Ⅲ. 研究成果の刊行物・別刷

Validation Study

Inter-laboratory Study of an LC-MS/MS Method for Simultaneous Determination of Deoxynivalenol and Its Acetylated Derivatives, 3-Acetyl-deoxynivalenol and 15-Acetyl-deoxynivalenol in Wheat

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To validate an LC-MS/MS method for simultaneous determination of deoxynivalenol (DON) and its acetylated derivatives, 3-acetyl-deoxynivalenol (3ADON) and 15-acetyl-deoxynivalenol (15ADON), in wheat using a multifunctional column, an inter-laboratory study was performed in 9 laboratories using one blank wheat sample, three spiked wheat samples (10, 50, 150 μg/kg) and one naturally contaminated wheat sample. The recoveries ranged from 98.8 to 102.6% for DON, 89.3 to 98.7% for 3ADON, and from 84.9 to 90.0% for 15ADON. The relative standard deviations for repeatability (RSDr) and reproducibility (RSDR) of DON were in the ranges of 7.2–11.3% and 9.5–22.6%, respectively. For 3ADON, the RSDr ranged from 5.3 to 9.5% and the RSDR ranged from 16.1 to 18.0%, while for 15ADON, the RSDr ranged from 6.2 to 11.2% and the RSDR ranged from 17.0 to 27.2%. The HorRat values for the three analytes ranged from 0.4 to 1.2. These results validate this method for the simultaneous determination of DON and its acetylated derivatives, 3ADON and 15ADON.

Key words: deoxynivalenol; acetyl deoxynivalenol; LC-MS/MS; inter-laboratory study; wheat

Introduction

Deoxynivalenol (DON), a trichothecene mycotoxin produced by *Fusarium* species, is frequently detected in cereals such as wheat, corn and barley[1]. DON causes adverse events in humans and animals. Acute effects of DON in humans include nausea, vomiting, diarrhoea and blood in the stool, and chronic effects at low dietary concentrations include growth retardation and reduced food consumption[2,3].

In 2001, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established the provisional maximum tolerable daily intake (PMTDI) of DON for humans at 1.0 μg/kg body weight per day[4]. To decrease human intake of DON, the Ministry of Health, Labour and Welfare of Japan set a provisional standard of 1.1 mg/kg of DON in wheat grain in 2002. However, few countries have established regulatory limits or guidance concentrations for DON in foods and feeds.

Two kinds of acetylated derivatives of DON, 15-acetyl-deoxynivalenol (15ADON) and 3-acetyl-deoxynivalenol (3ADON), are frequently found in DON-contaminated samples. For example, the relative concentration of 15ADON detected in corn-based food was about 15% higher than that of DON, while 3ADON was detected in wheat and barley at concentrations below 10 μg/kg[5,6]. A median lethal dose (LD_{50}) study indicated that the toxicity of acetylated DON compounds in mouse was similar to that of DON[7]. When pigs were fed a commercial diet supplemented with 3ADON, DON was detected in plas-

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ma as soon as 20 min after feeding started, but no trace of 3ADON was found in plasma, urine or feces[9]. Based on these toxicological and pharmacokinetics data, JECFA in 2010 evaluated the health risk of acetylated DON compounds and established that they were converted to DON *in vivo* and that they contribute to the total DON-induced toxicity. Consequently, the PMTDI was set at 1.0 μg/kg of body weight per day for DON and its acetylated derivatives[9]. In response to this evaluation, the occurrence of 3ADON and 15ADON has started to attract more attention.

Many methods for the quantification of trichothecene mycotoxins using GC-MS, LC-MS or LC-MS/MS have been reported[10)–12)]. In recent years, LC-MS/MS has become very popular and methods for the determination of several kinds of trichothecenes have been developed, although only a few methods enable precise quantification of 3ADON and 15ADON. As the two acetylated DONs are isomers and the only difference in structure is the position of the acetyl group, HPLC retention times and most MS/MS daughter ions are similar for the two compounds. This is the reason why it is difficult to distinguish and quantify them precisely, and an analytical method which enables reliable quantification of 3ADON and 15ADON is required worldwide. We previously developed optimized HPLC conditions for separating 3ADON and 15ADON and reported a method for the simultaneous determination of DON, 3ADON and 15ADON by LC-MS/MS[5]. In this study, we validated the method for quantifying DON and its acetylated derivatives in wheat through an inter-laboratory study.

Materials and Methods

Chemicals

Solid crystals of DON (purity: 99.0%), 3ADON (purity: 98.0%) and 15ADON (purity: 98.5%) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Each compound was dissolved in acetonitrile (each 50 μg/mL) and the solution was stored at −20℃. Portions of the three stock solutions were mixed and diluted with acetonitrile to make a mixed standard solution (10 μg/mL each of DON, 3ADON and 15ADON) and three concentrations of mixed spiking solutions (1, 5, 15 μg/mL each of DON, 3ADON, 15ADON). LC/MS-grade acetonitrile and water were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Autoprep MF-T 1500 (Showa Denko K. K., Tokyo, Japan) was used as a multifunctional column.

Preparation of samples

Blank wheat and naturally contaminated wheat were purchased from Trilogy Analytical Laboratory Inc. (Washington, MO, U.S.A.). The advertised DON concentrations in blank wheat and naturally contaminated wheat were "not detected" and "0.9±0.1 mg/kg", respectively. These samples were packed into bags of 30 g each. Each participant in the inter-laboratory study received the following: (a) blank samples for the fortification test; (b) two bags of naturally contaminated wheat; (c) a mixed standard solution and spiking solutions.

Sample 25.0 g
- CH_3CN–H_2O (85 : 15) 100 mL
- Stand (10 min)
- Shake (30 min)
- Centrifuge (1,500×*g*, 5 min)

Apply to 10 mL to Autoprep MF-T 1500 (without pre-conditioning)
- 0–3 mL Discard
- 3–<5 mL Collected

Transfer 2.0 mL into a glass tube
- Dry up under N_2 gas

Residue
- Dissolved with 500 μL of CH_3CN–H_2O (10 : 90)
- Centrifuge (10,000×*g*, 5 min)

LC-MS/MS

Fig. 1. Flow diagram for the determination of deoxynivalenol and its acetylated derivatives using LC-MS/MS coupled to a multifunctional column

Fortification procedure

For evaluating recovery, 250 μL of mixed spiking solution was added to 25.0 g of blank wheat in a 300-mL flask (final concentration, 0, 10, 50, 150 μg/kg) and kept at room temperature. After 1 hr, DON, 3ADON and 15ADON were extracted from the spiked samples and quantified according to the protocol defined next. The concentrations of the spiked samples were set on the basis of the results of a 2-year (2010–2011) surveillance for DON, 3ADON and 15ADON in wheat (unpublished data).

Protocol used by participants

The method validated in this study is based on our previous report[5] (Fig. 1). Briefly, 25.0 g of sample was extracted with 100 mL of acetonitrile–water (85 : 15) statically for 10 min, followed by shaking for 30 min. The extract was transferred to a 50 mL centrifuge tube and centrifuged at 1,500×*g* for 5 min. Aliquots of 10 mL of the supernatant were applied to a multifunctional column (Autoprep MF-T 1500) without pre-conditioning. The first 3 mL of eluate was discarded, and the next 2 mL was collected. The collected eluate was dried under nitrogen at approximately 40℃. The residue was dissolved in 500 μL of acetonitrile–water (10 : 90). After centrifugation (10,000×*g*, 5 min), the sample was subjected to LC-MS/MS analysis.

