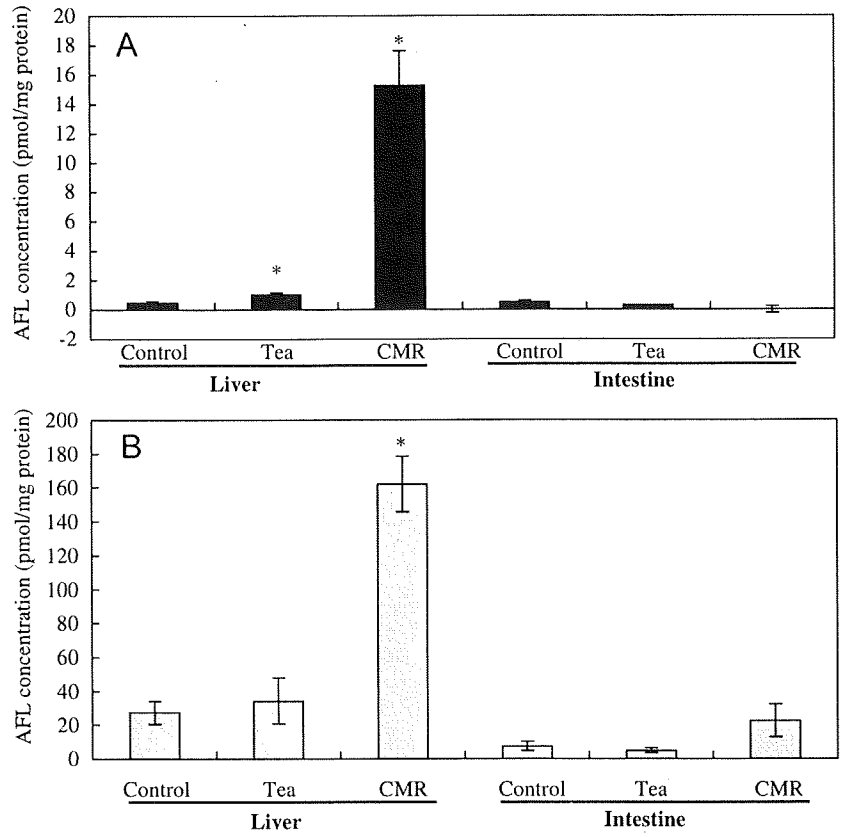

Fig. 3. Aflatoxicol formation from AFB1 incubation for 30 min with the microsome (A) or cytosol (B) fraction from the liver or intestine of control, or Sunphenon- or coumarin-treated piglets. *: Significantly different from each control ($p < 0.05$).

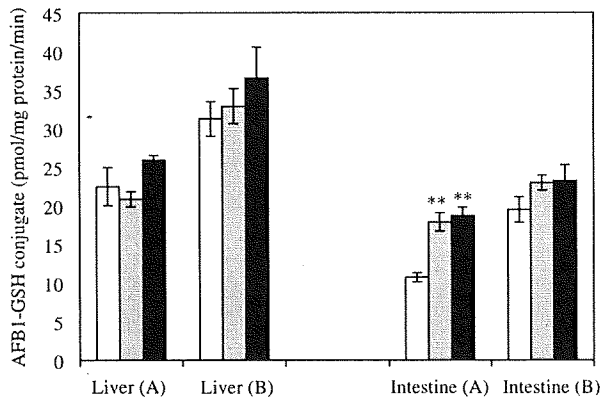

Fig. 4. AFB1–GSH conjugate formation by 30 min incubation of cytosol of the liver or intestine with 32 μM (A) and 128 μM (B) of AFB1-dialdehyde for 30 min: control (□), Sunphenon (▨) or coumarin (■). **: Significantly different from each control ($p < 0.01$).

metabolize AFB1 to AFQ1 and AFM1, respectively (Crespi et al., 1991; Gallagher et al., 1994).

As for the intestinal microsomes, coumarin treatment caused the reduction of the activity to form AF–DNA adduct and the enhancement of the activity to form aflatoxicol and B2α. Whether the same P450 enzymes as those in the liver are involved or not is uncertain.

The cytosolic GST activity to conjugate AFB1-epoxide with glutathione was increased by coumarin treatment in the intestine, but not in the liver, indicating that the detoxification activity toward AFB1 is enhanced by coumarin in the intestine but not in the liver. Knowing that AFB2α and aflatoxicol are less toxic than AFB1 (Lillehoj and Ciegler, 1969; Wong and Hsieh, 1980; Gurtoo et al., 1981; Loveland et al., 1987), all the results of AFB1 metabolism in the liver and intestinal tissue of the coumarin-treated piglets showed that coumarin may affect AFB1 metabolism toward the reduction of its toxic and carcinogenic activities through the suppression of P450 enzymes in the liver and intestine, and also through the enhancement of GST toward AFB1 in the intestine. The AFB1-aldehyde reductase activity was decreased in the intestine of coumarin-treated piglets, but its effect on AFB1 toxicity may be relatively small because the

contribution to AFB1 detoxification is far small compared with GST.

In contrast to coumarin, Sunphenon caused only a small change in the liver microsomal enzyme activity, as seen in the slight enhancement of the metabolic conversion of AFB1 to aflatoxicol. Neither the cytosol GST activity toward AFB1-epoxide nor the microsomal activity to form the AFB1–DNA adduct was affected by Sunphenon treatment. However, in the intestinal tissue, the GST activity toward AFB1-epoxide and the microsomal activity to convert AFB1 to AFQ1 were enhanced by Sunphenon. If it is taken into account that AFQ1 is less toxic than AFB1 (Hendricks et al., 1980), Sunphenon may affect AFB1 metabolism toward the reduction of AFB1 toxicity and carcinogenicity, although the effect may be less than that of coumarin.

The substrate specificities of class alpha, mu and pi isoenzymes of GST in pigs are characterized by a remarkable activity toward cumene hydroperoxide, *t*-PBO and EA, respectively (Dai et al., 1996; Kunze, 1997; Rouimi et al., 1996). Therefore, the result of the GST activity toward these substrates may indicate the enhancement of mu-class activity in the liver by Sunphenon and the reduction of pi-class activity in the intestine by both Sunphenon and coumarin. Both the compounds exerted no effect on the alpha-class activity. However, there were no changes in the GST activity toward AFB1 except for its increase in the intestine, indicating that the enhancement of some other isoenzymes activities may be involved in the change of the activity toward AFB1 in the intestine. Consistent with this, the GST activity toward CDNB, which is

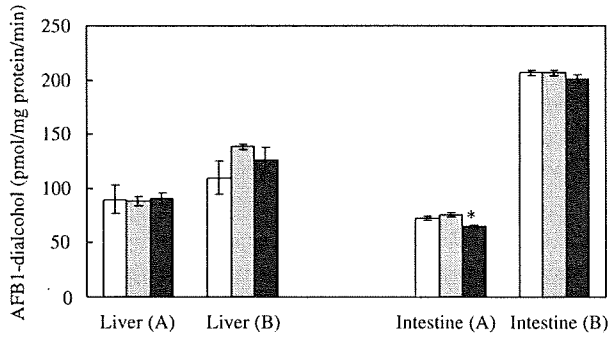

Fig. 5. AFB1-dialcohol formation from AFB1-dialdehyde in the liver or intestinal cytosol incubation with 15 μM (A) or 50 μM (B) AFB1-dialdehyde for 30 min: control (□), Sunphenon (▨) or coumarin (■). *: Significantly different from control ($p<0.05$).

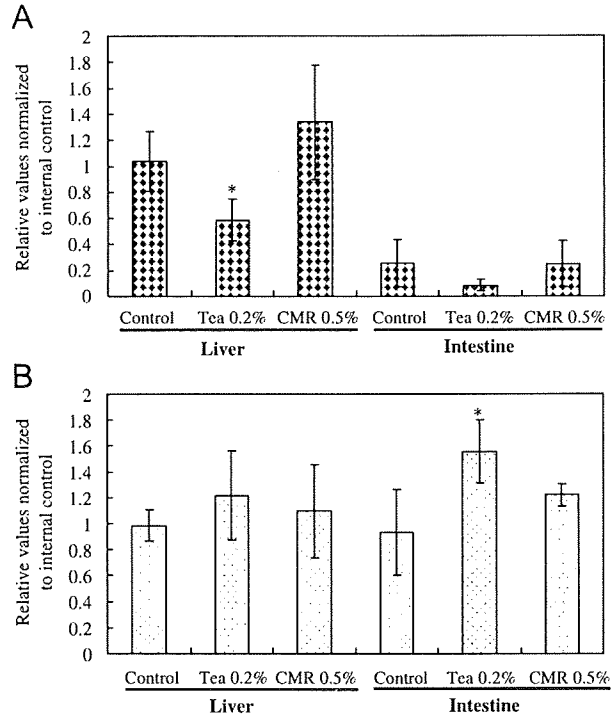

Fig. 6. The expression of GSTA2 and GSTO1 mRNA genes in piglets using real-time RT-PCR. The expression of GSTA2 (A) and GSTO1 (B) was normalized to the internal control (GADPH). *: Significantly different from the control of each organ ($p<0.05$).

