TABLE 2. *Natural fumonisins in retail food in fiscal years 2004 to 2007*[a]

Commodity	LOQ (μg/kg)	No. of analyzed samples					FB_1			FB_2			FB_3		
		2004	2005	2006	2007	Total	No. of contaminated samples	Mean of positives (μg/kg)	Maximum (μg/kg)	No. of contaminated samples	Mean of positives (μg/kg)	Maximum (μg/kg)	No. of contaminated samples	Mean of positives (μg/kg)	Maximum (μg/kg)
Raw corn	10	18	10	10	3	41	0	—	—	0	—	—	0	—	—
Frozen or Canned corn	10	51	32	30	14	127	2	26.4	36	1	14.8	14.8	0	—	—
Popcorn grain	2	15	13	15	14	57	49	67.5	354	44	17.9	94.0	40	12.4	64.0
Corn grits	2	10	13	15	8	46	46	104	1,380	42	36.5	590	39	22.8	358
Cornflakes	10	30	15	16	20	81	15	24.6	59.0	0	—	—	0	—	—
Corn soups	10	29	20	20	19	88	2	11.5	12.9	0	—	—	0	—	—
Cornstarch	2	NA	7	5	10	22	0	—	—	0	—	—	0	—	—
Corn snacks	2	NA	NA	20	30	50	41	113	1,670	40	31.8	597	36	19.5	281
Beer	2	NA	NA	10	20	30	10	4.7	12.9	0	—	—	0	—	—
Buckwheat flour	10	NA	10	NA	5	15	0	—	—	0	—	—	0	—	—
Buckwheat dried noodles	2	30	20	NA	NA	50	0	—	—	0	—	—	0	—	—
Flattened barley	10	20	20	NA	NA	40	0	—	—	0	—	—	0	—	—
Soybeans	2	NA	20	32	30	82	13	4.5	8.0	3	4.3	4.8	0	—	—
Polished rice	4	NA	11	10	10	31	0	—	—	0	—	—	0	—	—
Millet	2	NA	NA	10	20	30	6	5.1	6.5	0	—	—	0	—	—
Asparagus	2	NA	NA	NA	20	20	2	2.7	2.8	2	2.5	2.5	0	—	—

[a] LOQ, limit of quantification, which was determined from the height of the signal peak of fumonisin that corresponded to 10 times that of the background noise; —, no numerical data; NA, not analyzed.

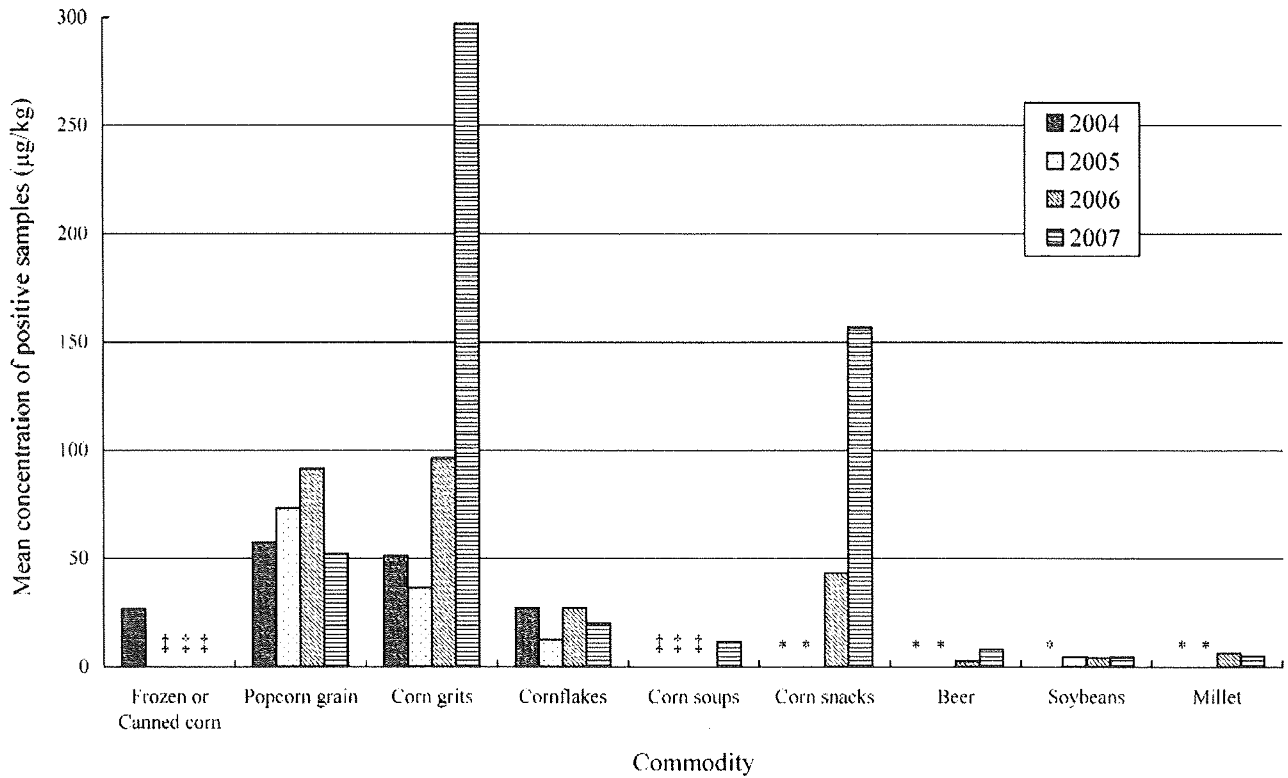

FIGURE 3. *Annual intergradation of the mean concentration of fumonisin B_1 contamination during fiscal years 2004 to 2007.* *, *Not analyzed;* ‡, *not detected.*

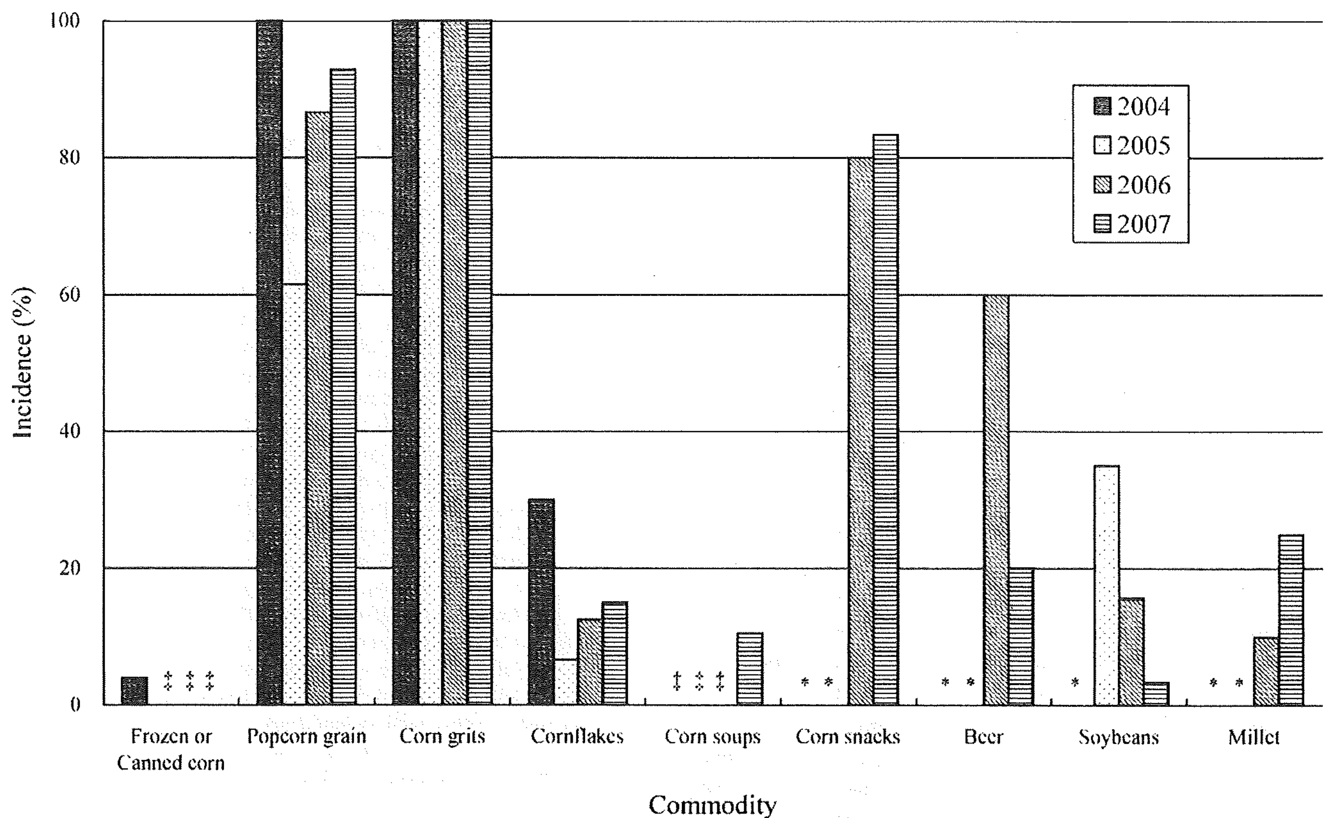

FIGURE 4. *Annual intergradation of the incidence of fumonisin B_1 contamination during fiscal years 2004 to 2007.* *, *Not analyzed;* ‡, *not detected.*

both the incidence of OTA contamination and its mean concentration were observed in oatmeal, wine, and roasted coffee beans. In oatmeal and wine, high mean concentrations tended to occur in years in which the incidence of OTA was high; this tendency was not observed in roasted coffee beans. This may indicate that the high levels of OTA detected in roasted coffee beans did not depend on the high incidence. The phenomenon could be due to differences in the processing stage of each sample of roasted coffee beans. On the other hand, over 90% of instant coffee and cocoa samples were constantly contaminated with OTA. The incidence and mean concentration of OTA in these samples varied significantly from year to year, highlighting the importance of continual monitoring.

The JECFA suggests that corn and corn products are the primary contributors of exposure to fumonisins. In this survey, fumonisins were detected in frozen and canned corn, popcorn grain, corn grits, cornflakes, corn soups, corn snacks, beer, soybeans, millet, and asparagus; it was not detected in the other products (Table 2). The mean recoveries for each commodity ranged from 61.3 to 124.5% for FB_1, 48.3 to 123.6% for FB_2, and 69.7 to 120.3% for FB_3. Fumonisins were detected in all of the samples of corn grits and in more than 80% of the samples of popcorn grain and corn snacks. The highest concentration was found in corn snacks, with maximums of 1,670 μg/kg for FB_1, 597 μg/kg for FB_2, and 281 μg/kg for FB_3. Tseng and Liu *(41)* have also reported that the highest values of FB_1 and FB_2 in Taiwan were detected in corn snacks (2,395 μg/kg for FB_1 and 715 μg/kg for FB_2). The European Union has set maximum levels for the total concentration of FB_1 and FB_2 in corn and corn products *(8)*. The maximum levels are at 2,000 μg/kg for unprocessed corn, 1,000 μg/kg for corn products that are not directly consumed by humans, and 400 μg/kg for corn-based foods that are directly consumed by humans. Although Japan has no regulatory limit for fumonisins, the contamination levels in most of the samples were relatively low *(7, 13, 15, 24, 31–35, 38, 41–43)*. Exceptions include a sample of corn grits (1,970 μg/kg for both FB_1 and FB_2), a sample of popcorn (448 μg/kg), and two samples of corn snacks (639 and 2,270 μg/kg), all of which exceeded the maximum level set in the European Union. Although fumonisins were not detected in domestic polished rice in our study, further surveillance is needed because of the contamination of rice in other countries, including Korea. Park et al. *(25)* have reported that 2 of 88 polished domestic rice samples were contaminated by FB_1 at levels of 48.2 and 60.6 μg/kg. These results showed that the commodities identified by the JECFA are the main contributors to fumonisin exposure in Japan.

