de-epoxy DON in rat excreta, following the oral administration of DON was the result of metabolism by microorganisms in the gut. Microbial degradation of DON in large intestine content of chickens and swine was investigated by He et al. (1992). They found microorganisms in large intestines of chickens completely transformed pure DON. A single metabolite was isolated and identified as de-epoxy deoxynivalenol, DOM-1. However, no alteration of the toxin by incubation with microbes from the contents of the large intestines of swine was detected. However, Danicke et al. (2004) found gut microbes in pig had de-expoxidation activity, and de-epoxy DON appeared in increasing proportions from the distal small intestine and reached approximately 80% of the sum of DON plus de-epoxy-DON in feces collected from the rectum. Research by Eriksen et al. (2003) showed that no de-epoxy-DON was found in plasma or urine of pigs after trichothecene exposure, even in pigs having fecal microflora with de-epoxidation activity. It was concluded that de-epoxidation of DON, which primarily occurs in the hindgut, probably does not contribute to DON detoxification in the pig.

It is unknown if DON can be transformed to DOM-1 by human gut microbes. One study done by Eriksen and Pettersson (2003) showed that 3-acetyl DON was metabolized to DON during the incubation period. However, no de-epoxidated metabolites were detected in the fecal incubation. Hedman (1997) observed that no metabolites of nivalenol (NIV) or DON
were formed in anaerobic incubation of the toxins with pig feces. However, after one week on a diet containing 2.5 or 5 ppm NIV, nearly all excreted NIV in feces had been de-epoxidated in five of six pigs. Thus, microbial adaptation to NIV and perhaps to DON as well might occur after exposure to these toxins.

Therefore, DON can be de-epoxided to DOM-1 by microbial in the alimentary tracts in cow, chickens after in vitro incubation of DON and contents of rumen and gastrointestinal (Swanson 1987; He et al. 1992). It was found that gut microorganisms in rats and pigs can transform DON to de-epoxy metabolite (Worrell et al. 1989; Danicke et al. 2004). In human, it is reported that DON can not be degraded to de-epoxy metabolite (Eriksen and Pettersson 2003).

(b) Bioavailability

DON bioavailability is used to describe the fraction of an administered dose of DON that reaches the systemic circulation. By definition, when DON is administered intravenously, its bioavailability is 100%. However, when DON is administered via other routes, its bioavailability decreases (due to incomplete absorption and first-pass metabolism).

Following administration of a single oral 10 mg/kg dose DON in male PVG rats, radioactivity excreted in the urine and feces accounted, respectively, for 25 and 64% of the administered dose within 96 h (Lake et al., 1987).

DON was quickly absorbed with t_{max} 4.0-5.3 h, but had a systemic bioavailability of only 7.5% due in part to its rapid and efficient metabolism by rumen microorganisms after oral dose of 5.0 mg/kg in sheep (Prelusky et al., 1985). Feeding dairy cows DON
contaminated diets for 5 d, approximately 20% of the DON fed was recovered in the urine and feces in the unconjugated forms as DOM-1 (96% of total DON excreted) and DON (4% of total). Preliminary data suggest that the remaining 80% may be excreted as a glucuronide conjugate (at least in urine) (Cote et al., 1986).

In swine, after consuming DON contaminated feed, DON was rapidly absorbed while passing through the stomach and the upper small intestine. A dynamic laboratory model simulating the gastrointestinal (GI) tract of healthy pigs was used to evaluate the small intestinal absorption of DON. In vitro intestinal absorption of DON was 51% of 170 µg DON ingested through contaminated (spiked) wheat. Most absorption occurred in the jejunal compartment. At the same time, activated carbon produced a significant reduction in the intestinal mycotoxin absorption (Avantaggiato et al., 2004). Eleven castrated male pigs weighing 88.1 ± 3.9 kg on average were adapted to a diet containing DON (4.2 mg DON/kg) over a period of 7 days. Feed was given restrictively with 1.1 kg per meal (two meals per day). DON was rapidly and nearly completely absorbed while passing through the stomach and the proximal small intestine. Maximum serum concentration appeared 4.1 h after the DON-containing meal and half of the systemically absorbed DON was eliminated after 5.8 h. (Danicke et al., 2004).

After intragastric (0.60 mg and 0.60 µCi DON/kg) administration of 14C-labeled DON in swine, DON was very rapidly absorbed, reaching near peak plasma levels within 15-30 min. Levels remained elevated (63-325 ng/ml) for approximately 9 hr, and began declining slowly (t1/2 β = 7.1 h). The calculated systemic bioavailability was between 48 and 65% based on comparison of iv and intragastric AUC, although urinary and biliary recoveries indicated marginally greater absorption actually occurred (54-85%) (Prelusky et al. 1988).
Toxicokinetics of DON has been investigated in 16 castrated male pigs (41.5 ± 2.0 kg) through feeding DON from naturally contaminated wheat (5.7 mg DON/kg) after chronic exposure (5-8 weeks) 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). 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. The mean bioavailability for free DON was 89% for the chronic group and 54% for the acute oral group (Goyarts and Danike, 2006a).

The disposition of [14C] DON administered to hens as either a single oral dose or consumed in spiked feed over a 6-day period was determined by tracing the specific radioactivity of tissues and excreta. Following a single intubated dose of 2.2 mg [14C] DON with 2.4 µCi/bird, the toxin was found to be poorly absorbed; peak plasma levels (2-2.5 h post-treatment) accounted for less than 1% of the administered dose (Prelusky et al., 1986).

As a conclusion, DON has poor absorption in sheep with systemic bioavailability only 7.5% (Prelusky et al., 1985) and poor absorption in chicken with maximum plasma levels less than 1% of the administered dose (Prelusky et al., 1986). Feeding cows for 5 days on DON contaminated foods showed systemic bioavailability of about 1% DON in urine (Cote et al., 1986). DON could be rapidly absorbed while passing through the stomach and the upper small intestine in swine (Avantaggiato et al., 2004; Danicke et al., 2004) with systemic bioavailability of 50% from a single oral dose or 89% from chronic intake (4-8 weeks) with 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.
After intravenous or oral dose of DON, how fast DON goes to tissue, what kind of tissue DON is distributed and tissue binding of DON are the research contents of distribution.

Sprague-Dawley rats received a single dose of $[^{14}C]$ DON (5.0 ± 0.1 mg/kg body weight, 5.5 ± 0.1 microCi/kg) and the distribution of DON in body fluids was investigated over 72 h. DON and its metabolites were detectable in the plasma of rats with the highest levels at 8 h, at which time approximately 9% was bound to plasma protein (Meky et al., 2003).

The disposition of $[^{14}C]$ DON administered to hens as either a single oral dose or consumed in spiked feed over a 6-day period was determined by tracing the specific radioactivity of tissues and excreta. Maximum tissue residues were measured at 3 h in all tissues (liver, kidney, brain, heart, spleen, proventriculus, gizzard, small intestine) except for fat, muscle, and oviduct, which occurred at 6 h postdosing. Among the organs, the highest activities were measured in kidney, liver, and spleen; however, these levels were equal to less than 500 ng DON equivalents/g tissue, and declined quickly. Clearance of radioactivity from tissue had an average half-life of 16.83 ± 8.2 h (range 7.7-33.3 h, depending on the tissue). Elimination of the labeled toxin in excreta occurred rapidly; recovery of radioactivity accounted for 78.6, 92.1, and 98.5% of the dose by 24, 48, and 72 h, respectively. In continuously dosed birds fed 2.2 mg unlabeled DON for 6 days followed by 2.2 mg (1.5 µCi) $[^{14}C]$ DON for 6 days, accumulation of radioactivity in tissues did not occur. Maximum residual levels, which occurred in the kidneys, were only 60 ng DON.
equivalents/g. Estimated level of residues contained in the edible tissues amounted to only 13-16 µg DON/1.5 kg hen (Prelusky et al., 1986).

After intravenous (iv) administration DON (0.5 mg/kg) in sheep, plasma levels were found to decrease biexponentially, showing a rapid distribution phase \( t_{1/2 \alpha} = 12-23 \) min). DON was confined mainly to extracellular fluid, and did not appear to undergo any significant binding or uptake by tissue (Prelusky et al., 1985). After feeding sheep (DON tolerant animals) and pigs (DON sensitive animals) diets including DON, DON could be detected in their cerebral spinal fluid. After iv administration, DON was detected very rapidly (less than 2.5 min) in the CSF of both species, but whereas peak levels (t-max) occurred at 5-10 min in sheep, in swine it was 30-60 min. It would appear that the very rapid and extensive tissue distribution of DON in swine \( V_d \gamma = 1.13 \) L/kg) may be slowing the rate of diffusion of the toxin into the CSF compared to sheep \( V_d \beta = 0.19 \) L/kg) where the toxin is confined essentially to the extracellular compartment. A good relationship between blood and CSF DON levels was apparent in both species, although limitations in detection methods made it impossible to resolve a slow terminal phase (gamma) in swine CSF that was evident in the plasma profile after iv administration. The toxin could be detected in CSF for up to 20 h post-dosing (Prelusky, 1990).

\((d)\) Excretion

Urine and feces are main excretion routes after consumption of DON contaminated diet. In different animals, the ratio of DON in urine and feces is different.

Following administration of a single dose of \([14C]\) DON (5.0 ± 0.1 mg/kg body weight, 5.5 ± 0.1 µCi/kg) in Sprague-Dawley rats, a total of 37% of the administered DON was excreted in the urine (Meky et al., 2003).
After feeding dairy cows DON contaminated feed (66 mg/kg) for 5 day, urine and feces were the main excretion route. DOM-1, the de-epoxy DON, was found in milk at concentrations up to 26 ng/ml. Detectable concentrations of unconjugated DOM-1 were found in urine and feces up to 72 h after the last oral dose (Cote et al., 1986).

Excretion of DON has been studied in sheep. After iv dosing (0.5 DON/kg b.w.), urinary DON levels declined in a biphasic fashion with an average elimination half-life (terminal phase) of 1.2 h, diminishing to baseline concentrations by 8 hr. Maximum urinary excretion rates for the two major metabolites identified (conjugated DON, conjugated DOM-1) occurred 0.5-1.5 h after dosing, exhibiting elimination half-lives of 2.2 and 3.1 h, respectively. Total recovery accounted for only about 66.5% of the dose: 63.0% in the urine and 3.5% in bile. Following oral administration (5.0 mg DON/kg body wt), urinary excretion rates of the major metabolites (DON, conjugated DON, conjugated DOM-1) reached maximum 6-9 hr post-treatment, and declined exponentially with t ½ values of 3.2, 4.0, and 5.0 h, respectively. Urinary and biliary recovery of administered DON averaged approximately 7.1%: 7.0% in urine and 0.1% in bile (Prelusky et al., 1986).

The excretion kinetics of DON was investigated in swine. Serum DON levels declined slowly with an elimination half-life (t ½β) of 6.28 and 5.32 h after feeding castrated male pigs (n = 16, 41.5 ± 2.0 kg) on DON from naturally contaminated wheat (16.6 mg DON/kg) chronic exposure or one single oral dose (acute) (Goyarts T and Danike, 2006). The pigs were fed a commercial diet with 3- acetyl DON added in a concentration of 2.5 mg/kg feed for 2.5 d (fed five times with feed corresponding to 2% of their body weight). The excretion of DON was mainly in urine (45 ± 26% of the toxin ingested by the pigs) and only low amounts of metabolites of 3-acetyl DON (2 ± 0.4%) were recovered in feces. De-
epoxidated DON constituted $52 \pm 15\%$ of the total amount of 3-acetyl DON-metabolites detected in feces. The remaining part in feces was DON. DON was still present in the urine and feces at the end of the sampling period 48 h after the last exposure with concentration between 58 and 131 ng/ml (Eriksen et al. 2003).

DON was found in human urine through collecting urine sample from women in suspected high and low risk region in China. DON was detected in all 15 samples following beta-glucuronidase treatment and immunoaffinity columns (IAC) enrichment with the identity of DON being confirmed by mass spectrometry. The mean levels of DON from the suspected high and low exposure regions of China were 37 ng/ml (range 14-94 ng/ml) and 12 ng/ml (range 4-18 ng/ml), respectively. This is estimated to correspond to daily DON exposures of 1.1-7.4 µg/kg/day and 0.3-1.4 µg/kg/day, respectively. (Meky et al., 2003)

(e) Transmission into eggs and milk

It is concerned if DON is transmitted to eggs and milk after cows and hens were fed on high DON contaminated diet.

Corn contaminated with DON was added to the diets of three dairy cows for 5 d with dietary concentrations of DON averaging 66 mg/kg. Unconjugated de-epoxy DON (DOM-1), a metabolite of DON, was present in milk at concentrations up to 26 ng/ml. DON was not detected in the milk (Cote et al., 1986).

DON is seemingly not transmitted to egg after feeding hens DON contaminated feed. Twenty single comb white Leghorn hens, approximately 26 weeks of age, were individually housed in metabolic cages and fed a control or DON-contaminated (82.8 mg/kg) diet for 27 days. No significant DON residues were found in the yolk, albumin and eggshell (Lun et al., 1986). The transmission of DON and of its metabolite de-epoxy DON into eggs has
been investigated by (Valenta and Danicke (2005). Laying hens were fed a maize-based diet with a DON concentration of 11.9 mg/kg dry matter for 16 weeks. Eggs were collected during weeks 2, 4, 8, and 16 of the experiment, and DON and its metabolite de-epoxy DON were analyzed in freeze-dried yolk and albumen. All samples were incubated with β-glucuronidase prior to extraction. Yolk and albumen were extracted with acetonitrile-water, and the extracts were purified with IACs after a precleaning step. The toxins were determined by high-performance liquid chromatography (HPLC) with UV detection. The detection limits of both toxins were 5 and 8 µg/kg in freeze-dried yolk and albumen, respectively, corresponding to approximately 2.5 and 1 µg/kg in fresh samples (calculated based on dry material of 51% for yolk and 13% for albumin). The recovery of DON and de-epoxy-DON in yolk spiked was 80% and 78%, respectively, and in albumin 77 and 72%. Neither DON nor de-epoxy DON or glucuronide conjugates of both substances could be detected in any of the samples. These results indicate that eggs do not contribute to the dietary DON intake of humans.