Table 2
The GST activity in the liver and intestinal cytosol toward various substrates in control and Sunphenon- and coumarin-treated piglets

Substrate	Liver (nmol/min/mg protein)			Intestine (nmol/min/mg protein)		
	Control	Tea	CMR	Control	Tea	CMR
CDNB	27.08±6.25	129.17±15.35**	270.14±31.30**	124.31±18.01	263.89±46.04*	291.67±33.74*
EA	196.0±91.65	229.33±63.59	389.33±132.83	528.0±0	309.33±35.27*	316.0±41.63*
t-PBO	2.96±0.53	7.52±1.49*	4.84±0.80	7.80±1.34	5.38±0.71	5.11±0.71
Cumene hydroperoxide	356.99±99.55	400.0±18.90	662.37±130.37	76.34±14.93	89.25±7.05	120.43±25.14

*Significantly different from the control value at $p<0.05$.
**Significantly different from the control value at $p<0.01$.

a substrate for a broad range of GST isoenzymes, was elevated by both Sunphenon and coumarin.

The DNA sequence of pig GST has been known only for GSTA2 and GSTO1 (Tosser-Klopp et al., 1997; Rouimi et al., 2001; Miao et al., 2003). Real-time RT-PCR showed that the expression of GSTA2 in the liver was reduced by Sunphenon, and that the expression of GSTO1 in the liver was enhanced by coumarin. However, no effects were noted in the intestine. Taken together with the finding that the GST activity toward AFB1 in liver was unaffected, while that in the intestine was enhanced, the observed changes of mRNA expression indicate that both GST isoenzymes are not involved in conjugation of AFB1-epoxide to glutathione.

Extensive studies have been done using rodents. However, the findings in piglets show some different features from those seen in rodents. In rodents, alpha-class GST plays an important role in the toxic and carcinogenic effects of AFB1 (Judah et al., 1993), while it is not the case in piglets as described above. Also, in contrast to the finding in piglets, coumarin has been observed to increase the hepatic reductase activity toward AFB1-dialdehyde and GST activity toward AFB1-epoxide in rats (Kelly et al., 2000a, b). Only the finding that epigallocatechin gallate, the main ingredient of green tea extract, is a strong inducer of GSTM2 in the rat liver (Chou et al., 2000; Ahmed et al., 2002) is consistent with the Sunphenon-induced elevation of the GST activity toward *t*-PBO in piglet liver. Thus, GST isoenzymes toward AFB1-epoxide or the effects of tea extracts and coumarin on GST isoenzymes may be different among different species so that the methods for the chemoprevention of toxic effects of aflatoxins in farm animals could not be directly based on the finding in rodents.

In conclusion, coumarin may affect AFB1 metabolism toward the enhancement of detoxification through the suppression of P450 enzymes in the liver and intestine, and through the enhancement of GST toward AFB1 in the intestine. Sunphenon may affect AFB1 metabolism toward the enhancement of AFB1 detoxification through the enhancement of intestinal GST activity although its effect is not as prominent as coumarin.

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Nivalenol and the targeting of the female reproductive system as well as haematopoietic and immune systems in rats after 90-day exposure through the diet

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Nivalenol (NIV) is considered to be an important trichothecene mycotoxin produced by *Fusarium* species because of its frequent contamination in wheat and barley worldwide. The present study examined the subchronic toxicity of NIV in male and female F344 rats fed diets containing 0, 6.25, 25 and 100 mg kg^{-1} of the toxin for 90 days. During the experimental period there was a decrease in the white blood cell count at 100 mg kg^{-1} in males and at ≥6.25 mg kg^{-1} in females. Histopathologically, treatment-related changes were observed in the haematopoietic and immune systems in both sexes and in the female reproductive system at 100 mg kg^{-1}. Flow cytometric analysis of splenic cells revealed an elevation in the ratio of helper/cytotoxic T-lymphocytes at 100 mg kg^{-1}. In summary, NIV targets the female reproductive system as well as haematopoietic and immune systems in rats fed NIV for 90 days. Based on a significant decrease in white blood cells in female rats relative to controls, the lowest observable effect level was calculated as 0.4 mg kg^{-1} body weight day^{-1}.

Keywords: nivalenol; toxicity; F344 Fisher rat; reproductive system

Introduction

Trichothecenes produced by *Fusarium* species are found in crops such as wheat, maize, barley, oats, and rye grown in the temperate regions of America, Europe and Asia (European Commission, Scientific Committee on Food 2000). They have long been a serious concern for human and animal health (Ali et al. 1998; Sudakin 2003). The type B trichothecenes, deoxynivalenol (DON) and nivalenol (NIV; Figure 1) are commonly detected together in Japanese wheat (Yoshizawa and Jin 1995).

Although DON has been evaluated by establishing a toxicity profile and a provisional maximum tolerable daily intake (PMTDI) by the WHO/FAO Joint Export Committee on Food Additives (WHO 2001), such a profile for NIV has not yet been established because there is a lack of adequate information about its toxicity.

There have been scarce data regarding the toxicity profile of this toxin. Ohtsubo et al. (1989) reported growth retardation and leukopenia in female mice that were given mouldy rice containing NIV in the diet for two years and determined a low observed effect level (LOEL) of 0.7 mg kg^{-1} body weight day^{-1}. Kawasaki et al. (1990) found that the NOEL of NIV was 0.4 mg kg^{-1} body weight day^{-1} when given to rats by gavage for 30 days. Embryo lethality was observed in mice given intraperitoneal or subcutaneous injections with 0.5 mg kg^{-1} body weight of NIV (Itoh et al. 1986). There was an increase in serum IgA and induction of IgA deposits in the glomerular mesangium of mice given 6 or 12 mg NIV kg^{-1} in feed (Hinoshita et al. 1997). In ovalbumin-specific T-cell receptor $\alpha\beta$-transgenic mice, production of IgE and interleukin (IL)-4 was inhibited by NIV, whereas IL-2 production was increased (Choi et al. 2000). While the Scientific Committee on Food of the European Commission (2000) has recently established a temporary total daily intake (TDI) of 0.7 μg kg^{-1} body weight day^{-1} based on the LOEL determined by Ryu et al. (1987) and Ohtsubo et al. (1989) and a safety factor of 1000, more detailed studies of NIV toxicity are urgently needed.

The objective of this study was therefore to examine the general toxicity and immunotoxic effects of subchronic dietary exposure to purified NIV in F344 rats according to OECD (1998). The results indicate that exposure to NIV caused changes in the white blood cell count in females fed ≥0.4 mg kg^{-1} body weight day^{-1}, suggesting that the estimated LOEL of NIV is 0.4 mg kg^{-1} body weight day^{-1}.

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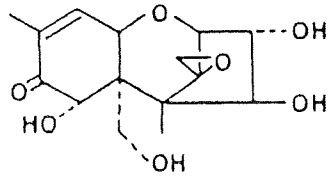

Figure 1. Chemical structure of nivalenol (NIV).

Materials and methods

Chemicals

Standards of nivalenol (NIV), Fusarenon X (FX), deoxynivalenol (DON) and zearalenone (ZER) were purchased from Sigma-Aldrich (St Louis, MO, USA). De-epoxy NIV was a gift from Dr Onji, Nara Prefectural Institute for Hygiene and Environment.

Purification of NIV

For purification of NIV, FX was extracted and purified from the culture media of *Fusarium kyushuense* (Fn-2B). A small portion of fungal stock was pre-cultured on Tsunoda's agar plate (2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 2.5 g yeast extract, 5 g polypeptone, 50 g sucrose, 20 g agar and 1 L tap water) at 25°C for 7 days. Thereafter, the precultured colonies were inoculated into Tsunoda's liquid medium (2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 2.5 g yeast extract, 5 g polypeptone, 50 g sucrose, and 1 L tap water) to perform a large-scale culture. After stationary cultivation at 25°C for 2–3 weeks, the liquid culture media was collected and strained (three times) through cotton gauze, mixed well by vigorous shaking with an equal volume of acetonitrile and 62.5 g ammonium sulfate. Then the acetonitrile layer was recovered, subjected to evaporation, and the residue dissolved in 20 ml of methanol/chloroform (2 : 18, v/v). The solution was applied to a florisil column (Wako Pure Chemical Industries, Ltd, Osaka, Japan), the column eluted with methanol/chloroform (2 : 18, v/v) and the eluate evaporated to dryness. The residues were then dissolved in the acetonitrile–methanol–water (10 : 5: 85) mobile phase and applied to the preparative column for high-performance liquid chromatography (Shodex® ODS Silica C18M 20E, Showa Denko K.K., Kawasaki, Japan; flow rate of 10 ml min^{-1}, detection by absorbance at 220 nm, and a column temperature at 40°C) and the fraction containing FX collected. The fraction was lyophilized, dissolved in 1 N ammonia–methanol solution and allowed to stand overnight to convert the FX to NIV. After evaporation, the resultant condensate was dissolved in hot methanol and then kept at 4°C overnight to form powdery crystals. The precipitated crystals were filtrated, washed with cold methanol, and air-dried in a desiccator. The crystallization was repeated three times per preparation.