LC-MS/MS conditions

Each laboratory determined the DON, 3ADON and 15ADON concentrations by LC-MS/MS. Ten microliters of the final solution was loaded on an Inertsil ODS-3 column (2.1×150 mm, 3 μm; GL Sciences Inc., Tokyo, Japan) at 40℃. Gradient elution was conducted with mixtures of acetonitrile and water. Initial eluent composition was acetonitrile–water (5 : 95). A linear gradient was set to reach 65% acetonitrile after 7 min, and

the flow rate was set at 0.4 mL/min. Electrospray ionization in the negative mode was used. The selected reaction monitoring mode of LC-MS/MS and all other conditions were set by each laboratory so that the height of the signal peak of 5 ng/mL standard solution was more than 10 times larger than the background noise level.

Calibration curve

The mixed standard solution was diluted with acetonitrile–water (10 : 90). The concentration of DON, 3ADON or 15ADON and the peak area were plotted for eight standard solutions with different concentrations (1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 ng/mL). The concentrations of DON, 3ADON and 15ADON in the sample solution were calculated from the calibration curve. Limits of detection (LODs) and limits of quantification (LOQs) were calculated based on signal-to-noise ratios of 3 : 1 and 10 : 1, respectively.

Inter-laboratory study design

To validate the method, an inter-laboratory study was carried out using five materials (three spiked samples of wheat, naturally contaminated wheat, and a blank sample). The study involved 9 laboratories in Japan and one laboratory in Taiwan.

Statistics

The results from participants were initially evaluated for evidence of outliers using statistical Cochran (between duplicates) and Grubbs single and Grubbs pair value tests (between laboratory means)[13]. The relative standard deviations for repeatability (RSDr) and reproducibility (RSDR), and the HorRat value, which is the ratio of the RSDR to the predicted RSDR, were obtained using an analysis of variance according to the AOAC guideline[14]. The predicted RSDR of the HorRat value was calculated according to the Thompson report[15]. The criteria for analytical methods mentioned in Commission Regulation (EC) No. 401/2006[16] were used for evaluation of these parameters. The homogeneity and average concentrations of DON, 3ADON and 15ADON in the naturally contaminated wheat supplied in this study were tested before the materials were distributed. *F*-Tests at the 95% confidence level showed that the naturally contaminated wheat could be regarded as homogeneous because the calculated *F*-value was less than the critical value. The mean DON, 3ADON and 15ADON concentrations calculated from the homogeneity test were 891.5±31.2 μg/kg, 7.7±0.3 μg/kg and 19.8±0.1 μg/kg, respectively. In the blank sample, 4.7±0.6 μg/kg of DON was detected, but the concentrations of 3ADON and 15ADON were below LOD (1 μg/kg).

Results and Discussion

The measurement conditions of each laboratory are shown in Table 1. Laboratory A changed the HPLC solvent from water to 0.5 mmol/L aqueous ammonium acetate solution containing 0.1% acetic acid and two laboratories (B and D) changed it from water to 10 mmol/L aqueous ammonium acetate solution because the three laboratories could not obtain sufficient sensitivity to detect 5 ng/mL of 15ADON by the method described in the protocol. In order to separate 3ADON and 15ADON, three laboratories (B, D and E) change the gradient HPLC method and laboratory D changed the HPLC solvent from acetonitrile to methanol.

The results of the inter-laboratory study are shown in Table 2 (DON), Table 3 (3ADON) and Table 4 (15ADON). The result of one laboratory was eliminated because of a protocol violation. In the DON fortification test, eight laboratories detected DON in the blank sample and the mean value was 3.3 μg/kg. To calculate recovery, the mean value of the blank sample was subtracted from each measured value for each laboratory. One outlier was observed in the sample spiked at the 10 μg/kg level. The recovery, RSDr and RSDR were in the ranges of 98.8–102.6%, 7.2–11.3% and 9.5–22.6%, respectively. The HorRat value ranged from 0.4–0.9. The LOQs of eight laboratories were below 1.0 μg/kg, but that of one laboratory was 12 μg/kg.

In the fortification test of 3ADON and 15ADON, no laboratory detected more than 1 μg/kg of these analytes in the blank sample. One outlier was observed in the sample spiked at the 10 μg/kg level of 3ADON. The recovery, RSDr and RSDR were in the ranges of 84.9–98.7%, 5.3–11.2% and 16.1–27.2%, respectively. The HorRat value ranged from 0.5–1.2. LOQs of all laboratories were below 5.0 μg/kg.

According to the criteria for analytical methods mentioned in Commission Regulation (EC) No. 401/2006, the recovery, RSDr and RSDR for >100–≤500 μg/kg of DON are required to be in the ranges of 60–110%, ≤20% and ≤40%, respectively; for >500 μg/kg of DON, the levels are required to be in the ranges of 70–120%, ≤20% and ≤40%, respectively[16]. Although criteria for 3ADON and 15ADON, and concentrations less than 100 μg/kg were not provided, the values obtained from this study were in good agreement with the criteria. A range of 0.5–1.5 for the HorRat value is acceptable according to AOAC International[14]. The HorRat value of 10 μg/kg in the DON-spiked sample was less than 0.5, but no problems were recognized throughout this inter-laboratory study. For naturally contaminated wheat, all parameters satisfied the above-mentioned criteria, although one outlier was observed in the DON result.

Aoyama *et al.* reported an inter-laboratory study for the determination of DON and nivalenol in wheat using an LC-MS(/MS) method coupled with a multifunctional column[17]. In their study, the concentrations of the fortification test were set at 100, 500 and 1,000 μg/kg and the results for DON were: recovery: (90–110%), RSDr (3.8–6.9%), RSDR (10.8–17.5%), HorRat value (0.6–0.8). Because the concentrations of the spiked sample were quite different from those in our study, it is not reasonable to compare the results of the two studies, though the results of the methods in this paper are similar to theirs. With regard to DON acetylated derivatives, some single laboratory studies have outlined a method to de-

Table 1. Measurement conditions of each laboratory

Laboratory		A	B	C	D	E	F	G	H	I
LC-MS/MS	Manufacturer	AB SCIEX	AB SCIEX	AB SCIEX	Waters	Waters	AB SCIEX	AB SCIEX	AB SCIEX	AB SCIEX
	Instrument	API 3000	API 4000	API 4000	Quattro micro API	Xevo TQ MS	API 4000	API 3200 QTRAP	API 4000 QTRAP	API 4000 QTRAP
Mobile phase	Solvent A	0.5 mM aquous ammonium acetate solution containing 0.1% acetic acid	10 mM aqueous ammonium acetate solution	Water	10 mM aqueous ammonium acetate solution	Water	Water	Water	Water	Water
	Solvent B	Acetonitrile	Acetonitrile	Acetonitrile	Methanol	Acetonitrile	Acetonitrile	Acetonitrile	Acetonitrile	Acetonitrile
Gradient method	% of solvent B	5% (0 min) → 65% (7 min)	5% (0 min) → 65% (14 min)	5% (0 min) → 65% (7 min)	10% (0–1 min) → 60% (23 min)	5% (0 min) → 60% (10 min)	5% (0 min) → 65% (7 min)	5% (0 min) → 65% (7 min)	5% (0 min) → 65% (7 min)	5% (0 min) → 65% (7 min)
Flow rate (mL/min)		0.3	0.2	0.4	0.2	0.4	0.4	0.4	0.4	0.4
Precursor ion (*m/z*)	DON	355 $[M+CH_3COO]^-$	355 $[M+CH_3COO]^-$	295 $[M-H]^-$	355 $[M+CH_3COO]^-$	295 $[M-H]^-$	295 $[M-H]^-$	295 $[M-H]^-$	295 $[M-H]^-$	295 $[M-H]^-$
	3ADON	397 $[M+CH_3COO]^-$	397 $[M+CH_3COO]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$
	15ADON	397 $[M+CH_3COO]^-$	397 $[M+CH_3COO]^-$	337 $[M-H]^-$	339 $[M+H]^+$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$	337 $[M-H]^-$
Quantifier ion (*m/z*)	DON	59	59	265	295	265	265	265	265	265
	3ADON	337	173	307	307	307	307	307	307	307
	15ADON	59	59	150	321	150	150	150	150	150