Figure 3 shows the annual mean concentrations of FB_1 in nine commodities, and Figure 4 shows the incidence of contamination. Significant annual variations in the mean concentrations of FB_1 were observed in corn grits (36.4 to 297 μg/kg), corn snacks (43.2 to 157 μg/kg), and beer (2.5 to 8.0 μg/kg). Significant annual variations in the incidence of contamination were observed in cornflakes (7 to 30%), beer (20 to 60%), and soybeans (3 to 35%). There is considerable variation in the concentration of fumonisins in corn. The JECFA has reported on the annual variation of FB_1 in corn collected in Iowa between 1988 and 1996 *(13)*. Each year between 1988 and 1991, the mean concentration of FB_1 was higher than 2,000 μg/kg, whereas between 1992 and 1996 the concentration was lower than 450 μg/kg. Shephard et al. *(33)* reported on levels of fumonisins in white and yellow corn in South Africa between 1989 and 1993. The average levels of FB_1 in white corn ranged from 320 to 570 μg/kg; the average level in yellow corn ranged from 170 to 190 μg/kg between 1989 and 1992, but was 680 μg/kg in 1993. Although the levels of FB_1 in white corn were consistently higher than those in yellow corn from 1989 to 1992, this trend was reversed in 1993; these results indicate that corn and corn products must be continually monitored for fumonisin contamination.

The average level of mycotoxins is different between the single-year (2004) survey *(36)* and the multiyear survey. The average concentration of OTA in raisins was 1.54 μg/kg in 2004 but 0.93 μg/kg over the years 2004 to 2007. Similarly, the average concentration of FB_1 in corn grits was identified as 51.1 μg/kg in 2004 but 104 μg/kg between 2004 and 2007, and the average concentration of FB_1 in popcorn grain was 57.2 μg/kg in 2004 and 67.5 μg/kg between 2004 and 2007.

For OTA and fumonisins, the incidence and level of contamination vary greatly from year to year. Therefore, continual monitoring is necessary, and it is valuable to use average contamination levels obtained from multiyear surveillance studies to more accurately assess exposure.

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Immunotoxicity of nivalenol after subchronic dietary exposure to rats

Atsutaka Kubosaki [a], Maki Aihara [a], Bong Joo Park [a], Yoshitsugu Sugiura [b], Makoto Shibutani [c], Masao Hirose [c], Yoshihiko Suzuki [d], Kosuke Takatori [a], Yoshiko Sugita-Konishi [a,*]

[a] *Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Department of Food Chemistry, Kobe Institute of Health, 4-6 Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan*
[c] *Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[d] *Department of Veterinary Science, Azabu University, 1-17-1 Fuchinobe, Sagamihara-shi, Kanagawa 229-0006, Japan*

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Abstract

Immunobiological effects of nivalenol (NIV), a trichothecene mycotoxin produced by *Fusarium nivale*, were examined in male F344 rats after 90-day dietary exposure at doses of 0, 0.4, 1.5, and 6.9 mg/kg body weight/day (0, 6.25, 25 and 100 ppm, respectively) in a subchronic toxicity study. With regards to the serum immunoglobulin levels, a slight increase of IgM was observed only at 6.9 mg/kg (26% increase), while levels of IgG and IgA did not fluctuate at any dose. Flow cytometric analysis of splenic cells revealed a dose-dependent decrease of T lymphocyte/B lymphocyte ($CD3^+/B220^+$) ratio from 1.5 mg/kg and an elevated $CD4^+$helper/$CD8^+$cytotoxic T lymphocyte ratio at 6.9 mg/kg. Furthermore, increases of natural killer (NK) activity of splenic lymphocytes against YAC-1 target cells were observed at all doses, while the magnitude of changes was similar between 1.5 and 6.9 mg/kg. At 6.9 mg/kg, the reduction of the ratio of NKR-P1A$^+$ splenic cells, which is an indicator of NK cells in the spleen, was apparent. As with other previous studies of NIV, decreased body weight was observed from 1.5 mg/kg during the experiment in the present study. In summary, NIV affected immune function in rats after 90-day dietary exposure, the effects being apparent from 0.4 mg/kg judging from the increase of NK activity, although nutritional suppression might have influenced the immunological changes appeared from 1.5 mg/kg.
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Keywords: Nivalenol; Subchronic exposure; Immunotoxicity; Flow cytometry; Natural killer activity; F344 rats

1. Introduction

Mycotoxins are secondary metabolites of fungi and are detrimental to the health of both animals and human. A number of different mycotoxins of the class of trichothecenes, zearalenone and fumonisins are produced by a variety of *Fusarium* fungi (European Commission, 2000a,b,c). A couple of strains of the *Fusarium* genus (e.g. *Fusarium cerealis* and *Fusarium poae*) produce a type B trichothecene mycotoxin, nivalenol (NIV). These fungi are abundant in various cereal crops such as wheat, maize, barley, oats, and rye and processed grains such as malt, beer and bread (European Commission, 2000a). The fungi producing trichothecenes are important pathogens that grow on the crop in the field in temperate regions of America, Europe and Asia.

Hitherto, NIV has been shown to exert diverse toxicities. A decrease of body weight gain as well as a reduction in the liver and kidney weights were found in female mice that were given NIV in feeds at up to 0.7 mg/kg body weight (bw)/day for two years (Ohtsubo et al., 1989). Embryolethality was also detected in pregnant mice injected 0.5 mg/kg bw of NIV by a single dose (Ito et al., 1986). Mice given 3.5 mg/kg bw NIV in the diet for 24 days showed toxicity

Abbreviations: NIV, nivalenol; FITC, fluorescein isothiocyanate; ANOVA, analysis of variance; NK, natural killer.

* Corresponding author. Tel.: +81 3 3700 1141x535; fax: +81 3 3700 9527.
E-mail address: ykonishi@nihs.go.jp (Y. Sugita-Konishi).

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targeting bone marrow and lymphoid organs, resulting in erythrocytopenia and leukocytopenia, but without changes in the weights of the body, spleen and thymus (Ryu et al., 1987). Mice given 6 or 12 ppm NIV in the food exhibited an increase in serum IgA and induction of IgA deposits in the glomerular mesangium (Hinoshita et al., 1997). In ovalbumin (OA)-specific T cell receptor αβ-transgenic mice, production of total and OA specific IgE in serum was inhibited by drinking water containing 6 ppm of NIV for 4 weeks (Choi et al., 2000). Cytokine production in the splenocyte showed that NIV inhibited interleukin 4 but increased interleukin 2. In addition, Poapolathep et al. (2003, 2004) revealed that NIV can induce apoptosis in lymphoid organs of mice and affected the viability of $CD4^{+}CD8^{+}$ thymocytes. However, the effect of NIV exposure for longer periods on immunological functions has scarcely been examined.

We recently examined the subchronic toxicity of NIV using F344 rats according to the Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 for 'Repeated dose 90-day oral toxicity study in rodents' (Takahashi et al., 2008). By exposure to NIV at dietary concentrations of 6.25, 25 and 100 ppm, a decrease of white blood cell counts was hematologically found at 100 ppm in males (6.9 mg/kg bw/day) and from 6.25 ppm in females (0.4 mg/kg bw/day). Histopathologically, treatment-related changes were predominantly observed in the hematopoietic and immune organs as well as the anterior pituitary in both sexes and the female reproductive system at 100 ppm (6.4–6.9 mg/kg bw/day), such as thymic atrophy, hypocellularity in the bone marrow, diffuse hypertrophy of basophilic cells in the anterior pituitary, and increase of the ovarian atretic follicles. Based on the hematological data, the no-observed-adverse-effect level of NIV was determined to be less than 6.25 ppm (0.4 mg/kg bw/day for both males and females).

The objective of the present study was to examine the immunotoxic effects of NIV after subchronic dietary exposure to F344 rats sharing tissue and blood samples with above described subchronic toxicity study. Because of the immunomodulatory potentials of estrogens (Jansson and Holmdahl, 1998; Lengi et al., 2007), male animals were selected in the present study.

2. Materials and methods

2.1. Chemicals

For purification of NIV, Fuzarenon X was extracted and purified from culture media of *Fusarium kyushuense* (Fn-2B). The identity and purity of NIV was determined by liquid chromatography/mass spectrometry (LC/MS; LCMS-2010A; Shimadzu Corp., Kyoto Japan), with an Atmospheric Pressure Chemical Ionization interface and the LC system (LC-2010CHT; Shimadzu Corp.), and the purity was estimated to be >98% from the area percentage of the chromatogram (data not shown).

For administration to rats, NIV was dissolved in a small quantity of ethanol first and then well mixed into powdered CRF-1 basal diet (Oriental Yeast Co., Tokyo, Japan). Stability of the test compound 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. CRF-1 is a grain-based (52% in total) regular rodent diet, in which deoxynivalenol and aflatoxins were under the detection levels (deoxynivalenol: <0.1 mg/kg; aflatoxins: <0.5 μg/kg).

2.2. Animals and treatment

Male and female F344/DuCrj rats, 5 weeks old, obtained from Charles River Japan Inc. (Yokohama, Japan), were used in a subchronic repeated oral dose toxicity study of NIV (Takahashi et al., 2008). They received powdered CRF-1 and tap water *ad libitum* during the 1 week acclimatisation period, housed 4 per polycarbonate cage with sterilized softwood chips as bedding in a barrier-maintained animal room conditioned at 23–25 °C and 50–60% humidity, and on a 12-h light/dark cycle.

In a preliminary dose-finding study, a total of 24 male F344/DuCrj rats at 6 weeks of age were fed diet containing NIV 0, 150 or 300 ppm for 2 weeks. Reduction of body weights was apparent at 150 and 300 ppm from week 1, and one animal in the 300 ppm group died at week 2. Body weights were reduced to 70% and 47% of the control value at the end of week 2 in the groups receiving 150 and 300 ppm, respectively. Therefore, the concentration of 100 ppm was determined to be the highest dose for the subsequent subchronic study.

Animals, weighing 109.8 ± 3.8 g in males and 94.0 ± 2.9 g in females (mean ± SD), were randomly divided into 4 groups, each consisting of 10 males and 10 females, housed 3 or 4 per cage and given 0 (control), 6.25, 25 or 100 ppm NIV in the powdered diet for the treatment period of 90 days. The test diets were available *ad libitum*, except for one-night fasting prior to the scheduled sacrifice. 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 the experiment, all animals were anesthetized with ether, weighed, and blood samples were collected from the abdominal aorta. Animals were then killed by exsanguination from the abdominal aorta. Since there was a published study that has examined in vivo effect of ochratoxin A on rat immune functions with variable number of animals ranging 4–10/per group for statistical comparison (Dortant et al., 2001), we, in the present study, also used six males per group and spleens of these animals were removed for analyses of immune functions as well as the histopathological analysis for the toxicity study (Takahashi et al., 2008). Initial six animals in the order of animal numbering in each group were selected for immune assays.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.3. Spleen cell subset analysis

Removed spleens were immediately minced, and passed through mesh to obtain single cell suspensions. Cells were incubated with mouse monoclonal antibody (mAb) D34-485 (subclass IgG2b) against FcγRII(CD32) to block non-specific staining and were double-stained with following 50 × diluted fluorescein isothiocyanate (FITC)-conjugated mouse mAbs and phycoerythrin (PE)-conjugated ones in combination: FITC anti-rat-CD8a (OX-8; IgG1), FITC anti-rat-CD3 (G4.18; IgG3), FITC anti-rat-NKR-P1A (10/78; IgG1), PE-conjugated anti-rat-CD4 (OX-35; IgG2a), PE anti-CD45RA (OX-33; IgG1) or PE anti-CD3 (1F4; IgM), purchased from BD Biosciences (San Jose, CA). Flow cytometric analysis was performed using a FACS Calibur (BD Biosciences).