As a conclusion, de-epoxication of DON is main metabolism of DON in gastrointestinal, especially in ruminant’s animals. After oral dose, chicken has a poor absorption with maximum plasma levels less than 1% of the administrated dose (Prelusky et al. 1986). The free DON in urine in cow and sheep are ~1% and 7.5% of dose due to de-epoxidation of DON by microbial in rumen fruit (Cote et al. 1986; Prelusky et al. 1985). In pigs, the bioavailability of DON was much higher than in these animals. The mean bioavailability for free DON was 89% after chronic exposure (5-8 weeks) of DON from naturally contaminated wheat based on the area under the curves after oral and intravenous application of pure DON (Goyarts and Danike 2006a). Distribution of DON to tissues is
very quickly at 3 h in all tissues, and accumulation of DON in tissues did not occur (Prelusky et al. 1986; Prelusky et al. 1990). At the same time, it is possible that DON does not be transmitted to eggs and milk or the concentration lower than detection limit results in no DON detected in eggs or milks (Lun et al. 1986; Cote et al. 1986).

**Biotransformation**

Besides some evidence showing that DON can be degraded to DOM-1 by microorganisms in rumen and gut of ruminant animals such as cow and sheep and in gut in animals such as pig and chicken (discussed in section “Gastrointestinal metabolism”), DON can be transformed in liver or intestine by UDP-glucuronosyltransferase (UGT) to DON glucuronide conjugate. At the same time, some evidence showed that DOM-1 can be transformed to DOM-1 glucuronide.

Glucuronidation of DON has been found in rat liver. An isolated rat liver was perfused with DON at a dose of 3 mg in a recirculating perfusion system. To identify glucuronide conjugates, equal amounts of bile samples, perfusate and liver homogenates in phosphate buffer solution (pH = 6.8), were incubated with and without a β-glucuronidase preparation and analyzed by thin layer chromatography and capillary gas liquid chromatography-chemical ionization mass spectrometry. A total of 40.4% of the administered dose of DON was found to be conjugated with glucuronic acid (perfusate 20.4%, bile 19.2%, liver 0.8%), while only 1.3% of the parent DON (perfusate 1.1%, bile 0.2%) was detected. The cleavage of DON glucuronide was demonstrated by incubating DON glucuronide containing bile samples and intestine contents under anaerobic conditions. To evaluate the effect of intestine microflora on the metabolism, glucaro-1, 4- lactone, a β-glucuronidase inhibitor, was added to the bile incubation. After 3 days incubation, a total of 1 µg DON/50 µL of bile
was detected in the bile sample with feces group, while only a total of 0.25 µg DON/50 µL was detected in the glucaro-1, 4-lactone group. This difference could be explained by the inhibitory effect of glucaro-1, 4-lactone on bacterial β-glucuronidase. From these findings in vivo a hydrolytic cleavage of conjugated toxin metabolites by the action of microbial β-glucuronidase seems to be possible and free metabolites then undergo enterohepatic circulation. About 50% of dose is apparently reabsorbed after β-glucuronidase action (Garesis et al., 1987)

Sprague-Dawley rats received a single dose of [14C] DON (5.0 ± 0.1 mg/kg body weight, 5.5 ± 0.1 µCi/kg) and the distribution of DON in body fluids was investigated over 72 h. A total of 37% of the administered DON was excreted in the urine and DON-glucuronide was implicated as the major urinary metabolite based on reverse-phase HPLC analysis of β-glucuronidase- and sulfatase-treated samples (Meky et al., 2003).

Corn contaminated with DON was added to the diets of three dairy cows for 5 d. Dietary concentrations of DON averaged 66 mg/kg. After incubating urine with beta-glucuronidase, the concentration of unconjugated DOM-1 increased by 7 to 15-fold whereas unconjugated DON increased 1.6 to 3-fold. Detectable concentrations of unconjugated DOM-1 were found in urine and feces up to 72 h after the last oral feeding (Cote et al., 1986)

After administrating intravenous and oral doses (0.5 and 5.0 mg/kg, respectively) in sheep, the metabolic formation of the glucuronide conjugate after iv and oral administration of DON appeared to occur quite efficiently (iv, 21%; oral, 75%) with longer elimination half-lives than that of parent toxin. Detection in plasma of the de-epoxide metabolite, DOM-1, accounted for only a minor portion of the dose after either dosing regimen (iv, less
than 2.0%; oral, less than 0.3%), but occurring predominantly as the glucuronide conjugate (Prelusky et al., 1985). In male sheep following either intravenous (iv) or oral administration of DON at levels of 0.5 and 5.0 mg DON/kg body wt, respectively, the peak biliary excretion rate for glucuronide conjugated DOM-1 was found to occur within 1 hr postdosing, which rapidly declined to baseline levels by 5 h. Following oral administration, urinary excretion rates of the major metabolites (DON, conjugated DON, and conjugated DOM-1) reached maximum 6-9 h post-treatment, and declined exponentially with $t_{1/2}$ values of 3.2, 4.0, and 5.0 h, respectively. Urinary and biliary recovery of administered DON averaged approximately 7.1%: 7.0% in urine (2.1% DON, 3.6% conjugated DON, 0.06% DOM-1, 1.2% conjugated DOM-1) and 0.11% in bile (predominantly conjugated DOM-1) (Prelusky et al., 1986). In lactating sheep, using a combination of radioisotopic counting and chromatographic detection techniques, the kinetics and metabolic fate of DON in plasma, urine and bile were studied, its major plasma metabolite, DON-glucuronide conjugate accounted for 13% of the plasma radioactivity levels (Prelusky et al., 1987).

Five castrated male Swedish Landrace pigs were fed a commercial diet with 3-acetyl DON added in a concentration of 2.5 mg/kg feed for 2.5 days. No traces of 3-acetyl DON or its de-epoxide metabolite were found in plasma, urine or feces. A significant part of the DON in plasma was in a glucuronide-conjugated form (42 ± 7%). Only low amounts of metabolites of 3-acetyl DON (2 ± 0.4%) were recovered in feces. De-epoxide DON constituted 52 ± 15% of the total amount of 3-acetyl DON-metabolites detected in feces. The remaining part in feces was DON (Eriksen et al., 2003).

Evidence obtained by comparing DON concentration of urine before and following β-glucuronidase treatment shows DON could be biotransformed in humans. An
immunoaffinity column (IAC)-HPLC method was subsequently developed to measure urinary metabolites, with a view to establishing a urine-based human biomarker. Urine samples were collected and tested from female inhabitants of Linxian County, China, a high risk region for esophageal cancer (OC) and an area of potentially high DON exposure, and Gejiu, a low risk region in China. DON was detected in all 15 samples following β-glucuronidase treatment and IAC enrichment with the identity of DON being confirmed by mass spectrometry. The increase in parent DON recovered following enzyme treatment ranged from 1.2- to 2.8-fold (Meky et al., 2003).

However, in some research, DON has been found to be poorly metabolized. After feeding 16 crossbred German Landrace × Pietrain castrated male pigs (41.5 ± 2.0 kg) on naturally contaminated wheat (5.7 mg DON/kg) with chronic exposure or one single oral dose (acute), glucuronide conjugation of DON 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 feces, whereby the metabolite de-epoxy DON was found only in the trials after pigs were fed DON contaminated diets over 6 weeks, no de-epoxy DON was found with 4 weeks of DON exposure. (Goyarts T and Danike, 2006a). Similar results were found in an in vitro study. Through incubating Sprague-Dawley rat hepatic microsomes with DON and 14C-labelled uridine 5'-diphosphoglucuronic acid (UDPGA), no radioactivity was detected in the TLC zone where the glucuronide was expected. Three rats and one pig were dosed orally with 2 mg DON/kg and samples of their urine and feces were extracted and incubated with β-glucuronidase or with buffer only. No differences in DON or DOM-1 concentrations were detected between samples incubated
with or without β-glucuronidase. Neither DON- nor DOM-1 glucuronides were formed either in *in vitro* liver systems or in vivo (Cote et al., 1987). It is possible no glucuronidation of DON found in their studies because the following reasons: 1) although the microsome was working in that p-nitrophenyl was used as a positive control to assess the validity of the glucuronidation system. However, the p-nitrophenyl glucuronide which served as a reference and the expected DON glucuronide are expected to migrate at the same place at the TLC plate. Different compound glucuronide conjugate couldn’t show at the same retention time. For example, using the same HPLC column, YMC-PACK C18 column, the retention time of DON glucuronide was 3.7 min using 6-9% methanol over 40 min as mobile phase, while daidzein and genistein glucuronide were 15.5 and 22.1 min, respectively using 20-30% acetonitrile over 25 min as mobile phase (Zhang et al., 1999) 2) It is possible that the time (30 min) for microsome reaction was not long enough for glucuronidation of DON. 3) pH 3.8 buffer used in β-glucuronidase reaction was too acidic. The optimal pH is 4.5-7 based on the β-glucuronidase resource. Therefore, incorrect chemical analytical method and conditions for enzyme reaction could result in no glucuronidation of DON in vitro and in the *in vivo* study of Cote et al. (1987).

Glutathione- S-transferases (GSTs) could catalyze formation of a DON glutathione conjugate because the structure 12, 13 -epoxide could be the target of GST. Gouze et al. (2006) found that DON competitively inhibited 1-chloro-2, 4-dinitrobenzene (substrate) conjugation to GST, suggesting that DON is a substrate of GSTs.

As a result, in ruminant animals, the major metabolite in plasma was the glucuronide conjugate of DON or the glucuronide conjugate of DOM-1 (Cote et al., 1986; Prelusky et al., 1987). The glucuronidation of DON has been found in pigs after feeding 3-
acetyl DON at a concentration of 2.5 mg/kg feed for 2.5 days (Eriksen et al., 2003) and oral single exposure to DON from naturally contaminated wheat (Goyarts T and Danike, 2006a). DON glucuronide has been found in human urine as a biomarker together with parent DON for biomonitoring DON exposure (Meky et al., 2003). No glucuronidation of DON was found in pigs after iv administration DON in Goyarts study (2006a), whereas glucuronidation of DON after oral exposure indicates DON could be conjugated in the intestine of pigs before absorption in this study. No glucuronidation of DON was observed in the Cote et al. (1987) study after incubation UDPGA, microsome, buffer and DON. It was possible that chemical analytical method (TLC) was not correct at all or incubation time was not long enough to produce DON glucuronide. Further studies should investigate whether DON can conjugate with glutathione.

**Toxicity**

Acute toxicity of DON is characterized by vomiting particularly in pigs, feed refusal, weight loss and diarrhea. Acute intoxication may produce necrosis in various tissues such as the gastrointestinal tract, bone marrow and lymphoid tissues. Subchronic oral exposure in experimental animals, pigs, mice and rats may also lead to reduced feed intake and slowed weight gain and changes in some blood parameters including serum immunoglobulins. Studies suggest that DON may have effects on the immune system. There are no indications of carcinogenic, mutagenic or teratogenic effects (‘Opinion on *Fusarium* Toxins’, European commission, 1999). The following reviews the immunotoxicity of DON studied in different cell models, animal models and in human epidemiological studies to evaluate the risk of DON to human health.
Cellular and molecular effects

(a) Inhibition of cell proliferation

*In vitro*, different cell model has been used to study DON toxicity. It is observed that DON inhibited lymphocyte proliferation due to apoptosis and cytotoxicity.

It is reported that DON binds to the ribosomal peptidyltransferase site in eukaryotic cells and inhibits protein and DNA synthesis, consequently exposure resulting in decreased cell proliferation and apoptosis. Shifrin and Anderson (1999) found that selected trichothecenes at 10 µM strongly activate JNK/p38 kinases and induce rapid apoptosis in Jurkat T cells. Although the ability of individual trichothecenes to inhibit protein synthesis and activate JNK/p38 kinases is dissociable, both effects contribute to the induction of apoptosis. At the same time, the research results show the peptidyltransferase site could be a regulator of both JNK/p38 kinase activation and apoptosis.

Human and animal cell lines have been used extensively to study the mechanisms of in vitro toxicity of DON. Cell type is an important determinant of susceptibility to DON toxicity as well as determined IC₅₀. Cetin et al. (2005) investigated DON cytotoxicity in mammalian cell lines, such as CHO-K1, Caco-2, C5-O, V79, and HepG2 cell lines, to find the most sensitive cell line. Sensitivities of cell lines to DON were found in decreasing order of CHO-K1 > V79 > C5-O > Caco-2 > HepG2 cells with final concentration of DON from 0.04 to 20 µg/ml.

Meky et al. (2001) reported DON effectively inhibited mitogen-induced lymphocyte proliferation. Human peripheral mononuclear cells were obtained from healthy male and female blood donors. The cells were treated with phytohemagglutinin-p (PHA-p) at 10 µg/ml or 0 µg/ml, and DON was applied at a range 50-500 ng/ml. Cell cultures were
incubated in humidified chamber for 5 d and cell proliferation was evaluated by methylthiazol tetrazolium (MTT) assay. A decrease in proliferation was observed with DON at concentrations greater than 100 ng/ml and 50% inhibition at 216 ng/ml. However, DON had no effect on the viability of unstimulated lymphocytes at these concentrations as measured by the MTT assay. In another study, the lymphocytes were treated with 5 µg/ml PHA or 1 µg/ml Pokeweed mitogen (PWM). The lymphocytes were cultured for 72 h and cell proliferation was evaluated by \[^{[3]}\text{H}\]-thymidine assays. The IC50s of DON for the proliferation responses of human lymphocytes were 124 ng/ml (female) and 130 ng/ml (male) after PHA and 113 ng/ml for both female and male after PWM. Therefore, there were no statistically significant differences in sensitivity to the DON between lymphocytes from females and males (Thuvander et al., 1999).

U-937, a human monocyte-like histiocytic lymphoma (CRL-1593.2) was differentiated into macrophages using PHA and was treated with different concentrations of DON for 24 h. The cytotoxic effects of DON on U937 macrophage cells were measured using MTT assay. Cell proliferation was significantly reduced at concentrations of 50-1000 ng DON/ml (Sugita-Konishi et al., 2001).

The effects of DON in the human K562 erythroleukemia cell line were evaluated. DON inhibited K562 cell proliferation. The IC\textsubscript{50}s were 650 ng/ml and 474 ng/ml after measured by MTT colorimetric assay and BrdU uptake with 48 h treatment. Fluorescent propidium iodide staining and flow cytometry revealed DON induced programmed cell death at the highest concentration of DON (23.7 µg/ml), particularly chromatin condensation and hyperchromasia. Protein synthesis inhibition may play a role in DON induced apoptosis (Minervini, 2003).
Uzarski et al. (2003) reported DON induced apoptosis and reduced B cell proliferation in an immature (CH31, WEHI-23) and mature cells lines of B cells (CH12.LX). DON induced cytotoxicity and apoptosis in WEHI-23 cells after exposure at 100-1000 ng/ml for 18h, with EC50 at 500 ng/ml for apoptosis and 300 ng/ml for cytotoxicity, respectively. Additionally, immature and mature B cells are equally sensitive to DON induced cell death. Investigating activation marker expression among subpopulations of human T cells from freshly collected blood found DON inhibited lymphocyte proliferation in the early cell cycle and its inhibition is before or in conjunction with CD25 (the IL-2 receptor) expression (Johannisson, et al. 1999).