Table 2. Results of the inter-laboratory study on the determination of deoxynivalenol by LC-MS/MS in wheat

Laboratory	Spiked sample								Naturally contaminated wheat (μg/kg)		LOD[a] (μg/kg)	LOQ[b] (μg/kg)
	Blank		10 μg/kg		50 μg/kg		150 μg/kg					
A	4.2	6.8	12.2	13.7	52.2	59.5	151.3	175.8	828.3	864.2	0.3	1.0
B	2.8	3.0	12.4	13.2	56.2	63.1	145.2	182.8	680.6	679.8	0.2	0.5
C	4.2	4.2	13.7	13.5	54.1	58.9	229.2	176.8	861.5	867.9	0.1	0.5
D	<LOD	<LOD	14.5	18.3	67.7	67.7	175.4	178.2	803.4	744.1	4.0	12
E	4.8	4.0	12.5	12.0	43.2	43.5	112.2	112.9	673.0	859.4	0.05	0.1
F	4.5	4.4	11.0	12.6	50.4	44.2	131.6	130.8	857.6	859.1	0.02	0.06
G	<LOD	1.2	6.6[c]	6.7	34.9	25.4	142.0	164.0	794.0	910.0	0.08	0.3
H	6.6	5.8	14.1	14.7	54.2	51.1	140.0	134.6	869.3	826.6	0.2	0.8
I	1.4	1.5	13.1	14.7	68.5	63.6	163.6	183.9	1366.0	1391.5	0.2	0.5
Mean (μg/kg)	3.3		13.5		53.3		157.2		811.2			
Mean recovery (%)			98.8		99.8		102.6					
Outlier (Cochran parameters)	—		0		0		0		0			
Outlier (single Grubbs parameters)	—		0		0		0		1			
Outlier (paired Grubbs parameters)	—		1		0		0		0			
Repeatability relative SD [RSDr, %]	—		9.0		7.5		11.3		7.2			
Reproducibility relative SD [RSDR, %]	—		12.4		22.6		19.1		9.5			
HorRat	—		0.4		0.9		0.9		0.6			

[a]: Limit of detection
[b]: Limit of quantification
[c]: Underline means an outlier

Table 3. Results of the inter-laboratory study on the determination of 3-acetyl-deoxynivalenol by LC-MS/MS in wheat

Laboratory	Spiked sample								Naturally contaminated wheat (μg/kg)		LOD[a] (μg/kg)	LOQ[b] (μg/kg)
	Blank		10 μg/kg		50 μg/kg		150 μg/kg					
A	<LOD	<LOD	9.9[c]	13.1	53.8	54.4	162.8	157.2	10.8	9.9	0.3	1.0
B	<LOD	<LOD	9.9	9.2	60.4	45.0	141.7	136.8	10.9	10.6	0.6	2.0
C	<LOD	<LOD	10.7	11.0	51.2	51.2	118.7	143.3	10.1	10.3	0.2	0.7
D	<LOD	<LOD	10.2	10.3	44.2	40.4	165.8	149.0	10.2	10.3	1.0	4.0
E	<LOD	<LOD	7.2	6.9	34.6	33.2	93.6	96.9	10.0	9.4	0.05	0.1
F	<LOD	<LOD	7.6	8.4	49.5	45.9	133.9	135.7	7.5	7.8	0.02	0.06
G	<LOD	<LOD	7.6	9.2	63.4	58.5	182.0	138.0	8.1	5.8	0.1	0.2
H	<LOD	<LOD	10.5	10.6	50.6	42.9	127.6	123.9	9.6	10.5	0.2	0.7
I	<LOD	<LOD	6.8	7.0	54.5	54.8	141.9	158.3	12.0	11.3	0.02	0.07
Mean (μg/kg)	—		8.9		49.4		139.3		9.7			
Mean recovery (%)			89.3		98.7		92.9					
Outlier (Cochran parameters)	—		1		0		0		0			
Outlier (single Grubbs parameters)	—		0		0		0		0			
Outlier (paired Grubbs parameters)	—		0		0		0		0			
Repeatability relative SD [RSD_r,%]	—		5.3		8.9		9.5		6.7			
Reproducibility relative SD [RSD_R,%]	—		18.0		17.2		16.5		16.1			
HorRat	—		0.6		0.7		0.8		0.5			

[a]: Limit of detection
[b]: Limit of quantification
[c]: Underline means an outlier

Table 4. Results of the inter-laboratory study on the determination of 15-acetyl-deoxynivalenol by LC-MS/MS in wheat

Laboratory	Spiked sample								Naturally contaminated wheat (μg/kg)		LOD[a] (μg/kg)	LOQ[b] (μg/kg)
	Blank		10 μg/kg		50 μg/kg		150 μg/kg					
A	<LOD	<LOD	10.6	9.6	55.4	55.6	167.6	155.6	24.8	28.2	0.3	1.0
B	<LOD	<LOD	9.6	9.5	45.5	38.6	123.3	110.8	27.4	25.4	1.0	3.0
C	<LOD	<LOD	8.4	8.5	45.9	42.7	76.0	124.4	21.0	18.0	1.0	3.3
D	<LOD	<LOD	4.1	4.4	27.2	24.1	117.0	89.3	26.7	21.7	0.3	1.0
E	<LOD	<LOD	6.3	6.3	34.0	32.8	87.2	91.9	20.7	19.5	0.05	0.1
F	<LOD	<LOD	8.3	8.8	47.3	45.9	135.6	141.8	20.6	20.4	0.03	0.1
G	<LOD	<LOD	7.3	8.4	49.4	45.1	138.0	140.0	21.9	20.6	0.1	0.4
H	<LOD	<LOD	9.9	10.4	45.4	39.4	119.9	114.8	24.1	23.5	0.2	0.7
I	<LOD	<LOD	10.8	12.3	70.4	65.6	179.9	194.1	31.6	30.8	0.1	0.4
Mean (μg/kg)	—		8.5		45.0		128.2		23.7			
Mean recovery (%)			84.9		90.0		85.4					
Outlier (Cochran parameters)	—		0		0		0		0			
Outlier (single Grubbs parameters)	—		0		0		0		0			
Outlier (paired Grubbs parameters)	—		0		0		0		0			
Repeatability relative SD [RSDr, %]	—		6.2		6.4		11.2		7.3			
Reproducibility relative SD [RSDR, %]	—		26.2		27.2		25.8		17.0			
HorRat	—		0.8		1.1		1.2		0.6			

[a]: Limit of detection
[b]: Limit of quantification

termine them[18), 19)], but an inter-laboratory study has not yet been reported. The present one is the first. Because the Codex general standard for DON and these derivatives is now under consideration, information about analytical methods to determine them is important.

Our results provide a validated method to study the status of contamination with DON and its acetylated derivatives in wheat. Data on the occurrence of these mycotoxins is important for dietary exposure assessment and establishment of a standard value.

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Original Article

Induction of ovarian toxicity in a subchronic oral toxicity study of citrinin in female BALB/c mice

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ABSTRACT — The present study was performed to elucidate toxicity profile of citrinin (CTN) after repeated oral doses for 90 days, especially on the kidneys and female reproductive organs using female BALB/c mice. We first performed a 70-day repeated oral dose toxicity study of CTN by setting the doses at 1.25 and 7.5 ppm in the drinking water (Experiment 1). As a result, CTN did not produce any toxicity in the kidneys, liver, and female genital organs/tracts, except for a slight increase of relative ovary weight. We, next, performed 90-day repeated oral dose toxicity study of CTN by increasing the dose levels at 15 and 30 ppm in the drinking water. The results suggested that CTN did not produce any toxicity in the kidneys, liver, and female genital organs/tracts, except for increase of both absolute and relative ovary weights accompanying increase of large follicles at ≥ 15 ppm. On the basis of these findings, the lowest-observable-adverse-effect level of CTN was 15 ppm (2.25 mg/kg body weight/day) in the drinking water for female BALB/c mice after 90-day oral treatment.