2.4. Analysis of natural killer (NK) activity

Splenic NK activity was assayed according to the method as described previously (Arase et al., 1999). Briefly, YAC-1 target cells (1×10^7 cells/ml) were labeled with PKH2 green fluorescent dye (Sigma, Saint Louis, MO) for 3 min. The labeled target cells (100 μl at 1×10^5/ml) were plated into round bottomed 96 well microplates (TPP, Trasadingen, Switzerland), then splenocytes as effector cells in 100 μl 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 at 37 °C in 5% CO_2, dead cells were stained with propidium

iodide (PI; Dojindo, Kumamoto, Japan), and the proportion of PI-stained cells among PKH2 dye-stained cells was determined by analysis with a FACS Calibur. Spontaneous and maximum cell death ratios were determined by incubating target cells with either medium or with 30 μg saponin (MP Biomedicals, Eshwege, Germany) in the absence of effecter cells. The percent cytotoxicity was calculated as [(experimental cell death – spontaneous cell death)/(maximum cell death – spontaneous cell death)] × 100.

2.5. Determination of immunoglobulin subclasses in serum

To measure the levels of IgG, IgM and IgA subclass antibodies in the serum samples, purified immunoglobulins, rat IgG_1 (BioLegend, San Diego, CA), rat IgM (eBioscience, San Diego, CA) and rat IgA (BD Bioscience), were used to generate standard curves for each subclass, respectively. Individual wells of flat-bottom 96-well plates (Corning, Corning, NY) were coated with anti-rat IgG/IgM/IgA (Open Biosystems, Huntsville, AL) or anti-rat IgA (Sigma) overnight at 4 °C. Nonspecific binding sites were blocked with phosphate-buffered saline (PBS) containing 1% BSA (Serological Proteins, Kankakee, IL) for 90 min at room temperature. After washing of plates, diluted serum and standards were added to individual wells. After incubation for 90 min at room temperature and washing of plates, horseradish peroxidase-conjugated anti-rat IgG (Invitrogen, Carlsbad, CA), anti-rat IgM (Stressgen Bioreagents, Ann Arbor, MI) or anti-rat IgA (Bethyl, Montgomery, TX) antibody was added to appropriate wells. All plates were washed and added with tetramethylbenzidine substrate reagent (BD Bioscience), and color development was recorded at 450 nm using EL800 Universal microplate reader (BIOTEK, Winooski, VT).

2.6. Statistical analyses

Differences between groups were evaluated using the following methods. Numerical data were analyzed for homogeneity of variance using Bartlett's test. When the variance was homogenous among the groups, a one-way analysis of variance (ANOVA) was carried out. If significant differences were found, the mean value for each treatment group was compared to that of the controls using Dunnett's test. When the variance was heterogeneous based on Bartlett's test, the Kruskal-Wallis's H test was used to check for differences among the groups. If significant differences were found, a Dunnett-type rank-sum test was performed.

3. Results

3.1. In-life parameters

In males, dose levels of NIV at dietary concentrations of 0, 6.25, 25 and 100 ppm were calculated to be 0, 0.4, 1.5 or 6.9 mg/kg bw/day, respectively. Dose level at each dietary concentration was used for group description hereafter.

No deaths occurred throughout the feeding period. Although loose stools were found at 6.9 mg/kg from the start of the experiment, no other clinical signs were observed during the study. On the other hand, lower body weights with statistical significance were evident at 6.9 mg/kg by parametric method as compared with the untreated controls from week 1 of the experiment. This was also evident at 1.5 mg/kg from week 6 to the end of experiment with parametric method. Mean final body weights (g) at necropsy in the groups of control, 0.4, 1.5, and 6.9 mg/kg were 303.9 ± 10.5, 306.5 ± 13.9, 282.2 ± 10.5, and 227.7 ± 8.4, respectively. Body weights of the 1.5 and 6.9 mg/kg groups were decreased to 93% and 75% of the control value, respectively. Although values were approximate due to the small sample size in each group ($n = 3$ cages/group; statistical analysis, not applied), suppressed food consumption/animal was only observed during the initial six weeks of the study at 6.9 mg/kg.

3.2. Spleen cell subset analysis

T and B lymphocyte populations in the spleen were analyzed by flow cytometry with cell surface marker proteins CD3 and B220, respectively. T lymphocyte/B lymphocyte ratios ($CD3^+/B220^+$) were dose-dependently decreased with statistical significance from 1.5 mg/kg by parametric method (Fig. 1). To examine helper T lymphocyte ($CD4^+$) and cytotoxic T lymphocyte ($CD8^+$) populations, $CD4^+/CD8^+$ ratios were analyzed, and the mean of $CD4^+/CD8^+$ ratios did not change at 0.4 and 1.5 mg/kg when compared with the untreated control value, but significantly increased at 6.9 mg/kg by parametric method (Fig. 2). When the mean percentage of $NKR\text{-}P1A^+$ cells containing $NKR\text{-}P1A^{high}$ and/or $NKR\text{-}P1A^{dull}$ cells was examined, it was significantly decreased at 6.9 mg/kg by parametric method (Fig. 3). NKR-P1A is the type II integral membrane protein that is known to express on all rodent NK cells essentially (Chambers et al., 1989; Ryan et al., 2001).

3.3. Natural killer activity

As shown in Fig. 4, splenic NK activities against YAC-1 target cells in all NIV-treated groups were significantly higher than that in the control group at any E:T ratio by parametric method. The activities at 6.9 mg/kg were similar to those at 1.5 mg/kg. For example, at an E:T ratio of 25:1, the mean levels of cytolytic activity in splenic cells from

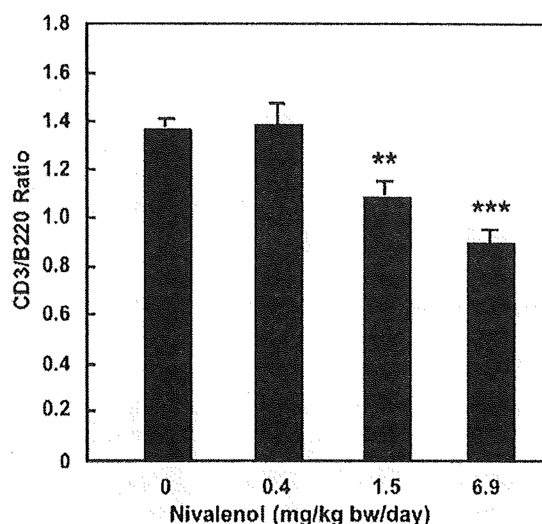

Fig. 1. Flow cytometric analysis of T lymphocyte/B lymphocyte ratios ($CD3^+/B220^+$) in splenic cells of male rats treated with NIV for 90 days. Data are expressed the average ± SEM as a ratio of CD3 to B220 using six animals per group. Asterisks indicate significant differences from control. $^{**}P < 0.01$, $^{***}P < 0.001$ by Dunnett's test.

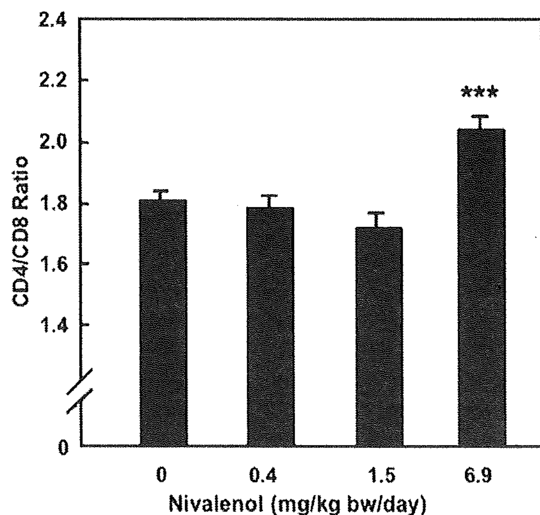

Fig. 2. Flow cytometric analysis of helper T lymphocyte/cytotoxic T lymphocyte ratios ($CD4^+/CD8^+$) observed in splenic cells of male rats treated with NIV for 90 days. Data are represented the average ± SEM as a ratio of CD4 to CD8 using six animals per group. Asterisks indicate significant differences from control. $^{***}P < 0.001$ by Dunnett's test.

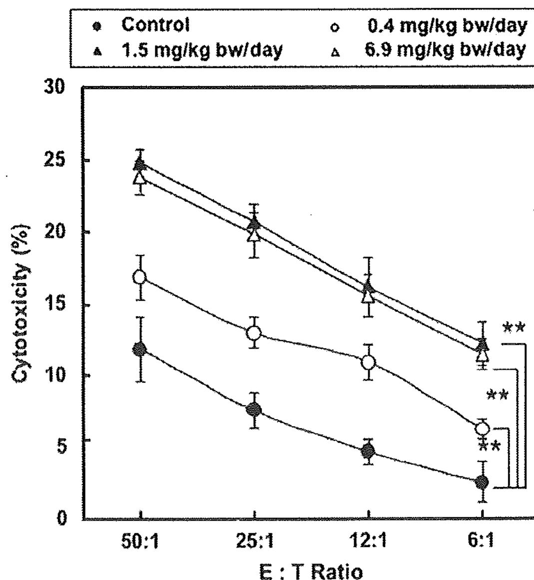

Fig. 4. Splenic NK activity of male rats treated with NIV for 90 days. Data are presented as the average ± SEM, and the number of rats per group was six.

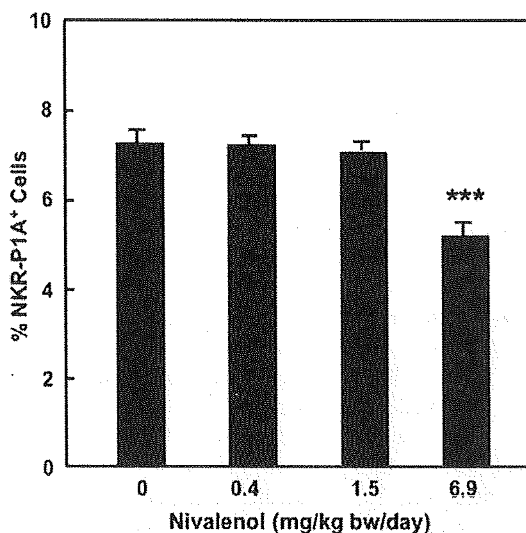

Fig. 3. The mean percentage of $NKR\text{-}P1A^+$ cells in splenic cells of male rats treated with NIV for 90 days. Data are presented the average ± SEM using six animals per group. Asterisks indicate significant differences from control. $^{***}P < 0.001$ by Dunnett's test.

rats treated with NIV at 0.4, 1.5 and 6.9 mg/kg showed 1.7-, 2.7- and 2.6-fold enhancement, respectively.

