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VITELLOGENIN DETECTION AND CHICK PATHOLOGY ARE USEFUL ENDPOINTS TO EVALUATE ENDOCRINE-DISRUPTING EFFECTS IN AVIAN ONE-GENERATION REPRODUCTION STUDY

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Abstract—To investigate additional endpoints for screening of endocrine disruptors in birds, effects of 17β-estradiol (E2) on one-generation reproduction in the Japanese quail (*Coturnix japonica*) were assessed. Pairs of the 10-week-old Japanese quail were fed a low-phytoestrogen diet containing E2 at 0 (control), 10, 100, and 1,000 ppm for six weeks. In the E2 100- and 1,000-ppm groups, the parental quail represented marked toxic changes including high mortality, decreased food consumption, decreased gonad weights, gross and histologic toxic changes in the reproductive and other organs, and inhibition of the reproduction. However, no adverse effects were observed in the parental quail from the E2 10-ppm group. In the parental males, serum vitellogenin (VTG) concentrations were increased significantly in the E2 10-ppm group, disclosing that serum VTG concentration is one of the highly sensitive endpoints for evaluating estrogenic endocrine activities. In the E2 10-ppm group, number of eggs laid, number of eggs with abnormalities, eggshell strength and thickness, fertility, early and late viabilities of embryos, normal hatchling rate, and clinical signs, mortality, viability, and body weight of chicks at 14 d of age were not affected. However, histopathology of the chicks in the E2 10-ppm group revealed meaningful morphological changes in the reproductive organs, such as cystic dilatation of seminiferous tubules, increased interstitial cells in the testis, and decreased theca cells in the ovary. The present study suggests that serum VTG concentration in the parental quail and histopathology of reproductive organs in the offspring are sensitive endpoints and are useful as additional endpoints in the avian one-generation reproduction test using the Japanese quail for evaluating estrogenic endocrine-disrupting effects.

Keywords—Chick pathology Endocrine disruption Japanese quail One-generation reproduction study Vitellogenin

INTRODUCTION

In the past two decades, endocrine-disrupting chemicals (EDCs), which are any chemicals known or suspected to cause adverse endocrine effects in organisms or their progeny, have been investigated with great attention because of their potential to alter normal functioning of the endocrine system in wildlife and humans. Endocrine-disrupting chemicals have been considered to possess estrogenic or other endocrine activity that could have disruptive endocrine effects in a variety of wildlife species, such as fish, birds, and mammals, as well as humans. Therefore, it has been necessary to develop assessment systems to evaluate any adverse effects of EDCs in a variety of wildlife species. Birds are one of the important wildlife species exposed by environmental pollutants, which may have endocrine-disrupting potential [1]. Birds are fundamentally different from mammals in the control of their sexual differentiation and reproduction system. Aspects of sexual differentiation in birds may make them uniquely sensitive to the effects of EDCs with estrogenic activity [2]. Consequently, separate testing for assessing impact of chemicals with endocrine-disrupting potential to birds is required.

The Organization for Economic Cooperation and Development (OECD) already published the guidelines for testing chemicals in birds, such as Avian Dietary Toxicity Test (Testing Guideline 205) and Avian Reproduction Test (Testing Guideline 206) [3,4]; these guidelines were for the evaluation of any chemical toxicity in birds. Hence, the OECD developed a revised guideline for the Japanese quail (*Coturnix japonica*) or northern bobwhite in 2000, called the Avian Reproduction Toxicity Test (ARTT 2000), to assess the effects of pesticides and other chemicals upon avian health and reproduction [5]. On the other hand, the U.S. Environmental Protection Agency recently proposed a revised draft review paper for the Avian Two-Generation Toxicity Test in 2003 [6]. The protocol is designed for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. It is essential that the two-generation toxicity test be able to assess the impact of endocrine-disrupting chemicals on endocrine-mediated processes as systems organize during embryonic development and they are activated in adult birds [6]. However, the avian two-generation toxicity test is a huge experiment. It is likely that the test should be performed based on a general concern for saving resources, animal usage, and time, and validation of the data obtained is required.

The OECD Guideline 206 and its revised protocol (ARTT 2000) are not yet sufficient to detect mimic endocrine-disrupting effects [4,5]. In the present study, therefore, we investigate new endpoints for evaluating estrogenic endocrine-disrupting effects in the avian one-generation reproduction test, essentially according to the OECD protocol. Additional new endpoints, such as blood levels of test substances, vitel-

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Table 1. Microbiological certification of the WE strain of Japanese quail

Examination items	Methods	Results
Newcastle disease	HI[a]	Negative
Infectious bronchitis	SN[b]	Negative
Avian leukosis (Subtype A and B)	SN	Negative
Avian encephalomyelitis	AGP[c]	Negative
Avian nephritis	SN	Negative
Infectious laryngotracheitis	SN	Negative
Reticuloendotheliosis	AGP	Negative
Marek's disease	AGP	Negative
Infectious Bursal disease	AGP	Negative
Avian reovirus	AGP	Negative
Avian adenovirus	AGP	Negative
Egg drop syndrome–76 virus	HI	Negative
Avian influenza	AGP	Negative
Avian paramyxovirus	HI	Negative
Chicken anemia	SN	Negative
Fowl pox	CO[d]	Negative
Infectious coryza	HI	Negative
Salmonelosis	CO	Negative
Pullorum disease	AG[e]	Negative
Avian mycoplasmosis	AG	Negative
Coccidiosis	FE[f]	Negative

[a] HI = hemagglutination inhibition reaction.
[b] SN = serum neutralization test.
[c] AGP = agarose gel diffusion precipitation reaction.
[d] CO = clinical observation.
[e] AG = agglutination reaction.
[f] FE = fecal examination.

Table 2. Nutrient compositions and contaminants of phytoestrogen-low diet

Examination items	Values	Detection limits
Moisture	10.60%	NA[a]
Crude protein	22.60%	NA
Crude fats	4.10%	NA
Crude fibers	2.30%	NA
Crude ash	8.50%	NA
Carbohydrate	51.90%	NA
Daizin	ND[b]	0.05 mg/100 g
Daizein	ND	0.05 mg/100 g
Genistin	ND	0.05 mg/100 g
Genistein	ND	0.05 mg/100 g
Coumesterol	ND	0.1 mg/100 g
Estradiol	ND	0.01 ppm
Total mercury	ND	0.01 ppm
Cadmium	0.04 ppm	NA
Lead	0.16 ppm	NA
Arsenic	0.7 ppm	NA
Selenium	0.52 ppm	NA
Hexachlorocyclohexane	ND	0.005 ppm
DDT	ND	0.05 ppm
Aldorin	ND	0.01 ppm
Dieldrin	ND	0.01 ppm
Endrin	ND	0.01 ppm
Heptachrol	ND	0.01 ppm
Malathion	ND	0.05 ppm
Parathion	ND	0.05 ppm
Aflatoxins	ND	5 ppb
Polychlorinated biphenyls	ND	0.01 ppm
Nitrosodimethylamine	ND	0.01 ppm
Nitrosodiethylamine	ND	0.01 ppm

[a] NA = not applicable.
[b] ND = not detected.

logenin and histopathology of parental birds, egg weights, eggshell strength, gross pathology, size of gonad, and histopathology of chicks, were included and the natural estrogen 17β-estradiol (E2), which is recommended as a positive control chemical in the assessments of estrogenic endocrine effect elsewhere, was used in the study.

MATERIALS AND METHODS

Chemicals

Technical-grade (98%) E2 was obtained from Sigma Chemical (St. Louis, MO, USA).

Birds

Eighty males and 80 females of the WE strain of Japanese quail (*C. japonica*) were purchased from the Laboratory Animal Research Station of Nippon Institute for Biological Science (Yamanashi, Japan) at six weeks of age. The WE strain has been maintained in the facility under the specific pathogen-free condition (Table 1), and the quail possess wild plumage color and produce eggs with white eggshell. Body weights on the purchase day ranged from 70 to 115 g (97 ± 8 g) in males and 88 to 142 g (124 ± 11 g) in females. After the two-week acclimatization period, the birds, at eight weeks of age, were paired on a one-to-one basis in order of body weights. Subsequently, during the two-week pretreatment period, pairs with no egg laying, aggression, or any adverse clinical signs were removed from the study. The birds were cared for and treated humanely during the experiments in accordance with the Guidelines for Care and Use of Laboratory Animals at the Nippon Institute for Biological Science (1999), Tokyo, Japan.

