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Suppressive effect of polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and dioxin-like polychlorinated biphenyls transfer from feed to eggs of laying hens by activated carbon as feed additive

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ABSTRACT

In this study, we investigated the suppressive effect of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (DL-PCBs) transfer from the feed to the eggs of laying hens by using activated carbon as a feed additive. Four groups of six hens (White Leghorn egg-layers; age, 11 weeks) were housed as two control groups and two exposure groups for a period of 20 weeks. Two control groups were fed with either the basal feed "Control" or basal feed additing activated carbon "Control + C". Another two exposure groups were fed with feed contaminated (about 6 ng TEQ kg^{-1} feed) by standard solutions of PCDDs/PCDFs and DL-PCBs "Exposure" alone and contaminated feed adding activated carbon "Exposure + C". There was no significant effect on each groups for the growth rate, biochemical blood components, and egg production: these were around the standard levels for poultry in general. Moreover the results in this study showed the availability of activated carbon as a feed additive owing to the reduction in the risk of food pollution by PCDDs/PCDFs and DL-PCBs. The concentration in the eggs of the Exposure group gradually increased following the start of egg-laying but reached a steady state after about 1 month. In contrast, the concentration for the Exposure + C group was stationary and below the maximum EU level (6 pg TEQ g^{-1} fat). In comparison to the Exposure group, the Exposure + C group showed a significant decline in the percentage of bioaccumulation into the egg. This reduction due to activated carbon was also observed in the muscle and abdominal fat. The reductions were compound- and congener-dependent for DL-PCBs as follows: PCDDs/PCDFs, non-ortho-PCBs, and mono-ortho-PCBs were more than 90%, 80%, and 50%, respectively, irrespective of the type of tissues. Fat soluble vitamin concentrations in the eggs of the Exposure + C group showed lower trends than the Exposure group. The γ-tocopherol and α-tocopherol concentrations in eggs of Exposure + C group showed a significant reduction of about 40%. However, the addition of activated carbon into animal feed could obviate the remote potential for accidents causing unintentional food pollution with PCDDs/PCDFs and DL-PCBs.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (DL-PCBs), which are dubbed as "PCDDs/PCDFs and DL-PCBs," are unintentionally generated by-products from many industrial processes such as combustion, metal refining, pesticide formulation, and municipal incineration. PCDDs/PCDFs and DL-PCBs exhibit high chemical stability and are fat-soluble. Therefore, they spread widely in the environment, entered into animal and human bodies through the food chain, and then bioaccumulated. More than 90% of the dietary intake of PCDDs/PCDFs and DL-PCBs is through food consumption. In particular, fish, seafood, meat, eggs and meat products have been reported to be the principal routes for human exposure to PCDDs/PCDFs and DL-PCBs. Since the World Health Organization (WHO) established a tolerable daily intake (TDI) of 1–4 pg TEQ kg^{-1} bw^{-1} in 1998 (Leeuwen et al., 2000), Western countries have implemented many strategies to reduce environmental emissions and human intake levels to below this threshold (European Commission, 2001, 2006b). Japan passed special measures to ensure low concentrations of PCDDs/PCDFs and DL-PCBs in 2000 to protect human health from the adverse effects of

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PCDDs/PCDFs and DL-PCBs, and PCB treatment began in 2004. As a result, the concentrations of PCDDs/PCDFs and DL-PCBs in emissions from industries, the environment, and foods are trending downward, which suggests that efforts to control emissions and reduce exposure are succeeding (Hays and Aylward, 2003; Charnly and Doull, 2005; European commission, 2006a). Nevertheless, humans in industrialized countries are exposed to relatively high concentrations of PCDDs/PCDFs and DL-PCBs from the food chain. From the late 1990s to the early 2000s, several serious incidents of the contaminations of PCDDs/PCDFs and DL-PCBs in foodstuffs in Europe and North America were caused by feed contaminated with PCDDs/PCDFs and DL-PCBs in the livestock industry. In 1997, extremely high concentrations of PCDDs/PCDFs and DL-PCBs in the human diet were detected in Germany; this was attributed to the pellet feed of Brazilian citrus pulp (Malisch, 2000). Two years later in 1999, a tragic accident occurred in Belgium where feed for laying hens and a broiler were accidentally contaminated by PCB oil, which spread widely in eggs and chicken meat for human consumption (Bernard et al., 2002). More recently, at the end of 2010, feedstuffs from a feed production plant company in Germany were contaminated by PCDDs/PCDFs and DL-PCBs, which had far-reaching negative impacts around the world (Bundesinstitut fur Risikobewertung, 2011). Moreover, a considerable number of studies have been conducted on seafood; farmed fish has especially high concentrations of persistent organic pollutants (POPs) due to the use of contaminated fish oil as a feedstuff (Fisk et al., 1998; Jacobs et al., 2002). WHO also revealed that hazardous potential exposure is associated with the contaminated fish oil (World Health Organization, 1999). To prevent health hazards caused by PCDDs/PCDFs and DL-PCBs, it is essential to control the concentrations of POPs in food production and to ensure food safety.