The identity and purity of NIV was determined by liquid chromatography/mass spectrometry (LC/MS, LCMS-2010A; Shimadzu Corp., Kyoto, Japan) equipped with an atmospheric pressure chemical ionization (APCI) interface and an LC system (LC-2010CHT; Shimadzu Corp.). Chromatographic separation was achieved on ZORBAX XDB-C18 (i.d., 2.1 × 150 mm, 3.5 μm) from Agilent Technologies (Santa Clara, CA, USA) at 40°C, using an isocratic elution in which the mobile phase consisted of methanol/water containing 5 mM ammonium acetate at a ratio of 65/35 (v/v). The flow rate was 0.2 ml min^{-1} and the injection volume was 3 μl. The APCI interface was used in both negative- and positive-ion modes at 400°C, 4.5 kV with the following settings: nebulizer gas (2.5 l min^{-1}), drying gas (0.02 MPa), CDL voltage (10 V), CDL voltage (10 V), and CDL temperature (250°C). The total ion chromatogram of purified material showed one peak under both negative- and positive-ion modes. The mass spectrum was identical to that of the authentic NIV standard and without any other peaks, including FX and ZER. The purity of the NIV was estimated to be >98% from the percentage area of the chromatogram (data not shown).

NIV was administered in a powdered basal diet CRF-1 (Oriental Yeast Co., Tokyo, Japan). Briefly, NIV was weighed and mixed with a small quantity of fresh diet, and the mixture was well ground in a mortar to obtain a homogenous premix. The 100 mg kg^{-1} diet was then prepared by vigorously shaking the premix with fresh basal diet. For the preparation of diets at lower doses, aliquots of the 100 mg kg^{-1} diet were further diluted with basal diet. Stability of the test compound in the diet was confirmed for up to 2 weeks at room temperature (>92%). Therefore, test diets were prepared every 2 weeks, and stored at 4°C before use.

Animals

Male and female F344/DuCrj rats at 5 weeks of age, purchased from Charles River Japan Inc. (Kanagawa, Japan), were used in this study. They received powdered CRF-1 and tap water *ad libitum* during the 1-week acclimation period, housed four per cage in a barrier-maintained animal room at 23–25°C, 50–60% relative humidity, and a 12-h light/dark cycle.

Experimental design

In a preliminary dose-finding study, groups of eight male F344/DuCrj rats, 5 weeks of age, were fed a diet containing NIV at zero, 150 and 300 mg kg^{-1} for 2 weeks. Reduction of body weights was apparent

at 150 and 300 ppm from week 1, and one animal in the 300 mg kg^{-1} group died during week 2. Therefore, 100 mg kg^{-1} was determined to be the highest dose for the subsequent subchronic study. For the subsequent experiment, F344/DuCrj rats were randomly divided into four groups, each consisting of ten males and ten females, housed three or four per cage and given zero (control), 6.25, 25 or 100 mg kg^{-1} NIV in the powdered diet for 13 weeks. The test diets were available *ad libitum*, except for a one-night fasting before the scheduled necropsy. The animals were observed daily for clinical signs and mortality, and body weights were measured every week during the study period. At the end of week 13, all animals were anaesthetized with ether, weighed, and blood samples were collected by cardiac puncture for haematology and serum biochemistry tests. From male rats, samples of liver and spleen were collected for xenobiotics metabolism and immunotoxicity studies, respectively. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Haematology and serum biochemistry

Haematological analysis was performed using an automated haematology analyser, K-4500 (Sysmex Corp., Hyogo, Japan). Aliquots of whole blood were analysed for the following parameters: red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (Plt), and white blood cell count (WBC). For the measurement of differential leukocyte counts and reticulocyte counts, aliquots of whole blood were analysed with a Microx HEG-120A (Omron Tateishi Electronics Co., Ltd, Tokyo, Japan). Parameters for serum biochemistry, analysed at SRL, Inc. (Tokyo, Japan) were: total protein (TP), albumin (Alb), the Alb : globulin ratio (A/G), total bilirubin (total Bil), conjugated bilirubin (conjugated Bil), free bilirubin (free Bil), glucose, triglyceride (TG), total cholesterol (TC), blood urea nitrogen (BUN), creatinine (CRN), sodium (Na), chloride (Cl), potassium (K), calcium (Ca), inorganic phosphate (IP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT).

Necropsy and histopathology

The brain, heart, lungs, thymus, spleen, liver, kidneys, adrenal glands, and testes were removed and weighed at necropsy. In addition, the pituitary, nasal cavity, eyes, Harderian glands, spinal cord (cervical, thoracic, and lumbar portions together with corresponding vertebral bones), salivary glands, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (caecum, colon, and rectum), pancreas, urinary bladder, skin, male and female mammary glands, mesenteric lymph nodes, trachea, oesophagus, thyroid glands, tongue, thigh muscle, sciatic nerve, epididymides, seminal vesicles, prostate (ventral and dorso-lateral lobes), uterus, ovaries and vagina were also removed. All organs/tissues were fixed in 10% buffered formalin for 3 days, except for testes which were fixed in Bouin's solution overnight. Tissues that needed decalcification, such as the nasal cavity, spinal cords with bones, sternum, and femur, were treated with a mixture of 10% formic acid and 10% buffered formalin. The fixed tissues were paraffin embedded, sectioned, and stained with haematoxylin and eosin by routine methods and examined microscopically.

Immunoglobulins in serum

IgG, IgM and IgA subclass of antibodies in the serum samples were measured by enzyme-linked immunosorbent assay with standards of rat IgG_1 (BioLegend, San Diego, CA, USA), rat IgM (eBioscience, San Diego, CA, USA) and rat IgA (BD Bioscience). Ninety-eight well microplates (Corning, Corning, NY, USA) were coated with 100 µl of anti-rat IgG/IgM/IgA (Open Biosystems, Huntsville, AL, USA) or anti-rat IgA (Sigma) in a 0.05 M bicarbonate buffer (pH 9.6) at a concentration of 10 µg ml^{-1} overnight at 4°C. Non-specific binding sites were blocked with 200 µl of PBS containing 1% BSA (Serological Proteins, Kankakee, IL, USA) for 90 min at room temperature. After washing of plates, diluted serum and standards were added to the individual wells. After incubation for 90 min at room temperature, the plates were washed, 100 µl of horse radish peroxidase (HRP)-conjugated anti-rat IgG (Invitrogen, Carlsbad, CA, USA), anti-rat IgM (Stressgen Bioreagents, Ann Arbor, MI, USA) or anti-rat IgA (Bethyl, Montgomery, TX, USA) antibodies were added to wells. The plates were incubated for 60 min, washed with PBS, and then 100 µl of tetramethylbenzidine (TMB) substrate reagent (BD Bioscience) was added to each well. The reaction was stopped after 15 min by adding 100 µl of 2 M sulphuric acid, and then the absorbance of the developed colour was determined at 450 nm using an EL800 Universal microplate reader (BIOTEK, Winooski, VT, USA).

Spleen cell subset analysis

After necropsy (same day), the spleens from treated male rats were minced and passed through a mesh to obtain single cell suspensions. Cells were incubated with D34-485 mAb (anti-FcγRII) to block non-specific

staining and then stained with a combination of the following mAb mouse monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-rat-CD8a (OX-8), FITC anti-rat-CD3 (G4.18), FITC anti-rat-NKR-P1A (10.78), phycoerythrin (PE)-conjugated anti-rat-CD4 (OX-35), PE anti-CD45RA (OX-33) or PE anti-CD3 (1F4), purchased from BD Biosciences (San Jose, CA, USA). Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences).

Natural killer (NK) activity

NK activity in spleen cells was assayed according to a method described previously (Arase et al. 1999). Briefly, YAC-1 target cells (10^7 cells ml^{-1}) were labelled with PKH2 green fluorescent dye (Sigma) for 3 min. The labelled target cells (100 µl at 10^5 ml^{-1}) were plated into round-bottomed 96-well microplates (TPP, Trasadingen, Switzerland), then splenocytes (effector cells) suspended in 100 µl of RPMI 1640 were added to the wells at effector-to-target (E : T) cell ratios of 50 : 1, 25 : 1, 12 : 1 and 6 : 1. Following a 2-h incubation (37 °C; 5% CO_2) dead cells were stained with propidium iodide (PI; Dojindo, Kumamoto, Japan), and the proportion of PI-stained cells and PKH2-stained cells was determined by flow cytometric analysis (FACSCalibur). Spontaneous and maximum cell death was determined by incubating target cells in medium or in medium supplemented with 30 µg of saponin (MP Biomedicals, Eshwege, Germany) in the absence of effector cells.

Activities of xenobiotic enzymes in liver

The livers were weighed, frozen in liquid nitrogen, and then stored at −80 °C. Microsomal and cytosolic fractions in each liver sample were prepared according to the method of Esaki and Kumagai (2002). Protein concentrations of both fractions in the liver were measured with a spectrophotometer using a reagent for protein assays (Bio-Rad Protein Assay, Bio-Rad Laboratory, Hercules, CA, USA). The activities of microsomal cytochrome P450, isotype CYP1A2 and CYP3A4 were determined using P450-Glo ™ Assays kits (Promega Corporation, Madison, WI, USA). Cytosolic glutathionine *S*-transferase (GST) activity was determined spectrophotometrically at 340 nm using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the procedure of Habig et al. (1974).