Environmental conditions, diets, and water

Environmental conditions of the study were controlled according to OECD Guideline 206 and its revised protocol (ARTT 2000) [4,5]. During the two-week acclimatization period, quail of the same sex were housed five per cage (15 cm wide × 40 cm depth × 14 cm height). In the present study, quail cabinets used, including wire-mesh cages with slanting floors and egg-catchers, water providers and food containers, and brooders, were all stainless steel construction. In the two-week pretreatment period and six-week treatment period, a pair of the quail was housed in each same-type cage. The birds were maintained in a barrier-sustained room controlled at 20 to 25°C, 35 to 69% relative humidity, and a 16:8-h light:dark cycle throughout the study. The room air was ventilated with filtered fresh air at 10 to 15 times/h. The parental quail and their offspring had free access to a phytoestrogen-low diet for Japanese quail (Oriental Yeast Industry, Tokyo, Japan) as a basal diet and chlorinated tap water. Component and contaminants of the diet were analyzed at Japan Food Research Laboratories (Tokyo, Japan; Table 2). Contaminants of tap water were analyzed at Yakult Center Institute (Tokyo, Japan).

Eggs were incubated in an incubator controlled at 38.4 to 38.6°C, 56.4 to 70.2% relative humidity, and once per hour of egg-turning cycle. Eggs were transferred to hatching conditions at 15 d of incubation and hatching was completed by 18 d of the incubation. Hatchlings were housed in groups according to pen of origin and maintained in a pen controlled at 35 to 38°C in the first week and 30 to 35°C in the second week. Each hatchling was identified by a foot ring, by which the parent number, treatment week, and laying date were recorded.

Treatment

Before the present study, the dose-finding dietary toxicity test using 16-d-old chicks of the WE strain of Japanese quail

Table 3. Concentration of 17β-estradiol (E2) in the diets. Values represent concentration (ppm)

Preparation time	Groups	Sampling points in the bowl		
		Upper	Middle	Lower
1st preparation (Before the treatment)	Control	NM[a]	Not detected	NM
	E2 10 ppm	9.8	9.7	9.5
	E2 100 ppm	89	88	89
	E2 1,000 ppm	870	860	860
2nd preparation (Treatment week 3)	E2 10 ppm	NM	9.6	NM
	E2 100 ppm	NM	91	NM
	E2 1,000 ppm	NM	910	NM

[a] NM = not measured.

was performed according to the OECD guideline (Testing Guideline 205) [3]. At the termination of the three-week administration period, mortalities of the chicks were 0, 30, 50, 0, and 20% in the E2 0, 625, 1,250, 2,500, and 5,000 ppm groups, respectively. The mortality of the highest dose (E2 5,000 ppm) was less than 50%, so that the median lethal concentration was determined greater than E2 5,000 ppm. In the OECD Guideline 206 [4], the maximum recommended test concentration has been described as 1,000 ppm. Consequently, E2 1,000 ppm was selected as the highest concentration and other concentration levels were decided at E2 100 and 10 ppm, respectively, in accordance with a ratio of 1/10 in the present one-generation reproduction study.

Sixty-four healthy pairs were allocated to four groups. Each group consisted of 16 pairs. Quail in the control group were given a basal diet; quail in the E2 10-, 100-, and 1,000-ppm groups were fed the phytoestrogen-low diet containing 10, 100, and 1,000 ppm of E2, respectively. The E2 diets were prepared two times, such as just before the start of administration and before treatment week 4, and fed for three weeks. Concentrations and homogenous distributions of E2 in the diets were measured by high-performance liquid chromatography at Japan Food Research Laboratories and confirmed (Table 3).

Examinations

Adult birds. During the acclimatization, pretreatment, and treatment periods, the parental birds were observed daily to detect any clinical signs, health conditions, or deaths. Body weights were measured at arrival, coupling (two weeks after the arrival), and the start of treatment period and, subsequently, once a week during the treatment period and at necropsy. Daily food consumption per pair was measured weekly during the pretreatment and treatment periods, and individual test substance intake was calculated with body weight and food consumption. At necropsy, blood samples of all adults that survived to the end of the treatment period were collected under anesthesia of ether inhalation, and sera were separated for measuring concentrations of E2 and VTG. The measurements of serum E2 and VTG were performed in Trans Genic (Kumamoto, Japan) by using enzyme-linked immunosorbent assay kits. Validation of VTG concentrations was based on the standard curve of the concentrated solutions of the standard Japanese quail VTG. The duplicate validation assays of the standard VTG concentrations were performed in each measurement. The birds then were euthanized by ether anesthesia and subjected to a complete necropsy. The testis, ovary, oviduct, liver, spleen, and cloacal gland were weighed and the organ-to-body weight ratios were calculated based on the body weight measured at necropsy. Adult birds that died during the course of the treatment period were subjected to the same procedures, except for the collection of blood samples or measurement of organ weights. The testis, ductus deferens, ovary, oviduct, liver, spleen, kidney, cloacal gland, and other organs with abnormal findings were fixed in 10% neutral buffered formalin. These organs were embedded in paraffin wax, processed routinely to prepare hematoxylin and eosin-stained sections, and examined histopathologically.

Eggs. All eggs produced in the pretreatment and treatment periods were collected daily, numbered individually, and weighed. A total number of eggs was counted in each group, and stored in a cold storage facility until incubation. Before placing the eggs in the incubator, all eggs were candled to check for abnormalities and fine cracks. Abnormal eggs that were cracked, broken, or abnormal externally were numbered, their findings were recorded, and then they were discarded. In each treatment week, the first and second eggs without any abnormalities were used to measure eggshell thickness and strength, respectively, and the remaining eggs were equilibrated to room temperature and set, artificially incubated, and allowed to hatch. To determine eggshell thickness, each egg was cut open longitudinally and washed out and, subsequently, the shells were left to dry with the membrane intact for at least 48 h at room temperature. Eggshell thickness was measured at four points, including both polar portions and two opposite equatorial portions, using a calibrated micrometer. Eggshell strength at the equator of the egg was measured using a strength tester (Harding Tester, Intesco, Chiba, Japan).

Embryos. Fertility and early viability of embryos were checked at 7 d of incubation. Late viability of embryos was checked at 14 d of incubation. All eggs were candled and those that appeared to contain live embryos were placed back into the incubator. The eggs that did not appear to contain a live embryo were opened and examined in order to distinguish between infertility and early embryonic death with a stereoscopic microscope. Fertility, infertility, viability, and embryonic death were recorded. At 14 d of incubation, the eggs were candled for viability of embryos. All live embryos were transferred to a hatcher at 15 d of incubation. Chicks that did not hatch until 18 d of incubation were considered unhatched. Hatchability was calculated as rates of normal hatchlings in fertile eggs.

Chicks. All offspring were maintained until 14 d after hatching and observed daily for clinical signs and mortality. Chicks were housed together by each group and each treatment week. They were weighed at 14 d and were euthanized by ether anesthesia and subjected to a complete necropsy. Briefly, the heart, liver, spleen, stomachs, intestines, and pancreas were removed after the abdomen was opened. These organs and the

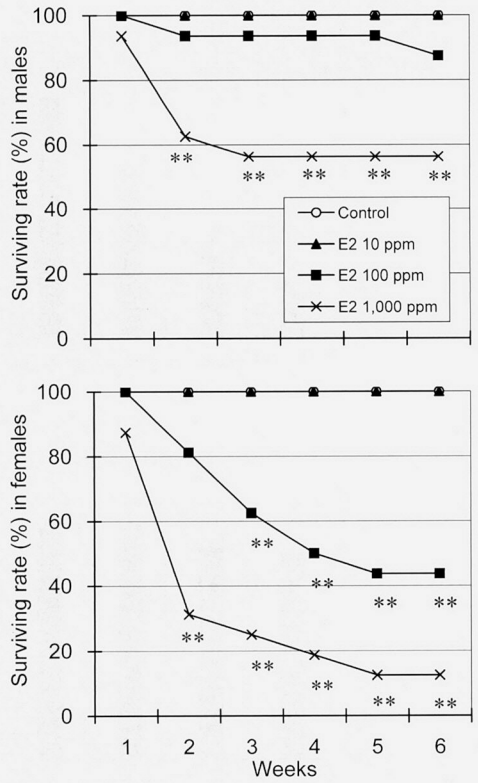

Fig. 1. Surviving rate of male and female parental quail fed the 17β-estradiol (E2)–containing diets. Note significant decreases (**$p < 0.01$) in males from the E2 1,000-ppm group at treatment weeks 2 to 6 and in females from the E2 100- and 1,000-ppm groups at treatment weeks 3 to 6 and treatment weeks 2 to 6, respectively.