Previous studies in laboratory animals have shown that using several dietary supplements to inhibit the absorption of harmful substances and promote excretion (e.g., activated charcoal, cholestyramine, dietary fiber, and chlorella) are effective means to prevent health hazards and accumulation (Yoshimura et al., 1986a,b; Takenaka et al., 1991; Takekoshi et al., 2005). Activated charcoal, cholestyramine, and dietary fibers have the same mode of action of promoting excretion of PCDDs/PCDFs and DL-PCBs; these supplements work by hastening excretion of these compounds through the adsorption of aromatic ring structured compounds and bile acids, which are required for reabsorption of PCDDs/PCDFs and DL-PCBs in the intestines. Liquid paraffin and squalane can dissolve these organic compounds, but the intestine poorly absorbs them. Almost all of these studies were based on therapeutic or pharmaceutical viewpoints for post-exposure in humans; so far, no study has tried to reduce the risk of food pollution accidents prior to above those accidents. Technologies to prevent exposure of PCDDs/PCDFs and DL-PCBs in animals and humans should be established and put into practical use as soon as possible.

The purpose of our study was to prevent hen eggs from being polluted by contaminated feed. In this study, an animal exposure study using activated carbon as a feed additive was implemented based on the results of previous studies (Iwakiri et al., 2007). In addition, as a practical application, plasma biochemistry, histopathological examination, measurement of fat-soluble vitamins in eggs, and other biological measurements were conducted to evaluate the hens' health and the nutrients in the eggs.

2. Experimental

2.1. Chemicals and reagents

All of the following standard solutions were purchased from Wellington Laboratories Inc., Canada. A mixture of 17 native congeners of PCDDs/PCDFs (1.0, 2.0, and 5.0 μg mL^{-1} of tetra- to pentachlorinated, hexa- to heptachlorinated and octachlorinated congeners, NK-ST-B4) and a mixture of 14 native congeners of DL-PCBs (2.0 μg mL^{-1} of each congener, PCB-ST-A) were used due to compound-contaminated feed. $^{13}C_{12}$ labelled PCDDs/PCDFs standard solution (1.0 and 2.0 μg mL^{-1} of tetra to heptachlorinated and octachlorinated congener, DF-LCS-A) and $^{13}C_{12}$ labelled DL-PCBs standard solution (2.0 μg mL^{-1} of each congener, PCB-ST-A) were used as a cleanup spike solution for quantification. $^{13}C_{12}$ labelled PCDF standard solution (1.0 μg mL^{-1} of 1,2,7,8-, 1,2,3,4,6-, 1,2,3,4,6,9-, and 1,2,3,4,6,8,9-PCDF congener, DF-IS-I) was used as the syringe spike solution to calculate recoveries. Analytical grade solvents for pesticide residue and PCBs (ethanol, *n*-hexane and toluene) and HPLC grade solvents (methanol, 2-propanol, ethanol and *n*-hexane) were purchased from Wako Pure Chemical Industries Ltd., Japan, and potassium hydroxide for alkaline treatment was obtained from Kanto Chemical Co. Inc., in Japan.

2.2. Animal studies

Twenty-four hatchlings (Julia strain of White Leghorn layers) were obtained from a local farm in Ibaragi prefecture, Japan. The basal feeds were purchased from NOSAN Corporation, Japan. The amount of feed during all feeding periods was calculated according to the feed requirements for each growing stage and laying period as described in the "Japanese Feeding Standard for Poultry, 1997" and prepared in advance. There are three growing stages: (stage-1) 0–4 weeks, (stage-2) 5–10 weeks, and (stage-3) 11–20 weeks. The laying period (stage-4) is 21–30 weeks. All of the chicks were fed with a basal chick feed in stage-1 and 2. They were then divided into four groups of six hens each, and were housed individually. Two groups were then fed with either the basal feed (Control group) or basal feed containing activated carbon (Control+C group). Another two groups were fed with either the contaminated feed with standard of PCDDs/PCDFs and DL-PCBs (Exposure group) or the contaminated feed containing activated carbon (Exposure+C group). The "control" groups were prepared to confirm whether there were any negative effects due to the addition of the standard solutions. The component and the concentrations of PCDDs/PCDFs and DL-PCBs in each diet of stage 3 and 4 are shown in Table 1. The activated carbon additive in the feed made up 0.5% (w/w) with reference to Iwakiri et al. (2007).

During the animal study, the whole body weights of laying hens were measured once a week. After 30 weeks, each laying hen was dissected; blood plasma samples were immediately collected, and parts of the breast muscle and abdominal fat were stored at −20 °C to analyze PCDDs/PCDFs and DL-PCBs. The biochemical parameters were analyzed in plasma using a Hitachi model 7020 auto analyzer with standards from Wako Pure Chemical Industries Ltd., Japan. The following tissues were fixed in 10% phosphate buffered formalin and processed for histological examination: esophagus, crop, glandular stomach, gizzard, duodenum, liver, pancreas, small intestine, cecal tonsil and rectum. During egg laying, all of the eggs were collected daily; after weight measurement, the whole raw eggs without the shell were mixed five by five in order into a PP sample tube and then stored at −20 °C for analysis. This experiment was conducted according to the guidelines for animal experiments of the National Institute of Animal Health, Tsukuba, Japan.

2.3. Chemical analysis

2.3.1. PCDDs/PCDFs and DL-PCBs

2.3.1.1. Extraction. For liquid–liquid extraction of PCDDs/PCDFs and DL-PCBs in breast muscle and abdominal fat, 50 g and 10 g of the sample was accurately weighed into a separatory funnel, respectively. Then, 150 mL of ethanolic 2 M KOH and $^{13}C_{12}$ labelled

Table 1
Ingredients of the basal feed and concentration of contaminated feed (pg g^{-1}).