Key words: Citrinin, Mycotoxin, Nephrotoxicity, Ovarian toxicity, Mouse

INTRODUCTION

Citrinin (CTN) is a mycotoxin produced as a secondary metabolite by several fungal species, including *Penicillium, Aspergillus* and *Monascus*. CTN contaminates maize, wheat, rye, barley, oats, and rice (Flajs and Peraica, 2009). Red mold rice, the common fermented product of *Monascus* producing CTN as an active ingredient, has been widely used as a natural coloring agent for many kinds of food in China and Japan (Su *et al.*, 1973).

CTN is known as a nephrotoxic compound that has been implicated as a potential causative agent in human endemic Balkan nephropathy (IARC, 1986). CTN produces nephrotoxic lesions in most animal species including mice, rats, dogs and poultry (Carlton *et al.*, 1974; Ames *et al.*, 1976; Berndt and Hayes, 1978; Jordon *et al.*, 1977). Porcine nephropathy, associated with feeding moldy cereals to farm animals, has been reproduced by feeding either artificially contaminated barley-rye or purified CTN to pigs (Krogh *et al.*, 1970). Acute toxicity study of CTN by single oral administration at 112 mg/kg caused nephrotoxicity such as renal tubular dilatation, scattered protein casts, cortical tubular necrosis of slight to moderate degree and a slight increase in mitotic activity in the medullary tubular epithelium in mice (Jordon *et al.*, 1977). Similar acute renal tubular toxicity of CTN has also been detected in rats with a single dose at 50 mg/kg (Lockard *et al.*, 1980). Renal tubular toxicity by CTN appeared to be selective through organic anion transporter expressing at the proximal tubular epithelial cells (Berndt, 1998). However, a recent 90-day toxicity study of rats by treatment with *Monasucus*-fermented products including CTN at 200 ppm in the drinking water did not produce renal toxicity (Lee *et al.*, 2010).

On the other hand, reproductive toxicity and developmental toxicity have also been reported on CTN. CTN at 5 μM (1.25 ppm) in the drinking water for 4 days resulted in decreased oocyte maturation in mice (Chan, 2008).

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Ex vivo culture of mouse blastocysts with CTN at 15 or 30 μM decreased cell viability causing apoptosis and suppression of implantation success rate (Chan and Shiao, 2007). CTN also appears to inhibit proliferation of macrophage-derived cell line (Oh *et al.*, 2012). On the other hand, repeated intraperitoneal injections of CTN at 3.0 mg/kg body weight decreased peripheral lymphocytes (Reddy *et al.*, 1988); however, this decrease was judged to be attributable to the inflammatory responses to injected CTN (Ambrose and Deeds, 1946; Friis *et al.*, 1969). In addition, there were no effects on the weights of primary or secondary immune organs of mice in the same study (Reddy *et al.*, 1988).

All the data that have shown positive toxicity in the kidney and reproductive systems are derived from short-term studies of CTN. On the other hand, the negative results with a 90-day repeated oral toxicity study above-mentioned administered fermented mold product and did not use purified CTN (Lee *et al.*, 2010). The present study was thereby performed to elucidate toxicity profile of CTN after repeated oral doses for 90 days, especially on the kidneys and female reproductive organs using female BALB/c mice.

MATERIALS AND METHODS

Chemical

CTN used in this study was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) with purity of > 98%.

Animals and experimental design

Female 4-week-old BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a twelve-hour light/dark cycle (room temperature, 23 ± 3°C; relative humidity, 55 ± 15%), and given free access to a MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water during acclimatization period for 1 week. All procedures of this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee at the Tokyo University of Agriculture and Technology. All efforts were made to minimize animal suffering.

In Experiment 1, animals were divided into 3 groups, each consisting of 15 animals. Mice at 5-week-old were given 0 (untreated controls), 1.25 or 7.5 ppm CTN in tap water for 70 days. The dosage was determined based on the results of previous study, in which 5 μM (1.25 ppm) CTN in the drinking water for 4 days resulted in decreased oocyte maturation in mice (Chan, 2008), and blastocyst treated *ex vivo* with 30 μM (7.5 ppm) CTN showed significant increase in apoptosis and decrease in total cell number (Chan and Shiao, 2007). In Experiment 2, animals were similarly divided into 3 groups (N=15/group), and given 0 (untreated controls), 15 or 30 ppm CTN in tap water for 90 days. Because only an increase of relative ovary weights was observed without accompanying histopathological alterations at 7.5 ppm in Experiment 1, we decided to increase the dose level of CTN for administration in Experiment 2. In both Experiment 1 and 2, CTN solution at each dose was prepared twice a week, and drinking water containing CTN was provided with shading bottles. Urinalysis was performed using UROPAPER 'EIKEN' (Eiken Chemical Co. Ltd., Tokyo, Japan) in Experiment 2 without restriction of water and food supply. Urine samples were collected by forced excretion with handling stimulation of animals, and were immediately applied to test paper. At the end of animal study, mice were euthanized by exsanguination from the abdominal aorta under deep anesthesia after whole blood collection.

Stability of CTN in the drinking water was examined using high pressure liquid chromatography. CTN at 15 ppm (14.7 μg/ml) and 30 ppm (29.3 μg/ml) kept for 4 days in shade at room temperature resulted in CTN retention of 97.3% and 92.5% of the original concentration, respectively.

In Experiment 1, the kidneys, liver, ovaries and uterus were excised and fixed in 10% phosphate-buffered formalin for histopathological and immunohistochemical evaluations. Before fixation, weights of the kidneys and liver were measured. Ovaries and uterus were weighed after fixation. In Experiment 2, the kidneys, liver, ovaries, uterus, vagina, spleen, thymus, heart, lung, pancreas, intestine, brain, pituitary gland, adrenal glands, aorta, trigeminal nerve, muscle, bone, mesenteric lymph node, esophagus, trachea and thyroid gland were excised and fixed in 10% phosphate-buffered formalin. Before fixation, weights of the kidneys, liver, spleen, thymus, heart, lung, brain and adrenal glands were measured. Ovaries and uterus were weighed after fixation.

All tissues were trimmed after fixation for 24 hr and were subjected to additional fixation for 24 hr and paraffin-embedding after dehydration.

Serum biochemistry

In both Experiment 1 and 2, the whole blood samples collected from the abdominal aorta at necropsy were centrifuged (3,000 rpm, 15 min) and sera obtained were stored in -80°C. Blood biochemical values were deter-

mined in 5 animals in each group in terms of blood urea nitrogen (BUN) with urease-leucine dehydrogenase method, creatinine with enzymatic method, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) with JSCC transferable method, albumin with BCG method, and total protein (TP) with biuret method using an BioMajesty autoanalyzer (JCA-BM6050; JEOL Co. Ltd., Tokyo, Japan) at Mitsubishi Chemical Medience Corp. (Tokyo, Japan). In Experiment 1, because induction of nephropathy was suggested by CTN-treatment (Carlton *et al.*, 1974; Ames *et al.*, 1976; Berndt and Hayes, 1978; Jordon *et al.*, 1977), serum level of IgG and IgA antibodies were determined in 4-5 pooled blood samples of 2-3 animals in each group employing an immunoturbidimetric method at SRL, Inc. (Tokyo, Japan).