Analysis of the metabolites of NIV in faeces

Faeces were collected at weeks 2, 8 and 12 from control and treated males. NIV and its metabolites were extracted from the faeces with 85% acetonitrile–water and cleaned up with Multisep #227 (Romer Labs, Inc., Union, MO, USA) according to the method of Sugita-Konishi et al. (2005). The cleaned up samples were analysed by GC-MS as described by Tanaka et al. (2000) and the concentrations of NIV and a de-epoxy NIV calculated by comparison to calibration curves prepared with NIV and de-epoxy NIC standards.

Statistics

Body weight, haematology, serum biochemistry, and organ weight (both absolute and relative weights) data were checked for homogeneity of variance using Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal–Wallis test was used. When statistically significant differences were indicated, Dunnett's multiple test was used to compare the control and treatment groups. Histopathological incidences were compared using the Fisher's exact probability test and lesion severity data were analysed with the Mann–Whitney's *U*-test.

Results and discussion

A preliminary study indicated that NIV targeted the haematopoietic and immune systems (Ryu et al. 1987; Ohtsubo et al. 1989). The present study not only examined the toxicity of NIV to these systems, but also evaluated NIV detoxification by hepatic xenobiotic metabolic enzymes. In both sexes, body weight gain was significantly suppressed at 100 mg kg^{-1} from week 1. From week 6 to the end of the experiment, suppression of body weight gain was also evident at 25 mg kg^{-1} in males and from week 4 to the end in females (Table 1). The feed consumption of both sexes was suppressed at 100 mg kg^{-1}. Suppression was evident throughout the feeding period in females whereas a significant decrease in feed consumption was observed in males only during the initial 6 weeks of feeding (data not shown). As a result, while total feed consumption of males fed 100 mg kg^{-1} NIV during the experimental period was slightly lower than the controls (Table 1), the difference was not significantly significant. The NIV intakes calculated from feed consumption and body weight data (Table 2) indicated that 6.25, 25 and 100 mg kg^{-1} corresponded to 0.4, 1.5 and 6.9 mg kg^{-1} body weight day^{-1} in males and 0.4, 1.6 and 6.4 mg kg^{-1} body weight day^{-1} in females.

With regard to organ weights (data not shown), decreases in absolute liver weight and increases in the relative weights of the brain, lungs, heart, kidneys and testes were found in males from 25 mg kg^{-1}.

Table 1. Body weight of male and female F344 rats fed nivalenol (NIV) for 90 days.

Diet	Week 0	Week 1	Week 3	Week 5	Week 7	Week 9	Week 11	Week 13
Control	109.73 ± 3.9	145.56 ± 7.8	206.69 ± 9.3	236.19 ± 11.0	264.98 ± 9.1	290.00 ± 10.5	305.81 ± 11.2	316.87 ± 10.6
NIV 6.25 mg kg^{-1}	110.15 ± 4.2	142.69 ± 5.3	206.97 ± 8.5	238.63 ± 9.7	266.75 ± 9.5	294.18 ± 11.4	312.03 ± 13.5	318.55 ± 14.5
NIV 25 mg kg^{-1}	109.99 ± 4.1	139.61 ± 5.6	197.34 ± 5.3	225.67 ± 6.2	249.28 ± 9.8*	272.66 ± 9.2**	285.76 ± 9.9**	295.46 ± 10.7**
NIV 100 mg kg^{-1}	109.41 ± 3.6	119.75 ± 7.2**	168.16 ± 13.4**	186.28 ± 11.1**	207.82 ± 9.8**	226.84 ± 9.8**	240.66 ± 9.3**	243.62 ± 8.5**
Control	93.9 ± 3.8	113.38 ± 4.9	138.97 ± 6.9	154.5 ± 8.2	162.92 ± 7.7	169.38 ± 8.0	175.32 ± 8.6	178.26 ± 8.7
NIV 6.25 mg kg^{-1}	93.87 ± 2.7	111.81 ± 4.5	134.89 ± 5.0	151.2 ± 5.5	159.61 ± 4.6	167.49 ± 5.5	172.81 ± 4.8	175.19 ± 4.3
NIV 25 mg kg^{-1}	94.13 ± 2.6	109.32 ± 3.5	133.11 ± 4.4	148.11 ± 4.6	157 ± 5.2	165.61 ± 5.9	170.85 ± 6.2	170.56 ± 5.2
NIV 100 mg kg^{-1}	94.09 ± 3.0	93.15 ± 4.1**	115.44 ± 5.1**	124.49 ± 6.5**	134.93 ± 9.0**	143.04 ± 8.3**	148.15 ± 7.4**	150.19 ± 8.8**

Notes: Values are the mean ± standard deviation (SD), $n = 10$. *$p < 0.05$. **$p < 0.01$ compared with untreated controls.
Data are from by Takahashi et al. (2008).

Table 2. Feed consumption and intakes of nivalenol (NIV) in F344 rats fed diets containing NIV for 90 days.

Sex	NIV in diet ($mg\,kg^{-1}$)	Number of rats examined	Average feed consumption ($g\,day^{-1}$)		Mean daily NIV intake ($mg\,kg^{-1}$ body weight day^{-1})[a]	Total NIV intake ($mg\,kg^{-1}$ body weight)[b]
Males	0	10	14.7	(60.5)[c]	0	0
	6.25	10	14.7	(60.1)	0.4	33.8
	25	10	14.2	(61.5)	1.5	138.4
	100	10	13.6	(68.6)	6.9	617.4
Females	0	10	10.1	(65.6)	0	0
	6.25	10	10.0	(66.0)	0.4	36.9
	25	10	9.6	(64.2)	1.6	148.5
	100	10	8.2*	(63.6)	6.4	572.4

Notes: [a]Values were calculated by multiplying the feed consumption ($g\,kg^{-1}$ body weight day^{-1}) by the dietary level of NIV ($mg\,g^{-1}$ diet).
[b]Values are the sum of NIV administered during the experimental period and were calculated by multiplying the mean daily intake of NIV ($mg\,kg^{-1}$ body weight day^{-1}) by 90 (days).
[c]Values in parentheses are for feed consumption calculated as $g\,kg^{-1}$ body weight day^{-1}.
*Significantly different from control at $p < 0.01$, respectively (Dunnett's test or Dunnett-type rank-sum test).

Table 3. Haematological data of F344 rats fed diets containing nivalenol (NIV) for 90 days.

	NIV in diet ($mg\,kg^{-1}$)			
	0	6.25	25	100
Male				
RBC ($\times 10^4\,ml^{-1}$)	906.5 ± 18.3[a]	927.1 ± 29.7	901.2 ± 25.3	855.7 ± 38.2**
Hb ($g\,dl^{-1}$)	15.3 ± 0.3	15.6 ± 0.5	14.8 ± 1.1	15.0 ± 0.7
HCT (%)	48.3 ± 1.0	49.6 ± 1.4	48.9 ± 1.4	47.9 ± 2.0
MCV (fl)	53.3 ± 0.5	53.5 ± 0.5	54.3 ± 0.2*	55.9 ± 0.4**
MCH (pg)	16.9 ± 0.4	16.8 ± 0.3	16.4 ± 1.0	17.5 ± 0.3*
MCHC ($g\,dl^{-1}$)	31.7 ± 0.6	31.4 ± 0.6	30.3 ± 1.8	31.3 ± 0.6
Plt ($\times 10^4\,ml^{-1}$)	66.1 ± 16.7	70.5 ± 3.8	66.3 ± 14.8	59.2 ± 3.4**
WBC ($\times 10^2\,ml^{-1}$)	38.5 ± 12.2	37.0 ± 12.2	36.9 ± 6.1	21.6 ± 3.9**
Female				
RBC ($\times 10^4\,ml^{-1}$)	919.2 ± 25.8[a]	917.4 ± 51.2	900.0 ± 23.7	874.6 ± 59.2
Hb ($g\,dl^{-1}$)	16.3 ± 0.6	16.2 ± 0.8	15.5 ± 1.1	15.3 ± 1.0*
HCT (%)	51.3 ± 1.7	51.4 ± 2.7	50.3 ± 1.3	48.8 ± 3.3
MCV (fl)	55.8 ± 0.4	55.9 ± 0.4	55.9 ± 0.4	55.8 ± 0.5
MCH (pg)	17.8 ± 0.7	17.6 ± 0.5	17.3 ± 1.2	17.5 ± 0.6
MCHC ($g\,dl^{-1}$)	31.9 ± 1.2	31.5 ± 0.9	30.9 ± 2.0	31.3 ± 1.0
Plt ($\times 10^4\,ml^{-1}$)	76.6 ± 6.5	73.5 ± 3.0	74.2 ± 3.3	60.7 ± 5.3**
WBC ($\times 10^2\,ml^{-1}$)	38.8 ± 7.7	30.5 ± 10.1*	29.7 ± 5.5*	19.6 ± 4.5**

Notes: [a]Mean ± standard deviation (SD).
*, ** Significantly different from the controls at $p < 0.05$ and $p < 0.01$, respectively.
Data are from Takahashi et al. (2008).