Ingredient			Composition (%)	
			Stage-3	Stage-4
Corn, Polished white rice			64	59
Soybean oil and corn gluten meal			17	21
Rice barn			12	8
Fish meal			2	3
Other			5	9
Concentration	Basal feed	Contaminated	Basal feed	Contaminated
2,3,7,8-TeCDD	ND	0.74	ND	0.78
1,2,3,7,8-PeCDD	ND	1.7	ND	1.8
1,2,3,4,7,8-HxCDD	ND	1.4	ND	1.7
1,2,3,6,7,8-HxCDD	ND	1.6	ND	1.9
1,2,3,7,8,9-HxCDD	ND	1.5	ND	1.7
1,2,3,4,6,7,8-HpCDD	0.16	1.6	0.18	2.0
OCDD	0.52	3.3	1.6	5.3
2,3,7,8-TeCDF	ND	0.55	ND	0.69
1,2,3,7,8-PeCDF	ND	1.4	ND	1.6
2,3,4,7,8-PeCDF	ND	1.5	ND	1.7
1,2,3,4,7,8-HxCDF	ND	1.5	ND	1.9
1,2,3,6,7,8-HxCDF	ND	1.5	ND	1.8
1,2,3,7,8,9-HxCDF	ND	1.6	ND	1.9
2,3,4,6,7,8-HxCDF	ND	1.5	ND	1.8
1,2,3,4,6,7,8-HpCDF	0.13	1.3	ND	1.6
1,2,3,4,7,8,9-HpCDF	ND	1.5	ND	1.8
OCDF	ND	3.1	ND	3.8
3,4,4′,5-TeCB (#81)	(0.04)	13	(0.23)	16
3,3′,4,4′-TeCB (#77)	0.50	13	3.80	19
3,3′,4,4′,5-PeCB (#126)	(0.12)	12	(0.19)	15
3,3′,4,4′,5,5′-HxCB (#169)	(0.04)	12	(0.07)	15
2′,3,4,4′,5-PeCB (#123)	0.21	12	0.62	15
2,3′,4,4′,5-PeCB (#118)	12	24	30	49
2,3,3′,4,4′-PeCB (#105)	4.1	16	9.8	25
2,3,4,4′,5-PeCB (#114)	0.29	12	1.20	16
2,3′,4,4′,5,5′-HxCB (#167)	0.92	14	1.70	17
2,3,3′,4,4′,5-HxCB (#156)	1.6	15	3.4	19
2,3,3′,4,4′,5′-HxCB (#157)	0.41	13	0.79	15
2,3,3′,4,4′,5,5′-HpCB (#189)	0.19	12	0.27	15

ND: Not detected.
Figure in parenthesis is provided between LOD and LOQ.

PCDDs/PCDFs and DL-PCBs cleanup spike were added into the funnel, and the solution was shaken and mixed for 1 h. After that, 150 mL of distilled water and 100 mL of *n*-hexane were each added into the funnel, which was shaken for 30 min. The aqueous layer was put into another separatory funnel and, 50 mL of *n*-hexane was again added; the funnel was then shaken for 10 min. This was repeated twice. All of the *n*-hexane layers were combined and washed with 100 mL of distilled water. The extracts were filtered through anhydrous Na_2SO_4, and were then concentrated to about 2 mL.

The extraction of PCDDs/PCDFs and DL-PCBs in eggs was performed with a microwave solvent extraction (ETHOS E touch control microwave solvent extraction Labstation; Milestone General K.K., in Japan). The TFM vessel was airtight, pressure resistant (up to 35 bar), and had a volume of 80 mL; 20 g of homogenized egg samples was accurately weighed into the MAE vessels. After the addition of $^{13}C_{12}$ labelled PCDDs/PCDFs and DL-PCBs cleanup spike, approximately 15 mL of the saponifying solution (ethanolic 2 M KOH) and 20 mL of *n*-hexane were added as an extractant. MAE was carried out at 120 °C for 20 min. After the vessels were cooled until room temperature, almost all of the *n*-hexane supernatant was filtered with anhydrous sodium sulfate into a separating funnel. The aqueous layer was transferred into a centrifuge tube, and 10 mL of *n*-hexane was added, after which the tube was shaken for 2 min. After being centrifuged at 3000 rpm for 5 min, almost all of the *n*-hexane supernatant was filtered with anhydrous sodium sulfate into the same funnel. The extracts were washed with 50 mL of distilled water; the *n*-hexane layer was then filtered with anhydrous sodium sulfate, and the extracts were concentrated to about 2 mL.

2.3.1.2. Purification method. The purification was performed fully automated by using SPD (SPD-600GC; Miura Co. Ltd., Japan), which can carry out accurate and rapid purification for extracts (Kishino et al., 2004; Miyawaki et al., 2007). The purification column was a multilayer silica gel column (200 mm × 12.5 mm I.D.) made from silica gel, 44% (w/w) H_2SO_4 silica gel, and 10% (w/w) $AgNO_3$ silica gel. Another column was the concentration column (30 mm × 6 mm I.D.) filled with an activated carbon dispersed silica gel. These columns were obtained from the Miura Institute of Environmental Science, Miura Co. Ltd., Japan. The crude extracts were applied on top of the purification column; this was followed by setting the column connecting parts and starting the program sequence. The details of the validation of the purification step were given in a previous publication (Fujita et al., 2009).