3.4. Immunoglobulin subclasses in serum

Changes in the serum level of IgG, IgM and IgA are shown in Table 1. Among immunoglobulins, treatment-related trend to increase was observed with IgM. Especially, the mean level at 6.9 mg/kg showed a 26% increase (statistically significant by parametric method). Although the mean level of IgG was slightly higher than that of untreated controls, neither a clear dose–response nor a statistically significant difference was detected. The mean IgA levels were not affected by the NIV treatment.

4. Discussion

In the present study, NIV targets cytotoxic T lymphocytes as a most sensitive cell population than other subpopulations of T and B lymphocytes in the spleen. According to previous reports, a decrease in cell numbers of cytotoxic T lymphocytes by NIV may be followed by apoptotic death (Poapolathep et al., 2001, 2002). On the other hand, Hedman and colleagues reported that exposure to 2.5 mg/kg bw of NIV for 30 days resulted in an increased plasma concentration of IgA and decreased concentration of IgG using young pigs (Hedman et al., 1997). Also, Hinoshita et al. (1997) reported that renal glomerular changes represented by mesangial IgA deposition resembling IgA nephropathy in human were induced in mice administered NIV at 12 ppm in diet for 8 weeks (daily dose level not shown). These animals exhibited profound elevation of serum IgA levels accompanied with increase of IgG levels of lesser degrees and unaltered IgM levels in the serum. We, however, found no changes in the serum IgG and IgA levels by NIV treatment, whereas IgM level at 6.9 mg/kg slightly increased as compared with that of untreated controls in rats. Considering the apparently higher level of dietary exposure to NIV in our rats as compared with that in mice exhibiting mesangial IgA deposition, there must be species differences in the development of IgA nephropathy. B

Table 1
Serum immunoglobulin levels in male F344 rats fed diets containing nivalenol for 90 days

Level of isotype (μg/ml)	Nivalenol (mg/kg body weight/day)			
	0	0.4	1.5	6.9
IgG	40,574 ± 7804	41,464 ± 5805	49,020 ± 6873	43,209 ± 6646
IgM	54.7 ± 2.9	55.5 ± 3.4	58.0 ± 4.1	69.1 ± 3.2*
IgA	0.35 ± 0.03	0.34 ± 0.03	0.31 ± 0.01	0.41 ± 0.03

Data are means ± SEM.
* Significantly different from corresponding control ($P < 0.05$ by Dunnett's test).

lymphocyte activity may be affected by NIV at higher doses than those affecting cytotoxic T lymphocytes.

Regarding genotoxicity of NIV, mixed results have been reported (reviewed in Bony et al., 2007). With regard to carcinogenicity, Hsia et al. (2004) reported that food in high-risk areas of esophageal cancers in China contained higher levels of NIV. These authors also observed papillomas and carcinomas in 47% of mice topically exposed to NIV during 60 weeks. However, in another study, carcinogenicity was not detected in female mice that have been administered NIV at dietary concentrations up to 30 ppm for two years (3.5 mg/kg bw/day; Ohtsubo et al., 1989). With regard to the immunomodulation on carcinogenesis, a possible role of suppressed NK activity for promotion of carcinogenesis has been reported (Zamai et al., 2007). In the present study, we observed decrease in the ratio of NKR-P1A$^+$ cells by NIV at 6.9 mg/kg. This result is 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 enhanced by exposure to NIV from the lowest dose level in the present study. Generally, increased NK activity is regarded to be a signature of beneficial defense responses (Zamai et al., 2007). On the other hand, enhanced NK activity that appears in athymic mice as compared to normal mice is considered to be the complementation to the depletion of T lymphocytes (Hasui et al., 1989). Although it could be a subtler indicator of immune dysfunction than the other changes noted at higher doses, increased NK activity in the present study may indicate an adaptive response against reduction of T lymphocytes. In any case, increased NK activity may not be related to immunosuppression-related carcinogenesis.

In the past, effects of mycotoxins derived from *Fusarium* fungi on the immune system have been reported (Ouyang et al., 1995; European Commission, 2000a,b,c; Pestka et al., 2004; Schlatter, 2004). Although most toxicity studies have addressed the effect of mycotoxins one by one, synergistic effect of exposure to multiple mycotoxins is poorly understood. Since co-occurrence of different mycotoxins derived from *Fusarium* fungi is usually encountered in natural human diets (Hsia et al., 2004), and the toxicity mechanisms such as those involving immune system are complex, further studies for the synergistic effects on immune functions with NIV and other co-occurring mycotoxins appear warranted.

In mice, body weight reduction was earlier evident on dietary administration of NIV (Ohtsubo et al., 1989; Ryu et al., 1988; Yamamura et al., 1989), while oral administration of NIV at doses up to 2 mg/kg to F344 rats by gavage for 15 or 30 days had no effect (Kawasaki et al., 1990). We here observed body weight reduction at 6.9 mg/kg. Although statistical analysis was not applied due to small number of samples in each group ($n = 3$ cages/group), the decrease of food consumption was observed at 6.9 mg/kg, but was limited to the initial half of the feeding period, suggesting that the reduction of body weights observed here was not simply a reflection of decreased food consumption, but rather due to toxicity of NIV, perhaps by malabsorption of nutrients induced by effects on the gastrointestinal tract or inhibition of protein synthesis. Decreased body weight even at 1.5 mg/kg from week 6 without affection on food consumption may support this hypothesis. Generally, immune function is easily influenced by nutritional conditions, suggesting that the body weight reduction observed here from 1.5 mg/kg dose level might have influenced the immunological changes appeared, due to nutritional suppression in these animals. However, induction of hematotoxic and/or immunotoxic effects was reported without affection on body weight in mice treated with NIV at 4 mg/kg bw/day (30 ppm in diet; Ryu et al., 1987). In the present study, increased NK activity that was observed from 0.4 mg/kg dose level without accompanying body weight reduction may be due to the direct action of NIV on the immune system.

In conclusion, NIV affected immune function in rats after 90-day dietary exposure, these effects being apparent from 0.4 mg/kg dose level judging from the increase of NK activity, although nutritional suppression might have influenced the immunological changes appeared from 1.5 mg/kg dose level.

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A 90-day subchronic toxicity study of nivalenol, a trichothecene mycotoxin, in F344 rats

Miwa Takahashi [a], Makoto Shibutani [a,*], Yoshiko Sugita-Konishi [b], Maki Aihara [b], Kaoru Inoue [a], Gye-Hyeong Woo [a], Hitoshi Fujimoto [a], Masao Hirose [a]

[a] *Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

[b] *Division of Microbiology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

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Abstract

A subchronic toxicity study of nivalenol (NIV), a trichothecene mycotoxin, was conducted in male and female F344 rats fed diet containing 0, 6.25, 25 or 100 ppm concentration for 90 days. Decrease of body weight and loose stools were observed at 100 ppm in both sexes from the start of the experiment, and body weight reduction was also observed at 25 ppm in males from week 6. At necropsy, many organs demonstrated reduced absolute weights at 100 ppm in both sexes, mostly due to the reduction in the body growth, with reduction of relative thymus weight also being evident in females. Hematologically, decrease of the white blood cell count was found at 100 ppm in males and from 6.25 ppm in females. In addition, decreased platelet counts in both sexes, red blood cell counts in males, and the hemoglobin concentration in females were detected at 100 ppm. Histopathologically, treatment-related changes were predominantly observed in the hematopoietic and immune organs and the anterior pituitary in both sexes and female reproductive organs at 100 ppm, such as thymic atrophy, hypocellularity in the bone marrow, diffuse hypertrophy of basophilic cells with increase of castration cells in the anterior pituitary, and increase of ovarian atretic follicles. Based on the hematological data, the no-observed-adverse-effect level of NIV was determined to be less than 6.25 ppm (0.4 mg/kg body weight/day for both males and females).
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Keywords: Subchronic toxicity; Nivalenol; Mycotoxin, trichothecene; F344 rats

1. Introduction

Nivalenol (NIV) is a trichothecene mycotoxin produced by *Fusarium* fungi which, along with deoxynivalenol (DON), is frequently detected in agricultural commodities such as wheat, rye, barley, oats, and other cereals. Thus contamination by these mycotoxins is now a serious concern for human and animal health (Ali et al., 1998; Sudakin, 2003), and it has become important to assess the health risks associated with exposure to the *Fusarium* mycotoxins.

It is well-known that trichothecene mycotoxins induce various toxic effects in animals such as suppression of body growth and immune function, diarrhea, and general loss of condition. The more potent trichothecene mycotoxins, T-2 toxin and diacetoxyscirpenol, can induce severe toxicosis, including hemorrhage and necrosis in the gastrointestinal tract, with destruction of hematopoietic and lymphoid tissues and irritation of the skin and oral cavity (Ryu et al., 1988; Hascheck et al., 2002; SCF, 2002; Rocha et al., 2005). Trichothecene mycotoxins exhibit inhibitory

Abbreviations: A/G, albumin: globulin ratio; Alb, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bil, bilirubin; BUN, blood urea nitrogen; BW, body weight; Ca, calcium; Cl, chloride; CRN, creatinine; DON, deoxynivalenol; Hb, hemoglobin; Ht, hematocrit; IP, inorganic phosphate; K, potassium; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; Na, sodium; NIV, nivalenol; NOAEL, no-observed-adverse-effect level; Plt, platelet; RBC, red blood cell; TC, total cholesterol; TG, triglyceride; TP, total protein; WBC, white blood cell.

* Corresponding author. Tel.: +81 3 3700 9821; fax: +81 3 3700 1425.
E-mail address: shibutan@nihs.go.jp (M. Shibutani).

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influence on protein synthesis by binding to ribosomes, and inhibition of DNA and RNA synthesis has also been reported (Hascheck et al., 2002; SCF, 2002). Therefore, organs/tissues showing high rate of cell turnover are regarded as particularly susceptible to trichothecenes, such as the lymphoid and hematopoietic tissues, and the gastrointestinal tract (Hascheck et al., 2002; SCF, 2002; Rocha et al., 2005). In addition, trichothecenes have been shown to affect immunological functions by deregulating production of cytokines and immunoglobulins and by inducing apoptosis (Thuvander et al., 1999; Bondy and Pestka, 2000; Pestka et al., 2004). With regard to *in vivo* toxicity of NIV, dietary administration to mice at concentrations of 12 ppm for up to 8 weeks resulted in elevation of serum IgA and its deposition in the glomerular mesangium, resembling IgA nephropathy in man (Hinoshita et al., 1997). Dose-related suppression of body weights was observed in mice fed diets containing NIV up to 30 ppm for up to 12 weeks (Yamamura et al., 1989). Body weight reduction was more apparent, with reduced feed consumption as well as reduction of white blood cell (WBC) counts, in female mice treated at lower doses for longer durations, i.e., 6 months or 1 year (Ryu et al., 1988), and therefore, a no-observed-adverse-effect-level (NOAEL) for NIV could not be established. However, the lowest-observed-adverse-effect-level (LOAEL) could be determined as 0.68 mg/kg body weight/day. Carcinogenicity was not detected in a study using female mice that were administered NIV at dietary concentrations up to 30 ppm for 2 years (3.49 mg/kg body weight/day; Ohtsubo et al., 1989). In rats, *in vivo* toxicity information is rather limited. Repeated oral doses of NIV by gavage at 0.4 or 2 mg/kg body weight/day for 15 or 30 days did not affect the body weight, and no histopathological lesions developed in either sex (Kawasaki et al., 1990). However, decrease of WBC counts was detected in males given doses from 0.4 mg/kg for 15 days, again without clear dose relation.