At $100\,mg\,kg^{-1}$, however, a significant increase in relative liver weight and a decrease in absolute weights of the brain, lung, heart, spleen and kidneys was observed. Both absolute and relative thymus weights decreased at $100\,mg\,kg^{-1}$. In females, relative weights of the lungs, heart, spleen and kidney were increased in a dose-dependent manner from $25\,mg\,kg^{-1}$. However, the absolute weights of these organs were either unchanged or decreased. Similarly, decreased absolute weights and increased relative weights were found for the brain, liver, and kidneys at $100\,mg\,kg^{-1}$. Here we observed an apparent reduction in body weight at 100 ppm in both sexes.

Regarding haematology, in males a slight but significant increase in MCV was observed from $25\,mg\,kg^{-1}$, and increased MCH and decreased RBC, Plt and WBC were detected at $100\,mg\,kg^{-1}$. In females, a dose-dependent decrease in WBC was observed from $6.25\,mg\,kg^{-1}$ and slight decreases in Hb and Plt were

Table 4. Treatment-related histopathology findings in F344 rats fed nivalenol (NIV) for 90 days.

Organ		Significant cellular/histological changes (mg kg^{-1} NIV*)	
		Male	Female
Pituitary	Castration cell (−/±/+)	100	100
	Hypertrophy of basophilic cell	100	100
Submandibular gland	Decrease of granules in secretory granular duct	100	100
Lung	Macrophage accumulation	100	100
Kidney	Eosinophilic body (−/±/+)	100	n.f.
	Hyaline droplet degeneration (−/±/+)	100	n.f.
Spleen	Extramedullary haematopoiesis (−/±/+)	n.f.	100
Mesenteric LN	Aggregation of macrophages and mast cells in the medullary sinuses	100	100
Thyroid	Increase of dilated follicles (or inactive follicles lined by flattened epithelium)	100	100
Adrenal	Size of vesicles in zona fasciculata of the adrenal cortex (uniformly small)	100	n.f.
Uterus	Atrophy with diestrus endometrial mucosal change	n.e.	100
Ovary/oviduct	Increase of interstitial gland	n.e.	100
	Loss of corpora lutea	n.e.	100
	Mineralization	n.e.	100
Nasal cavity	Increase of goblet cells in the epithelium of paranasal sinus (−/±/+)	n.e.	100
Mammary gland	Lobular hyperplasia	n.e.	100
Femur/marrow	Decrease of haematopoietic cells (pancytopenia)	100	100
	Decrease of metaphyseal trabeculae	100	100
Sternum/marrow	Decrease of haematopoietic cells (pancytopenia)	100	100
	Decrease of metaphyseal trabeculae	100	100
	Thinning of diaphysis	100	100

Notes: *Diet concentration of NIV at which the effect was found.
n.f., Not found; n.e., not examined.
Significantly different from control values were calculated by Fisher's exact probability test and Mann–Whitney's *U*-test.
Data are from Takahashi et al. (2008).

found at 100 mg kg^{-1} (Table 3). As for females, no differences in differential leukocyte counts or reticulocyte counts were found in males.

With the exception and aspartate aminotranferease and alanine aminotransferase, no differences were found for the serum biochemistry variables evaluated. Aspartate aminotransferase was significantly increased in males fed 100 mg kg^{-1} NIV and in females fed ≥25 mg kg^{-1} (data not shown). Alanine aminotransferase decreased from 25 mg kg^{-1} in males but did not change in females.

Treatment-related changes in histopathological data are summarized in Table 4. In both sexes, atrophy of the thymus and reduction of haematopoietic cells in the bone marrow were apparent at 100 mg kg^{-1}. In the spleen, an increased extramedullary haematopoiesis was observed at 100 mg kg^{-1}. In females, an increase in the amount of atretic ovarian follicles and interstitial glands was apparent at 100 mg kg^{-1}, and a lack of corpora lutea development was apparent in severely affected cases, although the number of secondary follicles remained unchanged. Uterine atrophy with diestrus endometrial mucosal change was observed at 100 mg kg^{-1} and the vaginal histology of these animals demonstrated changes indicative of proestrus or diestrus. An increase in castration cells, which is an indicator of depressed sex hormone production, and diffuse hypertrophy of basophilic cells in the anterior pituitary were observed at 100 mg kg^{-1} in both sexes. In the mammary gland, lobular hyperplasia was apparent only in females fed 100 mg kg^{-1}. In the thyroid, dilated follicles lined by a flattened epithelium were more common at 100 mg kg^{-1} in both sexes. In the mesenteric lymph nodes, increased accumulation of macrophages and mast cells was evident in the medullary sinuses at 100 mg kg^{-1} in both sexes. A decrease in metaphyseal trabeculae and thinning of the diaphysis were frequently observed in the femur and sternum of both sexes at 100 mg kg^{-1}. No significant changes were observed in organs other than listed up in Table 4.

There was a slight increase in serum levels of IgM at 100 mg kg^{-1} but the mean levels of IgG and IgA were not affected by NIV treatment. In mice, Hinoshita et al. (1997) found that NIV deregulated IgA production and resulted in IgA nephropathy

Table 5. Immunological effect of nivalenol (NIV) after oral administration for 90 days to male F344 rats.

NIV in diet (mg kg^{-1})	T-/B-cell ratio	CD4/CD8 ratio	NKR-P1A$^+$ cells (%)	NK activity[a] (E:T ratio = 25 : 1)
0	1.8	1.8	7.5	1.0
6.25	1.8	1.8	7.5	1.7**
25	1.1**	1.7	7.5	2.7**
100	0.9***	2.1***	5.8***	2.6**

Note: [a]Values are fold change from control.
Significantly different from the controls at $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively.
Data are from Kubosaki et al. (2008).

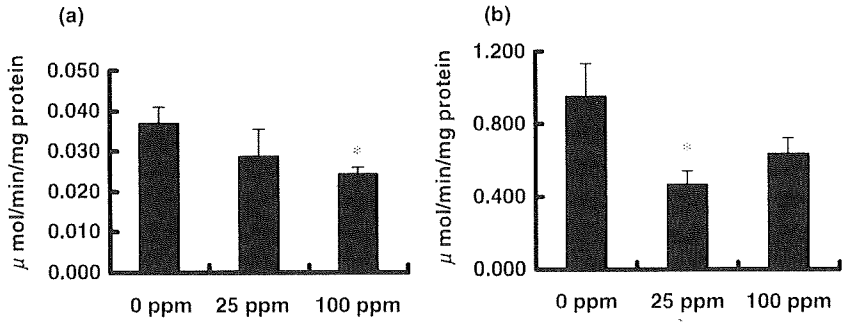

Figure 2. Activities of xenobiotic enzymes (glutathionine *S*-transferase) in the liver of F344 rats fed diets containing NIV for 90 days. Effect of NIV on the glutathionine conjugation of (a) t-PBO and (b) CDNB in rat liver cytosols. *Significant difference from corresponding control ($p < 0.05$).

similar to that seen in humans. In the present study with rats, there was no change in IgA production suggesting that there are species differences in the development of NIV-induced IgA nephropathy.

Table 5 summarizes the immunological effects of NIV in male rats. T- and B-lymphocyte populations in the spleen analysed by flow cytometry revealed that the T-lymphocyte/B-lymphocyte ratios ($CD3^+/B220^+$) were decreased in a dose-dependent manner at $\geq 25\,mg\,kg^{-1}$, while the mean $CD4^+/CD8^+$ ratios in groups fed 6.25 or $25\,mg\,kg^{-1}$ NIV did not differ from the controls; however, the CD4+/CD+8 ratio was increased significantly at $100\,mg\,kg^{-1}$. The population of NKR-P1A$^+$ cells, an indicator of NK cells, decreased at $100\,mg\,kg^{-1}$. These results obtained are parallel to the results of a previous *in vitro* study using human peripheral blood mononuclear cells (Berek et al. 2001). On the other hand, splenic NK activity was unexpectedly enhanced by exposure to NIV at all three dose levels in the present study. Since the activity of NK cells in athymic nude mice is enhanced compared with normal mice (Hasui et al. 1989), the enhanced NK activity observed in NIV-treated rats in the present study may reflect compensation to the reduction/depletion of T-lymphocytes. With regard to the immunotoxicity, an effect was apparent at $\geq 25\,mg\,kg^{-1}$ judging from the decreased T/B ratio while the increase in NK activity that was apparent at $\geq 6.25\,mg\,kg^{-1}$ was considered indicative of a beneficial host-defence response.