Histopathology and immunohistochemistry

In both Experiment 1 and 2, tissue sections of all organs were stained with hematoxylin and eosin (HE). Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed with the kidneys and ovaries in Experiment 1 and the kidneys, liver and ovaries in Experiment 2 using horseradish peroxidase avidin-biotin complex method using Vector M.O.M.™ Immunodetection Kit (Vector Laboratories Burlingame, CA, USA). Deparaffinized tissue sections were placed in an antigen retrieval solution (0.01 M citrate buffer, pH 6.0) for 15 min in a microwave oven at 97°C. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide with methanol for 5 min. Sections were incubated overnight with mouse monoclonal anti-PCNA antibodies (Dako, Glostrup, Denmark) at a dilution of 1:800 at 4°C, followed by incubation with the biotinylated secondary antibody for 10 min and with avidin peroxidase conjugates for 5 min at room temperature. Sections were developed in 0.05% 3, 3'-diaminobenzidine/ hydrogen peroxide as a chromogen. After staining, slides were lightly counterstained with hematoxylin.

In Experiment 1, renal tubular cells were subjected to analysis of cell proliferation activity. The number of PCNA-positive cells were counted in the cortex and outer stripe of the outer medulla of kidney sections under 100 × magnification and expressed as a percentage of total cells counted in 6 randomly selected fields per animal. In Experiment 2, renal tubular cells and liver cells were subjected to analysis of cell proliferation activity. In the kidney, the number of PCNA-positive cells was similarly counted under 200 × magnification in 10 randomly selected fields. In the liver, the number of PCNA-positive liver cells was counted under 100 × magnification and expressed as a percentage of total cells counted in 5 randomly selected fields.

In the ovaries in both Experiment 1 and 2, PCNA-staining was performed to detect nucleus of oocytes for differentiation of primordial follicles. For this purpose, color development with DAB was performed exceedingly to enhance background nuclear staining over the exact immunoreactivity against PCNA antigen (Yoshida *et al.*, 2009). Follicles and corpora lutea were counted at a 100 × magnification. Follicles in Experiment 1 were morphologically classified into (1) primordial follicles with oocyte and one flattened pregranulosa cells at the periphery of the follicle; (2) primary follicles with oocyte having one complete ring of cuboidal granulosa cells; (3) secondary follicles consisted of one large oocyte and several hundreds of granulosa cells showing more than one complete ring of granulosa cells with fluid-containing antral space; (4) atretic follicles characterized by frequent apoptotic cellular deaths in oocytes and granulosa cells; and (5) corpora lutea. In Experiment 2, follicles were further classified into (1) Small follicles consisting of primordial and primary follicles; (2) medium-sized follicles consisting of secondary follicles and preantral follicles; (3) large follicles consisting of preantral follicle, antral follicles and Graafian follicles; (4) currently formed corpora lutea defined as those formed within one estrous cycle after the latest ovulation; (5) previously formed corpora lutea remained in the ovary formed during several estrous cycles before their complete dissolution; and (6) atretic follicles (Pedersen and Peters, 1968).

The uteri in Experiment 1 and 2 were morphologically classified into following four categories of estrous cyclicity. At the proestrus, endometrial epithelial cells were medium-sized, low to high columnar with cytoplasmic to nuclear ratio being about 1.5 and a few mitotic figures. Stromal cells were spindle-shaped and lamina propria was inactive. At the estrus, endometrial epithelial cells were very large and tall columnar with cytoplasmic to nuclear ratio being greater than 2 and rare mitotic figures. Vacuolar degeneration and apoptosis were very frequent in both luminal and glandular epithelium. Stromal cells were spindle-shaped and lamina propria was inactive. At the metestrus, endometrial epithelial cells were large and tall columnar with cytoplasmic to nuclear ratio being about 2 and frequent mitotic figures. Vacuolar degeneration and apoptosis were occasional in luminal epithelium. Stromal cells were spindle-shaped and lamina propria was inactive. At the diestrus, endometrial epithelial cells were small and columnar with cytoplasmic to figures being less than 1. Mitotic figures were very common in the glandular epithelium. Stromal cells were

round to ovoid and lamina propria was active.

The vagina in Experiment 2 was also morphologically classified into following four categories of estrous cycle. At the proestrus, there were 7-9 cell layers of stratum germinativum, 2-3 cell layers of stratum granulosum and 2-3 cell layers of stratum mucification. Stratum corneum was absent. At the estrus, there were 7-8 cell layers of stratum germinativum and 1-2 cell layers of stratum granulosum. Stratum corneum was present, but stratum mucification was absent. At the metestrus, stratum germinativum was 5-6 layers of cells. All other stratum granulosum, stratum corneum, and stratum mucification were absent. At the diestrus, stratum germinativum was 8-9 layers of cells. All other stratum granulosum, stratum corneum, and stratum mucification were absent (Yuan and Foley, 2002).

Statistical analysis

Data regarding the body and organ weights, serum biochemistry and immunoglobulin levels, PCNA-positive cell index in the kidney and liver, morphologically classified ovarian follicles and corpora lutea were evaluated using the following methods. A Bartlett's test for equal variance was used to determine if the variance was homogenous between the groups. If the variance was homogenous, numerical date was assessed using Dunnett's multiple test. If a significant difference in variance was observed, the Steel's test was used instead. Urinalysis data were assessed between the control group and each treated group using Mann-Whitney's *U*-test. Incidence data of histopathological change were assessed between the control group and each treated group using Fisher's exact probability test.

RESULTS

Body weights, daily food intake and water consumption

In Experiment 1, during the dosing period, neither death nor marked change in general condition was observed in both treatment groups of CTN. There was no significant difference in the body weight, daily food intake and water consumption between the untreated controls and each CTN group (Table 1, Fig. 1). Thus, CTN was proportionally administered to animals dependent on the dose level (Table 1).

In Experiment 2, body weight decreased significantly at 1 and 2 weeks after starting the CTN-treatment of the 30 ppm group (Fig. 2). Water consumption decreased significantly at 5 weeks after starting the CTN-treatment of the 15 ppm group (Fig. 2).

Regarding daily food intake, there were no changes throughout the treatment period (Table 2, Fig. 2). Thus, CTN was proportionally administered to animals dependent on the dose level (Table 2).

Urinalysis data

In Experiment 2, there were no differences in the level of urinary protein and occult blood between the untreated controls and each CTN group (Table 3). The urine pH was significantly higher in the 15-ppm CTN group at week 8 after starting the CTN-treatment as compared with the untreated control group (Table 3).

Final body and organ weights

In Experiment 1, there were no statistically significant differences in the absolute and relative weights of the liver, kidney and uterus between the untreated controls and each CTN group. While absolute values did not attain statistically significant difference, relative ovary weight significantly increased in the 7.5-ppm CTN group (Table 4).

In Experiment 2, both absolute and relative ovary weights significantly increased in the CTN groups (Table 5). Relative liver weight in the 15-ppm CTN group decreased significantly, while the absolute value did not attain significant difference (Table 5). Regarding other organ weights, there were no differences between the untreated controls and each CTN group.

Serum biochemistry data

In Experiment 1, data on serum biochemical parameters, such as creatinine, BUN, AST, ALP, ALT, TP, albumin, and IgG and IgA are shown in Table 6. There were no significant differences in the values in these parameters between the untreated controls and each CTN group.

In Experiment 2, significantly decreased BUN and increased ALT were observed in the 30-ppm CTN group, as compared with untreated controls (Table 7). There were no differences in the values of creatinine, AST, ALP, TP and albumin between the untreated controls and each CTN group.