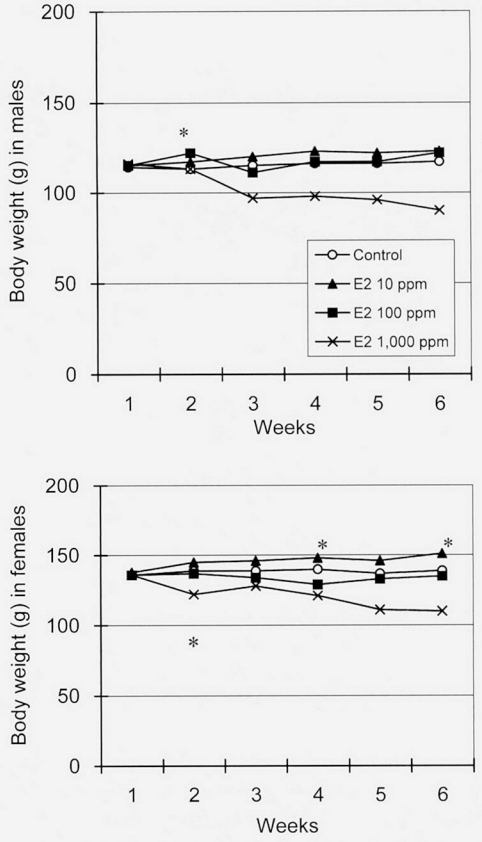

Fig. 2. Body weight of male and female parental quail fed the 17β-estradiol (E2)–containing diets. Note significant increases (* $p < 0.05$) in males from the E2 100-ppm group at treatment week 2 and in females from the E2 10-ppm group at treatment weeks 4 and 6, and a significant decrease (* $p < 0.05$) in females from the E2 1,000-ppm group at treatment week 2.

body of chicks, in which the reproductive and other organs were placed in situ, were fixed in 10% neutral buffered formalin. After fixation, chicks were observed grossly in detail with a stereoscopic microscope and major axis of the testes and ovary were measured using a calibrated micrometer. Chicks that died before necropsy at the termination of the study were not examined histopathologically. The testes and ovaries of the chicks from treatment weeks 1, 2, 3, and 6 were embedded in paraffin wax, processed routinely to prepare hematoxylin and eosin-stained sections, and examined histopathologically. Selected sections of the testis were immunohistochemically stained by the indirect immunoperoxidase method as described previously [7]. Monoclonal anti–proliferating cell nuclear antigen antibody (PC10, Dako, CA, USA) diluted 1:50 was used as a primary antibody and peroxidase-conjugated goat antimouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:800 was used as a secondary antibody. The sections after application of antibodies were visualized by 3,3′-diaminobenzidine and counterstained with methylgreen.

Statistical analysis

Quantitative data were analyzed initially by the Bartlett's test for homogeneity of variance (two-tailed, significance level: 5%). If the data distribution revealed homogeneity, the values were assessed by one-way analysis of variance (significance level: 5%) and, if significant difference was seen between groups, multiple comparisons were performed by the Dunnett's test (two-tailed, significance level: 5 and 1%). If the data distribution was not homogenous, the Kruskal-Wallis test was applied (significance level: 5%) and, if significant difference was seen between groups, ranking comparison was performed by the Dunnett's multiple comparison test (two-tailed, significance level: 5 and 1%). Data of incidences were analyzed by the Fisher's exact probability test. Values of $p < 0.05$ were considered significant.

RESULTS

Adult birds

In the E2 1,000-ppm group, male and female quail began to die at treatment week 1 (Fig. 1). Surviving rates of males and females in the E2 1,000-ppm group were significantly lower ($p < 0.01$) than those in the control group at treatment weeks 2 to 6, and surviving rates of females in the E2 100-ppm group were significantly lower ($p < 0.01$) than those in the control group at treatment weeks 3 to 6. No quail in the E2 10-ppm and control groups died during the treatment period.

Body weights of males in the E2 100-ppm group at treatment week 2 were significantly higher ($p < 0.05$) than those in the control group (Fig. 2). Body weights of females in the E2 10-ppm group at treatment weeks 4 and 6 were significantly higher ($p < 0.05$ and $p < 0.01$, respectively) than those in the control group. Body weights of males and females in the E2 1,000-ppm group decreased gradually during the course of the study (Fig. 2). Body weights of females in the E2 1,000-ppm group at treatment week 2 were significantly lower ($p < 0.05$) than those in the control group.

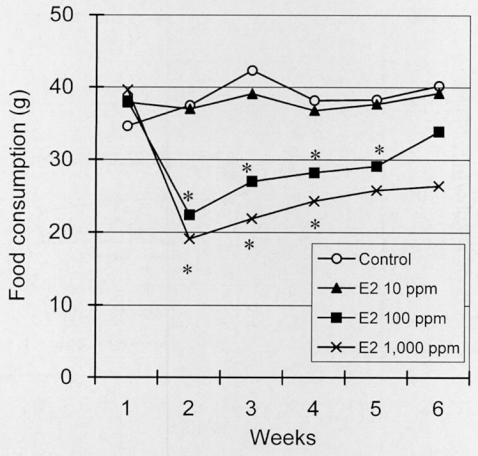

Fig. 3. Food consumption of pairs of parental quail fed the 17β-estradiol (E2)–containing diets. Note significant decreases (*$p < 0.05$) in the E2 100-ppm group at treatment weeks 2 to 5 and in the E2 1,000-ppm group at treatment weeks 2 to 4.

Food consumption of pairs in the E2 100-ppm group decreased markedly and there were significant differences ($p < 0.05$) of food consumption in the E2 100-ppm group at treatment weeks 2 to 5 and in the E2 1,000-ppm group at treatment weeks 2 to 4 (Fig. 3).

At the end of the study, serum E2 concentrations of males in the E2 100- and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group (Fig. 4). Serum E2 concentrations of females in the E2 10-, 100-, and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group. Serum VTG concentrations of males in the E2 10-, 100-, and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group (Fig. 4). Serum VTG concentrations of females in the E2 100- and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group.

Grossly, atrophy of the testis, ductus deferens, and the cloacal gland, swelling of the liver, and discoloration of the kidneys were observed in male birds from the E2 100- and 1,000-ppm groups with high incidences (Table 4). The incidences of these lesions in the E2 100- and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group. Fragility of the testis was detected in males from the E2 100-ppm group with a high incidence, which was significantly higher ($p < 0.01$) than that in the control group. On the other hand, males in the E2 10-ppm group did not show any abnormal findings, except for one male with testis atrophy. In female birds, atrophy and degenerate ova of the ovary and discoloration of the kidneys were observed in the E2 100- and 1,000-ppm groups with high incidences (Table 4). The incidence of ovarian atrophy in the E2 100-ppm group was significantly higher ($p < 0.05$) than that in the control group. The incidences of degenerative ova and discoloration of the kidneys in the E2 100- and 1,000-ppm groups were significantly higher ($p < 0.01$) than those in the control group.

In male birds, weights of the testis and their body-weight ratios in the E2 100- and 1,000-ppm groups were significantly lower ($p < 0.01$) than those in the control group (Table 5). Weights of the cloacal gland and their body-weight ratios in the E2 10-, 100-, and 1,000-ppm groups were significantly lower ($p < 0.01$) than those in the control group. Weights of the liver and their body-weight ratios in the E2 100- and 1,000-ppm groups were significantly higher ($p < 0.01$) than those

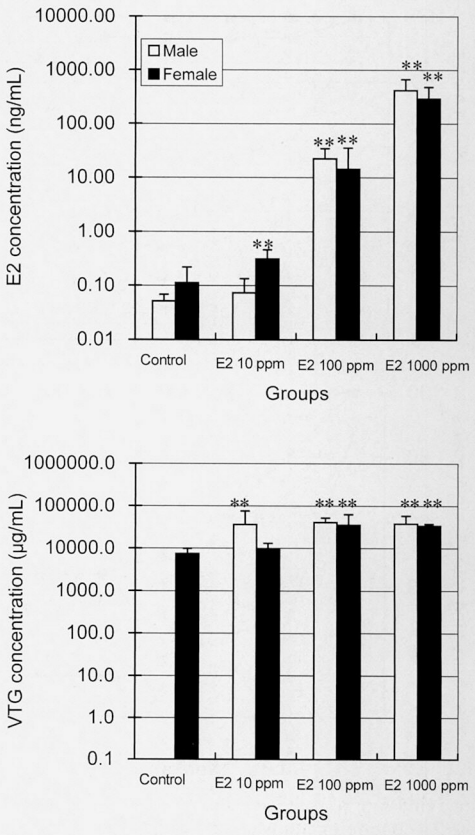

Fig. 4. Serum 17β-estradiol (E2) and vitellogenin (VTG) concentration of male and female parental quail after six-week treatment of E2–containing diets. Note significant increases (** $p < 0.01$) of E2 in males from the E2 100- and 1,000-ppm groups and in females from the E2 10-ppm and greater dose groups. Note significant increases (** $p < 0.01$) of VTG in males from the E2 10-ppm and greater dose groups and in females from the E2 100- and 1,000-ppm groups.

in the control group. Weight of the spleen and its body-weight ratio in the E2 100-ppm group were significantly higher ($p < 0.01$) than those in the control group. In female birds, weight of the ovary and its body-weight ratio in the E2 1,000-ppm group were significantly lower ($p < 0.01$) than those in the control group. The body-weight ratio of the ovary in the E2 10-ppm group was significantly ($p < 0.05$) lower than that in the control group.