Mikami et al. (2004) demonstrated the cytotoxicity of DON in porcine primary cultured hepatocytes and analyzed the mode of cell death. Livers were obtained from healthy 1-month old LWD piglets and porcine hepatocytes were cultured in vitro. The primary hepatocytes were treated with 100, 10, 1, 0.1 or 0.01 µg DON/ml. Cell viability was measured by MTT assay. Transmission electron microscopy was employed, and lactate dehydrogenase (LDH) release and caspase-3 activity were measured to analyze the mode of cell death. The cell death of the hepatocytes was observed with DON at 100 µg/ml and 10 µg/ml groups starting at 6 h after DON was added. There was dose-dependent effect of cell death in 24 h treatment for all tested concentrations. The dead hepatocytes showed characteristics of apoptosis. LDH was released from apoptotic hepatocytes, and increased caspase-3 activity was seen in higher concentrations DON groups. Additionally, albumin secretion was reduced in all treatment groups, and the concentrations of albumin in the medium in DON 100, 10 and 1 µg/ml groups were similarly low. The reduction of albumin secretion from the hepatocytes into medium was due not only to the loss of hepatocytes by
apoptosis but also to the inhibition of protein synthesis. Decreased concentrations of serum protein and albumin may be secondary to the reduced feed uptake. These results indicate that DON induced apoptosis through the caspase-3 activation pathway and caused functional disorder in porcine hepatocytes. Further research will be needed to determine if DON induced functional deficit in hepatocytes requires apoptosis. WCH-17 and PLHC-1 could be used as cell lines to study functional change of liver after DON exposure.

As a result, DON inhibited mitogen-induced lymphocytes proliferation with IC_{50} at 216 ng/ml (Meky et al. 2001). Cancer cell lines, such as K562 erythroleukemia cell line and different B cell lines have been used investigated DON cytotoxicity. DON inhibited cell proliferation in different cell lines with similar IC_{50} values from 216 ~ 650 ng/ml (Meky et al. 2001; Minervine 2003; Uzarski et al. 2003). Cytotoxicity of DON in hepatocytes has been observed and dead cells show apoptosis. DON exposure influences albumin in the medium (Mikami et al. 2004). Further research need to determine if DON induced functional deficit in hepatocytes requires apoptosis.

**(b) Hematological problems**

Hematotoxicity induced by xenobiotics involves altered blood cell production or function either in bone marrow or at the level of circulating blood cells. Human white blood cell progenitors, red blood cell progenitors and platelet progenitors are the target of the cytotoxic effects of DON.

Froquet et al. (2001) researched DON toxicity on human hematopoietic progenitors. Human umbilical cord blood samples were collected in heparinized tubes from placentas obtained after normal deliveries. Lineage cells were identified with immunocytochemical staining. A decrease of growth percentage of platelet progenitors and red blood cells
progenitors is detectable for $2.5 \times 10^{-7}$ M DON, whereas DON exhibits cytotoxic effects at $2.5 \times 10^{-7}$ M on white blood progenitors. Froquet et al. (2003) also researched whether DON inhibited the clotting process and compared the sensitivities of circulating blood cells and hematopoietic progenitors to DON. The results show the coagulation disturbances induced by DON were not statistically different from control in vitro for prothrombin time, activated partial thromboplastin time and thrombin time assays at measured concentrations ($10^{-5}$ M, $10^{-6}$ M and $10^{-8}$ M). The aggregation initiated by ADP or collagen did not show any statistically significant modification in the presence of increasing concentrations of DON. Red blood cells from human umbilical cord blood did not show significant haemolysis in the presence of increasing concentration of DON. A 50% decrease in leucocyte number was observed for $10^{-5}$ M DON. In vitro data confirmed that DON effects were likely to be predominantly on progenitor cell rather than on circulating mature blood cells. Further research will be needed to determine if the effect of DON is on differentiation or proliferation of progenitor cells, or both.

(c) Inhibition or stimulation of cytokine production

A number of studies suggest that mycotoxins are potent immunomodulators. Depending on the dose, timing and route of exposure, mycotoxins can be immunostimulatory or immunosuppressive agents. Immune modulation is related to changes in cytokine production. Elevated cytokine expression may play an important role in the pathophysiologic effects of DON and other trichothecenes. Cytokine superinduction by protein synthesis inhibitors is likely to involve transcriptional and/or post transcriptional mechanisms.
Zhou et al. (1997) found acute oral DON (5 and 25 mg/kg b.w.) exposure enhances mRNA expression of several cytokines, including IL-1β, IL-2, IL-4, IL-6, IFN-γ and IFN-α in spleen, Peyer's patches (indicators of systemic and mucosal immune compartments, respectively), and other organs in male B6C3F1 mice. The effects were more pronounced in spleen than in the Peyer's patches. Serum levels of IFN-α, IL-6, and IFN-γ were elevated 3 h after exposure to 25 mg/kg DON, thus suggesting that elevation of splenic and Peyer's patch mRNA abundance correlated with increases in systemic production of these cytokines.

To better understand molecular mechanisms by which DON superinduces cytokine gene expression, Li et al. (1997) studied the posttranscriptional effects of this mycotoxin on interleukin-2 (IL-2) gene expression in murine EL-4 thymoma cells stimulated with phorbol 12-myristate 13-acetate and ionomycin (PMA + ION). Northern analysis revealed that doses of 50 to 500 ng/ml DON superinduced IL-2 mRNA expression in a dose- and time-dependent manner in a synchronous model where DON was added at onset of PMA + ION stimulation. In accordance with the mRNA levels, IL-2 production was significantly elevated in the presence of 50 to 250 ng/ml DON. Superinduction of IL-2 mRNA was also observed in a delayed synchronous model (DON added 20 h after PMA + ION stimulation) and an asynchronous model (DON added 20 h after PMA + ION stimulation and removal). To assess the effects of DON (500 ng/ml) on IL-2 mRNA half-life, three transcriptional inhibitors (Actinomycin D, 5, 6-dichloro-beta-D-ribofuranosyl-benzimidazole, cyclosporin A) were used in the delayed synchronous model. The results suggest that DON can superinduce IL-2 at both the mRNA and the protein level and that this superinduction can be explained, in part, by posttranscriptional mechanisms such as enhanced mRNA stability.
Transcription factor NF-κB plays a critical role in regulation of transcription of several cytokine genes. DON exposure can enhance NF-κB binding activity by inhibiting the synthesis of IκBα, which is a negative regulator of NF-κB. Besides NF-κB, activator protein-1 (AP-1) is another critical transcription factor involved in transcriptional regulation of IL-2 and IL-6 gene expression. AP-1 is composed of members of the Jun (JunB, c-Jun and JunD) and Fos families (c-Fos, FosB, Fra-1 and Fra-2), which bind to the TPA response element (AP-1 site). It was demonstrated by gel supershift assay that DON (250 ng/ml) preferentially affected phosphorylated c-Jun, Jung, c-Fos and Fra-2 binding activities, whereas it did not alter Jun D and Fra-1 binding. These increasing binding activities are associated with enhanced AP-1 transactivation, which may contribute to cytokine dysregulation and immunotoxic effects associated with exposure to trichochecene mycotoxins such as DON (Li et al., 2000).

Evidence for cytokine dysregulation was found by Meky et al. (2001). Blood was collected from healthy donors and mononuclear cells were isolated. Duplicate cultures were prepared with or without phytohemagglutinin-p for each concentration of DON (0, 100, 200 and 400 ng/ml). Supernatants were collected every 24 h for 3 d. Cytokine levels were analyzed in supernatants by ELISA. The results show cytokine production could not be detected in cells that were treated with toxin alone or which did not receive PHA stimulation. Short-term treatment of PHA-stimulated lymphocytes with DON modulated the kinetics of IL-2, IL-4 and IL-6 production. IL-2 levels were up to 12-fold higher in comparison to control levels at DON concentrations of 200 and 400 ng/ml 72h after treatment. IL-4 levels were only slightly elevated and IL-6 levels were slightly inhibited by these DON concentrations. At the lower DON concentration of 200 ng/ml, IL-2 levels were
elevated with a concomitant mild elevation in IFN-γ. At 400 ng/ml, IL-2 levels were significantly elevated until 6 d post treatment.

Effects of DON on the production of the cytokines have been investigated in a human macrophage model (Sugita-Konishi et al., 2001). U-937, a human monocyte-like histocytic lymphoma, was differentiated into macrophages by incubating with PHA. Subsequently, the U-937 macrophage cells were incubated with different concentrations of DON alone or in combination with 0.2 µg/ml lipopolysaccharide (LPS). At appropriate time intervals, the cells were analyzed for IL-6, TNF-α and IL-8 production using ELISA. In the absence of LPS, DON at 500 or 1000 ng/ml upregulated TNF-α production as early as 3 h and up to 6 h, whereas 100 to 1000 ng/ml of DON significantly increased production of IL-6 from 3 to 24 h and IL-8 from 6 to 48 h. In the presence of LPS, DON at 500 or 1000 ng/ml markedly induced TNF-α and IL-8 production. However, for IL-6, 100 ng/ml of DON potentiated LPS-induced IL-6 production, while 500 or 1000 ng/ml of DON suppressed the LPS-induced IL-6 production. As a result, DON induces or superinduces cytokines production even at DON concentrations that are cytotoxic (as shown in section ‘inhibition of cell proliferation’). In another study, the monocyctic U937 cells were used as a model for human alveolar macrophages. The IL-8 cytokine in U937 cells peaked (0.6 ± 0.2 µg/ml) at the very same DON concentration (250 ng/ml) that inhibited 50% of the cells’ protein synthesis suggesting that IL-8 can be used be an additional index for cytotoxicity in mononuclear phagocytes (Instances et al., 2004).

These data suggest DON has potent effects on human lymphocyte cytokine production, which could be used to investigate DON exposure in human population. Low dose exposure of DON (50 ng/ml) induced increased IL-2 levels with mitogen stimulation and involved
transcriptional (increase mRNA level) and posttranscriptional (increase mRNA stability) mechanisms. DON exposure (100 ng/ml) also induced increased IL-6 and TNF-α level and chemokine IL-8 level (Li et al., 1997; Sugita-Konishi et al., 2001). Since IL-2 therapies have been associated with rheumatoid arthritis and vitiligo development (Massarotti et al., 1992; Richards et al., 1992) and IL-2 may mediate the number of T helper cells, which induce B lymphocytes to differentiate into plasma cells which secret IgA. Increased serum IgA levels were found to associate with autoimmune disease in mice because the immune-complex was deposited in kidney, similar to human IgA nephropathy (Bondy and Pestka, 1991). Therefore, increased serum IL-2 after DON exposure could be used as a biomarker to monitor chronic disease such as human Ig A nephropathy associated with DON exposure. At the same time, DON exposure induces IL-6 levels, which also stimulate plasma cells to secrete IgA (Sugita-Konishi et al., 2001). Therefore, DON exposure stimulates mitogen- induced lymphocytes secretion of these cytokines at lower concentrations. It is not clear if IL-2 and IL-6 could be used as biomarkers in plasma after chronic DON exposure because the level of lymphocyte stimulation and resultant cytokine levels would probably differ from the well-controlled levels in vitro.

(d) Changes in immunoglobulin

Human peripheral mononuclear cells were obtained from healthy male and female humans; pokeweed mitogen was added to the cultures at 1 µg/ml. The lymphocytes were cultured for 7 d and the medium was refreshed. Antibody production was tested using ELISA kit. The results show that immunoglobulin (Ig) A, IgG and IgM production were inhibited by exposure to DON. The IC_{50} of inhibitory effects of DON on Ig production was 0.37 µM, 0.39 µM, 0.4 µM, for IgG, IgM and IgA, respectively (Thuvander et al., 1999).
Another study in pigs showed similar results. ConA stimulated pig peripheral blood mononuclear cells were exposed to DON for 72 h with IC_{50} of inhibitory effects on Ig production of 0.41 µM, 0.28 µM and 0.24 µM, respectively (Goyarts et al., 2006b).

However, in vivo study (animals were fed DON and Ig levels measured in plasma or serum) or in vivo-vitro study (animals were fed DON, lymphocytes removed, and Ig levels measured in supernatant of cultured lymphocytes), IgG, IgM and IgA levels were inhibited or increased after DON exposure depending on species and exposure dose and time. In C57BL6 mice, a subchronic dose of 0.071 mg DON/kg b.w. for 3 d per week for 4 weeks (corresponding to daily mean intakes of 0.2 ppm contaminated feed applied for four consecutive weeks) provoked increases in plasma IgA (Gouze et al. 2006). In German Landrace x Pietrain pigs (both sexes), serum IgA levels was significantly increased after they were fed wheat naturally contaminated with DON (6.5 mg/kg diet) for 11 weeks ad libitum, with no change in restrictively fed pigs fed DON (Goyarts et al., 2005b). In the follow-up study, Goyarts et al. (2006) found IgA, IgM and IgG in the supernatant of cultured lymphocytes were not significantly affected after dietary DON exposure (5.7 mg/kg diet, 550 g diet) over 4 weeks or as a single dose (pigs fed restrictively). However, serum IgM and IgG were significantly increased in pigs fed a single dose of DON, although IgA was not affected (Goyarts et al., 2006b).

Variation in results that DON influences immunoglobulin levels could be due to various other factors than DON that may influence Ig production in animals, such as exposure time and dose, or cytokine levels. Firstly, IL-2, a TH1 cytokine expressed in T-lymphocytes following activation, plays a central role in T and B cell growth and differentiation. IL-2 can act as a stimulus for synthesis of IgA and also act in concert with
other cytokines to enhance IgA production. Ingestion of DON results in elevated serum IgA and IgA nephropathy in mice, which may be mediated through the superinduction of gene expression of several cytokines that include IL-2 and IL-6. Secondly, immunoglobulin secretion is dependent on the type of lymphocytes used. For example, IgA and IgG secretion was highest in ConA stimulated compared to LPS stimulated or unstimulated cultures (Pestka et al., 1990). Thirdly, IgA is important for mucosal immune system. Transcytosis of IgA antibody across epithelia is mediated by the poly-Ig receptor, a specialized transport protein. It is reported that DON exposure in the intestine modulated the activities of intestinal transporters (Maresca et al. 2002). Therefore, it is possible the serum IgA levels was increased partly due to dysfunction of poly-Ig receptor. Additionally, different species have different responses to DON exposure. For example, DON caused more diverse immunoglobulin disturbances in pigs than in mice.