The present study was thereby performed to evaluate the subchronic toxicity of NIV and to establish a NOAEL in rats with oral administration in the diet according to the OECD Test Guideline 408 for 'Repeated dose 90-day oral toxicity study in rodents' except for the lack of opthalmological examination and functional observations during the feeding experiment (OECD, 1998).

2. Materials and methods

2.1. Chemicals

For purification of NIV, Fuzarenon X was extracted and purified from culture media of *Fusarium kyushuense* (Fn-2B). The identity and purity of NIV was determined by liquid chromatography/mass spectrometry (LC/MS; LCMS-2010A; Shimadzu Corp., Kyoto Japan), with an Atmospheric Pressure Chemical Ionization interface and the LC system (LC-2010CHT; Shimadzu Corp.), and the purity was estimated to be >98% from the area percentage of the chromatogram (data not shown).

For administration to rats, NIV was dissolved in a small quantity of ethanol first and then well mixed into powdered CRF-1 basal diet (Oriental Yeast Co., Tokyo, Japan). Stability of the test compound 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.

2.2. Animals

Male and female F344/DuCrj rats at 5 weeks of age, purchased from Charles River Japan Inc. (Yokohama, Japan), were used in the present study. They received powdered CRF-1 and tap water *ad libitum* during the 1 week acclimatization period, housed 4 per polycarbonate cage with sterilized softwood chips as bedding in a barrier-maintained animal room conditioned at 23–25 °C and 50–60% humidity, and on a 12-h light/dark cycle.

2.3. Experimental design

In a preliminary dose-finding study, a total of 24 male F344/DuCrj rats at 6 weeks of age were fed diet containing NIV 0, 150 or 300 ppm for 2 weeks. Reduction of body weights was apparent at 150 and 300 ppm from week 1, and one animal in the 300 ppm group died at week 2. Body weights were reduced to 70% and 47% of the control value at the end of week 2 in the groups receiving 150 and 300 ppm, respectively. Therefore, the concentration of 100 ppm was determined to be the highest dose for the subsequent subchronic study.

Animals, weighing 109.8 ± 3.8 g for males and 94.0 ± 2.9 g for females (mean ± SD), were randomly divided into 4 groups, each consisting of 10 males and 10 females, housed 3 or 4 per cage and given 0 (control), 6.25, 25 or 100 ppm NIV in the powdered diet for the treatment period of 90 days. The test diets were available *ad libitum*, except for one-night fasting prior to the scheduled sacrifice. The animals were observed daily for clinical signs and mortality, and body weights were measured every week during the study period. The amounts of supplied and residual diet were weighed weekly in order to calculate the average daily consumption within each week, and then the overall mean throughout the treatment period was calculated from the determined weekly food consumption. At the end of the experiment, all animals were anesthetized with ether, weighed, and blood samples were collected from the abdominal aorta for hematology and serum biochemistry. Animals were then killed by exsanguination from the abdominal aorta.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.4. Hematology and serum biochemistry

Hematology analysis was performed using an automated hematology analyzer, K-4500 (Sysmex Corp., Hyogo, Japan). Differential leukocyte counts and reticulocyte counts were analyzed with a Microx HEG-120A (Omron Tateishi Electronics Co., Ltd., Tokyo, Japan). Parameters for serum biochemistry were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation of whole blood (×1000*g*, 10 min).

2.5. Histopathological assessment

At necropsy, the brain, heart, lungs, thymus, spleen, liver, kidneys, adrenal glands and testes were removed and weighed. 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 (cecum, colon, and rectum), pancreas, urinary bladder, skin, male and female mammary gland, mesenteric lymph nodes, trachea, esophagus, 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 three days, except for testes fixed in Bouin's solution overnight. Tissues that needed decalcification, such as nasal cavity, spinal cords with bones, sternum, and femur, were treated with the mixture of 10% formic acid and 10% buffered formalin. Histopathological assessment was first performed on all tissues of the control and highest

dose groups for both sexes, and for the liver and kidneys in all groups. If any chemical treatment-related change appeared at the highest dose, the relevant tissues from the lower dose groups were then also examined. The organs were routinely processed for paraffin embedding, sectioned and stained with hematoxylin and eosin.

2.6. Statistics

Variance in data for body weights during the feeding experiment, hematology, serum biochemistry, and organ weights (both absolute and relative weights) was checked for homogeneity by 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 applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between the control and treatment groups. For comparison of histopathological changes between the control and treatment groups, incidences and severity data were analyzed with the Fisher's exact probability test and Mann–Whitney's U-test, respectively.

3. Results

3.1. In-life parameters

No deaths occurred throughout the feeding period. Although loose stools were found at 100 ppm from the start of the experiment in both sexes, no other clinical signs of this type were observed during the study. In both sexes, body weights were decreased with statistical significance at 100 ppm from week 1 of the experiment (Fig. 1). This was also evident at 25 ppm from week 6 to the end of experiment in males and at week 4 in females. With regard to

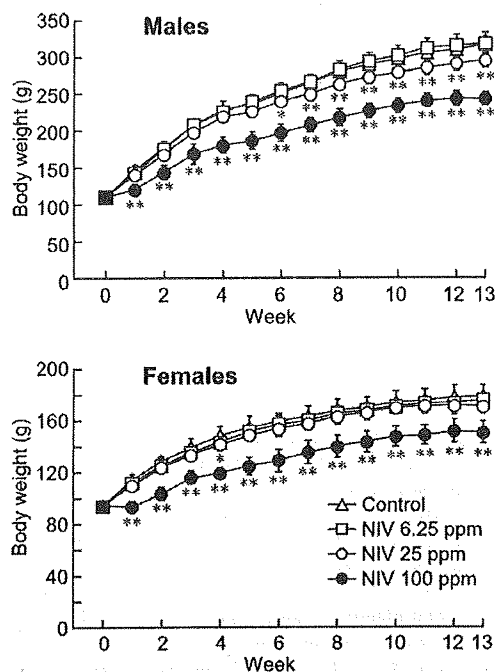

Fig. 1. Body weight curves for male and female F344 rats given nivalenol (NIV) for 90 days. Values are mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$, as compared with the untreated controls (Dunnett's test or Dunnett type rank-sum test).

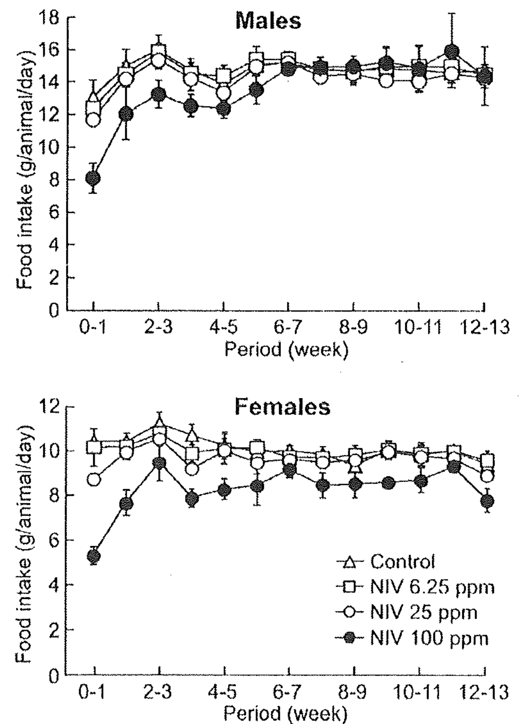

Fig. 2. Food consumption by male and female F344 rats given NIV for 90 days. Values are mean ± SD.

the food consumption, although values were approximate due to small number of samples in each group ($n = 3$ cages/group; statistical analysis, not applied), suppression was observed at 100 ppm throughout the feeding period in females (Fig. 2). In males, on the other hand, it was only observed during the initial 6 weeks of the study at the same dose. At 25 ppm, females also showed suppressed food consumption sporadically during the initial half of the study. In males, mean daily NIV intakes at 6.25, 25, and 100 ppm calculated after estimation of mean values of food consumption for the entire experimental period were 0.4, 1.5, and 6.9 mg/kg body weight/day, respectively. In females, NIV-intakes were 0.4, 1.6, and 6.4 mg/kg body weight/day, respectively.

3.2. Hematology/serum biochemistry

Hematology data are shown in Table 1. In males, statistically significant increase of mean corpuscular volume was observed from 25 ppm, and increase of mean corpuscular hemoglobin and decreases of the red blood cell (RBC), platelet (Plt) and WBC counts were detected at 100 ppm with statistical significance. Particularly, WBC count was reduced to the half of the control level. Differential leukocyte counts showed significant decrease and increase in the proportions of lymphocytes and segmented neutrophils, respectively, at 100 ppm. In females, significant decrease of WBCs was observed from 6.25 ppm in a dose-related

Table 1
Hematological data for F344 rats fed diets containing nivalenol for 90 days

	Nivalenol in the diet (ppm)			
	0	6.25	25	100
Males				
No. of animals examined	10	10	10	9[a]
RBC (×10⁴/μl)	906.5 ± 18.3[b]	927.1 ± 29.7	901.2 ± 25.3	855.7 ± 38.2**
Hb (g/dl)	15.3 ± 0.3	15.6 ± 0.5	14.8 ± 1.1	15.0 ± 0.7
Ht (%)	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)	31.7 ± 0.6	31.4 ± 0.6	30.3 ± 1.8	31.3 ± 0.6
Plt (×10⁴/μl)	66.1 ± 16.7	70.5 ± 3.8	66.3 ± 14.8	59.2 ± 3.4**
WBC (×10²/μl)	38.5 ± 12.2	37.0 ± 12.2	36.9 ± 6.1	21.6 ± 3.9**
Differential leukocyte counts				
Band form neutrophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Segmented neutrophils (%)	21.1 ± 5.0	20.5 ± 4.0	21.3 ± 2.1	29.7 ± 5.1**
Eosinophils (%)	0.6 ± 0.6	0.8 ± 0.8	0.8 ± 0.7	1.1 ± 1.2
Basophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes (%)	78.2 ± 4.7	78.4 ± 3.9	77.5 ± 2.2	68.8 ± 5.4**
Monocytes (%)	0.2 ± 0.2	0.3 ± 0.6	0.4 ± 0.6	0.4 ± 0.2
Reticulocytes (/100 cells)	1.1 ± 1.1	0.9 ± 0.7	1.0 ± 1.0	1.9 ± 1.8
Females				
No. of animals examined	9[a]	10	10	10
RBC (×10⁴/μl)	919.2 ± 25.8[b]	917.4 ± 51.2	900.0 ± 23.7	874.6 ± 59.2
Hb (g/dl)	16.3 ± 0.6	16.2 ± 0.8	15.5 ± 1.1	15.3 ± 1.0*
Ht (%)	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)	31.9 ± 1.2	31.5 ± 0.9	30.9 ± 2.0	31.3 ± 1.0
Plt (×10⁴/μl)	76.6 ± 6.5	73.5 ± 3.0	74.2 ± 3.3	60.7 ± 5.3**
WBC (×10²/μl)	38.8 ± 7.7	30.5 ± 10.1*	29.7 ± 5.5*	19.6 ± 4.5**
Differential leukocyte counts				
Band form neutrophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Segmented neutrophils (%)	19.3 ± 6.7	17.6 ± 3.8	18.3 ± 4.5	20.2 ± 3.5
Eosinophils (%)	1.3 ± 0.6	1.2 ± 0.9	1.0 ± 0.8	0.9 ± 0.9
Basophils (%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes (%)	79.0 ± 6.5	80.6 ± 3.6	80.1 ± 4.8	78.7 ± 3.7
Monocytes (%)	0.5 ± 0.5	0.6 ± 0.5	0.6 ± 0.5	0.2 ± 0.4
Reticulocytes (/100 cells)	1.9 ± 1.1	2.2 ± 1.6	2.0 ± 0.9	2.4 ± 1.6

Abbreviations: RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet; WBC, white blood cell.