Histopathologically, atrophy and degeneration of the seminiferous tubules and a marked decrease in spermatogenesis in the testis were observed in the E2 100- and 1,000-ppm groups with high incidences. The incidences of these findings were significantly higher ($p < 0.01$) than those in the control group (Table 6). Marked atrophy of the ductus deferens and decreased number of the sperm in the ductus deferens, which contained a sparse number of sperm, were observed in the E2 100- and 1,000-ppm groups, with high incidences and their incidences were significantly higher ($p < 0.01$) than those in the control group. Atrophy of the cloacal gland was observed in the E2 100- and 1,000-ppm groups with a high incidence, which was significantly higher ($p < 0.01$) than that in the control group. The glandular epithelial cells of the atrophic cloacal gland possessed few secretory granules in their cytoplasm, showing decreased secretory activity, which was significantly higher ($p < 0.05$ in the E2 10-ppm group, $p < 0.01$ in the E2 100- and 1,000-ppm groups) than that in the control group. Lymphoid cell depletion of the spleen, hepatocyte de-

Table 4. Gross pathology of parental quail fed the 17β-estradiol (E2)–containing diets. Values represent % of birds with findings. * = $p < 0.05$; ** = $p < 0.01$

Findings	Control	E2 10 ppm	E2 100 ppm	E2 1,000 ppm
No. of male birds examined	16	16	16	16
Testis				
Atrophy	0.0 (0/16)	6.3 (1/16)	75.0 (12/16)**	93.8 (15/16)**
Fragility	0.0 (0/16)	0.0 (0/16)	43.8 (7/16)**	0.0 (0/16)
Ductus deferens				
Atrophy	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)**	56.3 (9/16)**
Cloacal gland				
Atrophy	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)**	56.3 (9/16)**
Liver				
Swelling	0.0 (0/16)	0.0 (0/16)	81.3 (13/16)**	50.0 (8/16)**
Kidney				
Discoloration	0.0 (0/16)	0.0 (0/16)	93.8 (15/16)**	62.5 (10/16)**
No. of female birds examined	16	16	16	16
Ovary				
Atrophy	0.0 (0/16)	0.0 (0/16)	31.3 (5/16)*	25.0 (4/16)
Degenerate ova	0.0 (0/16)	6.3 (1/16)	37.5 (6/16)**	75.0 (12/16)**
Kidney				
Discoloration	0.0 (0/16)	0.0 (0/16)	81.3 (13/16)**	68.8 (11/16)**

generation of the liver, and glomerulonephropathy of the kidneys were observed in the E2 100- and 1,000-ppm groups with high incidences. The incidences of these findings were significantly higher ($p < 0.01$) than those in the control group. Glomerulonephropathy was detected in males with discolored kidneys and was characterized by severe scrolosis of the glomeruli, vacuolar degeneration of the renal tubules, and interstitial mineralization. In females, oocyte degeneration and ovum necrosis of the ovary were observed in the E2 100- and 1,000-ppm groups with high incidences. The incidence of oocyte degeneration was significantly higher ($p < 0.05$ in the E2 100-ppm group, $p < 0.01$ in the E2 1,000-ppm group) than that in the control group and the incidence of ovum necrosis in the E2 100- and 1,000-ppm groups was significantly higher ($p < 0.01$) than that in the control group. Atrophy of glandular epithelial cells in the oviducts was observed in the E2 10-ppm and greater dose groups with a high incidence and the incidence in the E2 100- and 1,000-ppm group was significantly higher ($p < 0.01$) than that in the control group (Table 6). Lymphoid cell depletion of the spleen, hepatocyte degeneration of the liver, and glomerulonephropathy of the kidneys were observed in females from the E2 100- and 1,000-ppm groups with high incidences. The incidences of these findings were significantly higher ($p < 0.01$) than those in the control group. On the other hand, the incidence of fatty change in the liver in the E2 100- and 1,000-ppm groups was significantly

Table 5. Organ weight and organ-to–body weight ratio of parental quail fed the 17β-estradiol (E2)–containing diets. Values represent mean ± standard deviation. * = $p < 0.05$; ** = $p < 0.01$

			Organ weight			
Group	Sex	No. of quail	Liver (g)	Spleen (mg)	Testis/Ovary (g)	Cloacal gland (g)
Control	Male	16	2.17 ± 0.58	37.0 ± 9.9	2.74 ± 0.77	3.13 ± 0.58
	Female	16	4.18 ± 0.74	64.4 ± 24.7	0.455 ± 0.140	1.42 ± 0.32
E2 10 ppm	Male	16	2.35 ± 0.71	46.4 ± 12.6	2.66 ± 0.62	2.52 ± 0.55**
	Female	16	4.29 ± 0.49	71.3 ± 41.0	0.364 ± 0.094	1.46 ± 0.47
E2 100 ppm	Male	14	4.32 ± 0.86**	54.5 ± 15.0**	0.79 ± 1.07**	1.63 ± 0.38**
	Female	6	4.63 ± 0.74	56.9 ± 19.0	0.335 ± 0.188	1.34 ± 0.53
E2 1,000 ppm	Male	9	4.13 ± 0.99**	35.1 ± 13.7	0.03 ± 0.01**	1.46 ± 0.33**
	Female	2	4.65 ± 1.20	76.5 ± 44.7	0.098 ± 0.042**	1.96 ± 0.49
			Organ to body weight ratio (%)			
Group	Sex	No. of quail	Liver	Spleen	Testes/ovary	Cloacal gland
Control	Male	16	1.83 ± 0.39	0.0316 ± 0.0080	2.36 ± 0.68	2.67 ± 0.40
	Female	16	2.96 ± 0.54	0.0457 ± 0.0179	0.322 ± 0.099	1.00 ± 0.23
E2 10 ppm	Male	16	1.89 ± 0.48	0.0377 ± 0.0104	2.16 ± 0.49	2.05 ± 0.46**
	Female	16	2.82 ± 0.30	0.0473 ± 0.0282	0.240 ± 0.063*	0.96 ± 0.32
E2 100 ppm	Male	14	3.48 ± 0.50**	0.0444 ± 0.0129*	0.62 ± 0.82**	1.32 ± 0.28**
	Female	6	3.40 ± 0.72	0.0405 ± 0.0115	0.233 ± 0.117	1.00 ± 0.51
E2 1,000 ppm	Male	9	3.99 ± 0.71**	0.0351 ± 0.0155	0.03 ± 0.01**	1.40 ± 0.22**
	Female	2	4.38 ± 1.07	0.0719 ± 0.0412	0.093 ± 0.039**	1.85 ± 0.49**

Table 6. Histopathology of parental quail fed the 17β-estradiol (E2)–containing diets. Values represent % of birds with findings. * = $p < 0.05$; ** = $p < 0.01$

Findings	Control	E2 10 ppm	E2 100 ppm	E2 1,000 ppm
No. of male birds examined	16	16	16	15
Testis				
Tubular degeneration	0.0 (0/16)	0.0 (0/16)	100.0 (16/16)**	100.0 (15/15)**
Tubular atrophy	0.0 (0/16)	0.0 (0/16)	68.8 (11/16)**	93.3 (14/15)**
Decreased spermatogenesis	0.0 (0/16)	6.3 (1/16)	100.0 (16/16)**	93.3 (14/15)**
Ductus deferens				
Atrophy	0.0 (0/16)	0.0 (0/16)	100.0 (16/16)**	33.3 (3/15)**
Decreased number of sperm	0.0 (0/16)	6.3 (1/16)	100.0 (16/16)**	100.0 (15/15)**
Cloacal gland				
Glandular atrophy	0.0 (0/16)	0.0 (0/16)	100.0 (16/16)**	80.0 (12/15)**
Decreased secretory activity	0.0 (0/16)	31.3 (5/16)*	100.0 (16/16)**	66.7 (10/15)**
Spleen				
Lymphoid cell depletion	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)**	93.3 (14/15)**
Liver				
Hepatocyte degeneration	0.0 (0/16)	0.0 (0/16)	50.0 (8/16)**	86.7 (13/15)**
Fatty change	50.0 (8/16)	75.0 (12/16)	43.8 (8/16)	6.7 (1/15)
Kidney				
Glomerulonephropathy	0.0 (0/16)	0.0 (0/16)	93.8 (15/16)**	86.7 (13/15)**
No. of female birds examined	16	16	16	16
Ovary				
Follicle degeneration	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)*	100.0 (16/16)**
Ovum necrosis	0.0 (0/16)	0.0 (0/16)	75.0 (12/16)**	56.3 (9/16)**
Oviduct				
Glandular atrophy	0.0 (0/16)	25.0 (4/16)	81.3 (13/16)**	85.7 (12/14)**
Cloacal gland				
Glandular atrophy	0.0 (0/16)	0.0 (0/16)	50.0 (8/16)**	14.3 (2/14)
Decreased secretory activity	0.0 (0/16)	0.0 (0/16)	12.5 (2/16)	0.0 (0/14)
Spleen				
Lymphoid cell depletion	0.0 (0/16)	12.5 (2/16)	75.0 (12/16)**	100.0 (12/12)**
Liver				
Hepatocyte degeneration	0.0 (0/16)	0.0 (0/16)	56.3 (9/16)**	80.0 (12/15)**
Fatty change	75.0 (12/16)	93.8 (15/16)	37.5 (6/16)*	0.0 (0/15)**
Kidney				
Glomerulonephropathy	0.0 (0/16)	0.0 (0/16)	87.5 (14/16)**	100.0 (15/15)**

lower ($p < 0.05$ in the E2 100-ppm group, $p < 0.01$ in the E2 1,000-ppm group) than that in the control group.