Take together, DON influence IgA, IgG and IgM levels after exposure to natural contaminated DON. Serum IgA level was significantly increased in mice fed 0.2 ppm DON for 4 weeks (Gouze et al. 2006). In pig, serum IgA level was not influenced after fed restrictively with DON contaminated food at 5.7 mg/kg diet as a single time or 6.5 mg/kg diet for 11 weeks (Goyarts et al., 2005b; Goyarts et al., 2006b). Serum IgG and IgM was observed to increase after single dose at 5.7 mg/kg diet in pig (Goyarts et al., 2006b). Effects of DON on immunoglobulin need further study to decide its effect in human.

(e) Inhibition of macrophage microbicidal killing and NK cells activity

Macrophage phagocytosis of microorganisms is important in host immunity and activated macrophages kill ingested pathogens by production of reactive oxygen and nitrogen metabolites. DON shown previously to exert immunosuppressive effects on the
immune system was examined for in vitro effects on some functions of murine peritoneal macrophages (Ayral et al., 1992). Peritoneal exudate cells (macrophage cells) in peritoneal cavity of Swiss Female mice (6 ~ 8 weeks) were collected. The cells were pre-incubated for 4 h with the mycotoxin (0.1 ~1000 ng/ml). At concentrations that did not affect cell viability (based on Specific Lactate Dehydrogenase test), DON suppressed microbicidal activity of phagocytic cells against model microbes, opsonized *Saccharomyces cerevisiae*. DON inhibited phagocytosis, microbicidal activity and superoxide anion production at 1 ng/ml whereas phagosome-lysosome fusion was reduced above 100 ng DON/ml. These results suggested that microbicidal activity inhibition by DON was independent of non-oxidative pathway (phagosome-lysosome fusion) impairment.

Berek et al. (2001) investigated the effects of DON on lymphocyte blast transformation activity, antibody-dependent cellular cytotoxicity (ADCC, destruction of antibody-coated target cells by NK cells) and natural killer (NK) cell activity. Human peripheral blood mononuclear cells (PBMC) were isolated from healthy blood donors. The cultures were incubated in the presence or absence of mitogens: PHA and Con A. [³H] thymidine assay were used to investigate lymphocyte blast transformation (T lymphocyte proliferation assays). The research compared the difference between incorporated activity of transformed cells and control cells (without mitogens). In the ADCC test, ⁵¹Cr labelled freshly collected Rh (D, C) - positive O red blood cells (RBC) were used as target cells and PBMC macrophages as an effector in a 1:10 ratio. The reaction was mediated by RBC-specific anti-D antibodies. The amount of released ⁵¹Cr in supernatant was determined using a gamma counter after incubation at 37°C for 16 h. In NK assay, ⁵¹Cr- labeled K562 cells were used as the target and PBMC used as effector in a 1:50 ratio. The amount of
radioactivity released into culture supernatants was determined and the results were expressed as percentage of inhibition after incubation at 37°C for 4 h. The investigated concentrations of DON were 0.1~1800 ng/ml. The results showed that DON exerted immunosuppressive effects on human peripheral blood mononuclear cells in vitro manifested as depressed T or B lymphocyte activity and inhibited NK cell activity at 50 ng DON/ml, suggesting that protection against tumor development may also be attenuated.

Taken together, these results show DON exposure exerted an inhibitory effect on the resident macrophage cell and NK cells at concentrations which occur in human peripheral blood. Natural killer cells are an early component of the host response to virus infection. Inhibition of NK cell activity occurs after DON exposure, the levels of some viruses will be much higher in the early days of the infection. Macrophage cells as phagocytic cells provide a first line of defense against infection and act as one of important antigen-presenting cells. Inhibition of macrophage cell activity and lymphocyte activity will cause inhibition of microbicidal killing and alteration of T_H1-T_H2 balance.

(f) Induction of inflammation-associated genes

DON and other trichothecene mycotoxins mediate a broad range of immunotoxic effects via the induction of inflammation-associated genes in leukocytes. Moon and Pestka (2002) tested the hypothesis that DON induces cyclooxygenase-2 (COX-2) gene expression in macrophages and they determined that DON-increased COX-2 is regulated at the level of mitogen-activated protein kinases (MAPKs). Exposure of the murine macrophage cell line RAW 264.7 to 50-250 ng/ml DON for 24 h markedly enhanced the production of prostaglandin E_2 (PGE_2), a major COX-2 metabolite. PGE_2 elevation was preceded by increases in COX-2 mRNA (2 h) and COX-2 protein (15 h) in DON-treated cells. DON
induced rapid (15 min) and persistent (up to 240 min) phosphorylation of extracellular, signal regulated protein kinases 1 and 2 (ERK1/2) and p38 MAPK as well as a rapid (15 min) but transient (up to 60 min) phosphorylation of c-Jun N-terminal kinases 1 and 2 (JNK1/2). The ERK inhibitor PD98059 and p38 inhibitor SB203580 suppressed DON-induced PGE₂ and COX-2 protein expression, whereas impairment of JNK function by transient transfection with a dominant negative (dn) JNK vector had no effect on COX-2 protein expression after DON treatment. In cells transfected with a COX-2 promoter-luciferase construct, PD98059- and SB203580-, but not dnJNK-treatment, suppressed DON-induced luciferase transcription. DON also increased COX-2 mRNA stability, and this was inhibited by PD98059 but not by SB203580. Taken together, these results indicate that DON-induced PGE₂ production and COX-2 expression by elevating transcriptional activity and mRNA stability. Enhanced transcriptional activity was modulated by ERK and p38 signaling pathways, whereas mRNA stability was promoted exclusively by DON-activated p38 phosphorylation. These data provide insight into possible general mechanisms by which DON and other trichothecenes upregulate proinflammatory genes and impart immunotoxicity.

Animal studies

(a) DON toxicity effect on inflammation

Kinser et al. (2004) reported acute exposure to DON modulates gene expression profiles in murine spleen by microarray analysis. B6C3F1 mice were treated orally with 25 mg DON/kg body weight. Two hours later, the spleens were collected for microarray
analysis. DON was found primarily to modulate genes associated with immunity, inflammation, and chemotaxis.

Endotoxin is a biologically active component of the Gram-negative bacterial cell wall that exists as complexes of LPS and protein. Mononuclear phagocytes are main targets for LPS. Islam and Pestka (2006) reported LPS priming sensitized a host to DON-induced proinflammatory cytokine induction and apoptosis in mice. Male B6C3F1 Mice (7 weeks) were primed with LPS (1 mg/kg b.w.) i.p. and were treated with DON (0.5 to 25 mg/kg b.w) p.o. 8 h later. For cytokine protein and mRNA determination, blood was collected at 2 h after DON treatment. The results show the minimum DON doses for inducing IL-1α, IL-β, IL-6 and TNF-α serum proteins and mRNAs in spleen were significantly lower than the DON doses required for vehicle-primed mice. DON doses from 2 to 25 mg/kg b.w. significantly induced plasma IL-1β, and LPS pre-treatment lowered the threshold for DON to 0.5 mg/kg. An identical pattern was observed for IL-6 in plasma. DON at 12.5 and 25 mg/kg b.w. but not lower doses induced TNF-α; LPS-pre-treatment lowered this threshold to 2 mg/kg in plasma. Although IL-1α was not detectable in serum of any experimental group, thresholds for induction on splenic IL-1α mRNA by DON alone was 12.5 mg/kg b.w.; thresholds for DON-induced IL-1α mRNA decreased to 5 mg/kg following LPS priming. Increased apoptosis in the thymus was observed in mice primed with LPS i.p. at DON concentration of 12.5 mg/kg. As a result, LPS potentates the effects of DON in vivo and in vitro. These results indicate DON exposure could increase sensitivity in chronically sick or malnourished individuals.

*Salmonella enteritidis*, a bacterium causing food-borne infection, is often used for studying mechanisms of bacteria invasion into intestinal cells in vivo and in vitro. S.
*Salmonella enteritidis* infects intestinal cells at first, and then transfers to the other organs via blood stream. The effect of DON on *Salmonella enteritidis* infection was investigated in male Balb/c mice. Mice were given water containing 2 ppm DON or no toxin for 3 weeks. The results of bacteria counting showing that *Salmonella* increased more rapidly in the mesenteric lymph node, liver and spleen of the DON-treated mice than in untreated mice. These finding suggest that DON promotes invasion or/and growth of *Salmonella* in the lymph node, liver and spleen. These results indicate that DON reduces the resistance of the organs and enhanced infection through inhibition of cell-mediated immune function (Hara-Kudo et al., 1996).

Atroshi et al. (1994) investigated the effect of dietary exposure to DON on host resistance and serum immunoglobins of normal and mastitis lactating inbred Han:NMRI mice. The influence of DON on mastitis was evaluated by examining, pathologically and immunologically, the degree of infection in the presence of *S. hyicus* and *M. avium*. After feeding a single dose of DON (12.5 mg/kg b.w.) or a week of dosing of DON (6.25 mg/kg b.w. per day), the mice were infected with mastitis. The levels of serum IgA, IgG and IgM were increased in mice fed 12.5 mg/kg b.w. DON. A single treatment of DON and infection didn’t show significantly difference from control, whereas one-week treatment of mice with DON had a significant mediating effect on the severity of mastitis. Macroscopic examination of the mammary glands revealed severe mastitis in 70% of the glands of control mice, compared with only 20% in the glands of the DON treatment mice. These results indicate that DON is able to modulate the acute-phase response to infection through non-specific host-resistance factors.
(b) DON toxicity on humoral and cellular immunity

Weanling male Swiss Webster mice were administered by gavage 0.75, 2.5, and 7.5 mg of DON per kg body weight once per day for 5 weeks. Serum antibody (IgM) levels to sheep red blood cells (SRBC) were significantly reduced in the treatment groups compared to the control groups. Plaque-forming cell (PFC) numbers were also lower in the treated groups compared to the control groups. Furthermore, DON at a dose of 0.75 mg/kg b.w. resulted in a significant increase in the albumin, albumin/globulin ratio and a decrease in the alpha-2 globulin fraction compared to the control groups. Administration of 7.5 mg/kg b.w. of DON resulted in deaths, due to toxicity, in all animals of this group within 3 weeks. These findings indicate a potential effect of DON on the immune system which could have serious implications for humans (Tryphonas et al., 1984). Tryphonas et al. (1986) gave male Swiss Webster mice sublethal doses (0, 0.25, 0.5 and 1 mg/kg b.w./day corresponding to 0, 1, 2 and 4 ppm of DON in feed for 5 weeks to study DON effects on humoral and cellular immunity. DON at 1.00 mg/kg resulted in a statistically significant reduction in the serum levels of alpha 1 and alpha 2-globulins, an increase in total serum albumin, and a reduction in feed consumption and body weight gain compared to the control group. The 0.50 mg/kg dose of DON resulted in significantly reduced serum levels of alpha 2- and beta-globulins while a significant reduction of feed consumption was evident only during Week 4. Similarly, body weight gain in this group of mice was significantly reduced during Week 2 but increased to normal levels during Week 3 and remained parallel to the control for Week 4 and 5. Both levels (0.50 and 1.00 mg/kg) of DON resulted in a reduced, dose-related, time-to-death interval following a challenge with L. monocytogenes and increased proliferative capacity of splenic lymphocyte cultures stimulated with the
phytohemagglutinin P (PHA-P) mitogen compared to the control group of mice. The 0.25 mg/kg dose of DON did not have any significant effects on the parameters studied. A reasonable estimation of a 'no effect' level for immunologic effects in mice based on these and previous immunological studies would seem to be between 0.25 and 0.50 mg/kg b.w./day.

Male Balb/c mice (4~6 weeks) were used to investigate the immunosuppressive effect of DON. Mice were daily fed DON for 1 or 2 weeks at doses of 2.5 to 100 ppm in the diet. The 100ppm exposure of DON in diet was lethal to all animals within a few days. After oral administration of DON to mice, liver and kidney weights were not changed whereas thymus weight was reduced significantly at concentration >10 ppm. Histologically, the structure of the thymus was damaged and the high doses produced an atrophy of this organ. The spleen weight was reduced less than thymus weight. Serum levels of anti-sheep red blood cell antibodies (in vivo T-dependent synthesis of anti-sheep red blood cells antibodies) were significantly reduced with dose-response effect. The stimulation of B and T cells by mitogens was depressed more for thymic cells than for splenic cells. In vino, DON inhibited cell proliferation estimated by $[^3H]$ thymidine incorporation: murine splenocytes were more sensitive than XP human fibroblasts. These results indicate that the immune system is the primary target of DON toxicity (Robbana-Barnat et al., 1988). Thymus weight reduction and structural damage after DON exposure suggested children could be more sensitive to DON exposure than adults because in the fetus and the juvenile the thymus is the source of large numbers of new T lymphocyte. In mature individuals, the development of new T cells in the thymus slows down and T cell numbers are maintained through T cells outside the central lymphoid organs.
(c) DON interactions with other mycotoxins

The effects of dietary exposure to DON and zearalenone (ZEA) at naturally occurring levels (DON 0.5, 5 and 25 ppm; ZEA 10 ppm; DON 5 ppm or 25 ppm plus ZEA 10 ppm) on the immune function were evaluated for 2-3 and 8 week feeding periods in B6C3F1 weanling female mice, and the effects of immune parameters after exposure to DON and ZEA in combination were evaluated. The immune assays included resistance to *Listeria monocytogenes*, plaque-forming cell (PFC) response to sheep blood cells and delayed hypersensitivity response by measuring the degree of footpad swelling response to keyhole limpet hemocyanin. The results showed that dietary DON depressed the plaque-forming response to sheep red blood cells, the delayed hypersensitivity response to keyhole limpet haemocyanin and the ability to resist *Listeria monocytogenes* after 2 weeks feeding. Resistance to *Listeria* was reduced to a greater extent by co-administration of DON and ZEA than by DON alone, whereas the ability of DON to inhibit the delayed hypersensitivity response was significantly lessened in the presence of ZEA. Immunosuppression can thus result from ingestion of *F. graminearum*-infected agricultural staples, the suppression being attributable to interactions between direct immunotoxic effects of DON and ZEA and nutritional effects associated with DON-induced food refusal. Dietary exposure to naturally occurring levels of DON and ZEA caused significant effects on host resistance, humoral immunity and cell-mediated immunity in the mouse (Pestka et al., 1987).