*, **: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively (Dunnett's test or Dunnett type rank-sum test).

[a] Blood sampling failed in one animal.

[b] Mean ± SD.

manner, and decreases of Hb and Plt were found at 100 ppm. Differential leukocyte counts were unchanged in females.

Serum biochemistry data are shown in Table 2. In males, dose-related decreases of total cholesterol (TC), creatinine (CRN) and alanine aminotransferase (ALT) were observed from 25 ppm with statistical significance. Although dose relation was not apparent, similar significant decrease was observed in triglyceride (TG) from 25 ppm. Significant decreases of total protein (TP), glucose, K and aspartate aminotransferase (AST) were observed at 100 ppm, together with increases of the albumin (Alb): globulin ratio (A/G), Alb and Cl. In females, significant increase of A/G and decreases of TP and CRN were detected from 25 ppm dose-relatedly. Although no clear dose-relation was apparent, TC and AST were also significantly decreased from 25 ppm.

3.3. Organ weights

Data for the final body and organ weights are shown in Table 3. In males, significant decrease of final body weights was dose-relatedly observed from 25 ppm. With regard to organ weights, statistically significant decrease of the absolute liver weight and increases of the relative weights of the brain, lungs, heart, kidneys and testes were found from 25 ppm in a dose related manner. At 100 ppm, however, decreases of the absolute weight of the brain, lung, heart, spleen and kidneys, and increases of the relative weight of the spleen and liver were observed with statistical

Table 2
Serum biochemical data for F344 rats fed diets containing nivalenol for 90 days

	Nivalenol in the diet (ppm)			
	0	6.25	25	100
Males				
No. of animals examined	9[a]	10	9[a]	9[a]
TP (g/dL)	6.46 ± 0.27[b]	6.41 ± 0.19	6.27 ± 0.18	6.22 ± 0.10*
A/G	2.12 ± 0.15	2.03 ± 0.07	2.26 ± 0.09	2.76 ± 0.25**
Alb (g/dL)	4.37 ± 0.17	4.30 ± 0.14	4.33 ± 0.12	4.57 ± 0.14*
Total Bil (mg/dL)	0.041 ± 0.006	0.038 ± 0.004	0.042 ± 0.007	0.042 ± 0.004
Conjugated Bil (mg/dL)	0.021 ± 0.003	0.020 ± 0.008	0.027 ± 0.007	0.026 ± 0.005
Free Bil (mg/dL)	0.020 ± 0.007	0.018 ± 0.006	0.016 ± 0.011	0.017 ± 0.005
Glucose (mg/dL)	151.1 ± 8.7	160.3 ± 21.1	144.6 ± 4.4	129.0 ± 10.0**
TG (mg/dL)	62.8 ± 17.8	71.8 ± 33.7	34.8 ± 10.0*	38.3 ± 10.1*
TC (mg/dL)	66.1 ± 4.8	64.6 ± 4.6	58.7 ± 2.8**	56.0 ± 3.8**
BUN (mg/dL)	18.4 ± 1.1	18.4 ± 2.1	16.7 ± 1.4	18.2 ± 1.2
CRN (mg/dL)	0.28 ± 0.03	0.29 ± 0.04	0.25 ± 0.03*	0.22 ± 0.01**
Na (mEQ/L)	144.7 ± 2.1	144.2 ± 2.0	144.3 ± 1.3	145.0 ± 1.0
Cl (mEQ/L)	103.2 ± 0.4	103.2 ± 1.5	104.4 ± 1.8	107.1 ± 2.1**
K (mEQ/L)	4.69 ± 0.17	4.61 ± 0.31	4.58 ± 0.19	4.31 ± 0.24**
Ca (mg/dL)	10.72 ± 0.43	10.62 ± 0.48	10.60 ± 0.19	9.59 ± 2.90
IP (mg/dL)	6.30 ± 0.29	6.25 ± 0.47	6.36 ± 0.61	5.93 ± 0.55
AST (IU/L)	98.0 ± 14.8	91.9 ± 6.6	87.8 ± 10.7	66.8 ± 6.0**
ALT (IU/L)	52.4 ± 16.6	46.1 ± 5.9	37.9 ± 5.9**	36.0 ± 5.3**
Females				
No. of animals examined	9[a]	10	10	10
TP (g/dL)	6.19 ± 0.21[b]	6.10 ± 0.21	5.95 ± 0.21*	5.72 ± 0.19**
A/G	2.32 ± 0.16	2.41 ± 0.11	2.58 ± 0.21*	3.13 ± 0.24**
Alb (g/dL)	4.31 ± 0.18	4.31 ± 0.14	4.28 ± 0.09	4.33 ± 0.14
Total Bil (mg/dL)	0.049 ± 0.009	0.052 ± 0.008	0.051 ± 0.009	0.050 ± 0.007
Conjugated Bil (mg/dL)	0.027 ± 0.007	0.031 ± 0.007	0.025 ± 0.01	0.028 ± 0.009
Free Bil (mg/dL)	0.022 ± 0.004	0.021 ± 0.007	0.026 ± 0.005	0.022 ± 0.009
Glucose (mg/dL)	103.0 ± 9.6	103.2 ± 10.3	107.0 ± 5.5	110.4 ± 18.9
TG (mg/dL)	19.8 ± 10.1	14.5 ± 4.8	16.5 ± 5.2	24.0 ± 6.4
TC (mg/dL)	92.8 ± 10.5	85.9 ± 6.1	78.2 ± 8.1**	80.9 ± 7.3**
BUN (mg/dL)	15.6 ± 1.0	14.8 ± 1.1	15.2 ± 1.8	16.6 ± 1.9
CRN (mg/dL)	0.27 ± 0.02	0.26 ± 0.02	0.23 ± 0.02**	0.21 ± 0.02**
Na (mEQ/L)	143.1 ± 1.2	143.2 ± 1.2	142.8 ± 0.6	143.7 ± 0.9
Cl (mEQ/L)	105.6 ± 1.3	104.7 ± 1.3	105.9 ± 1.1	105.6 ± 1.3
K (mEQ/L)	4.32 ± 0.31	4.48 ± 0.40	4.29 ± 0.22	4.50 ± 0.37
Ca (mg/dL)	10.14 ± 0.24	10.18 ± 0.23	10.01 ± 0.13	10.04 ± 0.30
IP (mg/dL)	5.28 ± 0.45	5.50 ± 0.60	5.54 ± 0.63	5.96 ± 0.56
AST (IU/L)	78.9 ± 4.6	72.1 ± 4.5	67.5 ± 5.3**	67.7 ± 10.9**
ALT (IU/L)	31.4 ± 3.0	29.3 ± 3.4	28.8 ± 2.3	30.7 ± 3.8

Abbreviations: TP, total protein; A/G, albumin: globulin ratio; Alb, albumin; Bil, bilirubin; TG, triglyceride; TC, total cholesterol; BUN, blood urea nitrogen; CRN, creatinine; Na, sodium; Cl, chloride; K, potassium; Ca, calcium; IP, inorganic phosphate; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

*, **: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively (Dunnett's test or Dunnett type rank-sum test).

[a] Number of effective animals was reduced to nine due to the failure of blood sampling or insufficient blood volume for serum biochemistry.

[b] Mean ± SD.

significance. Additionally, absolute thymus weights were significantly decreased at 100 ppm. In females, final body weights were significantly decreased at 100 ppm. Although statistically non-significant, decrease was also evident at 25 ppm. As for organ weights, relative weights of the lungs, heart, spleen and kidneys were increased significantly and dose-relatedly from 25 ppm. However, in these organs, absolute weights were rather unchanged (lungs, heart, and spleen) or significantly decreased (kidneys at 100 ppm). Similarly, decrease of absolute weights and increase of relative weights were observed for the brain and liver with statistical significance at 100 ppm. Both absolute and relative thymus weights were significantly decreased at 100 ppm.

3.4. Histopathology

Histopathological data are summarized in Table 4. In both sexes, atrophy of the thymus and reduction of hematopoietic cells in the bone marrow were apparent and statistically significant at 100 ppm (Fig. 3A–D). In the spleen, although an increase of extramedullary hematopoiesis was

Table 3
Final body and organ weights for F344 rats fed diets containing nivalenol for 90 days

		Nivalenol in the diet (ppm)			
		0	6.25	25	100
Male					
No. of animals examined		10	10	10	10
BW	(g)	303.9 ± 10.5[a]	306.5 ± 13.9	282.2 ± 10.5**	227.7 ± 8.4**
Brain	(g)	1.93 ± 0.03	1.92 ± 0.05	1.91 ± 0.04	1.88 ± 0.03**
	(g%)	0.636 ± 0.021	0.627 ± 0.029	0.677 ± 0.025**	0.826 ± 0.027**
Thymus	(g)	0.157 ± 0.045	0.187 ± 0.030	0.172 ± 0.019	0.092 ± 0.039**
	(g%)	0.052 ± 0.015	0.061 ± 0.008	0.061 ± 0.006	0.041 ± 0.017
Lungs	(g)	0.977 ± 0.034	1.004 ± 0.083	0.974 ± 0.042	0.924 ± 0.050
	(g%)	0.322 ± 0.009	0.328 ± 0.020	0.345 ± 0.015**	0.406 ± 0.013**
Heart	(g)	0.928 ± 0.049	0.940 ± 0.075	0.912 ± 0.053	0.824 ± 0.043**
	(g%)	0.305 ± 0.012	0.307 ± 0.015	0.323 ± 0.017*	0.362 ± 0.017**
Spleen	(g)	0.622 ± 0.040	0.622 ± 0.032	0.618 ± 0.031	0.557 ± 0.032**
	(g%)	0.205 ± 0.011	0.203 ± 0.004	0.219 ± 0.006	0.245 ± 0.011**
Liver	(g)	7.113 ± 0.45	7.24 ± 0.49	6.54 ± 0.26*	5.81 ± 0.68**
	(g%)	2.34 ± 0.09	2.36 ± 0.08	2.32 ± 0.05	2.55 ± 0.24*
Adrenals	(mg)	34.70 ± 7.617	32.10 ± 4.067	34.30 ± 8.616	33.80 ± 11.98
	(mg%)	11.4 ± 2.6	10.5 ± 1.5	12.2 ± 3.2	14.9 ± 5.4
Kidneys	(g)	1.87 ± 0.08	1.88 ± 0.11	1.82 ± 0.10	1.62 ± 0.08**
	(g%)	0.616 ± 0.023	0.615 ± 0.021	0.644 ± 0.017*	0.710 ± 0.032**
Testes	(g)	3.18 ± 0.10	3.20 ± 0.20	3.21 ± 0.09	3.27 ± 0.07
	(g%)	1.05 ± 0.04	1.04 ± 0.03	1.14 ± 0.05**	1.44 ± 0.04**
Females					
No. of animals examined		10	10	10	10
BW	(g)	167.7 ± 8.1[a]	167.9 ± 11.6	160.9 ± 5.3	138.5 ± 7.6**
Brain	(g)	1.79 ± 0.04	1.82 ± 0.05	1.80 ± 0.04	1.70 ± 0.11*
	(g%)	1.07 ± 0.05	1.09 ± 0.08	1.12 ± 0.04	1.23 ± 0.10**
Thymus	(g)	0.175 ± 0.043	0.157 ± 0.019	0.154 ± 0.015	0.091 ± 0.020**
	(g%)	0.105 ± 0.024	0.094 ± 0.011	0.096 ± 0.008	0.065 ± 0.013**
Lungs	(g)	0.699 ± 0.049	0.738 ± 0.034	0.730 ± 0.042	0.731 ± 0.091
	(g%)	0.417 ± 0.018	0.441 ± 0.032	0.454 ± 0.026*	0.529 ± 0.067**
Heart	(g)	0.566 ± 0.035	0.594 ± 0.050	0.599 ± 0.032	0.536 ± 0.020
	(g%)	0.338 ± 0.015	0.354 ± 0.031	0.373 ± 0.015*	0.388 ± 0.015**
Spleen	(g)	0.387 ± 0.021	0.400 ± 0.016	0.413 ± 0.025	0.380 ± 0.033
	(g%)	0.231 ± 0.012	0.238 ± 0.018	0.257 ± 0.013**	0.275 ± 0.026**
Liver	(g)	3.67 ± 0.25	3.84 ± 0.26	3.68 ± 0.19	3.38 ± 0.51*
	(g%)	2.19 ± 0.11	2.30 ± 0.22	2.28 ± 0.07	2.45 ± 0.42*
Adrenals	(mg)	37.20 ± 5.224	47.60 ± 13.64	40.20 ± 6.215	33.70 ± 15.81
	(mg%)	22.2 ± 3.0	28.5 ± 9.0	25.0 ± 4.2	24.2 ± 10.8
Kidneys	(g)	1.08 ± 0.06	1.13 ± 0.05	1.14 ± 0.07	1.01 ± 0.03*
	(g%)	0.646 ± 0.022	0.677 ± 0.040	0.711 ± 0.037**	0.729 ± 0.034**