Eggs and embryos

Number of eggs laid in the E2 100-ppm group at treatment weeks 2 to 6 was significantly lower ($p < 0.01$ at treatment weeks 2 to 5, $p < 0.05$ at treatment week 6) than those in the control group (Table 7). No eggs were laid in the E2 1,000-ppm group in treatment weeks 2 to 6 and the number of eggs laid in the E2 1,000-ppm group at treatment week 1 was significantly lower ($p < 0.01$) than that in the control group. An incidence of eggs with abnormalities in the E2 10-ppm group was significantly higher ($p < 0.01$) than that in the control group (Table 7). Egg weights in the E2 10-ppm group at treatment weeks 4 to 6 were significantly higher ($p < 0.05$) than those in the control group (Table 7). No statistically significant changes of eggshell thickness or eggshell strength were detected in any E2 treatment groups at any treatment weeks.

Fertility of the E2 10-ppm group at treatment weeks 2 and 5 was significantly lower ($p < 0.01$) than that in the control group, and fertility of the E2 100-ppm group at treatment weeks 2 to 6 was significantly lower ($p < 0.01$) than that in the control group (Table 7). Only four eggs were incubated in the E2 1,000-ppm group from treatment week 1 and fertilized, whereas no eggs were incubated during treatment weeks 2 to 6.

Viability of embryos at 7 d of incubation in the E2 10-ppm group at treatment week 5 was significantly lower ($p < 0.05$) than that in the control group (Table 8). No statistically significant changes of viability of embryos at 14 d of incubation (Table 8) or normal hatchling rates were detected in any E2 treatment groups at any treatment weeks.

Chicks

Viability of offspring at 14 d old in the E2 10-ppm group at treatment week 4 was significantly higher ($p < 0.05$) than that in the control group (Table 9). No statistically significant changes of body weights of offspring at 14 d old were detected in any E2 treatment groups (Table 9).

Grossly, cyst formation in the testis was observed in six of 77 chicks (7.8%) in the E2 10-ppm group, and the incidence of this finding in the E2 10-ppm group was significantly higher ($p < 0.05$) than that in the control group (Table 10). Markedly decreased size of the testis was observed in two out of 80 chicks (2.5%) in the control group and six out of 77 chicks

Table 7. Comparison of eggs from parental quail with 17β-estradiol (E2) treatment. * = $p < 0.05$; ** = $p < 0.01$

	No. of eggs laid[a] at administration week					
Group	1	2	3	4	5	6
Control	4.9 ± 2.0 (16)	5.8 ± 1.1 (16)	5.8 ± 1.3 (16)	5.3 ± 1.7 (16)	5.4 ± 2.1 (16)	5.4 ± 2.0 (16)
E2 10 ppm	5.0 ± 1.5 (16)	5.6 ± 1.3 (16)	5.7 ± 1.0 (16)	5.6 ± 1.5 (16)	5.6 ± 1.8 (16)	5.6 ± 1.1 (16)
E2 100 ppm	4.5 ± 1.8 (16)	1.2 ± 1.8 (16)*	0.8 ± 2.0 (12)*	1.6 ± 2.3 (8)**	1.7 ± 2.4 (7)**	1.3 ± 2.3 (6)*
E2 1,000 ppm	2.6 ± 1.2 (16)*	0.0 ± 0.0 (9)**	0.0 ± 0.0 (4)**	0.0 ± 0.0 (3)**	0.0 (1)	0.0 (1)
	Incidence of eggs with abnormalities (%)[b] at administration week					
Group	1	2	3	4	5	6
Control	7.7 (6/78)	5.4 (5/92)	7.6 (7/92)	9.4 (8/85)	15.1 (13/86)	14.0 (12/86)
E2 10 ppm	6.3 (5/80)	14.4 (13/90)*	4.4 (4/91)	6.7 (6/89)	7.9 (7/89)	6.7 (6/90)
E2 100 ppm	4.2 (3/72)	5.6 (1/18)	10.0 (1/10)	23.1 (3/13)	8.3 (1/12)	25.0 (2/8)
E2 1,000 ppm	4.9 (2/41)	NA[c] (0/0)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
	Egg weight (g)[a] at administration week					
Group	1	2	3	4	5	6
Control	10.1 ± 0.6 (13)	10.2 ± 0.7 (16)	10.5 ± 0.6 (15)	10.5 ± 0.6 (15)	10.5 ± 0.5 (14)	10.5 ± 0.6 (14)
E2 10 ppm	10.0 ± 1.0 (14)	10.9 ± 0.7 (14)	11.0 ± 1.0 (15)	11.3 ± 0.9 (14)*	11.3 ± 0.9 (14)*	11.4 ± 0.8 (14)*
E2 100 ppm	10.4 ± 0.7 (14)	10.6 ± 1.3 (3)	11.6 ± 0.6 (2)	11.2 ± 1.4 (3)	11.5 ± 1.3 (3)	12.2 (1)
E2 1,000 ppm	10.2 ± 0.7 (6)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)
	Fertility (%)[d] at administration week					
Group	1	2	3	4	5	6
Control	100.0 (32/32)	96.4 (53/55)	90.2 (46/51)	91.5 (43/47)	97.8 (44/45)	90.9 (40/44)
E2 10 ppm	91.4 (32/35)	66.7 (32/48)**	83.6 (46/55)	86.8 (46/53)	79.6 (43/54)**	86.5 (45/52)
E2 100 ppm	92.3 (24/26)	28.6 (2/7)**	0.0 (0/5)**	0.0 (0/4)**	0.0 (0/6)**	0.0 (0/3)**
E2 1,000 ppm	100.0 (4/4)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)

[a] Values represent mean ± standard deviation; values in parentheses represent number of pairs examined.
[b] Values in parentheses represent number of eggs with abnormalities/number of eggs laid.
[c] NA = not available because of no eggs laid.
[d] Values in parentheses represent number of fertile eggs/number of eggs set.

(7.8%) in the E2 10-ppm group. Enlargement of the testis was observed in two out of 77 chicks (2.6%) in the E2 10-ppm group and two out of five chicks (40.0%) in the E2 100-ppm group, and the incidence in the E2 100-ppm group was significantly higher ($p < 0.01$) than that in the control group. In female chicks, malocclusion, cyst formation, and partial defect of the oviduct were observed in one chick each in the E2 10-ppm group, and no chicks with those findings were detected in the control, E2 100-ppm, and E2 1,000-ppm groups (Table 10).

Histopathologically, cystic dilatation of the seminiferous tubules in the testis was observed in the E2 10- and 100-ppm groups with high incidences, which were significantly higher ($p < 0.01$) than those in the control group (Table 10, Fig. 5). The lesion was characterized by aberrantly dilated seminiferous tubules, which were covered with a single layer of columnar, ciliated epithelial cells (Fig. 5A, B), and by multiple cysts of the tubules, which were sized variously (Fig. 5C). The luminal surfaces of the cysts were covered with a single layer of flattened squamous epithelial cells (Fig. 5D). In the im-

Table 8. Viability of embryos. Values represent % of viable embryos. * = $p < 0.05$

	Early viability (7-d incubation) at administration week					
Group	1	2	3	4	5	6
Control	93.8 (30/32)[a]	98.1 (52/53)	100.0 (46/46)	97.7 (42/43)	95.5 (42/44)	97.5 (39/40)
E2 10 ppm	93.8 (30/32)	100.0 (32/32)	97.8 (45/46)	91.3 (42/46)	79.1 (34/43)*	93.3 (42/45)
E2 100 ppm	95.8 (23/24)	100.0 (2/2)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
E2 1,000 ppm	100.0 (4/4)	NA[b] (0/0)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
	Late viability (14-d incubation) at administration week					
Group	1	2	3	4	5	6
Control	86.7 (26/30)[c]	88.5 (46/52)	91.3 (42/46)	97.6 (41/42)	97.6 (41/42)	94.9 (37/39)
E2 10 ppm	93.3 (28/30)	100.0 (32/32)	93.3 (42/45)	100.0 (42/42)	94.1 (32/34)	95.2 (40/42)
E2 100 ppm	91.3 (21/23)	100.0 (2/2)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
E2 1,000 ppm	75.0 (3/4)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)

[a] Values in parentheses represent number of viable embryos at 7-d incubation/number of fertile eggs.
[b] NA = not available because of no fertile eggs or no eggs laid.
[c] Values in parentheses represent number of viable embryos at 14-d incubation/number of embryos at 7-d incubation.