As a result, DON immunotoxicity was influenced by exposure time and interaction with other mycotoxins. For example, the dietary DON depressed the plaque-forming response and delayed hypersensitivity response after 2 weeks feeding, whereas no effect at
these terms after 8 weeks feeding. At the other hand, interaction with ZEA influences DON toxicity. Take delayed hypersensitivity response as an example, after 3 weeks feeding, DON alone decreased the delayed hypersensitivity response, whereas no effect at this term after interaction with ZEA. DON immunotoxicity interaction with other mycotoxins such as aflatoxin or fumonisin, need further research.

(d) Other DON toxicity research

Instanes and Hetland (2004) reported that DON did not increase levels of allergen-specific IgE or IgG1 in a mouse model (female Balb/c mice, 6-8 weeks old). However, DON reduced cellular protein synthesis, proliferation and survival rate dose-dependently in human colonic (Caco-2), lung (A549) and monocytic (U937) cell lines and increased the production of IL-8 in U937 cell line. These results suggest that DON may have toxic effect on human alveolar macrophages and epithelial cells in lungs and colon. So DON may have some potential environmental problem at work places where cereals are processed and food harvest.

Male C57BL6 mice (6 weeks old) were fed low doses of DON through 4 wks (0.014, 0.071, 0.355 and 1.774 mg of DON/kg b.w. applied three times for a week) corresponding to daily average intakes of 0.05, 0.2, 1 and 5 ppm of contaminated feed. Plasma IgA concentrations were significantly increased by 66%, 48% and 47% in animals receiving 0.2, 1 and 5 ppm DON. No significant change occurred in serum IgG and IgM concentrations in any treatment group. Liver biotransformation enzymes were examined. The two liver microsomal mixed-function oxidases ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) were unaffected, whereas pentoxyresorufin O-depentylase (PROD) activity was increased in 0.05, 0.2 and 1 ppm DON treatment groups.
Cytochrome P450 (P450) 2b subfamily expression was significantly increased in the liver of mice receiving 0.2 and 1 ppm DON, and P450 2b expression had significant correlation with PROD activity. Western Blot analysis of cytosolic GST α, μ or π subfamilies expression demonstrated an induction (56-65%) of the GST π subfamily in liver of mice receiving these doses of DON, a slight increase in GST α expression was observed at 1 ppm DON, whereas GST μ remains unchanged. A significant competitive inhibition of DON on 1-chloro-2, 4-dinitrobenzene (substrate) conjugation in vitro suggests that DON will bind to the same site on the enzyme and could likely to be a potential substrate of liver cytosolic GST π subfamily. These results also indicate that a subchronic low dose exposure could cause changes in the liver metabolism of xenobiotics (Gouze, et al. 2006). Further study should investigate DON metabolism by GST. In theory, the 12, 13 epoxide group characterizing all epoxy-trichothecene toxins could be a target for these for GST conjugate enzymes.

Pigs are the most sensitive animals to DON. Ingestion of naturally contaminated DON induced vomiting and reduced feed consumption. Goyarts et al. (2005) investigated the effects of a chronic DON intoxication on performance, hematological and serum parameters of pigs when diets were offered either ad libitum or restrictively. Female and castrated male crossbred German Landrace × Pietrain pigs were fed DON concentrations of 395 µg/kg diet and 16643 µg/kg diet for control group and DON contaminated diet group, respectively. The growth experiment covered the live weight range between 26 and 100 kg and lasted 11 weeks in total. The results shows there were not significant differences in feed intake, live weight gain and feed to gain ratio in restrictively fed groups. In ad libitum fed control group, feed intake was 2.90 kg/day, live weight gain 987 g/day and feed to gain ratio 2.77
kg/kg; in ad libitum fed DON group, consumed 15% less feed and gained 13% less live weight, while the feed to gain ratio was unaffected. The sex of pigs significantly affected live weight gain and feed intake. Castrated males of the ad libitum fed groups had a significantly higher feed consumption and the live weight gain than the females, while under restrictive feeding conditions no marked differences. Restrictively DON fed animals needed more time to consume the restrictive feed than the control group. A total of 85% of the control pigs consumed their feed within the first hour after feeding, while only 39% of the DON group did this. At the same time, serum IgA levels show significant difference in ad libitum groups, while no difference in restrictive groups. Additionally, serum clinical-chemical parameters, such as total protein, glutamate dehydrogenase, γ-glutamyltransferase, aspartate aminotransferase and creatinine kinase concentrations, were not affected by dietary treatment, except concentrations of alkaline phosphatase significantly lower in the ad libitum DON group than its counterparts. As a result, in restrictively fed groups, there was no significant different in feed consumption, sex, serum IgA levels and serum clinical chemical parameters in both control and DON groups, except longer time to be required consume restrict feed in DON group than control group. In ad libitum groups, there was significant different in these terms between DON group and control group. These results indicated ad libitum and restrictive consumption influenced the feeding consumption, and influenced DON concentrations, and then influence hematological parameters.

Low dose DON exposure (0.2 ppm diet) in mice induced liver Cytochrome P450 (P450) 2b subfamily and GST π subfamily expression suggested low doses of DON causes changes in the normal liver metabolism of xenobiotics. Therefore, cytochrome P40 and
GST π could be used as biomarkers to monitor the exposure of DON in human and liver enzyme induction after DON exposure need further research in other species (Gouze et al., 2006). In Goyarts et al. (2005) study, in restrictively fed groups DON exposure has less influence to pig in serum IgA levels and serum clinical chemical parameters than these terms in pigs in *ad libitum* group. Investigation of the difference between restrictively consumption and *ad libitum* after chronic consumption of low dose DON contamination food need further research in other species.

**Potential effects on the human population**

Outbreaks of DON mycotoxicosis are associated with consumption of mold damaged food products all over the world, in countries such as India and China. The main clinical symptoms are nausea, vomiting, dizziness, abdominal pain and diarrhea; some cases show headache and fever (Pestka, et al., 2005 review). In an epidemiological study, an outbreak in India in 1989 was associated with the consumption of bread made from mold-damaged wheat. The disease was not age or sex specific. Evidence of mold damage of wheat consisted of the presence of fungi, such as *Fusarium* sp. and trichothecene mycotoxins, such as DON.

In order to obtain a no observed adverse effect level (NOAEL) and acceptable daily intake (ADI) for human, the data in animal studies could be useful. If taking DON toxicity to humoral and cellular immunity as an endpoint (Tryphonas et al., 1986), sublethal doses of 0, 0.25, 0.5 and 1 mg/kg b.w./day corresponding to 0, 1, 2 and 4 ppm DON in food were investigated after 5 weeks of feeding male Swiss Webster mice. The results showed that the 0.25 mg/kg dose of DON did not have any significant effects on the serum levels of alpha 1 and alpha 2-globulins, serum albumin, feed consumption and challenge with *L.*
monocytogenes. Combining uncertainty factors (interspecies and interindividual, for a factor of 46, Kodell and Gaylor 1999 NYAcad Sci), NOAEL or ADI would be 5.4 µg/kg b.w. /day for humans. In another study (Gouze et al., 2006), male C57BL6 mice were fed 0.014, 0.071, 0.355 and 1.774 mg of DON /kg b.w., corresponding to daily average intakes of 0.05, 0.2, 1 and 5 ppm contaminated feed for 4 weeks, plasma IgA levels were significantly increased by 66%, 48% and 47% in groups fed 0.071, 0.355 and 1.774 mg of DON /kg b.w. If taking IgA levels as an endpoint, NOAEL or ADI will be 0.3 µg/kg b.w. /day for humans. The threshold for serum IgA elevation was at an oral dose of 0.071 mg/kg given three times a week for 4 weeks, which seems quite a low dose for human, about 20 µg per person. It is possible that increased serum IgA levels is a specific and early toxicological endpoint. At the cellular level, DON (50 ng/ml) regulated T helper cell cytokines, such as IL-2 (Li et al. 1997), and DON (100 ng/ml) stimulated macrophages to secrete IL-6 (Sugita-Konishi et al., 2001). IL-2 and IL-6 can stimulate B lymphoblast to differentiate to plasma cell to secrete IgA. Therefore, monitoring serum IgA is a realizable method to investigate the environmental DON exposure. NOAEL or ADI will be 0.3 µg DON/kg b.w./day for humans with serum IgA as endpoint.

It is of obvious concern that exposure to DON might produce chronic effects such as reduced weight gain and immunotoxicity in human population. Nowadays, a lot of studies have been done in animal models to help investigate the immunotoxicity of DON. Feeding male C57BL6 mice 0.071 mg of DON /kg b.w. for 4 weeks (corresponding to 0.2 ppm diet) significantly increased serum IgA (Gouze et al., 2006). Feeding male Swiss Webster mice 1ppm diet for 5 weeks did not have significant effects on the α-1 and α-2 globulins, total serum albumin, feed consumption, body weight gain and proliferation capacity of splenic
(Tryphonas et al., 1986). Combining uncertainly factors (interspecies and interindividual), the safe guideline maximum level 500 µgDON/kg diet (Codex Alimentarius Commission FAO/WHO, 2003) is still too high because of increase of serum IgA levels after 200 µgDON/kg diet exposure in mice. Further research should investigate what level of increase of IgA seriously affects human health.

Epidemiological studies have not yet targeted immunotoxicity of DON in humans. In lab situation, cells were cultured in medium and cell growth conditions were environmentally stable, whereas the human system is more complicated. In cell lines, in recent studies, the exposure concentration of DON was 50–1000 ng/ml (some up to 10 µg/ml) in order to research effects of DON on cell proliferation inhibition and cytokine levels. Significant inhibition of cell proliferation was reported at 100 ng DON/ml and cytokine level (e.g. IL-2) upregulation at 50 ng DON/ml (Meky et al. 2001; Li et al., 1997). Investigation of DON exposure through detection of DON and its glucuronide in human urine in high risk region found that the concentrations of DON were lower at 37ng/ml (this amount included DON glucuronide) (Meky et al. 2003). Therefore, it is an interesting question as to whether the level of DON in human serum is really of concern in terms of cytokine dysfunction and lymphocyte proliferation inhibition.

**Conclusion**

**(a) Summary**

DON is a mycotoxin produced by *Fusarium graminearum* and *Fusarium culmorum*. It is commonly found on cereals grown in the temperate regions of America, Europe and Asia (review by WHO 1990). The substance is a very stable compound; it does not degrade at
high temperatures and is found in raw and processing cooking of food (Castells et al., 2005). It could be a DON detoxification route to incubate seeds with biotransforming microorganisms from the gut in chicken/ruminant animals or the soil (He et al. 1992; Swanson 1987; Binder et al. 1998). Essential oils could be useful as alternatives to food preservatives currently in use (Velluti et al. 2004).

Numerous methods have been developed to monitor DON contamination in foods or detect DON residue in tissue, such as chemical method GC, HPLC-UV, LC-APCI-MS, and screening methods ELISA and cell proliferation assay. ELISA assay has a lowest detect limit with 0.2 ng/ml in buffer without clean up after extraction (Ngundi et al. 2006), however ELISA has crossreactivity between DON and other trichothecenes (Krska et al. 2001). Immunoaffinity column enrichment and HPLC-MS/UV detector has a very low detect limit at 12-20 ng/ml can detect individual compound specifically (Janes and Schuster 2001; Meky et al. 2003). The cheapest method is clean up using alumina and charcoal SPE column combined with cell proliferation assay. At the same time, it was reported this method has a good correlation with chemical method such as GC and HPLC (Landgren 2005).

DON has poor absorption in sheep with systemic bioavailability only 7.5% (Prelusky et al., 1985) and poor absorption in chicken with maximum plasma levels less than 1% of the administered dose (Prelusky et al., 1986) and about 1% free DON in cow urine (Cote et al., 1986). DON could be rapidly absorbed while passing through the stomach and the upper small intestine in swine (Avantaggiato et al., 2004; Danicke et al., 2004) with systemic bioavailability of 50% from a single oral dose or 89% from chronic intake (4-8 weeks) with maximal plasma concentrations (C max) of 21.79 and 15.21 ng
DON/ml serum after (t$_{\text{max}}$) 88.4 and 99.1 min in the chronic (n = 5) and acute (n = 6) fed group, respectively (Prelusky et al. 1988; Goyarts and Danike, 2006a). DON can be degraded to DOM-1 by microorganisms in rumen and gut of ruminant animals such as cow and sheep and in gut in animals such as pig and chicken (Swanson 1987; Prelusky et al. 1986; He et al. 1992; Danicke et al. 2004), DON can be transformed in liver or intestine by UDP-glucuronosyltransferase (UGT) to DON glucuronide conjugate in cow, sheep, rat, pig and human (Cote et al. 1986; Prelusky et al. 1987; Mety et al. 2003; Eriksen et al. 2003). At the same time, some evidence showed that DOM-1, the metabolite in gastrointestinal of DON, can be transformed to DOM-1 glucuronide (Prelusky et al. 1986). Further study should investigate whether DON can conjugate with glutathione.

DON exposure (0.2 ppm diet) has immunostimulatory effects resulting in elevated serum IgA levels (Gouze et al. 2006) and upregulation of the expression of many immune related genes such as those coding for COX 2 at 50 ng/ml (Moon and Pestka, 2002) and cytokines (TH$_1$ and TH$_2$) (eg. IL-2, 50 ng/ml) (Li et al., 1997). DON exposure could severely injury actively dividing tissues including bone marrow, lymph nodes, spleen and thymus resulting in immunosuppression by lowering T and B cells numbers (IC$_{50}$ 216 –500 ng/ml) (Meky et al., 2001; Uzarski et al., 2003), decreasing Ig G and IgM levels at 118 ng/ml (Thuvander et al., 1999), decreased resistance to pathogens (2 ppm DON in drinking water) (Hara-Kudo et al., 1996), inhibition of antibody response to model antigens (sheep red blood cells) (2 ppm diet) (Tryphonas et al., 1986). Additionally, low dose exposure of DON could change hepatic xenobiotic metabolism (Gouze et al. 2006). If immunotoxicity is taken as an endpoint, a human NOAEL may be estimated at 0.3~5.4 μg/kg b.w. (Gouze et al. 2006; Tryphonas et al., 1986).
(b) Future work

It is often difficult to relate experimental results of studies of the toxicology of DON with what occurs under real life situations because invariably there are other related trichothecenes present. 3-acetyl DON and 15-acetyl DON are often present at levels of 10-20% of DON. These toxins are metabolized to DON at varying rates and to varying degrees. Nivalenol and T-2 toxins were found in varying quantities in the food poisoning outbreak in India in 1989. Fumonisin B1 and DON were extracted from non-harvested maize (Cvetnic et al., 2005). This information at least suggests immunotoxicity studies of DON need to include interactions between DON and other mycotoxins, such as aflatoxin and fumonisin.