Abbreviation: BW, Body weight.
*, **: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively (Dunnett's test or Dunnett type rank-sum test).
[a] Mean ± SD.

significantly observed at 100 ppm in females, there were no apparent changes in male spleens at this dose. In females, increases of the ovarian atretic follicles and interstitial glands were apparent with statistical significance at 100 ppm, lack of corpora lutea development being apparent and statistically significant in severely affected cases, although numbers of secondary follicles remained unchanged (Fig. 3E and F). Also, there was no change in the numbers of primary follicles in these cases. Atrophy with diestrus endometrial mucosal change of the uterus, showing reduction of the uterine size, with a slit-like uterine lumen lined by low columnar cells, was apparent and statistically significant at 100 ppm (Fig. 3G and H). The vaginal mucosa of these animals demonstrated diestrus or proestrus of the estrus cycle, showing an increase in thickness with polygonal-shaped cells of the outer layers (diestrus) or mucinous surface epithelia in addition to underlying cornified epithelia (proestrus). On the other hand, no treatment-related changes were found in the testis and male accessory reproductive organs. In the anterior pituitary, however, increase (males) or development (females) of castration cells and development of diffuse hypertrophy of basophilic cells were apparent and statistically significant at 100 ppm in both sexes. Similar changes were also found in a few animals at 25 ppm (non-significant), and focal hypertrophy of basophilic cells was observed in one male at 6.25 ppm. In the mammary glands, lobular hyperplasia was apparent and statistically significant only in females at 100 ppm. In

Table 4
Treatment-related histopathological changes in F344 rats fed diets containing nivalenol for 90 days

		Nivalenol (ppm)			
	Dose	0	6.25	25	100
	No. of animals examined	10	10	10	10
Males					
Thymus	Atrophy	0	0	0	8^{**}
Femur/marrow	Decrease of hematopoietic cells (Pancytopenia)	0	0	0	9^{**}
	Decrease of metaphyseal trabeculae	0	0	0	7^{**}
Sternum/marrow	Decrease of hematopoietic cells (Pancytopenia)	0	0	0	7^{**}
	Decrease of metaphyseal trabeculae	0	0	0	8^{**}
	Thinning of the diaphysis	0	0	0	10^{**}
Anterior pituitary	Castration cells (±/+)	4(4/0)	5(5/0)	5(4/1)	$9^{*}(0/9)^{\#\#}$
	Focal hypertrophy, basophilic cells	0	1	0	0
	Diffuse hypertrophy, basophilic cells	0	0	2	10^{**}
Thyroid	Increase of dilated follicles lined by flattened epithelium	0	0	0	5^{*}
Adrenal cortex	Vacuolar degeneration, fine vesicular, zona fasciculata	0	0	0	10^{**}
Kidneys	Eosinophilic bodies, proximal tubular epithelium (±/+)	10(0/10)	10(0/10)	10(0/10)	$2^{**}(2/0)^{\#\#}$
	Hyaline droplet degeneration, proximal tubular epithelium (±/+)	10(0/10)	10(0/10)	10(1/9)	$9(9/0)^{\#\#}$
Submandibular gland	Decrease of granules, secretory granular ductal cells	0	0	0	10^{**}
Mesenteric L.N.	Microgranuloma development	6	7	4	7
	Aggregation of macrophages and mast cells, medullary sinuses	0	0	1	6^{**}
Lungs	Macrophage accumulation, focal, alveolar walls	0	0	0	1
Nasal cavity	Increase of goblet cells, paranasal sinus epithelium (±/+)	0	0	2(2/0)	$10^{**}(3/7)^{\#\#}$
Females					
Thymus	Atrophy	0	0	0	7^{**}
Femur/marrow	Decrease of hematopoietic cells (Pancytopenia)	0	0	2	9^{**}
	Decrease of metaphyseal trabeculae	0	0	0	8^{**}
Sternum/marrow	Decrease of hematopoietic cells (Pancytopenia)	0	0	0	10^{**}
	Decrease of metaphyseal trabeculae	0	0	0	10^{**}
	Thinning of the diaphysis	0	0	0	9^{**}
Spleen	Increase of extramedullary hematopoiesis (±/+)	0	0	0	$5^{*}(3/2)$
Ovary/oviduct	Increase of interstitial glands	0	0	0	10^{**}
	No. of secondary follicles (0–9/10< per animal)	(6/4)	(6/4)	(8/2)	(8/2)
	No. of atretic follicles (0–9/10–19/20< per animal)	(8/2/0)	(7/3/0)	(5/5/0)	$(0/7/3)^{\#\#}$
	Loss of corpora lutea	0	0	0	6^{*}
	Mesothelioma	0	0	1	0
Uterus	Atrophy with diestrus endometrial mucosal change	0	0	0	8^{**}
Vagina	Mucosal status classified based on estrous cycle (P/E/M/D)	(2/4/2/2)	(1/3/5/1)	(2/2/6/0)	(4/0/0/6)
Anterior pituitary	Castration cells (±/+)	0	0	1 (1/0)	$6^{**}(5/1)^{\#}$
	Focal hypertrophy, basophilic cells	0	0	0	0
	Diffuse hypertrophy, basophilic cells	0	0	1	9^{**}
Mammary glands	Lobular hyperplasia	0	0	0	9^{**}
Thyroid	Increase of dilated follicles lined by flattened epithelium	0	0	1	7^{**}
Adrenal cortex	Vacuolar degeneration, fine vesicular, zona fasciculata	10	10	10	10
Submandibular gland	Decrease of granules, secretory granular ductal cells	0	0	0	10^{**}
Mesenteric L.N.	Microgranuloma development	6	6	7	6
	Aggregation of macrophages and mast cells, medullary sinuses	0	0	1	9^{**}
Lungs	Macrophage accumulation, focal, alveolar walls	1	0	0	6^{*}
Nasal cavity	Increase of goblet cells, paranasal sinus epithelium (±/+)	0	0	2(1/1)	$10^{**}(1/9)^{\#\#}$

Grade of change: ±: minimal, +: slight.
Abbreviations: L.N., lymph node; P, proestrus; E, estrus; M, metestrus, D, diestrus.
*, **: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively (Fisher's exact probability test).
$^{\#}$, $^{\#\#}$: Significantly different from the control values at $P < 0.05$ and $P < 0.01$, respectively (Mann–Whitney's U-test).

the thyroids, increase of dilated follicles lined by flattened epithelium was apparent with statistical significance at 100 ppm in both sexes. Vacuolar degeneration with fine vesicular vacuoles was apparent in the zona fasciculata of the adrenal cortex in males with statistical significance at 100 ppm, while hypertrophic cellular change was lacking. All females showed similar vacuolar degeneration without relation to NIV treatment. In the male kidneys, the incidence and severity of eosinophilic bodies, recognized as pale eosinophilic, homogenous and irregular-shaped inclusions in the cytoplasm (Onodera et al., 1994), and hyaline droplet degeneration, both spontaneously-occurring lesions in the proximal tubular epithelium in males, were significantly reduced at 100 ppm. Decrease of granules in the secretory granular duct of the submandibular gland was found with statistical significance at 100 ppm in both sexes. In the