Histopathology and immunohistochemistry data

In Experiment 1, microgranulomas developed in most animals of all groups including untreated controls (Table 8). Microgranulomas accompanied with scattered liver cell necrosis were observed in a few cases of the CTN-treatment groups, and cases showing inflammatory cell infiltration around the interlobular bile duct were increased in both CTN-treatment groups without statistically significant difference. In the kidneys, there were no treatment-related histopathological changes. With regard to endometrial changes in terms of estrous cyclicity, both CTN-treatment

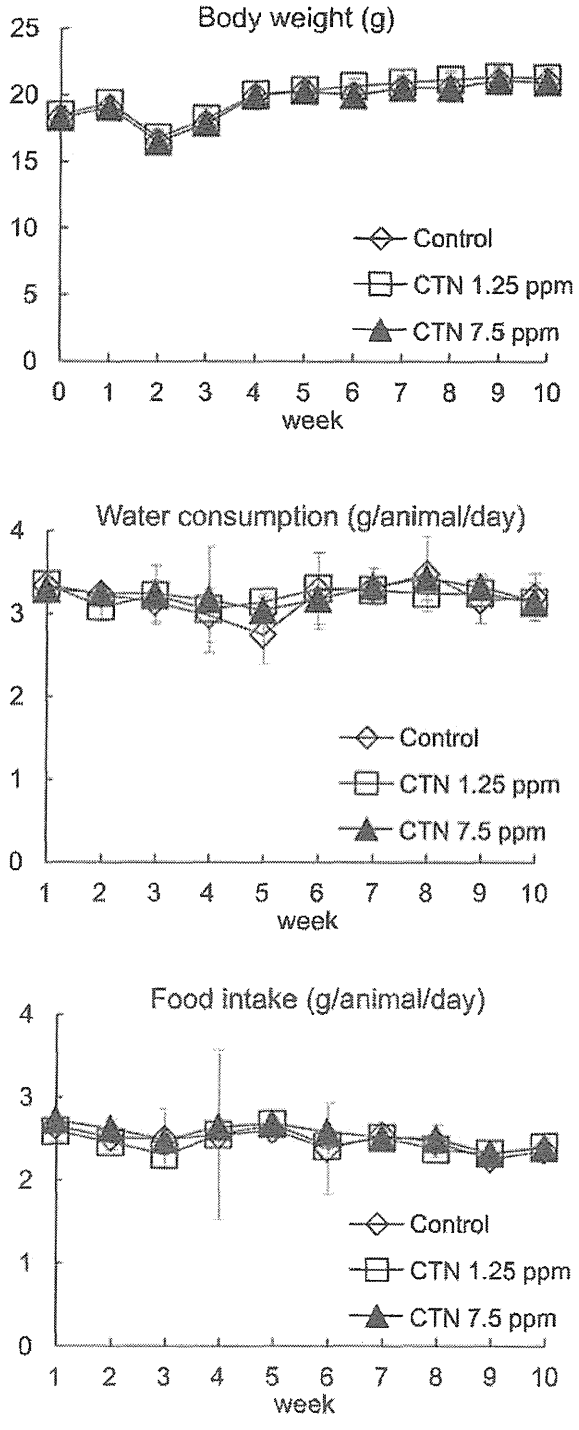

Fig. 1. Body weight, water consumption and food intake of female BALB/c mice given CTN in the drinking water for 70 days in Experiment 1.

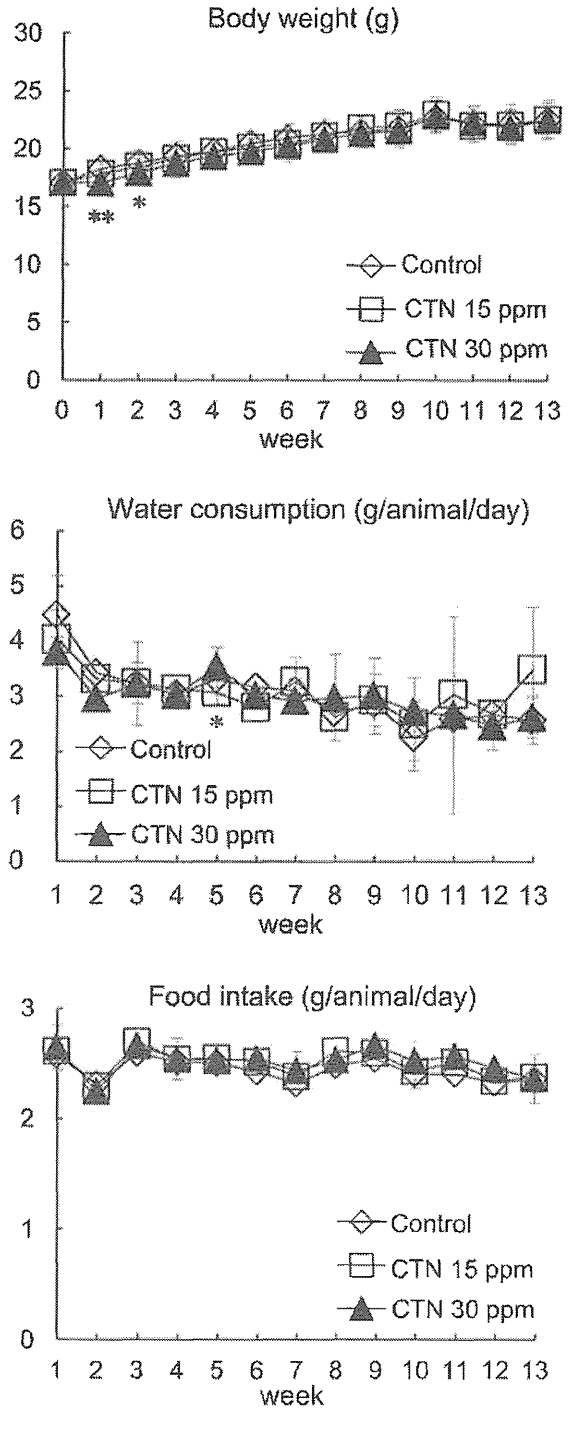

Fig. 2. Body weight, water consumption and food intake of female BALB/c mice given CTN in the drinking water for 90 days in Experiment 2. *, ** Significantly different from the untreated controls ($P < 0.05$, 0.01, Dunnett's multiple test or Steel's test).

Table 1. Final body weight, food intake, water consumption and CTN intake of BALB/c mice given CTN in the drinking water for 70 days in Experiment 1

	CTN (ppm)		
	0 (Control)	1.25	7.5
No. of animals	15	15	15
Final body weight (g)	21.33 ± 0.95[c]	21.07 ± 0.75	21.31 ± 0.82
Food intake (g/animal/day)[a]	2.48 ± 0.12	2.46 ± 0.12	2.54 ± 0.13
Water consumption (g/animal/day)[b]	3.20 ± 0.24	3.21 ± 0.16	3.24 ± 0.17
CTN intake (mg/kg body weight/day)	0	0.20 ± 0.01	1.23 ± 0.11

[a, b] Mean value of each week. [c] Mean ± S.D.
Abbreviation: CTN, citrinin.

Table 2. Final body weight, food intake, water consumption and CTN intake of BALB/c mice given CTN in the drinking water for 90 days in Experiment 2

	CTN (ppm)		
	0 (Control)	15	30
No. of animals	15	15	15
Final body weight (g)	22.56 ± 1.67[c]	22.62 ± 1.32	22.47 ± 1.56
Food intake (g/animal/day)[a]	2.44 ± 0.10	2.50 ± 0.10	2.52 ± 0.12
Water consumption (g/animal/day)[b]	3.03 ± 0.55	3.09 ± 0.41	3.00 ± 0.37
CTN intake (mg/kg body weight/day)	0	2.25 ± 0.46	4.47 ± 0.94

[a, b] Mean value of each week. [c] Mean ± S.D.
Abbreviation: CTN, citrinin.

groups increased cases showing estrus morphology, while the incidences of this increase were statistically non-significant.

In the renal tubular epithelia distributed in the cortex and outer stripe of the outer medulla, we found significant increases of PCNA-positive cells in both of the CTN-treatment groups (Table 9). In counting of follicles and corpora lutea of the ovaries, there were no apparent changes in the numbers of primordial follicles, primary follicles, secondary follicles, atretic follicles, and corpora lutea between the untreated controls and each CTN group (Table 9).