Table 9. Viability and body weight of 14-d-old chicks from parental quail with 17β-estradiol (E2) treatment. * = $p < 0.05$

	Viability (%)[a] at administration week					
Group	1	2	3	4	5	6
Control	80.8 (21/26)	75.6 (31/41)	82.1 (32/39)	67.5 (27/40)	81.1 (30/37)	73.5 (25/34)
E2 10 ppm	73.1 (19/26)	66.7 (18/27)	78.4 (29/37)	89.5 (34/38)*	73.3 (22/30)	74.4 (29/39)
E2 100 ppm	58.8 (17/10)	50.0 (1/2)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
E2 1,000 ppm	100.0 (2/2)	NA[b] (0/0)	NA (0/0)	NA (0/0)	NA (0/0)	NA (0/0)
	Body weight (g)[c] at administration week					
Group	1	2	3	4	5	6
Control	47.4 ± 5.0 (21)	46.7 ± 5.4 (31)	48.1 ± 5.1 (32)	48.0 ± 6.1 (27)	44.8 ± 4.3 (30)	46.3 ± 6.3 (25)
E2 10 ppm	47.2 ± 3.4 (19)	48.2 ± 5.8 (18)	46.5 ± 5.0 (29)	48.0 ± 5.8 (34)	47.0 ± 6.1 (22)	48.0 ± 5.1 (29)
E2 100 ppm	47.8 ± 5.0 (10)	48.5 (1)	NA (0)	NA (0)	NA (0)	NA (0)
E2 1,000 ppm	41.1 ± 0.1 (2)	NA (0)	NA (0)	NA (0)	NA (0)	NA (0)

[a] Values in parentheses represent number of surviving chicks/number of chicks hatched normally.
[b] NA = not available because of no chicks.
[c] Values represent mean ± standard deviation; values in parentheses represent number of chicks.

munohistochemical staining for proliferating cell nuclear antigen, these transformed epithelial cells covered with the dilated tubules and multiple cysts possessed very little proliferating activity, being compared with the normal seminiferous epithelial cells (Fig. 6). Marked proliferation of the testicular interstitial cells were observed in male chicks in the E2 10-ppm group and the incidence was significantly higher ($p < 0.01$) than that in the control group (Fig. 7). The lesion was accompanied with the cystic tubular dilatation and resulted in increased amount of the stroma in the lesions. Swelling of the seminiferous epithelial cells was observed in one out of 52 chicks (1.9%) in the E2 10-ppm group and two out of five chicks (40.0%) in the E2 100-ppm group, and the incidence of the lesion in the E2 100-ppm group was significantly higher ($p < 0.01$) than that in the control group. Pigment deposition (i.e., melanocyte infiltration) was detected in two out of 52 chicks in the E2 10-ppm group and one out of five chicks in the E2 100-ppm group. In female chicks in the E2-treated groups, the theca cells surrounding the follicles decreased in number, resulting in thinning of the theca (Fig. 8). The incidences of the lesion in the E2 10-, 100-, and 1,000-ppm groups were significantly higher ($p < 0.01$ in the E2 10- and 100-ppm groups, $p < 0.05$ in the E2 1,000-ppm group) than those in the control group (Table 10). Follicle hypoplasia, characterized by marked decrease of the primary and secondary follicles in the ovary, was observed in one chick in the E2 10-

Table 10. Gross pathology and histopathology of 14-d-old chicks from parental quail with 17β-estradiol (E2) treatment. Values represent % of chicks with findings. * = $p < 0.05$; ** = $p < 0.01$

Findings of gross pathology	Control	E2 10 ppm	E2 100 ppm	E2 1,000 ppm
No. of male birds examined	80	77	5	1
Testis				
Cyst formation	0.0 (0.80)	7.8 (6/77)*	0.0 (0/5)	0.0 (0/1)
Small in size	2.5 (2/80)	7.8 (6/77)	0.0 (0/5)	0.0 (0/1)
Large in size	0.0 (0/80)	2.6 (2/77)	40.0 (2/5)**	0.0 (0/1)
No. of female birds examined	86	74	6	1
Oviduct				
Retained right oviduct	0.0 (0/86)	1.4 (1/74)	0.0 (0/6)	0.0 (0/1)
Partial defect	0.0 (0/86)	1.4 (1/74)	0.0 (0/6)	0.0 (0/1)
External appearance				
Malocclusion	0.0 (0/86)	1.4 (1/74)	0.0 (0/6)	0.0 (0/1)
Findings of histopathology	Control	E2 10 ppm	E2 100 ppm	E2 1,000 ppm
No. of male birds examined	52	52	5	1
Testis				
Cystic dilatation of tubules	0.0 (0/80)	21.2 (11/52)**	40.0 (2/5)**	0.0 (0/1)
Increased interstitial cells	0.0 (0/52)	28.8 (15/52)**	0.0 (0/5)	0.0 (0/1)
Tubular epithelium swelling	0.0 (0/52)	1.9 (1/52)	40.0 (2/5)**	0.0 (0/1)
Pigment deposition	0.0 (0/52)	3.8 (2/52)	20.0 (1/5)	0.0 (0/1)
No. of female birds examined	57	43	6	1
Ovary				
Decreased theca cells	0.0 (0/57)	41.9 (18/43)**	66.7 (4/6)**	100.0 (1/1)*
Follicle hypoplasia	0.0 (0/57)	2.3 (1/43)	0.0 (0/6)	0.0 (0/1)
Oviduct				
Retained right oviduct	0.0 (0/57)	2.3 (1/43)	0.0 (0/6)	0.0 (0/1)