2.3.1.3. GC–MS measurement. The quantification for PCDDs/PCDFs and DL-PCBs in the sample were analyzed with a high resolution gas chromatograph/high resolution mass spectrometer (HRGC/HRMS; Agilent 6890N GC and JEOL JMS-700S) equipped with a DB-5 ms capillary column (60 m, 0.25 mm I.D., 0.25 μm; J&W Scientific, USA). Ultrahigh purity helium was used at a constant flow rate of 1.7 ml min^{-1} as a carrier gas. The oven temperature program was maintained at 150 °C for 1 min, increased to 180 °C at a rate of 20 °C min^{-1} and then to 280 °C at the rate of 2 °C min^{-1}, and finally maintained at 310 °C for 7 min. The inlet temperature was maintained at 250 °C, and 2 μL of the final sample solution was injected in the splitless injection mode. The HRMS was operated in electron ionization mode and used selected ion monitoring at an electron energy of 38 eV and ionizing current of 500 μA. The resolution of the instrument was routinely more than 10000 (10% valleys). Concentrations of the each congeners were calculated by using the mean relative response factors determined from standard calibration runs.

2.3.1.4. QA and QC. The criteria for quality of chemical analysis was conducted based on the Japanese industrial standard (JIS K0311:2005). The QA/QC procedure in this study suggested that chemical analysis reproducibility was high and that the identification of individual congeners and quantification of their concentrations in samples was reliable. Data were analyzed by the ANOVA/Tukey's multiple comparisons test to assess statistical significance in plasma biochemical parameters among groups. Student *t*-test was used to evaluate vitamin concentrations in eggs between Exposure group and Exposure+C group. Statistical analysis was performed using Data Analysis Tool Pack of Microsoft Office Excel (Microsoft, USA).

2.3.2. Nutrient component analysis

2.3.2.1. Measurement of lipid. Two grams of homogenized egg samples was accurately weighed into a 50 mL screw centrifuge tube. After the addition of 25 mL of chloroform/methanol (2:1, v/v), the tube was shaken for 3 min and centrifuged at 3500 rpm for 10 min. After that, the chloroform/methanol layer was filtered with

Table 2
Results of growth rate, egg production, and biochemical blood components.

Group	Growth rate (g/ 10 weeks)	Egg production				Biochemical blood component						
		Total number of egg	Egg weight (g)	Fat content (%)	Egg-laying rate (%)	AST (GOT) IU/L	ALP IU/L	NEFA (mEq L^{-1})	TG (mg dL^{-1})	T-Cho (mg dL^{-1})	HDL-Cho (mg dL^{-1})	Glu (mg dL^{-1})
Control	563 ± 52	43	52.7 ± 3.3	8.8 ± 0.57	95.3 ± 8.7	178 ± 66	1432 ± 330	0.54 ± 0.17	892 ± 170	84 ± 16	15 ± 5	253 ± 31
Control+C	547 ± 69	44	52.6 ± 3.0	8.4 ± 0.37	96.5 ± 11	197 ± 61	2020 ± 420	0.63 ± 0.12	850 ± 340	81 ± 19	17 ± 4	280 ± 25
Exposure	573 ± 32	44	53.2 ± 2.2	8.6 ± 0.56	97.0 ± 6.5	204 ± 52	1683 ± 550	0.96 ± 0.24	1110 ± 460	97 ± 28	20 ± 6	249 ± 29
Exposure+C	551 ± 43	45	53.2 ± 2.8	8.9 ± 0.56	95.5 ± 8.6	174 ± 32	2225 ± 660	0.74 ± 0.13	1016 ± 410	91 ± 30	15 ± 2	246 ± 31

Values are expressed as means ± SD, $n = 6$. None of values were significantly different, $p < 0.05$.
Growth rate: during exposure period between 11 and 20 weeks.
Egg-laying rate: For 1 month before slaughter.

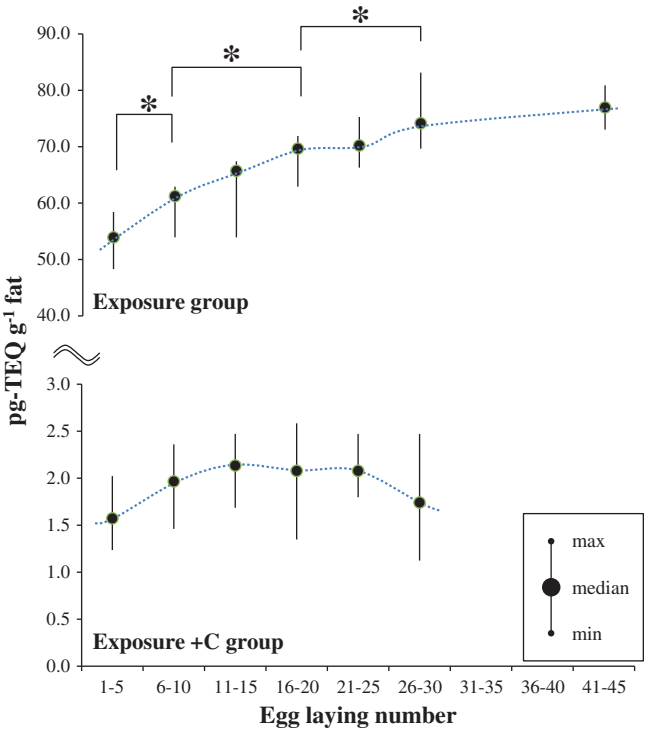

Fig. 1. Timerelated PCDDs/PCDFs and DL-PCBs concentration in egg. All data points are analytical composites of five egg samples in a laying order ($n = 6$). Each other's data points denoted by an asterisk indicate significant differences ($p < 0.05$, Student's t-test).

glass wool into another centrifuge tube; the residue was then washed with 5 mL of chloroform/methanol (2:1, v/v). The extracts was washed with 6 mL of NaCl aqueous solution (1%, w/v) and then centrifuged at 3500 rpm for 5 min. The aqueous layer was then removed, and the extracts were dehydrated with Na_2SO_4. The eluate was evaporated until dry, and the residue was dissolved with 1 mL of dichloromethane, washed three times with hexane, and placed into a vial whose weight was measured in advance. After completely removing the solvent under nitrogen, the entire vial was accurately measured.