In Experiment 2, inflammatory cell infiltration of portal areas and microgranulomas were observed in the liver in variable numbers of animals in all groups without statistically significant difference between the untreated controls and each CTN group (Table 10). In the kidneys, cases showing focally regenerating tubules were observed in all groups, showing increased incidence in CTN-treatment groups without statistically significant difference. In the heart, focal myocardial mineralization was observed in a few cases of animals of each group including untreated controls. With regard to changes in the endometrium and vaginal mucosa in terms of estrous cyclicity, there were no statistically significant differences in the incidence showing each estrous cycle stage. Estrous cycle stages between the endometrium and vaginal mucosa were identical in each animal.

With regard to the cell proliferation activity in the renal tubular epithelia and hepatocytes, there were no statistically significant fluctuations in the number of PCNA-positive cells between the untreated controls and each CTN group (Table 11). In counting of follicles and corpora lutea of the ovaries, number of large follicles increased in the CTN-treatment groups showing statistically significant difference in both groups (Table 11, Fig. 3).

DISCUSSION

In cultured renal tubular cells, CTN has shown to inhibit cell proliferation (Bouslimi *et al.*, 2008). However, we found significant increase of PCNA-positive proliferat-

Table 3. Urinalysis of BALB/c mice given CTN in the drinking water for 90 days in Experiment 2

	Time point (week)	Criteria		CTN (ppm) 0 (Control)	15	30
Urinary protein	4		No. of animals	14[a]	12[a]	15
		−		0	0	0
		±		1	0	0
		+		11	9	15
		++		2	3	0
		+++		0	0	0
	8		No. of animals	15	15	15
		−		0	0	0
		±		0	0	0
		+		15	13	15
		++		0	2	0
		+++		0	0	0
	12		No. of animals	12[a]	13[a]	14[a]
		−		0	0	0
		±		0	0	0
		+		12	12	13
		++		0	0	0
		+++		0	1	1
pH	4		No. of animals	13[a]	12[a]	15
		6		9	5	12
		6.5		0	3	0
		7		4	2	0
		8		0	2	3
		9		0	0	0
	8		No. of animals	15	13[a, *]	15
		6		11	3	7
		6.5		0	0	0
		7		3	10	5
		8		1	0	3
		9		0	0	0
	12		No. of animals	12[a]	13[a]	14[a]
		6		6	5	6
		6.5		0	0	0
		7		4	5	2
		8		2	1	5
		9		0	2	1
Occult blood	4		No. of animals	14[a]	12[a]	15
		−		14	12	15
		±		0	0	0
		+		0	0	0
		++		0	0	0
		+++		0	0	0
	8		No. of animals	15	14[a]	15
		−		14	14	15
		±		1	0	0
		+		0	0	0
		++		0	0	0
		+++		0	0	0
	12		No. of animals	13[a]	14[a]	14[a]
		−		12	12	13
		±		1	1	1
		+		0	0	0
		++		0	0	0
		+++		0	1	0

Grade of score: ±, minimal; +, slight; ++, moderate; +++, severe.

[a] 1, 2 or 3 animals could not be examined because of insufficient urine specimen.

* Significantly different from the untreated controls ($P < 0.05$, Mann-Whitney's U test).

Abbreviation: CTN, citrinin.

Table 4. Liver, kidney, ovary and uterine weights of BALB/c mice given CTN in the drinking water for 70 days in Experiment 1

		CTN (ppm)		
		0 (Control)	1.25	7.5
	No. of animals	15	15	15
Liver	Absolute (mg)	866.73 ± 64.49[a]	849.20 ± 46.95	853.13 ± 57.95
	Relative (% body weight)	4.07 ± 0.28	4.03 ± 0.16	4.00 ± 0.22
Kidneys	Absolute (mg)	235.40 ± 37.16	237.27 ± 12.91	244.93 ± 23.25
	Relative (% body weight)	1.14 ± 0.10	1.13 ± 0.06	1.15 ± 0.11
Ovaries	Absolute (mg)	7.23 ± 1.63	7.92 ± 1.90	8.75 ± 2.73
	Relative (% body weight)	0.034 ± 0.007	0.038 ± 0.009	0.041 ± 0.013*
Uterus	Absolute (mg)	99.53 ± 29.72	105.87 ± 40.85	98.33 ± 29.66
	Relative (% body weight)	0.47 ± 0.14	0.50 ± 0.20	0.46 ± 0.14

[a] Mean ± S.D.
* Significantly different from the untreated controls ($P < 0.05$, Dunnett's multiple test or Steel's test).
Abbreviation: CTN, citrinin.

Table 5. Organ weights of BALB/c mice given CTN in the drinking water for 90 days in Experiment 2

		CTN (ppm)		
		0 (Control)	15	30
	No. of animals	15	15	15
Liver	Absolute (mg)	1067.07 ± 103.67[a]	1019.70 ± 75.70	1046.73 ± 66.94
	Relative (% body weight)	4.73 ± 0.35	4.52 ± 0.33*	4.66 ± 0.16
Kidneys	Absolute (mg)	278.13 ± 21.13	290.80 ± 33.17	286.07 ± 24.59
	Relative (% body weight)	1.24 ± 0.08	1.28 ± 0.12	1.27 ± 0.10
Spleen	Absolute (mg)	116.13 ± 14.59	115.60 ± 13.39	110.73 ± 8.88
	Relative (% body weight)	0.52 ± 0.07	0.51 ± 0.04	0.49 ± 0.04
Thymus	Absolute (mg)	42.93 ± 11.55	45.67 ± 11.22	43.00 ± 11.01
	Relative (% body weight)	0.19 ± 0.05	0.20 ± 0.05	0.19 ± 0.05
Heart	Absolute (mg)	116.40 ± 14.56	117.27 ± 8.84	118.20 ± 15.26
	Relative (% body weight)	0.52 ± 0.06	0.52 ± 0.03	0.53 ± 0.05
Lungs	Absolute (mg)	180.93 ± 36.54	174.27 ± 18.79	180.53 ± 21.94
	Relative (% body weight)	0.80 ± 0.15	0.77 ± 0.11	0.81 ± 0.12
Brain	Absolute (mg)	474.00 ± 26.33	477.40 ± 19.48	479.00 ± 17.94
	Relative (% body weight)	2.11 ± 0.16	2.12 ± 0.16	2.14 ± 0.13
Adrenal glands	Absolute (mg)	9.55 ± 2.22	8.87 ± 1.45	9.70 ± 3.17
	Relative (% body weight)	0.043 ± 0.011	0.039 ± 0.0057	0.0426 ± 0.013
Ovaries	Absolute (mg)	7.93 ± 2.30	9.61 ± 2.10*	9.83 ± 1.70*
	Relative (% body weight)	0.035 ± 0.009	0.042 ± 0.008*	0.044 ± 0.007**
Uterus	Absolute (mg)	96.85 ± 27.37	89.95 ± 39.61	99.29 ± 27.73
	Relative (% body weight)	0.027 ± 0.022	0.023 ± 0.024	0.028 ± 0.021

[a] Mean ± S.D.
*,** Significantly different from the untreated controls ($P < 0.05$, 0.01, Dunnett's multiple test or Steel's test).
Abbreviation: CTN, citrinin.