The activities of the cytochrome P450 isoforms, CYP3A4 and CYP1A2 in the microsomal fraction and GST in the cytosolic fraction were examined in order to study the effects of NIV on xenobiotic metabolizing enzymes in the liver after 90 days' exposure (Figure 2). Both CYP3A4 and CYP1A2 were unaffected (data not shown). On the other hand, GST activity against t-PBO (Figure 2(a)) decreased in a dose-dependent way and the decrease was significant, at $100\,mg\,kg^{-1}$. Although the activity against CDNB (Figure 2b) was observed to decrease significantly at $25\,mg\,kg^{-1}$, this phenomenon did not seem to be a treatment-related change. Yabe et al. (1993) reported that NIV did not affect cytochrome P450 enzymes but increased total GST activity in mice receiving diets containing $6–12\,mg\,kg^{-1}$ NIV for 2 or 4 weeks. We obtained similar results in regard to cytochrome P450 in this study, therefore CYP3A4 and CYP1A2 activities were not affected by NIV exposure. While GST activities toward CDNB and t-PBO decreased in this study. GST has isozymes, and the alpha, mu and pi classes exhibited substrate specificities. It is reported that t-PBO is a substrate for the mu class (Shan and Armstrong 1994), whereas CDNB is a substrate for a broad range of GST isozymes (Kunze 1997). Our more detailed study revealed that exposure to NIV might

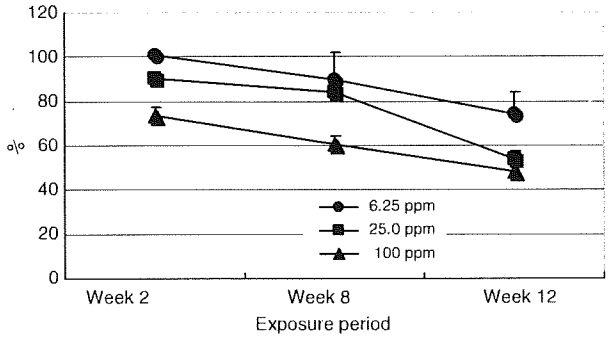

Figure 3. Time course of the ratio of NIV excretion in faeces of F344 rats fed diets containing NIV for 90 days. NIV concentrations in faeces were analysed by GC-MS, as described in the text. The ratio was expressed as the percentage of total faecal NIV (concentration × weight of faeces excreted)/total intake (NIV ppm in diet × feed consumption).

affect the activity of the mu class of GST isozymes rather than that of broad range of GST isozymes in rats.

Faecal metabolites were analysed by GC-MS by using NIV, FX and a de-epoxy NIV standard. The main peak found at various time points throughout the study was NIV and no peak corresponding to de-epoxy NIV was found (data not shown). The ratio of faecal excretion/dietary intake of NIV is shown in Figure 3. Interestingly, the longer the exposure period to NIV, the lower its percentage excretion from faeces. The reason is unknown and further studies on the uptake, tissue distribution and excretion of NIV and its metabolites are needed. This is important because if NIV remains in organs, it is possible that it could induce apoptosis in immunocompetent cells (Poapolathep et al. 2001, 2002, 2003, 2004).

De-epoxy NIV has been found in urine and faeces (Onji et al. 1989). In pigs, several investigators have reported that intestinal microorganisms might be able to transform trichothecenes to their corresponding de-epoxide (Swanson et al. 1988; Eriksen et al. 2002), while other results suggest that the microorganisms in some pigs lack this ability (Eriksen et al. 2002). The results are consistent with the latter as, although the rats were exposed to NIV for 90 days, no de-epoxy NIV could be found in the faeces of any treatment group.

In conclusion, NIV targeted endocrine organs, especially the anterior pituitary in both sexes and female reproductive organs, as well as the haematopoietic and immune organs when fed to rats for 90 days. NIV affected both T- and B-cell functions, with a decreased T-/B-cell ratio being apparent at $\geq 25\,mg\,kg^{-1}$, but also increased NK activity at diet concentrations of $6.25\,mg\,kg^{-1}$ and above. The latter was regarded to be a signature of beneficial host-defence responses. Based on the haematological data, specifically the decrease in WBC found in females at the low dose level, the lowest-observed effect level of NIV was determined to be $6.25\,mg\,kg^{-1}$ in the diet, which corresponds to a dose of $0.4\,mg\,kg^{-1}$ body weightday^{-1}. The results of this study provide essential information for human health risk assessment.

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Muscle tissue kinetics of oxytetracycline following intramuscular and oral administration at two dosages to giant freshwater shrimp (*Macrobrachium rosenbergii*)

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The giant river shrimp (*Macrobrachium rosenbergii*), a native species of Thailand, is either exported for commercial purposes or supplied to meet the local requirements in Thailand. Limited pharmacokinetic information of the major antibiotic, oxytetracycline (OTC), is available for this freshwater shrimp. The purpose of the present study was to investigate the muscle tissue kinetics of OTC in *M. rosenbergii* following either intramuscular (i.m.) or oral (p.o.) administration at two dosages of 11 and 22 mg/kg body weight (b.w.). The concentration of OTC in shrimp tissues was measured using high-performance liquid chromatography (HPLC) equipped with a fluorescence detector. Muscle tissue concentrations were below the detection limit (LOD, 0.1 μg/g) after 96 and 120 h, following i.m. and p.o. administration, respectively. Peak muscle concentrations (C_{max}) were 3.47 and 1.73 μg/g after i.m. and p.o. administration at a single dose of 11 mg/kg b.w. whereas they were 6.03 and 2.51 μg/g at a single dose of 22 mg/kg b.w., respectively. A noncompartment model was developed to describe the pharmacokinetics of OTC in the giant freshwater shrimp. The terminal half-lives of OTC were 28.68 and 28.09 h after i.m. and p.o. administration at a single dose of 11 mg/kg b.w., but 29.95 and 27.03 h at a single dose of 22 mg/kg b.w., respectively. The relative bioavailability was 82.32 and 64.67% following i.m. and p.o. administration, respectively. Based on the pharmacokinetic data, i.m. and p.o. administration with OTC at a dose of 11 mg/kg b.w. would be appropriate for use in giant freshwater shrimp farming. To avoid the OTC residue in shrimp muscle, it should take at least seven half-lives (8 days) to wash out the drug from the muscle of *M. rosenbergii*.

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INTRODUCTION

The giant river shrimp (*Macrobrachium rosenbergii*), a native species of Thailand, is the most popular freshwater shrimp for commercial cultivation (New, 1990). Freshwater shrimp farming is carried out to export the shrimp for commercial purposes or to supply for the local requirements in Thailand (Goss *et al.*, 1966; Singholka, 1978). With the increase in shrimp production, *M. rosenbergii* has become susceptible to various viral and bacterial diseases under adverse environmental conditions (Lalitha & Surendran, 2004). These infections affect shrimp production in hatchery, nursery and grow-out facilities (Lightner *et al.*, 2004). Antibiotics are used to improve disease control and treatment in shrimp farming. One of the major problems has arisen from incorrect methods of calculating the proper dose of these antibiotics. If the drug is administered in sub-therapeutic levels, the risk of therapeutic failure and/or bacterial resistance could be increased. On the other hand, over-dosing of the drug

exerts toxicity resulting in conditions such as soft shell or atrophy of hepato-pancreatic tissue (Lightner *et al.*, 2004). Antimicrobial agents including broad spectrum antibiotics, such as oxytetracycline (OTC), are widely used to treat bacterial infections in fish and shrimp (Sano & Fukuda, 1987; Phillip, 1996; Graslund *et al.*, 2003). The US Food and Drug Administration (FDA) and the US Environmental Protection Agency (EPA) approved three antibiotics, including OTC, for use in aquatic animals (channel catfish, salmonids and lobster).

The pharmacokinetic characteristics of OTC have been studied extensively in marine shrimp (Reed *et al.*, 2004; Sangrungruang *et al.*, 2004; Uno, 2004; Weifen *et al.*, 2004; Chiayvareesajja *et al.*, 2006), but only limited information of OTC residue is available in freshwater shrimp (Brillantes *et al.*, 2001; Poapolathep *et al.*, 2008). The lack of pharmacokinetic studies in giant freshwater shrimp obliges the veterinarian to use empirical approaches based on allometric calculations or extrapolate doses from marine species. The knowledge of drug disposition in giant freshwater shrimp is needed to implement appropriate dosage regimes and to minimize drug residues. The objective of this study was to elucidate pharmacokinetic patterns of OTC in *M. rosenbergii* muscle tissue following i.m. and p.o. administrations of the drug.

MATERIALS AND METHODS

Standard and chemicals

Oxytetracycline was purchased from Sigma Chemical Co. (St Louis, MO, USA). Other reagents and chemicals of analytical grade were purchased from Sigma Chemical Co. Purified water was produced using the Milli-Q water purification system from Millipore, Inc. (Bedford, MA.). A stock solution of OTC for HPLC analysis was prepared by dissolving 25 mg in 100 mL of methanol containing 1 M imidazole (77:23) solution. Oxytetracycline preparation for intramuscular and oral administration was dissolved in dimethyl sulfoxide (DMSO, Sigma) in 0.9% sterile saline to final concentration of 2 mg/mL.