2.3.2.2. Measurement of vitamins. Two grams of homogenized egg samples was put in a 50 mL screw centrifuge tube. Ten milliliters of pyrogallol/ethanol (6%, w/v), 0.5 mL of NaCl aqueous solution (1%, w/v) and 2.5 mL of KOH aqueous solution (60%, w/v) were added; the mixture was left in the water bath and kept at 70 °C for 60 min. After cooling and the addition of 20 mL of NaCl aqueous solution (1%, w/v), fat soluble vitamins in the mixture were extracted twice with 10 mL of hexane/ethyl acetate (9:1, v/v). The extracts was transferred to another centrifuge tube and washed with 10 mL of NaCl aqueous solution (1%, w/v); the extracts were then evaporated, and the residue was dissolved with 1 mL of hexane and filtered with glass wool into a graduated test tube. The eluate was removed by solvent under nitrogen, and the residue was finally dissolved with 4 mL of ethanol. 5 μL of the sample solutions was injected for liquid chromatography-tandem mass spectrometry (TSQ Quantum Access Triple Quadrupole LC/MS, ThermoFisher scientific Inc.). Separation of vitamins was achieved by using an L-column 2 ODS (150 mm × 2.1 mm, 3 μm, CERI, Japan) column under gradient elution conditions with *n*-hexane/2-propanol and methanol at a flow rate of 0.2 mL min^{-1}.

3. Results and discussion

3.1. Health and egg production of hens

First, we discuss the animal health and egg production conditions of this study in comparison to standards. Table 2 shows the growth rate of each feeding group during stage 3 (age: 11–20 weeks). The results agree almost completely with the growth curve model advocated by Miyoshi et al. (1995), indicating that it is the normal growth for general poultry industries. The egg production, egg weight, lipid content, egg laying rate, and plasma biochemical results are also shown in Table 2. AST; Aspartate aminotransferase (GOT; Glutamyl oxaloacetic transaminase) and ALP; Alkaline phosphatase gives an indicator of hepatic dysfunction. NEFA; non-esterified fatty acid, TG; triglyceride, T-Cho; Total-cholesterol, HDL-Cho; high-density lipoprotein-cholesterol and Glu; Glucose are concerned with lipid metabolism and nutritional status. These values show standard values for layers, and none of the diet groups showed any significant differences ($p < 0.05$). Moreover, based on the histopathological diagnoses of the dissected hens, no significant lesions in the digestive system or liver were seen relative to those from vehicle controls. Based on the reasons mentioned above, the hens' health was unaffected by the concentrations of PCDDs/PCDFs and DL-PCBs and addition of activated carbon in the feed during the exposure period.

3.2. Intake and bioaccumulation into egg

Because of several serious food pollution accidents in the late 1990s, accumulation studies of PCDDs/PCDFs and DL-PCBs from feed have added contaminated soil or fly ash. However, it is sometimes difficult to accurately determine the absorption and bioaccumulation because each congener has a different partition coefficient between the feed and soil or ash. In our study, we would be able to obtain exposure results closer to the normal excreta

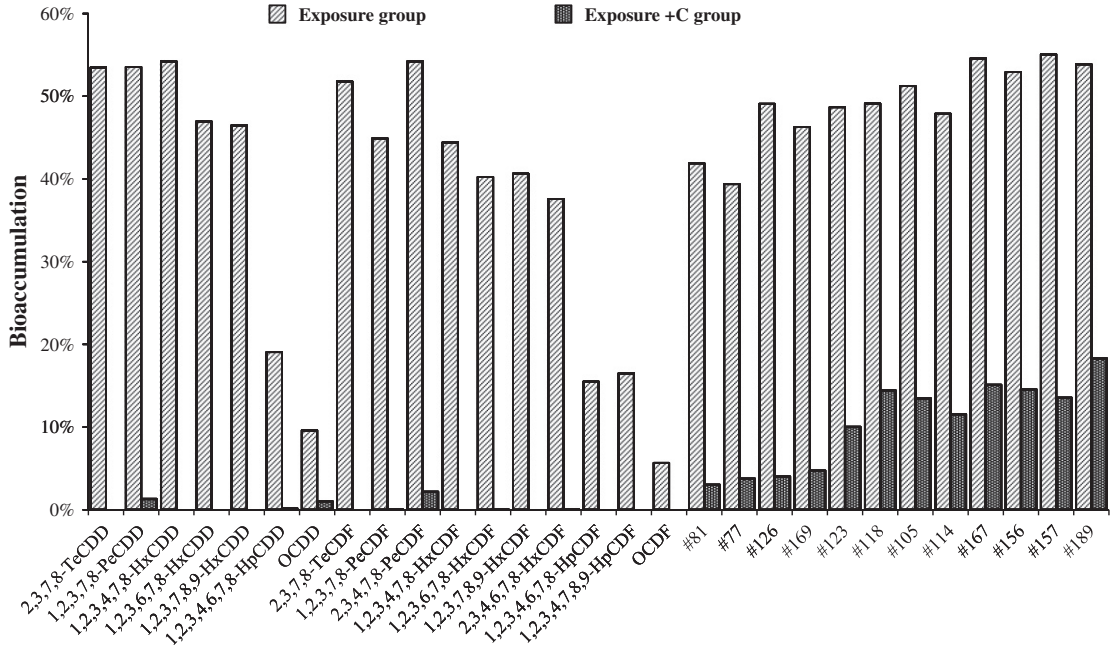

Fig. 2. Bioaccumulation into eggs after reaching the steady state. These values were estimated from the amount of intake and excretion through eggs between 24 and 30 weeks.