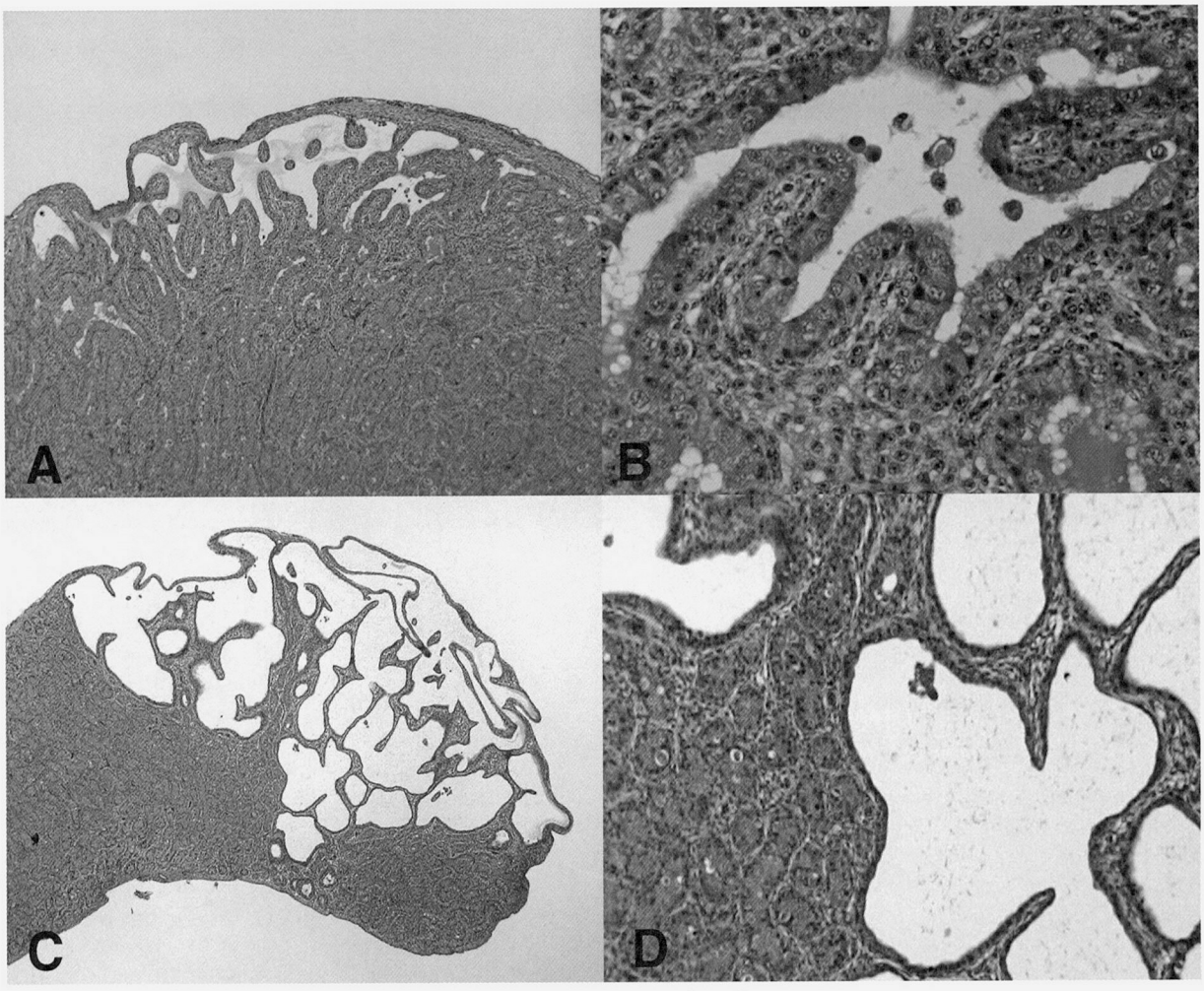

Fig. 5. Section of the testis in chicks from parental quail in the 17β-estradiol 10-ppm group. (**A**) Aberrant dilatation of the seminiferous tubules in the surface region (×150). (**B**) High magnification of (**A**). Note a single layer of columnar epithelial cell with cilia lining the tubules (×650). (**C**) Cystic dilatation of seminiferous tubules and multiple cysts formation (×80). (**D**) High magnification of (**C**). Note a single layer of squamous epithelial cells lining the tubules (×250). Hematoxylin and eosin staining.

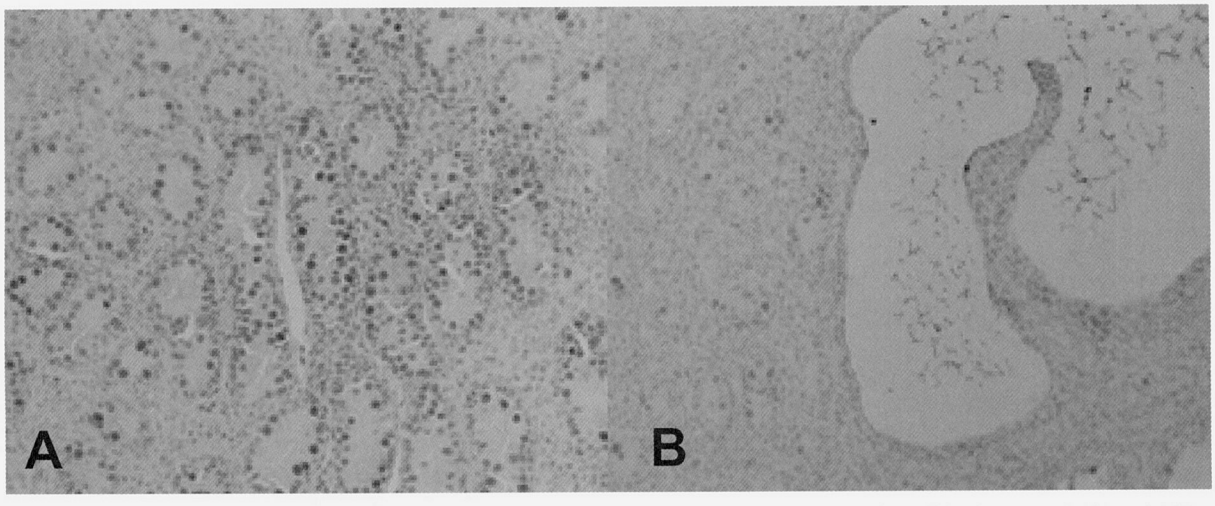

Fig. 6. Immunostaining for proliferative nuclear antigen (PCNA) in the testis of chicks from parental quail in the control (**A**) and 17β-estradiol 10-ppm (**B**) groups. (**A**) Note frequent PCNA positive reactions in the seminiferous tubular epithelium. (**B**) Note little PCNA positive reactions in the seminiferous tubular epithelium. Indirect immunoperoxidase method, counterstained with methylgreen (×300).

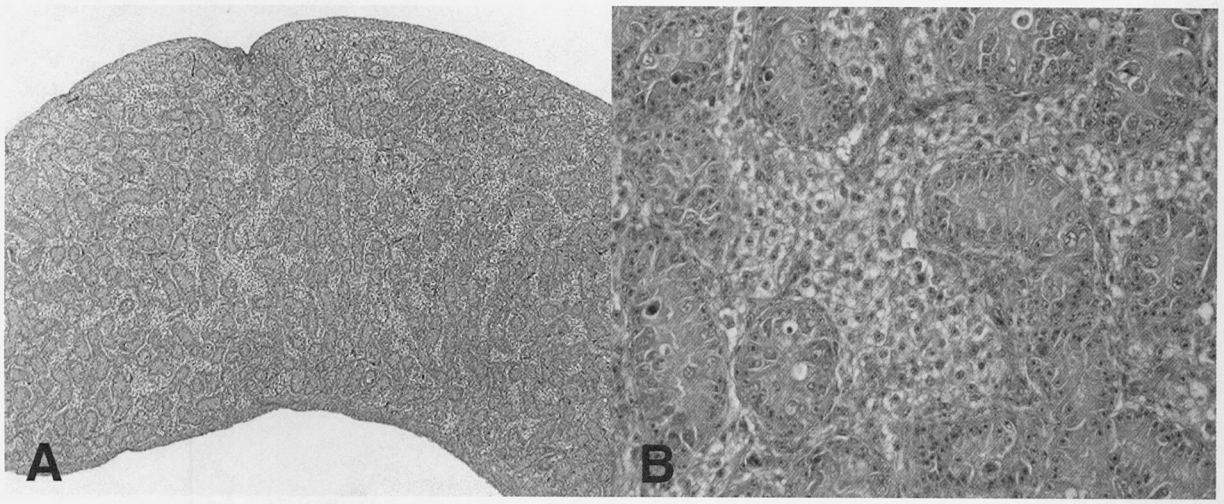

Fig. 7. Section of the testis in chicks from parental quail in the 17β-estradiol 10-ppm group. (**A**) Note marked proliferation of the interstitial cells (×80). (**B**) High magnification of (**A**) at ×450. Hematoxylin and eosin staining.

ppm group. Persistent right oviduct also was detected in another chick in the E2 10-ppm group.

DISCUSSION

The WE strain of Japanese quail (*C. japonica*) used in the present study has been developed as the standard strain in Japan and maintained under the barrier-sustained system. The WE strain is specific–pathogen-free (Table 1) and the WE quail produce plain whitish eggs, which are an advantage in candling. The Japanese quail has been used extensively in reproductive toxicity testing in the European community and, to a lesser extent, in the United States [6]. Therefore, the Japanese quail is an acceptable species in the OECD Guideline 206 [4]. Moreover, the Japanese quail is recommended as one of the preferred test species due to its small size, high fecundity, well-characterized reproductive biology, and, in particular, very rapid incubation and maturation stages [6,8]. In addition, it has been described that precocial species, such as quail, appear to be most sensitive to EDC effects during embryonic development [2,9].

In the present study, parental quail and their offspring were fed a phytoestrogen-low diet, which contained few components of phytoestrogens such as daizin, daizein, genistin, genistein, coumesterol, or estradiol (Table 2). It seems that any factors affecting endocrine systems should be as few as possible in the tests for evaluating endocrine-disrupting chemicals because natural phytoestrogens have been reported to reduce egg reproduction and delay onset of egg laying [10]. Concentrations of E2 in the diets of each treatment group ranged from 86.0 to 98.0% of the added doses, which seemed sufficient for use in the study, although the present study did not measure stability of the E2 in the diets. The guidelines of the OECD and the U.S. Environmental Protection Agency required that the dietary concentrations of the test substance should not fall below 80% of the initial concentrations [4,5,11] (http://www.epa.gov/oppts/oppts_harmonized/850_ecological_effects_test_guidelines/drafts/850–2300.pdf).