Animals

Giant freshwater shrimp (*Macrobrachium rosenbergii*) were purchased from a commercial farm in Garasin Province, Thailand. The average (±SD) body weight was 23.7 ± 3.8 g. The experimental shrimp were housed in cement ponds at the Chaiyaphum Inland Fisheries Station, which were operated as closed systems using well oxygenated fresh water. Throughout the study, important water quality parameters were monitored including temperature (28–30 °C), pH (7.5–8.5), ammonia (<0.3 p.p.m.), nitrite (<2 p.p.m.), total hardness (30–150 p.p.m.) and total alkalinity (20–60 p.p.m.). The shrimp were free from OTC as analyzed by HPLC and allowed to acclimatize in ponds for 1 week before dosing. They were fed with commercial antibiotic-free shrimp pellets (INTEQC FEED Co., Thailand).

Experimental design

Three hundred and fifty adult shrimp per group were either administered intramuscularly (i.m.) or orally (p.o.) with OTC at two dosages of 11 and 22 mg/kg b.w. For intramuscular injection, shrimp were injected into the mid-part of muscle tissue in the abdominal segment, whereas for oral administration, the drug was directly fed into the mouth using a feeding tube. Eight shrimp were collected at 5, 10, 20, 30, 45 min and 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 20, 24, 30, 36, 42, 48, 54, 60, 66, 72, 84, 96, 120, 144, 168, 192, 216, 240, 264, 268, 312, 336 and 360 h after i.m. and p.o. administration. Shrimp samples were kept at −20 °C until analysis. Prior to OTC assay, shrimp muscle tissues were separated and shells were completed removed from muscle tissues. The muscle tissues of eight shrimp of each collection time were pooled and blended for OTC extraction.

Tissue extraction and clean-up

The extraction method of OTC in shrimp muscle tissues was carried out as described previously (Yutaka *et al.*, 1989; Sokol & Matisova, 1994; Brillantes *et al.*, 2001). The extraction and clean-up were conducted in triplicate from each pooled sample. Briefly, 5 g of blended shrimp muscle was extracted with 20 mL of Na_2EDTA/McIlvaine buffer pH 4.0 and 2 mL of dichloromethane. After mixing in a vortex mixer for 1 min, samples were centrifuged at 1968 *g* for 15 min. Extraction with Na_2EDTA/McIlvaine buffer was repeated and the extract was filtered through a filter paper (Whatman No. 1, Whatman International Ltd., Tokyo, Japan). The filtrate was then applied to Sep-Pak plus C18 (Millipore) and washed with Milli-Q water. Prior to application, Sep-Pak cartridges were preconditioned and equilibrated with 10 mL of methanol and 10 mL of Milli-Q water. The OTC was eluted with 10 mL of methanol, and then evaporated to dryness under a nitrogen stream at 40 °C. The residue was dissolved in 4 mL of the mobile phase. After passing through an Acrodisc® Syringe filter (pore size 0.45 μm, Pall Life Sciences, Ann Arbor, MI), the sample was subjected to HPLC.

HPLC analysis

The analytical method was modified from those of previous described (Yutaka *et al.*, 1989; Sokol & Matisova, 1994; Brillantes *et al.*, 2001). Samples (20 μL) were injected on a model 09 autosampler (SIC Co., Tokyo, Japan) with a Symmetry C18 column (5 μm, 3.9 × 150 mm) and a Symmetry® Sentry™ guard column (5 μm, 3.9 × 20 mm) (Waters Corp., Bedford, MA). The HPLC column was maintained at a temperature of 30 °C using a column oven. The methanol:(1 M) imidazole (77:23, v/v) mobile phase was delivered at a flow rate of 1 mL/min using a PU-2080 HPLC pump (Jasco, Tokyo, Japan). Separation was achieved using a model FP-2020/2025 fluorescence detector (Jasco, Tokyo, Japan) set at λ_{ex} = 380 nm and λ_{em} = 520 nm. The limit of detection (LOD) was 0.1 μg/g. HPLC assays of the samples were conducted in duplicate.

Fortification procedure

To evaluate recovery, 5 g samples of shrimp muscle tissue were added to OTC solution to yield final drug concentrations of 0.1, 0.5, 1, 5 and 10 μg/g of tissue. The spiked samples were then analyzed in triplicate as described in the extraction procedure. The average (±SEM) recovery was 85.2 ± 3.3%.

Calculation of tissue kinetic parameters

The concentration of OTC in experimental shrimp with respect to time was pharmacokinetically analyzed by noncompartmental pharmacokinetic model using the PK Solutions 2.0™ Program including C_{max} (maximum concentration), T_{max} (maximum time concentration), AUC (area under the curve), $t_{1/2\beta}$ (terminal half-life), $t_{1/2ab}$ (absorption half-life), Cl (clearance), MRT (mean residence time) and F (relative bioavailability).

The relative bioavailability (F) was calculated using the equation:

$$F_{(i.m.)} = (AUC_{i.m.22} \times Dose_{i.m.11})/(AUC_{i.m.11} \times Dose_{i.m.22}) \times 100.$$

$$F_{(p.o.)} = (AUC_{p.o.22} \times Dose_{p.o.11})/(AUC_{p.o.11} \times Dose_{p.o.22}) \times 100.$$

RESULTS

The determination of OTC concentrations showed that OTC was detected in shrimp muscle tissue of *M. rosenbergii* following i.m. and p.o. administration. The drug, however, was below the LOD at 96 and 120 h after i.m. and p.o. administration, respectively, at either a dose level of 11 or 22 mg/kg b.w. Administration of 22 mg/kg b.w. of OTC to shrimp resulted in twice higher C_{max} values when compared with that of 11 mg/kg b.w. of OTC. The C_{max} values were noted at 1 and 4 h, respectively, after i.m. and p.o. administration at a single dosage of 11 mg/kg b.w. (Table 1). However, following i.m. and p.o. administration at a single dosage of 22 mg/kg b.w., the C_{max} values of OTC in shrimp muscle tissues were noted at 45 min and 4 h, respectively (Table 1). The tissue concentration–time profiles of OTC at dosages of 11 and 22 mg/kg b.w. after p.o and i.m. administration, respectively, are shown in Figs 1 & 2. The $t_{1/2\beta}$ values of OTC in shrimp muscle tissues were 28.68 and 29.95 h after i.m. administration and 28.09 and 27.03 h after p.o. administration at dose levels of 11 and 22 mg/kg b.w., respectively. The $AUC_{0-\infty}$ values were higher when shrimp were given 22 mg/kg b.w. of OTC than those when they were given 11 mg/kg b.w. of OTC (Table 1). The Cl values were higher in shrimp administered p.o. with 22 mg/kg OTC than those in the other groups (Table 1). The relative bioavailabilities of OTC were 82.32 and 64.67% following i.m. and p.o. administration, respectively. The other parameters did not differ among shrimp groups.

DISCUSSION

The disposition of OTC was investigated following i.m and p.o administration at a single dose of 11 and 22 mg/kg b.w. in giant freshwater shrimp. This is the first report on the muscle tissue kinetics of OTC in giant freshwater shrimp, *M. rosenbergii*, following i.m. and p.o. administration. Although, OTC has been practically administered by the medicated feed (Poapolathep *et al.*, 2008), dispositions and bioavailability of the drug must be evaluated by either direct p.o. or i.m. administration in order to devise an approach for the appropriate use of OTC in giant freshwater shrimp. Meanwhile, there have been extensive reports on oral availability and dispositions following either single or multiple oral dosing in marine shrimp species (Reed *et al.*, 2004; Sangrungruang *et al.*, 2004; Uno, 2004; Weifen *et al.*, 2004; Chiayvareesajja *et al.*, 2006) but the pharmacokinetic information are insufficient in giant freshwater shrimp.

The OTC concentration–time profiles show the curves after i.m. and p.o. administration to be parallel at either dose level, being consistent with a similar duration of detectable level after administration. The peak OTC level of shrimp muscle tissues after i.m. administration was approximately twice higher than that after p.o. administration at each dose level, and the OTC level after p.o. administration declined more slowly than that after i.m. administration.

The MRT, $t_{1/2ab}$ and $t_{1/2\beta}$ did not differ significantly with dosage following either i.m. or p.o. administration. After i.m. administration, the AUC of 22 mg/kg b.w. dose was 84.3 μg·h/g, which was 1.6 times that of 11 mg/kg b.w dose. The C_{max} of the 22 mg/kg b.w. was 1.7 times that of the

Table 1. Pharmacokinetic parameters for oxytetracycline following intramuscular and oral administration at two dosages of 11 and 22 mg/kg b.w. in *M. rosenbergii*

Pharmacokinetic parameters (units)	Intramuscular		Oral	
	11 mg/kg	22 mg/kg	11 mg/kg	22 mg/kg
$t_{1/2\beta}$ (h)	28.68 ± 2.43	29.95 ± 1.96	28.09 ± 2.24	27.03 ± 2.21
$t_{1/2ab}$ (h)	3.66 ± 0.38	3.85 ± 0.32	4.34 ± 0.44	5.25 ± 0.62
Cl (L/kg/h)	0.21 ± 0.02	0.26 ± 0.01	0.25 ± 0.01	0.38 ± 0.03
$AUC_{0-\infty}$ (μg·h/g)	51.2 ± 3.63	84.3 ± 3.73	44.3 ± 3.30	57.3 ± 2.46
MRT (h)	51.6 ± 2.25	54.4 ± 3.0	52.9 ± 3.79	53.9 ± 2.74
C_{max} (μg/g)	3.47 ± 0.38	6.03 ± 0.09	1.73 ± 0.03	2.51 ± 0.03
T_{max} (h)	1.0 ± 0.0	0.75 ± 0.0	4.0 ± 0.0	4.0 ± 0.0

Values ±SEMs of triplicate analyses for a pooled sample based on noncompartmental pharmacokinetic model.