There are few reports on developing a simple method to screen the human population in high-risk region of DON exposure. Until now, the only reported method is through detection of DON in human urine by HPLC method. Low dose DON exposure (0.2 ppm diet) in mice induced liver Cytochrome P450 (P450) 2b subfamily and GST π subfamily expression suggested low doses of DON causes changes in the normal liver metabolism of xenobiotics. Therefore, cytochrome P40 and GST π could be used as biomarkers to monitor the exposure of DON in human after DON exposure. Serum IgA level was observed to increase after 0.2 ppm DON exposure in mice indicating serum IgA could be a sensitive biomarker in human (Gouze et al., 2006). Additionally, the serum IgA levels were increased partly due to dysfunction of poly-Ig receptor. Thorough investigation of IgA levels in intestine could help explain or exclude this reason.

Determining the exposure concentrations of DON to cells relevant to real-life exposure is a crucial point when cell culture bioassays are employed to study the immunotoxicity of
DON. Future work should determine realistic doses when in vitro models are used for further toxicological investigations as possible alternatives to whole animal testing. Few epidemiological studies have examined immunotoxicity of DON in humans. The needed information would include the occurrence of DON in foods and human clinical samples associated with outbreaks of gastroenteritis and immune suppression, and comparison of incidences of acute (e.g. gastroenteritis) and chronic disease (e.g. IgA nephropathy) in geographical areas of high and low DON exposure.
MATERIAL AND METHODS

Chemicals

DON, uridine 5’-diphosphoglucuronic acid (trisodium salt, UDPGA), uridine 5’-diphospho-N-acetylglucosamine (sodium salt, UDP), L-glutamine, rat liver microsomes and Sephadex LH-20 were all obtained from Sigma Chemical Co., St. Louis, MO. RPMI-1640, gentamicin and fetal bovine serum (FBS)-heat inactivated were all from Invitrogen Corporation (Carlsbad, California). β-glucuronidase, type H-2 from Helix Pomatia was obtained from Sigma Chemical Co., St. Louis, MO. HPLC grade methanol and acetonitrile obtained from Fisher (Fair lawn NJ)

MTS, [3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) - 2-(4-sulfophenyl) - 2H -tetrazolium, inner salt was purchased from Promega Corporation, Madison, WI. Trypan blue solution (0.4%) was obtained from Sigma Chemical Co. St. Louis, MO.

DON glucuronide synthesis and purification

DON glucuronide synthesis

The final standard incubation mixture contained 0.3 mM DON, dissolved in water. Other reagents were 15 mM UDPGA, 50 mM Tris HCl buffer (pH 7.4), 5 mM MgCl₂, and 0.25 mM UDP to activate microsomes. The reaction mixture was prewarmed for 30s before adding 5 mg/mL rat liver microsomes. The volume of reaction mixture was 0.5 ml. The reaction mixtures were inverted a few times, and incubated at 37°C in a water bath for 1 h. Blank incubations contained all ingredients without UDPGA or microsomes. Incubation was terminated by addition of cold methanol (2-fold excess volume) and
centrifuged for 10 min at 10,000 rpm at room temperature (Eppendorf centrifuge 5415 C, Eppendorf North America, Inc. Westbury, CT) to pelletize the microsomal protein.

**Purification of DON glucuronide**

The 75 ml supernatant was concentrated by rotary evaporation (R-114; BÜCHI, Flawil, Switzerland). 5ml concentrated mixture was collected, and then 1 ml of concentrated reaction mixture was loaded onto a Sephadex LH-20 column (2.8 × 47.2 cm, bed vol. 292 ml) each time. The column was equilibrated and eluted with water. Fractions of 4 ml were collected and analyzed by spectrophotometer (Beckman DU 520 UV-Visible Spectrophotometer, Beckman Instruments Inc. Fullerton, CA) at 220 nm. All peak fractions eluting at fraction number 16-21, 23-25 and 33-37 (fraction volume of 4 ml) were combined. β-glucuronidase hydrolysis (in 37 °C water bath overnight, incubated with equal volume 0.2 M sodium acetate buffer (pH 5.5) and 50 µl on 10,000 unit β-glucuronidase, type H-2 from Helix pomatia, obtained from Sigma Chemical Co., St. Louis, MO) showed that fractions number 23, 24 and 25 included DON glucuronide. Therefore, these fractions were collected, lyophilized (Virtis Unitrap II freeze-dryer, Gardiner, NY) and then dissolved in water for further HPLC purification. The aqueous residue of DON glucuronide and some impurity (7 ml, 4 ml + 3 ml) was subjected to an analytical scale purification HPLC system by successive injections of 100 µl volume. The peaks corresponding to DON glucuronide (retention time 14 min) were combined and lyophilized to yield approximately 1.5 mg of material for MS, NMR analysis and cell culture. HPLC (HP1050, Hewlett Packard, Broken Arrow, OK) was equipped with photodiode array detector. Peaks were scanned across 190 nm to 400 nm. A reverse phase Atlantic dC_{18} column (5 µm, 250 × 4.5 mm) fitted with an in-line filter (Waters, Milford, MA) was used to separate DON
glucuronide from impurities. Mobile phase A was ultrapure water (Milli-Q, filtered by 0.2 
µM filter); mobile phase B was acetonitrile. The gradient was initially 0% B, linear to 
12% B over 15 min, switching to 50% B at 17 min, and holding at 50% B until 25 min then 
returning to initial conditions. Flow rate was 0.9 ml/min.

Identification of the structure of the synthesized DON glucuronide

The purity of the biochemically synthesized and purified DON glucuronide was 
determined using HPLC with photodiode array detection. Identification of the DON 
glucuronide was performed using HPLC-UV spectrum from 190 to 400 nm, comparative 
HPLC chromatograms between microsome reaction mixture and blank controls, β-
glucuronidase hydrolysis, mass and nuclear magnetic resonance (NMR) spectrometries.

β-glucuronidase hydrolysis combined with HPLC -UV detection In order to 
determine if rat liver microsomes catalyzed formation of DON glucuronide in vitro, 
reaction mixture after centrifugation was dried under N₂ to evaporate methanol, and then 
dissolved in 1.5 ml distilled deionized (dd) H₂O. Point two of ml reaction mixture residue 
was diluted with 0.3 ml ddH₂O and incubated with 0.5 ml 0.2 M sodium acetate buffer (pH 
5.5) and 50 µl β-glucuronidase (no enzyme for control) at 37°C water bath overnight. C₁₈ 
Sep-Pak cartridge (Waters, Milford, MA) was used to clean-up samples after β-
glucuronidase hydrolysis. After loading the sample, the cartridge was washed with 1 ml 
H₂O and 1 ml 1% methanol. DON or DON glucuronide eluted with 1 ml of 50% methanol. 
At the same time, blank incubations, which contained all ingredients without UDPGA or 
microsomes, were followed every step as the above. HP1050 series (Hewlett-Packard, 
Broken Arrow, Oklahoma) HPLC equipped with photodiode array detector was employed 
to detect the compounds. YMC-PACK C₁₈ column (10 cm × 4.6 mm, 5 µm particle size)
was used with a gradient of 6-9% methanol over 40 min and a flow rate of 1 ml/min. Four different chromatographs were obtained: microsome reaction mixture, blank controls (without UDPGA or without microsome) and after β-glucuronidase hydrolysis reaction. Photodiode array detector was used to check the wavelength across 190 nm to 400 nm for each peak.

**Mass spectrometer** MS analyses were performed with a Shimadzu LCMS-2010 system (Shimadzu, Kyoto, Japan), equipped with an electrospray ionization source (ESI) and atmospheric pressure chemical ionization (APCI) source. A myoglobin and MRFA mixture was used for tuning and routine calibration of the instrument. Purified sample was dissolved in 100% methanol about concentration at 2 mg/ml. The samples were introduced into the electrospray interface through an untreated fused-silica capillary with a 50 µm i.d. and 190 µm o.d. All samples were introduced via 5 µl loop injection.

**Nuclear magnetic resonance spectrometry (NMR)** All samples and spectra were run on a Varian VXR-400 NMR spectrometer equipped with a Bruker magnet, probes, shim supply with Varian console and software (Varian Inc. Palo Alto, CA). Samples of DON standard and purified DON glucuronide were dissolved in approximately 0.8 ml of d-methanol and placed into NMR microtubes. Proton and carbon NMR spectra were acquired on both samples.

**Extinction coefficient determination for DON glucuronide**

DON standard (1 mg, Sigma Chemical Co., St. Louis, MO) was dissolved in 1 ml 100% HPLC grade methanol and then diluted to concentrations from 10 to 100 mg/L. Purified DON glucuronide (0.53 mg) was dissolved in 1 ml 100% HPLC grade methanol and then diluted to 10.6 to 106 mg/L. Samples were transferred to a 1 ml cuvette and UV absorption
measured at 220 nm by spectrophotometer (Beckman DU 520 UV-Visible Spectrophotometer, Beckman Instruments Inc., Fullerton, CA).

**Comparative immunotoxicity of DON and DON glucuronide**

**Sample preparation**

DON standard (1 mg) was dissolved in 1ml methanol and diluted to 100 µg/ml stock solution and stored at -20°C until use. Aliquots of DON and/or DON glucuronide purified by HPLC were evaporated under N₂ and dissolved in cell culture medium with 1.7% ethanol (v/v) and 0.3% DMSO (v/v), and then filtered through 0.2 µm PVDF syringe filter (4 mm, Alltech. Co., Nicholasville, KY) into sterile microcentrifuge tubes and diluted serially twice with the medium plus 1.7% ethanol and 0.3% DMSO.

**Cell culture**

K-562 cells (ATCC® number CCL-243™ Manassas, VA) were cultured in RPMI medium 1640 (1×) supplemented with 25 mM HEPES buffer (pH 7.2), 2 mM L-glutamine, 10% heat inactivated fetal bovine serum and 50 µg/ml gentamicin. The cells were maintained in 75 cm² cell culture flasks in a humidified incubator at 37 °C in an atmosphere of 5% CO₂-95% air mixture. The cells were subcultured three times each week by replacement of fresh medium by starting new cultures at 2×10⁵ viable cells/ml. Cell suspensions of the cells were counted using a hemocytometer and 0.4% trypan blue.

To determine if there was a linear relation of cell numbers to optical density in MTS proliferation assay, single cell suspension containing cell densities ranging from 2×10³ to 2.6×10⁵ cells with viability of 98 % per 200 µl medium/well were added to 96-well plates after serial dilution in sterile centrifuge tubes. The medium was allowed to equilibrate for half an hour, and then the MTS bioassay was performed immediately. To determine the
number of seeding cells, single cell suspension containing cell densities ranging from 407 to 65000 cells per 200 µl medium/well were added to 96-well plates after serial dilution in sterile centrifuge tubes. MTS bioassay was performed after 48 h incubation of cells at 37 °C in an atmosphere of 5% CO₂-95% air mixture. There was a good correlation after seeding 407 to 25000 cells to wells for 48 h incubation. 25000 cells/well was selected for cell proliferation assay because a lower number of cells would not produce sufficient absorption after CellTiter™ was added; because DON inhibits cell proliferation, a strong absorption from the control cells improves the sensitivity of the assay.

The MTS cell proliferation assay

MTS cell proliferation assay is a colorimetric method for determining the number of viable cells in culture in multiwell plate format. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture.

The day of assay, cells in log phase growth were transferred to a 15 ml centrifuge tube and centrifuged 7 min at 1100 rpm, and the pellet was suspended and diluted to 2.5×10⁵ cells/ml (viability > 95 %). A 100 µl volume of cell suspension was added to each well of 96 well microtiter plate except the blank wells (Corning Inc. NY). A 100 µl sample of DON/glucuronide was added to cell cultures in triplicate. The final concentrations of DON and DON glucuronide were 0.13-135 µM, 1.1-270 µM, respectively. One row was used as control for cell growth (no DON or DON glucuronide) and one set of triplicate wells contained medium alone as a blank control. The total volume for each well was 200 µl. Equal concentration of DON and DON glucuronide at 0.5µM, 1.3 µM and 8.4 µM were combined to investigated if DON glucuronide influences DON toxicity. The microtiter
plate was incubated 48 h at 37°C in 5% CO₂/95% air and cell proliferation was determined by MTS cell proliferation assay. CellTiter 96® Aqueous One Solution Reagent (15 µl) was added to each well and swirled to mix. The plate was incubated for 90 min at 37°C in a humidified 5% CO₂/95% air atmosphere and read on an EL340 microplate reader (Bio-Tek Instruments, Winooski, VT) at 490 nm. A reference wavelength of 630 nm was used to subtract background contributed by cell debris and other nonspecific absorbance. The absorption of samples wells was converted to a percentage of control and plotted against the concentrations to obtain dose-response curves. Concentrations of DON that caused 50% inhibition of cell proliferation (IC₅₀) were derived by power trend line because of highest correlation between DON concentrations and cell proliferation in the power trend line (Microsoft Office Excel, Seattle, WA). The data were expressed as the mean of three independent experiments each with triplicate wells per treatment.

**Trypan blue exclusion**

The viability of the cells was estimated by the trypan blue exclusion method. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the cell membrane is damaged. Therefore, all the cells that exclude the dye are viable.

As with the MTS assay, the microtiter plate was incubated 48 h at 37°C in 5% CO₂/95% air. Trypan blue solution (0.4%) was added to cells at the ratio of 1:1. Cell/dye mixture was pipetted to hemocytometer slide and viewed with a light microscope at 100 times magnification. The live cells in each sample were converted to the percentage of live cells found in control incubations, and plotted against the DON/glucuronide concentration
to obtain dose-response curves. IC\textsubscript{50}s were derived by power trend line. The data were expressed as the mean of two independent experiments each with duplicate wells.

**DON and DON glucuronide addition and morphological and number observation**

The morphological observation and cell enumeration by inverted microscope was done at 48 h after DON and DON glucuronide addition. The Nikon TMS-F microscope systems have high-resolution Nikon Coolpix 995 digital cameras (Fryer Company INC, Scientific instruments, Huntley, IL) for the direct electronic capture of images.