Table 6. Serum biochemistry and immunoglobulin levels of BALB/c mice given CTN in the drinking water for 70 days in Experiment 1

	CTN (ppm)		
	0 (Control)	1.25	7.5
No. of animals	5	5	5
Blood urea nitrogen (mg/dl)	26.4 ± 3.8[a]	27.6 ± 3.5	29.6 ± 3.8
Creatinine (mg/dl)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Aspartate aminotransferase (IU/l)	53.4 ± 5.7	58.8 ± 4.3	54.8 ± 6.9
Alanine aminotransferase (IU/l)	24.8 ± 3.1	28.2 ± 3.4	26.2 ± 8.9
Alkaline phosphatase (IU/l)	292.6 ± 22.3	311.0 ± 17.4	283.0 ± 14.7
Albumin (g/dl)	3.48 ± 0.16	3.48 ± 0.13	3.40 ± 0.14
Total protein (g/dl)	4.64 ± 0.23	4.60 ± 0.19	4.54 ± 0.15
No. of animals	11[b]	9[b]	11[b]
IgG (mg/dl)	111.8 ± 48.4	97.3 ± 36.5	100.6 ± 28.1
IgA (mg/dl)	31.6 ± 6.0	29.0 ± 7.8	31.1 ± 5.3

[a] Mean ± S.D.

[b] Four or five pooled samples/group were subjected to analysis: Control, N = 4 pooled samples, each from 2, 2, 3, and 4 animals; 1.25 ppm CTN group, N = 5 pooled samples, each from 2, 2, 2, 2, and 1 animals; 7.5 ppm CTN group, N = 5 pooled samples, each from 3, 2, 2, 2, and 2 animals.

Abbreviation: CTN, citrinin.

Table 7. Serum biochemistry of BALB/c mice given CTN in the drinking water for 90 days in Experiment 2

	CTN (ppm)		
	0 (Control)	15	30
No. of pooled sample	7	8	8
Blood urea nitrogen (mg/dl)	23.9 ± 2.1[a]	44.8 ± 6.0	20.8 ± 2.5*
Creatinine (mg/dl)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Aspartate aminotransferase (IU/l)	56.4 ± 9.7	79.4 ± 45.5	71.9 ± 19.2
Alanine aminotransferase (IU/l)	28.7 ± 4.9	40.9 ± 15.5	39.0 ± 9.8*
Alkaline phosphatase (IU/l)	281.0 ± 13.9	237.6 ± 13.9	289.4 ± 19.9
Albumin (g/dl)	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
Total protein (g/dl)	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.1

[a] Mean ± S.D.

* Significantly different from the untreated controls ($P < 0.05$, Dunnett's multiple test or Steel's test).

Abbreviation: CTN, citrinin.

ing cells in the renal tubular epithelia in both of the CTN-treatment groups in Experiment 1, while we could not find any histopathological renal changes in relation with CTN-treatment in the present study. To confirm whether our observation regarding renal tubular proliferation by CTN-treatment was reproducible, we, in Experiment 2, again performed similar exposure study of CTN by setting high doses. As a result, we could not confirm such increases. In case of increased incidence of focal tubular regeneration in both CTN-treatment groups in the Experiment 2, this increase did not attain statistically significant difference with untreated controls, and the observed change itself was very minor one, suggestive of low toxicological relevance. A recent study reporting that 90-day treatment of rats with *Monascus*-fermented products in diet containing CTN at 200 ppm (100 mg/kg body weight/day) did

Table 8. Histopathological changes in BALB/c mice given CTN in the drinking water for 70 days in Experiment 1

	CTN (ppm)		
	0 (Control)	1.25	7.5
No. of animals	15	15	15
Liver			
Microgranulomas	8	11	9
Microgranulomas with liver cell necrosis	0	1	2
Inflammatory cell infiltration, bile duct	3	7	7
Kidneys			
Regenerating tubules, focal	1	0	1
Cytoplasmic vacuolation, proximal tubules	1	1	0
Hydronephrosis	1	0	2
Estrous cyclicity			
Proestrus	4	2	2
Estrus	3	6	6
Metestrus	4	3	5
Diestrus	4	4	2

Abbreviation: CTN, citrinin.

Table 9. The number of PCNA-positive renal tubular cells and ovarian follicles/corpora lutea of BALB/c mice given CTN in the drinking water for 70 days in Experiment 1

	CTN (ppm)		
	0 (Control)	1.25	7.5
No. of animals	15	15	15
Kidneys			
No. of PCNA-positive tubular cells[a]	0.11 ± 0.04[b]	0.16 ± 0.07*	0.17 ± 0.08*
Ovaries			
No. of primordial follicles/area	2.39 ± 1.64	2.33 ± 1.54	2.92 ± 1.88
No. of primary follicles/area	3.71 ± 2.31	3.01 ± 1.40	3.91 ± 2.23
No. of secondary follicles/area	7.12 ± 3.85	4.99 ± 1.43	6.41 ± 2.73
No. of atretic follicles/area	3.14 ± 1.27	2.74 ± 1.52	3.38 ± 1.79
No. of corpora lutea/area	1.14 ± 1.28	1.19 ± 0.69	1.08 ± 1.23

[a] Tubular cells in the cortex and outer stripe of the outer medulla.
[b] Mean ± S.D.
* Significantly different from control ($P < 0.05$, Dunnett's multiple test or Steel's test).
Abbreviation: CTN, citrinin.

not produce renal toxicity (Lee *et al.*, 2010). While animal species used in the present study was different from the study by Lee *et al.* (2010), we judge that repeated oral administration of CTN at doses used in our experimental conditions did not induce renal tubular cell toxicity in mice.

In Experiment 1, relative ovary weight was significantly increased in 7.5-ppm CTN group in the present study. However, there were no apparent changes in the numbers of follicles and corpora lutea of the ovaries between the untreated controls and each CTN-treatment group. With regard to endometrial changes in terms of estrous cyclicity, both CTN-treatment groups increased cases showing estrus morphology; however, the increases of the inci-

Table 10. Histopathological changes in BALB/c mice given CTN in the drinking water for 90 days in Experiment 2

	CTN (ppm)		
	0 (Control)	15	30
No. of animals	15	15	15
Liver			
Single cell necrosis, hepatocyte	0	0	1
Mononuclear cell infiltration, periportal	5	8	7
Microgranulomas	3	8	3
Mononuclear cell infiltration, focal	1	0	1
Coagulative necrosis, focal	1	0	0
Kidneys			
Regenerating tubules, focal	2	7	8
Heart			
Myocardial mineralization, focal	2	3	1
Thymus			
Eosinophil infiltration	0	0	1
Pancreas			
Microgranulomas	2	0	0
Stomach			
Inflammatory cell infiltration, submucosa	1	0	0
Small intestine			
Hyperplasia of foveolar epithelium, focal	1	0	0
Estrous cyclicity			
Proestrus	4	5	3
Estrus	6	3	3
Metestrus	5	5	8
Diestrus	0	2	1

Abbreviation: CTN, citrinin.

dence were statistically non-significant. These results may suggest that increased relative ovary weight at 7.5 ppm might be incidental. On the other hand, in Experiment 2, both absolute and relative ovary weights significantly increased in both of the CTN-treatment groups. Furthermore, in counting of follicles and corpora lutea of the ovaries, number of large follicles increased in both of the CTN-treatment groups showing statistically significant difference. On the other hand, there were no statistically significant differences in the incidence showing each estrous cycle stage judging from changes in the endometrium and vaginal mucosa. Also, estrous cycle stages between the endometrium and vaginal mucosa were identical in each animal. These results suggest that CTN exerted ovary toxicity; however, ovarian changes did not affect genital tract in terms of the weight and histopathological changes. Previously, it was reported that CTN at 5 μM (1.25 ppm) in the drinking water for 4 days resulted in decreased secondary oocytes, suggestive of decreased oocyte maturation in mice (Chan, 2008). In the present study, only large follicles increased with CTN-treatment. These follicles included those from primary antral follicles to Graafian follicles, the latter containing secondary oocytes. It may be possible that CTN affects the number of oocyte populations undergoing maturation other than the mature population. Possibility of decreased oocyte maturation by CTN-treatment may indicate the increase of antral follicles. Considering that CTN did not change the number of total oocytes, increase of large follicles may reflect delayed oocyte maturation in