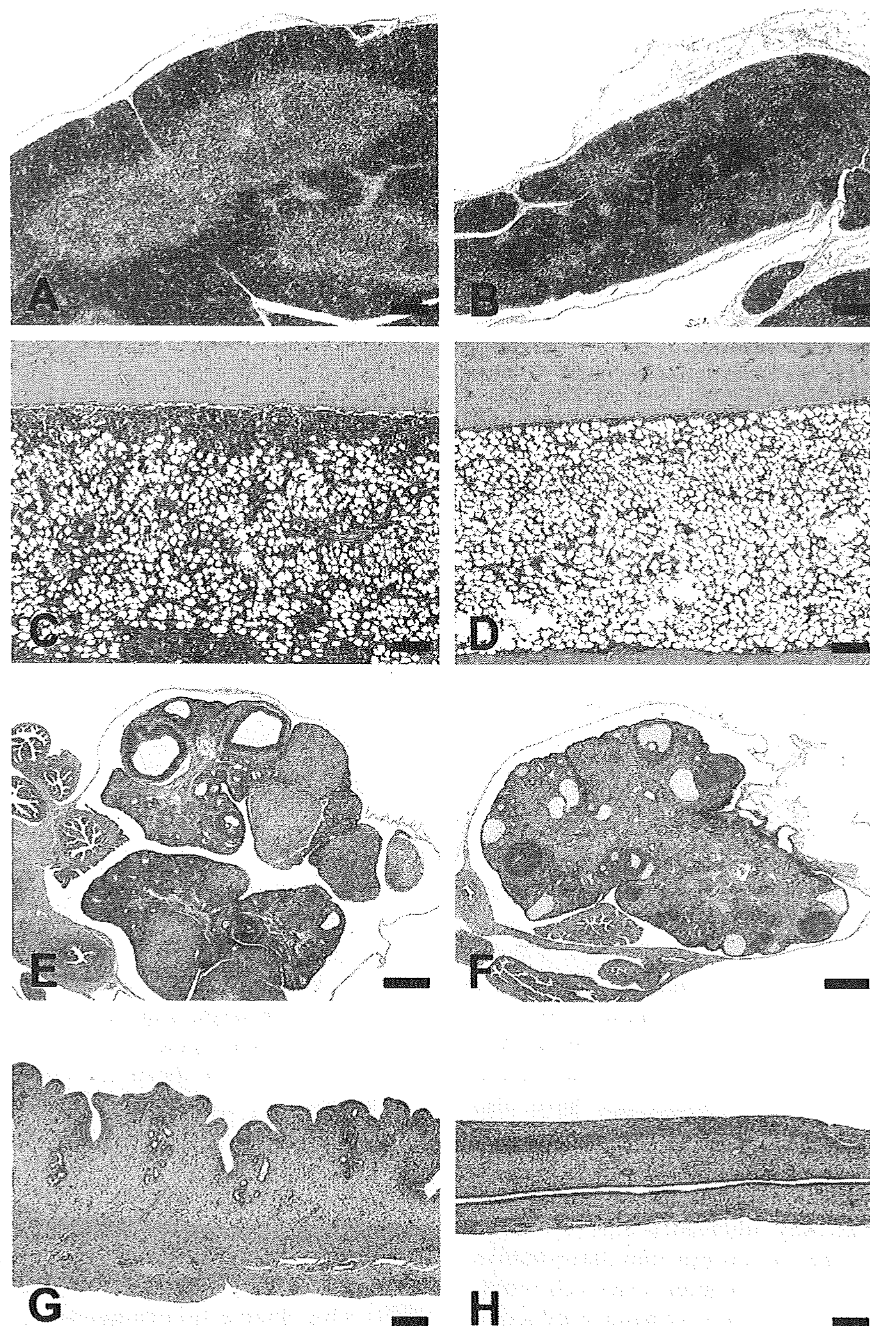

Fig. 3. Histopathology of the thymus, femur bone marrow, ovary and uterus of rats given 0 or 100 ppm NIV for 90 days. (A) Normal thymus of a male at 0 ppm. (B) Atrophy of the thymus observed in a male at 100 ppm. (C) Femur bone marrow of a male at 0 ppm. (D) Decrease of hematopoietic cells in the bone marrow found in a male at 100 ppm. (E) Normal ovary at 0 ppm. (F) Increase of atretic follicles and interstitial glands and lack of corpora lutea development observed at 100 ppm. (G) Endometrial mucosa in proestrus observed at 0 ppm. The uterine lumen is distended and the endometrium is lined by large columnar cells with many endometrial glands. (H) Atrophy with diestrus endometrial mucosal changes found at 100 ppm. The uterus is markedly reduced in size and the slit-like lumen is lined by low columnar cells. A–D, G, H: Bar = 200 μm. E, F: Bar = 500 μm. Hematoxylin and eosin.

mesenteric lymph nodes, increased severity of accumulation of macrophages and mast cells was evident in the medullary sinuses with statistical significance at 100 ppm in both sexes. The incidence of focal macrophage accumulation in the alveolar walls of the lungs was significantly elevated at 100 ppm in females, and increase of goblet cells in the epithelium of paranasal sinuses was statistically significant at 100 ppm in both sexes. In the femur and sternum at 100 ppm in both sexes, decrease of metaphyseal trabeculae and thinning of the diaphysis were frequently observed with

statistical significance. As spontaneously occurring lesions, subendothelial mineralization of the pulmonary arteries, microgranuloma development in the liver, focal mononuclear cell infiltration in the myocardium, basophilic or regenerative tubules in the kidneys and focal atrophy of the exocrine pancreas were also detected in both sexes, but their incidences and/or severities did not differ between the control and the NIV-treated groups.

4. Discussion

In the present study, we observed thymic atrophy and hypocellularity in the bone marrow at 100 ppm in both sexes and decrease of WBC counts at 100 ppm in males and from 6.25 ppm in females. In addition, decreased Plt counts in both sexes and RBC counts in males were detected at 100 ppm. It is well known that treatment-related stress can cause thymic atrophy (Gorski et al., 1988; Pearse, 2006). On the other hand, as mentioned earlier, trichothecenes are known to exert inhibitory effects on protein synthesis and thus suppress cell generation (Ueno et al., 1973; SCF, 2000, 2002; Haschek et al., 2002). Ryu et al. (1987) reported that 24 days administration of NIV to mice resulted in reduction of both RBC and WBC at 30 ppm in diet associated with polyribosomal breakdowns of the bone marrow cells. In view of this point, it is considered that such inhibitory actions of NIV targeting immune and hematopoietic systems might also be responsible for thymic atrophy and hypocellularity in the bone marrow and decreased levels of WBC, Plt and RBC in the present study.

With regard to the decrease of WBC counts in the present study, reductions were evident in lymphocyte population in males and universal populations in females judging from the differential leukocyte counts. However, it remains unknown regarding the sensitivity of WBC subpopulations and its sex-related difference against trichothecenes. On the other hand, trichothecenes have also been shown to stimulate immune functions apart from the immunosuppressive properties due to suppression of cell generation (Bondy and Pestka, 2000; Pestka et al., 2004). In fact, NIV effectively inhibited proliferation and immunoglobulin production in mitogen-stimulated human lymphocytes at high doses, but low levels enhanced proliferative responses, as well as immunoglobulin production (Thuvander et al., 1999). In another study investigating effects of NIV on immune functions using the same animals (males) as in the present study, as a beneficial host-defense response, increase of natural killer cell cytolytic activity of splenic lymphocytes against yeast artificial chromosome-1 lymphoma cells was apparent from 6.25 ppm (Dr. A. Kubosaki et al., manuscript submitted). In addition, decrease of globulin, causing increase of A/G ratio without decrease of the Alb level, was observed at 100 ppm in males and from 25 ppm in females in the present study, although definite evidence of reduction of the B cell population or immunoglobulins was not obtained (Dr. A. Kubosaki et al., manuscript submitted).

In mice, body weight reduction was earlier evident on dietary administration of NIV (Ohtsubo et al., 1989; Ryu et al., 1988; Yamamura et al., 1989), while oral administration of NIV at doses up to 2 mg/kg to male and female F344 rats by gavage for 15 or 30 days had no effect (Kawasaki et al., 1990). We here observed body weight reduction at 100 ppm in both sexes. The NIV intake at this dietary dose was 6.9 mg/kg/day in males and 6.4 mg/kg/day in females. Although statistical analysis was not applied due to small number of samples in each group ($n = 3$ cages/group), the food consumption during the course of feeding study was apparently decreased at 100 ppm in both sexes. However, the decrease in males was limited to the initial half of the feeding period, suggesting that the reduction of body weights observed here was not simply a reflection of decreased food consumption, but rather due to toxicity of NIV, perhaps by malabsorption of nutrients induced by effects on the gastrointestinal tract or inhibition of protein synthesis. Decreased body weight in males even at 25 ppm from week 6 without affection on food consumption may support this hypothesis. In addition, the loose stools found at 100 ppm in both sexes might be the effect of NIV on the gastrointestinal tract, while no corresponding histopathological changes were detected. It is known that malnutrition or protein deficiency can cause loss of the metaphyseal trabeculae without osteoclastic activity (Leininger and Riley, 1990), and such bone changes were observed in both males and females at 100 ppm in the present study.

Sprando et al. (2005) reported increased serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and decrease of serum testosterone in male rats administered DON orally at concentrations up to 5.0 mg/kg daily for 28 days, indicating an effect of DON on the pituitary-testicular axis. Increase in serum FSH was regarded to be due to the direct action of DON on the anterior pituitary or through the inhibition of Sertoli cells for production of inhibin, and the decrease in serum testosterone was considered to be induced by the stress resulting from the repeated administration of DON (Sprando et al., 2005). It has been reported that stress-like response such as increase of serum corticosterone level was induced after exposure to T-2 toxin in male mice (Taylor et al., 1989). Our finding of treatment-related histopathological lesions in the female reproductive organs at 100 ppm and changes in the anterior pituitary and mammary gland suggest possible alteration in serum hormone levels through stress-related responses and/or the direct action of NIV on the pituitary-gonadal axis. The reason for the sex difference in effects on reproductive organs, with no alteration in the testes, is unclear. Histopathological changes in the female mammary gland, thyroid in both sexes and male adrenal cortex may indicate diverse actions of NIV on the endocrine system.

In the present study, atrophy with diestrus mucosal changes of the uterus at 100 ppm might be associated with disturbance of follicular growth. Although estrus cycle was

not tracked throughout the study, proestrus or diestrus vaginal mucosal changes at this dose might also be linked to ovarian dysfunction. Zearalenone, a mycotoxin produced by several species of *Fusarium* fungi, is known to exert estrogenic potential due to the similarity of configuration of this toxin and estradiol (Kuiper et al., 1998; Hascheck et al., 2002). We here confirmed no zearalenone contamination in the NIV preparation by LC/MS analysis. Appearance of granules in the secretory granular ducts of the submandibular gland, known to be hormone dependent (Flynn et al., 1983; Neuenschwander and Elwell, 1990), was, however, decreased at 100 ppm in both sexes. Also, eosinophilic bodies and hyaline droplet degeneration in the renal proximal tubules, suggestive of accumulation of α_{2u}-globulin (Burnett et al., 1989; Ridder et al., 1990), were decreased at 100 ppm in males. α_{2u}-Globulin is a male rat specific protein that is synthesized by the liver cells under multi-hormonal control (Shapiro and Sachchidananda, 1982; Motwani et al., 1984; Yamasaki et al., 2002). Although the mechanisms responsible for changes in the submandibular gland and renal tubules are not clear, possible hormonal fluctuation due to NIV may here operated as well as the inhibitory potential on protein synthesis.

It was reported that renal glomerular changes represented by mesangial IgA deposition resembling IgA nephropathy in human were induced in mice administered NIV at 12 ppm in diet for 8 weeks (Hinoshita et al., 1997). Although these animals exhibited only mild mesangial expansion in the glomeruli light microscopically, profound elevation of serum IgA levels accompanied with increase of IgG levels of lesser degrees and unaltered IgM levels in the serum was evident. In the present study, we could not find changes suggestive of renal glomerular toxicity by NIV treatment by histopathological analysis and serum biochemistry. Importantly, in another study investigating immunotoxicity of NIV using male samples from the present study, serum levels of IgG and IgA did not change with NIV treatment, while the serum IgM level was slightly increased at 100 ppm (Dr. A. Kubosaki et al., manuscript submitted). Accordingly, it is suggested that there are species differences in the development of IgA nephropathy. In addition, fluctuations in serum biochemical parameters, such as decreases of TP, TG, TC, AST and ALT levels, were regarded to be of low or no toxicological relevance. Similarly, the alterations in the organ weights, including those for the brain, heart and liver, were considered to reflect the body weight decrease, and there were no treatment-related histopathological changes in these organs. We considered focal macrophage accumulation in the alveolar wall of the lungs and increase of goblet cells in the epithelium of paranasal sinus to be protective responses against irritation by respiratory exposure to NIV as a result of contact with powder diet (Pang et al., 1987; Rogers, 1994). The mechanism underlying macrophage and mast cell accumulation in the mesenteric lymph nodes is unknown, although there is a report suggesting that mast cell activation occurs with the T-2 toxin (Doebler et al., 1992).

In summary, NIV targets endocrine organs, especially the anterior pituitary in both sexes and the female reproductive organs, and the hematopoietic and immune organs in rats with 90 days dietary exposure. Based on the hematological data, the no-observed-adverse-effect level of NIV was determined to be less than 6.25 ppm (0.4 mg/kg body weight/day for both males and females).

Conflict of interest statement

We all authors disclose here that there are no conflicts of interest that could have inappropriately influenced the outcome of the present study.

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