**Statistical analysis of data**

All statistical analyses were performed using SAS 9.1 (Cary, NC, USA). One–way analysis of variance (ANOVA) was employed to compare immunotoxicity difference between DON and DON glucuronide and their combination, and to compare methods different between MTS cell proliferation assay and Trypan blue exclusion. A value of p<0.05 was considered significantly different.
RESULTS

DON glucuronide characterization and identification

DON glucuronide (DONGLU) synthesis incubations resulted in the formation of a peak with a retention time of 3.7 min on HPLC showing a similar UV spectrum to that of DON (Fig 4A, Fig 5). Preliminary identification showed that β-glucuronidase hydrolysis caused the formation of product with retention time of 13.1 min and UV spectrum similar to the DON standard (Fig 4A, 4B, Fig 5). Comparative HPLC chromatography of DON glucuronide reaction mix without UDPGA or without microsomes showed that only in the complete reaction mix was there a peak at a retention time of 3.7 min with a UV spectrum similar to DON, putatively DONGLU (Fig 4A, 4C and 4D).

DON glucuronide was purified by Sephadex LH-20 gel filtration. β-glucuronidase hydrolysis showed that fractions 23, 24 and 25 contained a product that was hydrolyzed to DON, putatively DONGLU (Fig 6A, Fig 7). When microsomal mixture was purified by a Sephadex LH-20 gel, DON was not eluted by water. Methanol and water solution of > 10% methanol (50% better) was required to clean the gel after collecting fractions including DON glucuronide. Preliminary study showed DON was eluted by 10% methanol from the Sephadex LH-20 gel after loading partial purified DON material (extract from extracted from *F. graminearum* culture in our lab) (Fig 7 B).

After Sephadex LH-20 fractionation, DON glucuronide was further purified by analytical HPLC. The retention time of DON glucuronide was at 14.6 min and the spectrum of DON glucuronide was similar to DON. The retention time of DON standard was 23.4 min (Fig 8)
Fig 4. Chromatograms showing DON glucuronide (DONGLU) (A) and the appearance of DON after DONGLU hydrolysis by 20 h β-glucuronidase treatment (B). (C) Incubation reaction without microsomes and (D) Incubation reaction without UDPGA show no DONGLU formation. YMC-PACK C\textsubscript{18} column (10 cm × 4.6 mm, 5 µm particle size) was used with a gradient of 6-9% methanol over 40 min, flow rate of 1 ml/min.
Fig 5: UV spectrum of each peak in the chromatogram of DON glucuronide (DONGLU) synthesis reaction (Fig 4A). Only the peak, retention time 3.7 min, shows a similar UV spectrum to DON, which has a maximum absorbance close to 220 nm. There is just one major peak from 190 to 300 nm for DON and DON glucuronide. Tentatively, the peak is DONGLU.
Fig 6: Elution profile of Sephadex LH-20 gel filtration and fractions measured by Spectrophotometer at 220 nm. (A) showed elution of DONGLU synthesis products by water; each fraction volume is 4 ml; Fraction number 23, 24 and 25 included DONGLU were collected and combined to further HPLC purification. (B) showed elution of a partial purified DON(extracted from F. graminearum culture) by 10% methanol; each fraction volume is 8ml.
Fig 7: Chromatograms shows fraction 16-21 did not include DON (a) and DON glucuronide (A, β-glucuronidase hydrolysis); fraction 23-25 did not include DON (b), but show DON peak after hydrolysis (B); fraction 33-37 did not include DON (c) and DON glucuronide (C, hydrolysis). YMC-PACK C18 column (10 cm × 4.6 mm, 5 µm particle size) was used with a gradient of 6-9% methanol (the rest is 0.1% acetic acid water) over 40 min, flow rate of 1 ml/min.
Fig 8: After Sephadex LH-20 fractionation, fraction number 23, 24 and 25 including DONGLU were combined and subjected to an analytical scale purification HPLC system. A reversal phase Atlantic dC₁₈ column (5 µm, 250 × 4.5 mm) fitted with an in-line filter was used to separate DONGLU and impurities. The retention time of DONGLU was 14.6 min (A), which was collected. The retention time of DON standard was 23.4 min (B). Mobile phase A was MQ water; mobile phase B was acetonitrile. The gradient was initially 0% B, linear to 12% B over 15 min, switching to 50% B at 17 min, and holding at 50% A until 25 min then returning to initial conditions at 30 min and holding 0% for 2 min. Total flow rate was a constant 0.9 ml/min. DON and DONGLU have a similar UV spectrum as seen in insert in chromatograms A + B, which have a maximum UV absorption close to 220 nm.
Fig 8 continued

The structure of DON glucuronide was further identified by MS and NMR. The expected molecular weight for DON glucuronide is 470 g/mol. The [DON-3-GLU-H]− peak for DON glucuronide with m/z of 471 was clearly observed on both ESI negative ion and APCI negative ion because of loss of −H at the group -COOH. APCI negative ion also shows m/z 471 peaks was fragmented to [DON-3-GLU-CH2O-H]− = 441 and [DON-3-DON-CH2O-O-H] = 425 as the main fractions. There are three-hydroxyl group at C-3, 7 and 15 of DON. The 3-and 15-hydroxyl groups are readily acylated through chemical synthesis or biosynthesis, while -7 hydroxyl of DON is never found acylated in foods. At the same time, it is possible to distinguish DON-3-glucuronide from DON-15-glucuronide due to the loss of CH2O (-30) from the - CH2OH group attached to the C-6 position of the DON-3-glucuronide. The fraction 441 in APCI negative ion shows that the DON glucuronide synthesized was likely to be DON-3 glucuronide (Fig 9, 10).
NMR spectroscopy is extremely useful for determining the structure of molecules because the local environment around a given nucleus in a molecule will slightly perturb the local magnetic field exerted on that nucleus and affect its exact transition energy. Therefore, addition of glucuronic acid to the hydroxyl group of DON influences the chemical shift of the local carbon, whereas the chemical shift of other carbons won't be changed after addition of a glucuronide conjugate. Because the amount of DON and DON glucuronide (~1 mg) were limited in this NMR assay, several carbon signals at higher chemical shifts did not appear in the $^{13}$C-NMR spectra, such as C=O. At the same time, the carbon signals of D-methanol (solvent) overlapped the carbon signals of the sample. However, analysis of the NMR spectrum of the purified DON glucuronide compared to that of the parent compound, DON, confirmed successful synthesis of DON glucuronide and the placement of the glucuronide moiety at the expected 3-OH position of DON for the following reasons: comparative $^1$H NMR DON and DON glucuronide showed two new double peaks (4.41 ppm and 3.73 ppm) and multiple peaks from 3.25-3.50 ppm corresponding to DON glucuronide NMR assignments (Fig 11). Comparative $^{13}$C NMR of DON and DON glucuronide showed new carbon peaks (103.4 ppm, 78.1 ppm, 75 ppm, 73.9 ppm) that could be assigned to DON glucuronide. The carbon of -COOH from the glucuronide is expected at 172~175 ppm (Fig 12). The same NMR spectrum for glucuronide moiety has been found in the glucuronide moiety of dobutamine monoglucuronides (Anna Alonen, 2005). At the same time, Savard (1991) reported that the only difference between DON-3-glucoside and DON-15-glucoside in chemical shifts of NMR was the carbon attached to glucose. This is in accordance with our finding that only a shift at carbon which carbon number 3 was expected for the carbon attached to glucuronide
moiety. A downfield chemical shift (δ ppm) (to the left) of 5.9 ppm was observed for carbon 3 (DON 70, DON glucuronide 75.9) between the spectra of DON and putative DON glucuronide, whereas no shift at carbon 15 further indicated that the DON glucuronide synthesized in our experiment was DON-3- glucuronide (Fig 9).

Extinction coefficient of DON glucuronide and DON standard were obtained through measuring the absorption at 220 nm of 10.6-106 µg DONGLU/ml and 10-100 µg DON/ml (Fig 13). The εₘ of the extinction coefficients was calculated as 4715.7 (L/cm/mol) for DON glucuronide (Fig 13A) and 6555.8 (L/cm/mol) for DON (Fig 13B). For spectrophotometric determination of type B trichothecenes, different values ranging from εₘ 4500 to 7040 for the molar absorption have been found in the literature, as a result, εₘ = 6400 is used as the molar absorptivity for all type B trichothecenes in acetonitrile (Krska et al., 2001). Krsta et al. (2004) found DON has maximum UV absorption at 217 nm. The mean of the extinction coefficients was calculated as 6727 (L/cm/mol) for DON (Sigma) AND 6825 (L/cm/mol) for DON (Biopure). The calculation for DON extinction coefficients in our research matched the literature.

![Fig 9: The chemical structure DON glucuronide (MW= 472 g/mol)](image)
Fig 10: ESI (negative mode) mass spectrum and APCI (negative mode) mass spectrum of purified DON glucuronide (DONGLU). The expected molecular weight for DON glucuronide is 472 g/mol. The [M]- peak for DONGLU with m/z of 471 was clearly observed on ESI and APCI negative ion because of loss of –H in the –COOH group. APCI negative ion also shows m/z 471 peaks was fragmented to [DONGLU-CH₂-O-H] = 441 and [DONGLU-CH₂-O-O-H] = 425 as the main fractions. The fraction 441 in APCI negative ion suggested DON glucuronide synthesized was likely to be DON-3 glucuronide.
Fig 11: $^1$H-NMR of DON (top) and DON glucuronide (bottom) spectra were run on a Varian VXR-400 NMR spectrometer. Comparative $^1$H NMR of DON and DON glucuronide showed two new double peaks (4.41 ppm and 3.73 ppm) and multiple peaks from 3.25-3.50 ppm assigned to DON glucuronide.
Fig 12. $^{13}$C-NMR of DON (top one) and DON glucuronide (bottom) spectra were run on a Varian VXR-400 NMR spectrometer. Comparative $^{13}$C NMR of DON and DON glucuronide showed new carbon peaks (103.4 ppm, 78.1 ppm, 75 ppm, 73.9 ppm) assigned to DON glucuronide.
Fig 13: The $\varepsilon_m$ of the extinction coefficients was calculated as 4715.7 (L/cm/mol) for DON glucuronide (A) and 6555.8 (L/cm/mol) for DON (B) in methanol at 220 nm absorbance and concentrations of 10-100 µg/ml.
Comparative immunotoxicty of DON and DON glucuronide

Effect of cell number on absorbance at 490 nm measured using the CellTiter 96® Assay showed a linear response with correlation coefficient, $r = 0.94$ for K562 cells in cell numbers from 2000 to 128000 (Fig 14). At the same time, linear regression analysis revealed high correlation between OD signals and the seeded cell number from 1626 to 26000 ($r = 0.98$) following 48h incubations (Fig 15). The numbers of cells seeded for MTS and trypan blue exclusion assays were 25000 cells per well/ 200µl because DON addition should inhibit cell viability.

The cytotoxicity of DON and DON glucuronide were measured in a concentration range from 0.13 µM to 135 µM and from 1.1 µM to 270 µM, respectively and results shown in Fig 16 and Fig 17. A significant decrease in cell numbers compared with controls was observed with DON at concentrations greater than 148 ng/ml (0.5 µM), with $IC_{50}$ of 389 ng/ml (1.31 µM) in MTS assay. As DON concentrations increased from 2.1 to 67.5 µM, inhibition of cell viability gradually increased with cell numbers from 32.5 to 18.1% control. There was no significant difference in cell viability in DON concentrations between 67.5 and 13.5 µM (Fig 16). Viable cells were observed at all concentrations of DON and DON glucuronide tested, using trypan blue exclusion assay, even at highest concentration of DON, 135 µM. The live cells within each sample were converted to the percentage of live cells of the control, and plotted against the concentration to get dose-response curve (Fig 17). The $IC_{50}$ values both as mass and molar concentrations for DON and DON glucuronide were 389 ng/ml (1.31 µM) and >127 µg/ml (>270 µM) measured by MTS assay (Table 2). No significant cytotoxicity was observed with up to 270 µM DONGLU by MTS assay (Fig 16). At the same time,
the IC₅₀ values both as mass and molar concentrations for DON and DON glucuronide were 257 ng/ml (0.87 µM) and >127 µg/ml (>270 µM) measured by trypan blue exclusion assay. Trypan blue exclusion assay showed DON glucuronide at 270 µM inhibited cell numbers with 72% cell growth. However, we could not decide whether or not significant different from controls because of only two separately experiments and duplicate wells for each experiment. ANOVA showed that there was a significant difference in immunotoxicity between DON and DON glucuronide (p <0.0001), with no significant difference between MTS and trypan blue exclusion assay (p = 0.8358). The cell number and necrosis was determined microscopically (Fig 18). The control was taken as 0% cell death. There was no significant cell death observed at 270 µM DONGLU. There was 60% cell death and 85% cell death at 2.1 µM DON and 135 µM DON, respectively after 48 h exposure. Effects of DON and DON glucuronide exposure, singly or in combination on K562 cells proliferation were measured by MTS assay after 48 h incubation. DONGLU did not influence DON toxicity at low, medium and high concentration combinations (0.5 µM, 1.3 µM and 8.4 µM) of each compound (Fig 19).
Fig 14: Effect of cell number on absorbance at 490 nm measured using the CellTiter 96® Assay. Various numbers of K562 cells were added to the wells of 96-well plate in complete RPMI medium. After 90 min at 37°C in a humidified, 5% CO₂ atmosphere, the absorbance at 490 nm was recorded (the background subtracted). Each point represents the mean ± SD of 3 replicates. The correlation coefficient (r) of the line was 0.94 for K562 cells between 2000 to 128000, indicating a linear response between cell number and absorbance at 490 nm.
Fig 15. Effect of cell number on absorbance at 490nm measured using the CellTiter 96® Aqueous One Solution Assay to decide the number of cells to be seeded. Different concentration cells in medium were added to 96 plates and incubated 48 h, and then 15 µl CellTiter was added. Each point represents the mean ± SD of 3 replicates. The correlation coefficient of the line was 0.98, indicating a linear response between cell number from 1626 to 26000 and absorbance at 490 nm after 48 h incubation. 25000 cells/200 µl well were seeded for the MTS assay and Trypan blue exclusion.
Fig 16: Proliferation dose/response curve of K562 cells exposed to DON and DON glucuronide (DONGLU) measured by MTS assay. The values are expressed as percent of control (0µM DON/ DONGLU) and each value is a result of three separate experiments with three replicates/treatment. Bars represent standard errors of the means. * mean significant difference from control, p<0.01; ** mean significant difference from control, p<0.001.
Fig 17: Dose/response curve of K562 cells exposed on DON and DON glucuronide (DONGLU) measured by Trypan blue exclusion assay after 48 h incubation. The values are expressed as percent of control (0 µM) and each value is the mean of two separate experiments (duplicate each time). Trypan blue exclusion assay showed DON glucuronide at 270 µM inhibited cell proliferation with 72% cell growth. However, we could not decide whether or not significant different from controls because of only two separately experiments and duplicate wells for each experiment.
Table 2 IC\textsubscript{50} values for DON and DON Glucuronide (DONGLU) in K562 cells after 48 h incubation measured by MTS proliferation assay and trypan blue exclusion. DON IC\textsubscript{50} values were calculated from derived powder line. DONGLU IC\textsubscript{50} was higher than the maximum detected concentration (270 µM, Fig 15 and Fig 16). DONGLU has at least 200 times less immunotoxicity than DON. Cell proliferation measured by Trypan blue exclusion or MTS were not statistically different.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>ng toxin/ml cell medium</th>
<th>µM toxin/ml cell medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypan blue exclusion</td>
<td>MTS assay</td>
</tr>
<tr>
<td>DON</td>
<td>257</td>
<td>389 ± 98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.87</td>
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<tr>
<td>DON</td>
<td></td>
<td>1.31 ± 0.33</td>
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<tr>
<td>DONGLU</td>
<td>&gt;127440</td>
<td>&gt;127440</td>
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<td></td>
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<td>&gt;270</td>
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Fig 18: Light microscopy (x100) of K562 cells exposed to 0 µM DON, 270 µM DON glucuronide, 135 µM DON and 2.1 µM DON for 48 h. The numbers of K562 cells did not change after exposure to the maximum concentration of DON glucuronide. In contrast, the number of K562 cells were reduced after exposure to 2.1 µM DON and the maximum concentration of 135 µM DON with cell number as 40% of control and 15% of control, respectively. Note: cell debris at 135 µM DON.
Fig19: The relative proliferation of K562 cells exposed on DON, DON glucuronide (DONGLU), and DON and DONGLU combined in all concentrations (0.5 µM, 1.3 µM and 8.4 µM). The values were expressed as percent of control (0 µM DON/DON glucuronide). This result is a mean of three separate experiments with triplicate wells per treatment. Bars represent standard errors of the mean. Letters denote significant differences, P<0.05. * mean significantly different from control (P<0.05); ** mean significantly different from control (p<0.01). The result shows DONGLU did not influence DON toxicity at low, medium and high concentration combinations (0.5 µM, 1.3 µM and 8.4 µM) of each compound.
DISCUSSION