Table 3
Estimated concentrations in eggs (pg TEQ g^{-1} fat) of Exposure and Exposure+C group by concentrations in feed (ng TEQ kg^{-1} fat) obtained from references.

Feed[a]		Estimated concentration in egg[b]		Reference		
		Exposure	Exposure+C	Actual concentration in egg[c]	Exposure period	
ΣPCDD DFs						
A	0.37	3.9	0.06	3.42	56 d	Hoogenboom et al. (2006)
B	0.90	9.5	0.15	9.29	56 d	Hoogenboom et al. (2006)
C	4.09	43.3	0.67	55.5	20 weeks	This study
D	21.03	212	4.14	287	10 weeks	Pirarad and Pauw (2005)
E	34.61	328	6.57	ca. 300	>60 d	Stephens et al. (1995)
ΣPCDD DFs + DL-PCBs						
A	0.67	6.9	0.4	7.27	56 d	Hoogenboom et al. (2006)
B	1.63	17	0.9	18.88	56 d	Hoogenboom et al. (2006)
C	5.66	59	2	79	20 weeks	This study
D	27.31	276	9.5	305	10 weeks	Pirarad and Pauw (2005)
E[d]	–	–	–	–	–	Stephens et al. (1995)

[a] These concentrations were recalculated with concentrations of individual congeners in feed obtained from references.
[b] These TEQ values were calculated with WHO 2005 TEF.
[c] These data shows the concentrations in eggs obtained from references.
[d] None of the data of DL-PCBs in this reference.

through the egg because we used feed contaminated with only a standard solution.

The cumulative total intake of PCDDs/PCDFs and DL-PCBs during exposure period was estimated from the concentrations of PCDDs/PCDFs and DL-PCBs in the feed (Table 1) and total amount of feed ingested in each growing stage based on the "Japanese Feeding Standard for Poultry, 1997.". The total intake of the exposure group was 43 ng TEQ kg^{-1} body. This value was a far lower than the LD50 for White Leghorn layers at 25–50 μg kg^{-1} body (Sawyer et al., 1986). Thus, the onset of toxicity could not be observed in this study, and no abnormalities were found at necropsy.

Fig. 1 shows the time related concentrations in eggs; all data points are analytical composites of five egg samples in laying order. In the earlier laying stage for the Exposure group, the average total concentration (first data point) was 53.4 pg TEQ g^{-1} fat. The concentrations gradually increased following the start of egg laying but reached a steady state after about 1 month (corresponding to "egg laying number; 26–30″ shown in Fig. 1). During the steady state period, the average concentration was 79.1 pg TEQ g^{-1} fat. On the other hand, the concentrations of the Exposure+C group were stationary and stayed at around 1.93 ± 0.21 pg TEQ g^{-1} fat on average, which is below the maximum EU level of 6 pg TEQ g^{-1} fat. As shown in Fig. 1, the result that the concentrations in egg reached a steady state is in agreement with other previous studies (Stephens et al., 1995; Hoogenboom et al., 2006; Pirarad and Pauw, 2006). Stephens et al. (1995) carried out an exposure examination by using adult hens (from age of 21 weeks) that had already been starting the egg laying period. The concentrations in eggs of two groups that were fed with diets containing soil contaminated with high or low concentrations of PCDDs/PCDFs and DL-PCBs showed a rapid rise for a few exposure days, a gradual increase after about 10 d, and then reached a steady state depending on the concentration in the feed after a month or two.

The bioaccumulation rate, R_{bio} (%), of individual congeners (i) for only 1 month during the steady state was calculate by the following expression:

Table 4
Average concentration (pg g^{-1}) in muscle, abdominal fat, and egg of hens.