Unfortunately, the parental Japanese quail fed 100 and 1,000 ppm of E2 for six weeks showed marked toxic changes, including high mortality, decreased food consumption, decreased gonad weights, high incidence of gross and histologic toxic changes in many organs, and inhibition of reproduction. However, no adverse effects were observed in the parental quail of the E2 10-ppm group. On the basis of these results,

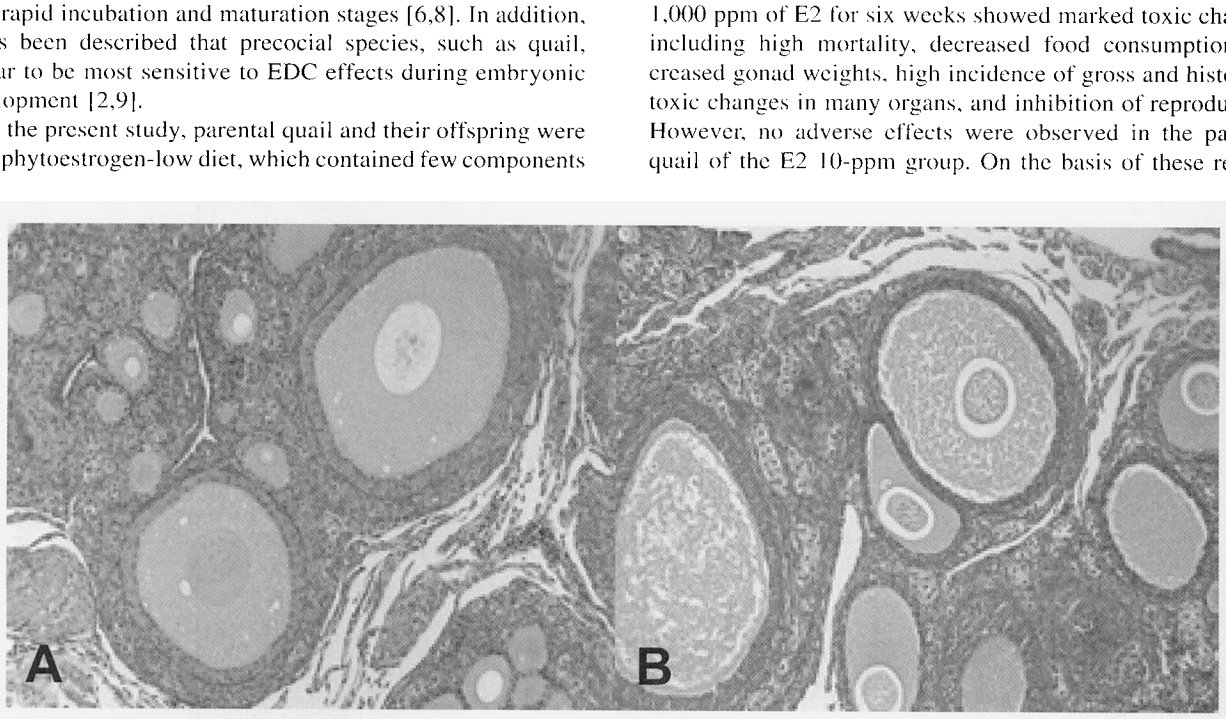

Fig. 8. Section of the ovary in chicks from parental quail in the control (**A**) and 17β-estradiol 10-ppm (**B**) groups. (**A**) Note no noticeable changes in the ovary. (**B**) Note decreased number of the theca cells in the ovary. Hematoxylin and eosin staining (×250).

it was estimated that the no-observable-adverse-effect level of E2 under the present study was 10 ppm. In the preliminary dose-finding dietary toxicity test, dose-dependent toxicity of E2 is unclear in 16-d-old Japanese quail. Mortalities of chicks were 30 and 50% in the E2 635-and 1,250-ppm groups, respectively, whereas mortalities were 0 and 20% in the E2 2,500- and 5,000-ppm groups, respectively. These results showed that dietary administration of E2 induced dose-independent toxicity in the quail chicks, suggesting that sensitivity of Japanese quail to E2 may be related to age. It has been reported that onset of egg laying, egg production, and egg fertility in adult Bobwhite quail were inhibited by dietary dosages of 1,000 mg/d of estriol, diethylstilbestrol, and β-estradiol-3-benzoate [12]. It is likely that adult Japanese quail should be used in the preliminary dose-finding test for chemicals with estrogenic actions, in accordance with draft guidelines of the avian reproduction toxicity test and avian two-generation toxicity test [5,6].

Severe renal toxicities, such as glomerulopathy, renal tubular degeneration, and interstitial calcification, were induced in the parental quail of the E2 100- and 1,000-ppm groups. It seems that these pathological changes of the kidney were major causes of death in the parental quail. Ethynyl estradiol and estrogenic endocrine-disrupting chemicals, such as nonylphenol and octylphenol, enhanced liver VTG II and very low-density lipoprotein (apoVLDL) II mRNA expressions [13]. Estradiol implants in chicks have been described to result in marked elevations of plasma lipoproteins [14]. Induction of VTG following intramuscular injections of E2 caused increased concentrations of total protein, total calcium, and protein-bound phosphorus concentrations in the plasma of mature male Japanese quail [15]. In the present study, VTG was induced in male quail of the E2 10-ppm and greater dose groups, and marked increases of serum VTG concentration were detected in female quail of the E2 100-ppm and greater dose groups. It is well-known that lipoproteins form large complexes in the blood and play the role of transporters of plasma lipids. Recently, several human cases of lipoprotein-related glomerulopathy or nephritic syndrome have been reported [16,17] and experimentally induced levels of apolipoproteins were related closely with glomerular injury [18]. It is presumed that elevated blood levels of VTG and lipoproteins in the adult quail fed E2 diets may contribute to develop glomerular lesions, although lipoproteins were not measured in the present study.

It is noteworthy in birds and other egg-laying animals that VTG is the yolk precursor phosphoprotein, which is synthesized in the liver, transported via the vascular system, taken up by the oocytes, and processed enzymatically into yolk proteins [19]. The egg yolk proteins do not appear to be synthesized by roosters or by immature chickens, but synthesis of these proteins is induced in the liver of such animals by administration of estrogen [20]. A variety of environmental contaminants with estrogenic actions have been described to induce vitellogenesis in mature male Japanese quail following intramuscular injections [15]. In the present study, serum VTG concentrations were increased significantly in the male birds of the E2 10-ppm group, which showed no adverse toxic effects. These results suggest that serum VTG concentration is one of the highly sensitive endpoints in male quail for evaluating estrogenic endocrine activities of the test substance.

In the E2 10-ppm group, egg reproduction, eggshell thickness, eggshell strength, late viability of embryos, or normal hatchability were not affected by the parental dietary administration of E2. However, in the E2 10-ppm group, egg weights increased at treatment weeks 4, 5, and 6, and the incidence of abnormal eggs was significantly high at treatment week 2. In addition, fertility and early viability of embryos in the E2 10-ppm group showed significant declines at treatment weeks 2 and 5 and at treatment week 5, respectively. It is not clear if these changes may be related to endocrine-disrupting effects of E2, because the changes were sporadic and not constant during the administration period.

In offspring, clinical signs, mortality, body weights at 14 d of age, or size of the gonad in the E2 10-ppm group showed no significant changes, compared with those in the control group. However, histopathology of the chicks examined as an additional new endpoint revealed morphological changes in the testis and ovary. In male chicks, cystic dilatation of the testicular tubules resulted from metaplastic changes of the seminiferous epithelium, in which a multilayered epithelium transformed into a single layer of squamous or ciliated epithelium. The immunohistochemical investigation using anti–proliferating cell nuclear antigen antibody revealed that the affected seminiferous epithelial cells in the lesions had very little proliferating activity. It is conceivable that these affected seminiferous epithelia of the chicks with the parental dietary treatment of E2 may develop imperfect spermatogenesis. In addition, increased number of the interstitial cells and swelling of the seminiferous epithelial cells in the testis were observed in the chicks of the E2 10-ppm group with a significantly high incidence. In female chicks, the theca cells decreased significantly in number in the E2-treated groups. It is well-known that the theca interna cells produce androgens and progestins and the theca externa cells produce estrogens using androgens as substrate [21]. It is likely that the female chicks with the parental dietary treatment of E2 may affect steroidogenesis of the ovary. These are the first descriptions of morphological changes in the testis and ovary in the chicks, which were examined in the avian one-generation reproduction test for testing parental dietary effects of E2 according to the draft guideline ARTT 2000. Further detailed characterizations of the testicular and ovarian lesions are needed to understand potential reproductive effects of E2 in the F1 generation of the Japanese quail. It is obvious that pathological investigation of offspring is one of the sensitive endpoints in the one-generation reproductive study for evaluating estrogenic endocrine disruptive effects in the Japanese quail.

CONCLUSION

In conclusion, the measurements of serum VTG concentration in the parental quail and histopathology of reproductive organs in the F1 chicks are very sensitive endpoints, which have the potential to disclose endocrine-disrupting effects, and are useful as additional endpoints in the avian one-generation reproduction test using the Japanese quail for evaluating estrogenic endocrine-disrupting effects.

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