This is the first study to synthesize DON glucuronide and compare the immunotoxicity of DON and DON glucuronide. Incubation of DON, UDPGA and rat liver microsomes led to glucuronidation of DON (Fig 4). The [DON-3-GLU-H] - peak for DON glucuronide with m/z of 471 was clearly observed on both ESI negative ion and APCI negative ion, in agreement with expected DON glucuronide molecular weight 472 g/mol. The fraction 441 in APCI negative ion shows that the DON glucuronide synthesized was likely to be DON-3 glucuronide because loss of CH2O (-30) from the - CH2OH group attached to the C-6 position of the DON-3-glucuronide produces this fraction (Fig 10). Comparative 1H NMR DON and DON glucuronide showed two new double peaks (4.41 ppm and 3.73 ppm) and multiple peaks from 3.25-3.50 ppm corresponding to DON glucuronide NMR assignments (Fig 11). Comparative 13C NMR of DON and DON glucuronide showed new carbon peaks (103.4 ppm, 78.1 ppm, 75 ppm, 73.9 ppm) that could be assigned to DON glucuronide. A downfield chemical shift (δ ppm) (to the left) of 5.9 ppm was observed for carbon 3 (DON 70, DON glucuronide 75.9) between the spectra of DON and putative DON glucuronide, whereas no shift at carbon 15 further indicated that the DON glucuronide synthesized in our experiment was DON-3- glucuronide (Fig 12). In accordance, Savard (1991) reported that the only difference between DON-3-glucoside and DON-15-glucoside in chemical shifts of NMR was the carbon attached to glucose.

Studies in vitro and on animal models have determined that DON can be rapidly metabolized to DON glucuronide. Garesis et al. (1987) reported a total of 40.4% of the administered dose of DON was found to be conjugated with glucuronic acid (hepatic perfusate 20.4%, bile 19.2%, hepatocytes 0.8% of DON administered, respectively) when
an isolated rat liver was perfused with DON at a dose of 3 mg in a recirculating perfusion
system. Sprague-Dawley rats received a single dose of $^{14}$C DON (5.0 ± 0.1 mg/kg body
weight, 5.5 ± 0.1 µCi/kg) and the distribution of DON in body fluids was investigated over
72 h. A total of 37% of the administered DON was excreted in the urine and DON-
glucuronide was implicated as the major urinary metabolite based on reverse-phase HPLC
analysis of β-glucuronidase and sulphatase-treated samples (Meky et al., 2003). Corn
contaminated with DON was added to the diets of three dairy cows for 5 d. After incubating
urine with β-glucuronidase, the concentration of unconjugated DOM-1 increased by 7 to
15-fold whereas unconjugated DON increased 1.6 to 3-fold (Cote et al., 1986). After
administering intravenous and oral doses (0.5 and 5.0 mg DON/kg, respectively) to sheep,
the metabolic formation of the glucuronide conjugate after iv and oral administration of
DON appeared to occur quite efficiently (iv, 21%; oral, 75%). The de-epoxide metabolite,
DOM-1, accounted for only a minor portion of the dose after either dosing regimen (iv, less
than 2.0%; oral, less than 0.3%), but DOM-1 occurred predominantly as the glucuronide
conjugate (Prelsuky et al., 1985). In lactating sheep, using a combination of radioisotopic
counting and chromatographic detection techniques, the kinetics and metabolic fate of DON
in plasma, urine and bile were studied, its major plasma metabolite, DON glucuronide,
accounted for 13% of the plasma radioactivity levels (Prelsuky et al., 1987). The pigs were
fed a commercial diet with 3-acetyl DON added in a concentration of 2.5 mg/kg feed for
2.5 days. A significant part of the DON in plasma was a glucuronide conjugate (42 ± 7%)
(Eriksen et al., 2003). Evidence shows DON could be biotransformed to DONGLU in
human through comparing DON concentration in urine following β -glucuronidase
treatment. Urine samples were collected from female inhabitants of Linxian County, China,
a high-risk region for esophageal cancer (OC) and an area of potentially high DON exposure, and Gejiu, a low risk region in China. DON was detected in all 15 samples following β-glucuronidase treatment with the increase in DON recovered following enzyme treatment ranging from 1.2-2.8-fold (Meky et al., 2003). Therefore, DON glucuronide has been found in urine or plasma in different animals and humans. The amounts of DON glucuronide in body fruits are range from 13% ~75%. In our study, the same concentration of DON and DON glucuronide combination were used to investigation of the interaction of both compounts.

Our present research demonstrated that DON was metabolized to DON-3-glucuronide in vitro through incubation of DON, UDPGA and rat liver microsomes. MTS proliferation assay and trypan blue exclusion assay have determined that DON glucuronide was a detoxification product and had no toxicity at up to 270 µM. The –OH group at C3 could be one of reasons of DON toxicity. Some evidence has shown less toxicity in DON-related compounds without –OH groups at carbon 3. Sundstol Eriksen reported (2004) that 3-acetyl DON was less toxic than DON and 15-acetyl DON and 15-acetyl DON was equal in toxicity to DON in Swiss mouse 3T3 fibroblasts cells. Poppenberger (2003) reported that UDP-glucosyltransferase, which catalyzes the transfer of glucose from UDP-glucose to the hydroxyl group at carbon 3 of DON, is able to detoxify DON. Additionally, DON can be biotransformed to 3-keto-4-DON by some bacteria in soil, and the metabolite exhibited a remarkably decreased (to less than one tenth) immunosuppression evaluated by means of a bioassay based on the mitogen-induced and mitogen-free proliferations of mouse spleen lymphocytes (Shima et al., 1997). These results are in accordance with our finding that
chemical groups conjugated with carbon 3 hydroxyl of DON are DON detoxification routes.

UDP-glucuronosyltransferases (UGTs) metabolize a wide range of endogenous and xenobiotic substrates, typically forming polar glucuronide conjugates. Glucuronidation is conventionally viewed as facilitating the toxicological deactivation and excretion of xenobiotics. DON glucuronide is very water soluble and more polar than DON. Comparative immunotoxicity in K562 cells proliferation assays determined that DON glucuronides had no toxicity at up to 270 µM. Lesser toxicity of xenobiotic glucuronides has been found in other cases. For example, resveratrol was cytotoxic at 30 µM, but no cytotoxicity was observed for its glucuronide metabolites at concentrations as high as 300 µM (Wang et al., 2004). Comparing cytotoxicity effect of PhIP between CHO cells and repair-deficient CHO cell transfected with human UGT1A1 expressing 5P3hUGT1A1, a form of UGT, Malfatti et al. (2005) showed that UDP-glucuronosyltransferase proteins (UGT1A1) detoxified food-borne carcinogenic heterocyclic amines through catalyzing the glucuronidation of N-hydroxy-PhIP. Research in our lab showed that glucuronidation was a detoxification reaction in that daidzein and genistein glucuronides had lesser biological activity than isoflavone aglycones (Zhang et al., 2004). Therefore, phase II biotransformation, glucuronidation is a detoxified route for some xenobiotic substrates.

In our research, K562 cells were used to a cell model to investigate immunotoxicity of DON and DON glucuronide. K562 cells, a human myeloid leukemia cell line, showed similar responses to DON as did human peripheral blood leukocytes but without exogenous mitogens. Other research showed that K562 cells are a human erythroleukemia continuous cell line with a lymphoblast-like morphology (Koeffler and Golde 1980), and K562 blasts
can differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series (Lozzio et al., 1981). K562 cells are easily cultured. Visconti et al. (1991) evaluated cytotoxicity of twenty-three *Fusarium* mycotoxins on human K-562 cells as well as their inhibitory effect on proliferation of phytohemagglutinin-stimulated human peripheral blood lymphocytes. The cytotoxicity of trichothecenes to this human cell line was similar to their inhibition of mitogen induced human lymphocyte blastogenesis with IC$_{50}$ of 430 ng DON/ml. Our method also showed that K562 cells were sensitive to DON with an IC$_{50}$ of 389 ng/ml in MTS assay. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Therefore the mechanism of the toxicity of DON appears to include decreased cellular metabolism and deceased/ inhibited cell proliferation.

The viability of the cells was estimated by trypan blue exclusion method. When exposed to different concentrations of DON, the viability of the cells was depressed in a dose-dependent manner, comparable to the result in MTS assay.

Inhibition of cell proliferation by DON has been observed in different cell lines. DON effectively inhibited mitogen-induced lymphocyte proliferation with 50% inhibition at 216 ng/ml (Meky et al., 2001). DON also inhibited macrophage cell proliferation at 50-1000 ng/ml (Sugita-Konishi et al., 2001). Exposure to DON induces immature and mature B cells apoptosis and cytotoxicity after exposure at 100-1000ng/ml for 18h, with EC$_{50}$ at 500 ng/ml for apoptosis and 300 ng/ml for cytotoxicity, respectively. (Uzarski et al., 2003). The cytotoxicity of DON in porcine primary cultured hepatocytes showed the cell death of the hepatocytes at 10 µg/ml groups starting at 6 h after DON was added (Mikami et al., 2004).
DON concentrations in peripheral blood could be evaluated from DON toxicokinetics in animals and human epidemiology. DON was detected in human urine collected from the suspected high and low exposure regions in China by Meky et al. (2003). The mean levels of DON were 37 ng/ml (n = 11, range 14-94 ng/ml) and 12 ng/ml (n = 4, range 4-18 ng/ml) in high and low exposure regions correspond to daily exposure of 1.1-7.4 µg/kg/day and 0.3-1.4 µg/kg/day respectively, following by β-glucuronidase treatment. The true DON concentrations were much lower since the increase in parent DON following enzyme treatment ranged from 1.2 to 2.8-fold. Castrated male pigs (n = 11) were adapted to a diet containing DON (4.2 mg DON/kg) over a period of 7 days, the maximum DON serum concentration was 14 ng/ml (t_{max} = 4.1 h) (Danicke et al., 2004). The maximal plasma concentrations of DON were 21.79 and 15.21 ng DON/ml after feeding castrated male pigs on naturally contaminated wheat (5.7 mg DON/kg) for 4-8 weeks (n = 5) or single doses (n = 6) (Goyarts and Danicke, 2005a). As a result, consuming naturally contaminated DON (4.2 - 5.7 mg DON/kg diet), the maximal plasma concentration of DON were 14 - 22 ngDON/ml. Therefore, in most of cases, after chronic exposure to DON contaminated food (up to 5.7 mg DON/kg diet), the lower serum concentration (22 ng/ml) should have little physiological effects in inhibiting peripheral blood cell proliferation.

On the other hand, doses of 50 to 500 ng/ml DON superinduced IL-2 mRNA expression in a dose and time-dependent manner (Li et al., 1997). Exposure of the murine macrophage cell line RAW 264.3 to 50 - 250 ng/ml DON for 24 markedly enhanced the production of prostaglandin E₂, a major COX-2 metabolite (Moon and Pestka, 2002). The LOAEL dose is 50 ng/ml for IL-2 and COX-2 gene expression dysfunction after DON exposure.
Therefore, determining the exposure concentrations of DON to cells is a crucial thing when cell culture bioassays are employed to study the immunotoxicity of DON. In our research, no significant cytotoxicity was observed for DON glucuronide at up to 270 µM. DON glucuronide did not influence DON toxicity at low, medium and high concentration combinations (0.5µM, 1.3µM and 8.4µM) of each compound. These data show that DONGLUC is a detoxification product of DON. Immunoassay would probably have DON/DON glucuronide crossreactivity because the antibodies used are designed against 3, 7, 15 triacetyl-DON (Krska et al. 2001). Therefore, the immunoassay would not accurately indicate what amount of DON was present. DON glucuronide had no effect on DON toxicity when cell proliferation assay was used to screen DON exposure. As a result, measuring DON alone without DON glucuronide in human urine or plasma would be a suitable biomarker of DON exposure and toxicity.
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