	Muscle (n = 6)			Abdominal fat (n = 6)			Egg (n = 6)		
	Exposure	Exposure+C	Inhibition	Exposure	Exposure+C	Inhibition	Exposure	Exposure+C	Inhibition
2,3,7,8-TeCDD	0.10	ND	–	8.1	0.30	0.96	0.54	(0.021)	0.96
1,2,3,7,8-PeCDD	0.22	ND	–	19	0.71	0.96	1.6	0.05	0.97
1,2,3,4,7,8-HxCDD	0.14	ND	–	15	0.51	0.97	1.5	(0.04)	0.97
1,2,3,6,7,8-HxCDD	0.13	ND	–	14	0.53	0.96	1.5	(0.04)	0.97
1,2,3,7,8,9-HxCDD	0.08	ND	–	9.1	0.20	0.98	1.19	(0.02)	0.98
1,2,3,4,6,7,8-HpCDD	(0.03)	ND	–	2.9	(0.13)	0.96	0.58	(0.02)	0.97
OCDD	0.03	ND	–	6.5	(0.4)	0.94	0.57	(0.05)	0.92
2,3,7,8-TeCDF	0.090	(0.009)	0.90	10	0.46	0.95	0.54	0.03	0.95
1,2,3,7,8-PeCDF	0.15	ND	–	13	0.41	0.97	1.4	0.04	0.97
2,3,4,7,8-PeCDF	0.20	(0.02)	0.91	15	0.79	0.95	1.4	0.07	0.95
1,2,3,4,7,8-HxCDF	0.11	ND	–	11	0.29	0.97	1.5	(0.03)	0.98
1,2,3,6,7,8-HxCDF	0.09	ND	–	9.4	0.25	0.97	1.4	(0.03)	0.98
1,2,3,7,8,9-HxCDF	0.10	ND	–	11	0.16	0.99	1.2	(0.02)	0.98
2,3,4,6,7,8-HxCDF	0.07	ND	–	7.4	0.27	0.96	1.18	(0.03)	0.97
1,2,3,4,6,7,8-HpCDF	0.02	ND	–	2.1	0.1	0.95	0.47	ND	–
1,2,3,4,7,8,9-HpCDF	(0.03)	ND	–	4.0	ND	–	0.56	ND	–
OCDF	ND	ND	–	5.9	ND	–	0.34	ND	–
3,4,4′,5-TeCB (#81)	1.9	0.20	0.89	180	21	0.88	11	1.1	0.90
3,3′,4,4′-TeCB (#77)	1.9	0.23	0.88	180	22	0.88	10	1.1	0.89
3,3′,4,4′,5-PeCB (#126)	1.8	0.21	0.88	180	23	0.87	13	1.4	0.89
3,3′,4,4′,5,5′-HxCB (#169)	1.6	0.23	0.86	150	24	0.84	13	1.7	0.86
2′,3,4,4′,5-PeCB (#123)	2.1	0.65	0.69	200	63	0.68	12	2.9	0.76
2,3′,4,4′,5-PeCB (#118)	6.9	2.8	0.60	620	260	0.58	38	14	0.64
2,3,3′,4,4′-PeCB (#105)	3.6	1.3	0.64	320	120	0.62	21	6.7	0.68
2,3,4,4′,5-PeCB (#114)	2.1	0.77	0.63	190	77	0.60	12	3.8	0.69
2,3′,4,4′,5,5′-HxCB (#167)	2.3	0.8	0.64	200	83	0.57	15	5.0	0.67
2,3,3′,4,4′,5-HxCB (#156)	2.6	1.0	0.60	220	98	0.55	17	6.0	0.64
2,3,3′,4,4′,5′-HxCB (#157)	2.0	0.71	0.65	180	74	0.59	14	4.3	0.69
2,3,3′,4,4′,5,5′-HpCB (#189)	1.9	0.87	0.53	150	68	0.54	15	5.7	0.61
ΣPCDDs + ΣPCDFs + ΣDL-PCBs ± SD**	32 ± 2.5	10 ± 2.6		2900 ± 100	940 ± 45		210 ± 32	54 ± 5.5	
ΣPCDDs/PCDFs/DL-PCBs TEQ* ± SD**	0.71 ± 0.07	0.025 ± 0.006		64 ± 2.3	4.4 ± 0.53		5.4 ± 0.46	0.27 ± 0.03	
ΣPCDDs/PCDFs/DL-PCBs TEQ* g^{-1} fat	76	2.7		75	5.1		63	3.0	

Figure in parenthesis is provided between LOD and LOQ.
* TEQ was calculated by WHO 2005 TEF.
** ±SD.

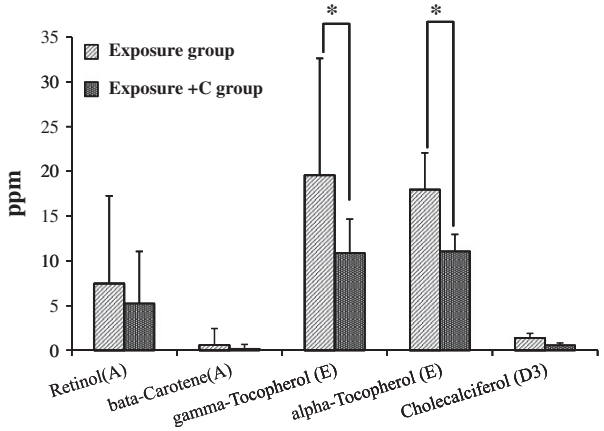

Fig. 3. Average concentrations of fat-soluble vitamins in egg (n = 6). There is a significant difference ($^{*}p < 0.05$) in the amount of vitamin E between Exposure and Exposure+C group.

$$R_{bio_{(i)}} = \left(\frac{Ce_{(i)} \times We \times Ne}{I_{f_{(i)}}} \right)$$

where *Ce* and *We* are an avarage of concentrations and weight per one egg. *Ne* is number of egg for 1 month during steady state. The total intake of PCDDs/PCDFs and DL-PCBs, I_f, was calculated based on the concentrations of feed contaminated for stage-4 and feed dosage. However, the effects of egg laying rate were taken into account in this because egg laying rate at this stage was almost of 100%. Results of the bioaccumulation rate are shown in Fig. 2. The data indicate that the bioaccumulation rate depended on the congener; that is, the lower chlorinated PCDDs/PCDFs congeners showed higher bioaccumulation than the higher chlorinated PCDDs/PCDFs congeners. Bioaccumulation of tetra to hexa chlorinated PCDDs/PCDFs congeners was in the 38–54% range, and hepta and octa chlorinated congeners showed a lower rate of 20% to over 5%. These patterns of individual congeners were similar to those in previous studies (Stephens et al., 1995; Hoogenboom et al., 2006; Pirarad and Pauw, 2006). On the other hand, when compared to the Exposure group, the Exposure+C group showed a significant decline in the percentage of bioaccumulation in the egg as follows: PCDDs/PCDFs, 0–2.2%; non-ortho-PCBs, 3–4.7%; and mono-ortho-PCBs, 10–18%. This difference in bioaccumulation rates for the DL-PCB congeners depended on the molecular structure and carbon properties. Kawashima et al. (2011) conducted an adsorption experiment for DL-PCBs in an *n*-hexane solution with 16 kinds of activated carbons. Their results showed that substances with a planar molecular structure such as non-ortho-PCBs have high adsorption, but ortho-substituted PCBs have lower adsorption. Furthermore, they reported that effective adsorption of DL-PCBs on activated carbon can be obtained depending on the presence of an appropriate pore size on the surface.