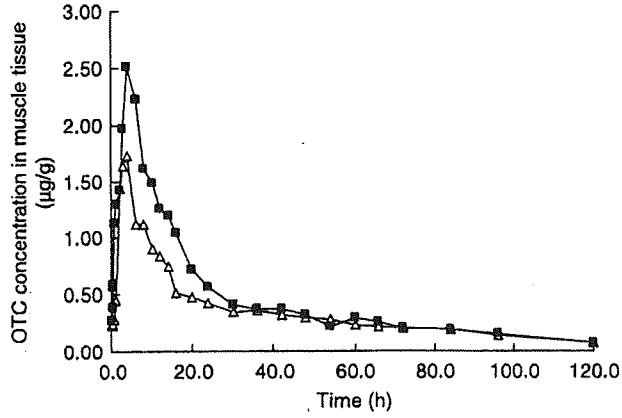

Fig. 1. OTC concentrations in muscle tissues of *M. rosenbergii* after oral administration at dosages of 11 (△) and 22 (■) mg/kg b.w. Each coordinate is value derived from triplicate analyses for a pooled sample at each time point.

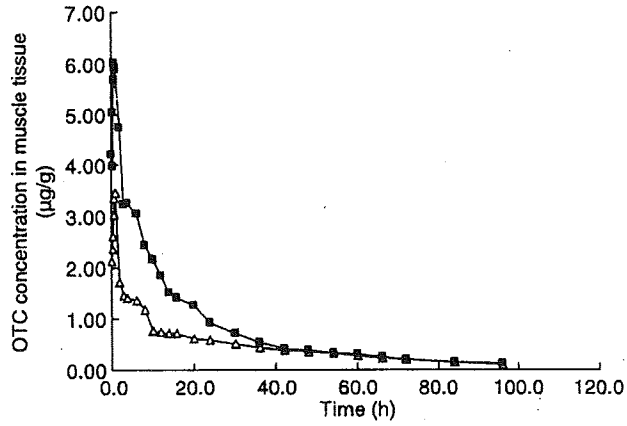

Fig. 2. OTC concentrations in muscle tissues of *M. rosenbergii* after intramuscular administration at dosages of 11 (△) and 22 (■) mg/kg b.w. Each coordinate is value derived from triplicate analyses for a pooled sample at each time point.

11 mg/kg b.w. dose following i.m. administration. The $t_{1/2\beta}$ values of OTC, 27–29 h, corresponded well with our previous investigation (Poapolathep *et al.*, 2008), showing 30.96 h as $t_{1/2\beta}$ in *M. rosenbergii* after medicated-feed treatment at the feeding level of 4 g/kg of feed for five consecutive days (1% of total prawn body weight, twice a day). On the other hand, following p.o. administration, the AUC of 22 mg/kg b.w. dose was 57.3 μg·h/g, which was 1.3 times that of the 11 mg/kg b.w. The C_{max} of 22 mg/kg b.w. was 1.5 times that of the 11 mg/kg b.w. dose. With increasing doses, the $t_{1/2\beta}$ and $t_{1/2ab}$ showed no significant changes after either i.m. or p.o. administration. This demonstrates that shrimp muscle is not the major organ for accumulation of OTC. The drug might have transferred into the digestive compartment such as the hepato-pancreatic tissues (Chiayvareesajja *et al.*, 2006). The tissue $t_{1/2\beta}$ in the present study is similar to that in the white shrimp, *Litopenaeus setiferus*, reported as 29.1 h (Reed *et al.*, 2004), while it is longer than that in *Litopenaeus vannamei* (20.9 h), *Penaeus monodon* (23.1 h) and *Penaeus japonicus* (24.7 h), following intrasinus injection, (Sangrungruang *et al.*, 2004; Uno, 2004; Chiayvareesajja *et al.*, 2006). However, Björklund and Bylund (1990) reported that the elimination of OTC was markedly temperature-dependent; the elimination of OTC from rainbow trout tissues was faster at higher water temperatures. The differences in pharmacokinetic parameters among species may be because of the differences in anatomical volumes, and binding affinity of drugs to plasma and tissue proteins. The degree of plasma protein and tissue binding has been observed to vary depending on aquatic species and drug used (Oie & Tozel, 1979; Guarino, 1986; Plakas *et al.*, 1988). Based on the ratio between C_{max} and MIC (Minimal Inhibitory Concentration) values and the terminal half-life of OTC in muscle tissue of shrimp, the dosage levels of 11 and 22 mg/kg b.w. can be maintained above the MIC. Takahashi *et al.*, 1985 reported the MIC of OTC was effective against 49 strains of *Vibrio* spp. in kuruma shrimp at <0.1 to 12.5 μg/mL.

The relative bioavailability of OTC was 64.67% under the described water quality and experimental conditions following p.o. administration. The oral bioavailability of OTC in giant freshwater shrimp was higher than that previously reported in finfish species. The bioavailability of OTC has been reported to be very low in rainbow trout (2.6%) following oral dosing with gelatin capsules at a dosage of 150 mg/kg b.w. (Rogstad *et al.*, 1991). The bioavailability was found to be 0.6% in carp with dry feed pellets at a dosage of 60 mg/kg b.w. (Grondel *et al.*, 1987), 5.6% in rainbow trout with suspension at a dosage of 75 mg/kg b.w. (Björklund & Bylund, 1991), 1.9% in Atlantic salmon with medicated feed containing a dose of 50 mg/kg b.w. (Elema *et al.*, 1996), 9.3% in ayu with medicated feed containing a dosage of 100 mg/kg b.w. (Uno, 1996) and 3.2–7.3% in Arctic charr with liposome/alginate particles (Haug & Hals, 2000). Abedini & Namdari, 1998 reported higher OTC bioavailabilities in salmon (24.8%) and trout (30.3%) with gelatin capsule containing a methanolic solution of OTC at a dose of 50 mg/kg b.w. Low oral bioavailability may be caused by complex-binding. Feed pellets may contain ions, which form complexes with OTC therefore absorption of complex-bound OTC cannot pass through lipid membranes (Elema *et al.*, 1996). Clive (1968) also reported that OTC in the intestine must be taken up from the liquid phase. Taken together, the oral bioavailability of OTC in *M. rosenbergii* determined in this study was almost higher than that found in marine shrimp. In kuruma shrimp (*P. japonicus*), the OTC bioavailability was 43.2% after oral administration. The maximal hemolymph concentration was attained within 24 h of oral dosing (Uno, 2004). In giant tiger prawn, *P. monodon*, the oral bioavailability was 59.9%. The maximal hemolymph concentration of OTC (20.2 μg/mL) was obtained after oral administration (Sangrungruang *et al.*, 2004), while it was 49.5% in white shrimp, *L.setiferus* (Reed *et al.*, 2006). Consistent with this, oral bioavailability of marine shrimp was lower than that of freshwater shrimp (our study); it may be

because of the higher levels of salinity, hardness and alkalinity in seawater (Uno, 2004; Chiayvareesajja *et al.*, 2006; Reed *et al.*, 2006). On the other hand, the water quality parameters are somewhat varied in our present study. The pH covers a 10-fold difference in hydrogen ion concentration and goes to a rather alkaline level (8.5), and a 5-fold difference in hardness can make a difference in OTC bioavailability. The relatively high value of oral bioavailability in giant freshwater shrimp obtained from our experiment might be caused by a redistribution of OTC from the other tissues of shrimp, particularly of the hepato-pancreas, as the drug might have been transiting in the hepato-pancreas (Weifen *et al.*, 2004; Chiayvareesajja *et al.*, 2006). Regarding the ability of oxytetracycline to form complexes with di- and trivalent cations has been known for a long time (Albert & Rees, 1956). Because OTC inevitably comes into contact with these ions when administered to seawater-adapted fish, the presence of $Mg^{2+}$and Ca^{2+} will affect the absorption. (Aksnes & Thingstad, 1990). Lunestad and Goksøyr (1990) also reported that 95% of OTC is bound by $Mg^{2+}$and Ca^{2+} present in seawater. In addition, Rogstad *et al.* (1991) reported that the tissue level of OTC in seawater rainbow trout was approximately 30% of that found in freshwater rainbow trout. These cations will also presumably make a difference in the bioavailability of OTC between seawater and freshwater shrimp.

In conclusion, OTC concentrations were detected in the muscle tissues of *M. rosenbergii* after i.m. and p.o. administration at dosages of 11 and 22 mg/kg b.w. Based on the pharmacokinetic data, i.m. and p.o. administration of OTC at a dose of 11 mg/kg b.w. would be appropriate for use in giant freshwater shrimp farming. To avoid the OTC residue in shrimp muscle, it should take at least seven half-lives (8 days) to wash out the drug from the muscle of *M. rosenbergii*.

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