In order to express more availability of this work, it is necessary to now discuss level of pollution risk with our results, privious studies and the maximum EU level. Assuming that laying hens are fed with a contaminated feed at least 30 d during steady state, their daily intake is about 100 g per day, and they lay one egg

containing 4.7 g lipid per day, the maximum concentrations estimated in egg, C (pg TEQ g^{-1} fat) were calculated by the equation:

$$C = \frac{\sum(R_{bio(i)} \times I_{f_{(i)}})}{4.7}$$

C shows the maximum concentration in the laying hen's egg exposured by each feed contaminated with the concentrations of PCDDs/PCDFs and DL-PCBs shown in Table 3 at least 30 d. The TEQ concentrations in the feed shown in Table 3 were recalculated by using WHO 2005 TEF and the concentrations of individual congener in feed obtained from previous literatures (Stephens et al., 1995; Pirarad and Pauw, 2005; Hoogenboom et al., 2006; Traag et al., 2006). The TEQ concentrations estimated in the eggs were close to these reference data shown in Table 3. It indicates in spite of hens are fed with animal feeds of less than the maximum EU level: 0.75 ng PCDDs/PCDFs TEQ kg^{-1}, the concentrations in the eggs might be possible to exceed the maximum EU levels: 3 pg PCDDs/PCDFs TEQ g^{-1} egg fat. In the contamination incident in Germany at the end of 2010, BfR (Bundesinstitute fur Risikobewertung) reported none of the PCDDs/PCDFs concentrations in animal feeds exceeded the maximum EU level, the maximum concentration reported in the feed was 0.468 ng PCDDs/PCDFs TEQ kg^{-1}. However, percentage of samples exceeding the maximum EU level of egg was 19%. According to the way of our prediction, if laying hens had been exposed with the lowest contaminated feed in Table 3 without addition of activated carbon at least 1 month, the concentrations in eggs could be 3.9 pg PCDDs/PCDFs TEQ g^{-1} fat which exceeded the maximum EU level. On the other hand, if it had been absorbed our technique, the concentrations in eggs might be less than 0.11 pg PCDDs/PCDFs TEQ g^{-1} fat. Whether this prediction can be proved or not is open to discussion, but we can easily predict that the food pollution risk in Germany might have been reduced by using our technique.

3.3. Distribution in tissues and egg

The concentrations of PCDDs/PCDFs and DL-PCBs in the breast muscle, abdominal fat, and mixed eggs laid from first laid egg to thirtieth are shown in Table 4. Individual congener concentrations are shown on a wet weight basis. Although the highest concentrations for the tissues were in abdominal fat followed by the eggs and then muscle, the congener profiles were similar in all tissues. "Inhibition" in Table 4 means the proportion of the accumulation inhibited compared to concentrations of the Exposure group. The inhibitive effects depended on the compound and congener for DL-PCBs as follows: PCDDs/PCDFs, non-ortho-PCBs, and mono-ortho-PCBs were inhibited by more than 90%, 84%, and 53%, respectively, regardless of the type of tissue. Maes et al. (2005) reported that activated carbon in fish oil had an effect similar to the present results. In present and previous studies, there is enough evidence to show that activated carbon could reduce PCDDs/PCDFs and DL-PCBs in animal bodies (Manara et al., 1984; Yoshimura et al., 1986a,b; Kamimura et al., 1988).

3.4. Fat soluble vitamins in egg

The above results clearly shows that the absorption of PCDDs/PCDFs and DL-PCBs ingested from feed by the intestine can be inhibited by the addition of activated carbon into the contaminated feed. However, the nutritional effects causes by the addition of activated carbon need to be determined to ensure that this technology is practical. Chicken eggs are a well-balanced food containing proteins, lipid, minerals and vitamins. In this study, retinol, beta-carotene, tocopherols and cholecalciferol were determined. These compounds are fat soluble vitamins that cannot be produced in the body and are absolutely essential for its growth. Fig. 3 shows retinol, beta-carotene, γ-tocopherol, α-tocopherol and cholecalciferol concentrations in the egg at steady state. These vitamin concentrations of the Exposure+C group showed lower trends than the Exposure group. The concentrations of γ-tocopherol and α-tocopherol for Exposure+C were significantly reduced by about 40%. When Maes et al. (2005) conducted the adsorption test of PCDDs/PCDFs and DL-PCBs in cod liver oil by using activated carbon, they also determined several oil qualities in the conditions tested. Although quality parameters such as fatty acids, the oxidative stability, and minerals were not significantly influenced by activated carbon (Kawashima et al., 2009; Usydus et al., 2009), retinol content was associated with a reduction in color parameters. There was a linear relationship between color parameters and retinol content. This suggests that discoloration is linked to the adsorption of vitamins by the activated carbon. Realizing a practical application of this new technique may require compensating for the vitamin shortfall caused by the addition of activated carbon.

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