J Toxicol Pathol 2004; 17: 245–252

Original

A New Screening Model Using F_1(AWE × WE) Japanese Quail Embryo for Evaluating Sex Reversal Effects

Kazumoto Shibuya[1], Makoto Mizutani[1], Masaru Wada[2], Kazuo Sato[1], and Tetsuo Nunoya[1]

[1]Nippon Institute for Biological Science, 9–2221–1 Shinmachi, Ome, Tokyo 198–0024, Japan
[2]College of Liberal Arts and Sciences, Tokyo Medical and Dental University, 2–8–30 Kohnodai, Ichikawa, Chiba 272–0827, Japan

Abstract: F_1(AWE × WE) strain of Japanese quail (*Corturnix japonica*) is produced from a mating between male AWE (albino plumage color) and female WE (wild plumage color) strains of Japanese quail. Male and female offspring exhibit wild and albino plumage colors, respectively, ruled by a criss-cross inheritance. F_1(AWE × WE) eggs received 17 beta-estradiol (E_2) or methyltestosterone (MT) at 0, 20, 200, 2000, and 20000 ng/egg just before incubation. At 16 days of incubation, embryos were subjected to a complete necropsy and their gonads were grossly observed and examined histopathologically. Viabilities of the embryos at 16 days of incubation were not significantly different between the control and all E_2 groups and between the control and the MT 20, 200 and 2000 ng groups, whereas viability of the MT 20000 ng group was significantly lower than that of the control group. Grossly, genetic sex confirmed by plumage colors coincided completely with sex phenotype of the gonads in all embryos of the control and treated groups. Histopathologically, E_2 exposure induced a dose-dependent feminization such as ovotestis of the left testis. No abnormalities were detected in any male embryos of all the MT groups. E_2 and MT exposures induced no noticeable changes in the ovaries of any embryos. The present study suggests that the sex reversal test using F_1(AWE × WE) Japanese quail embryos may be a rapid and cost-effective tool to evaluate screening feminization effects of the estrogenic endocrine disrupters. (J Toxicol Pathol 2004; **17**: 245–252)

Key words: estradiol, F_1(AWE × WE) Japanese quail embryo, methyltestosterone, ovotestis, screening model, sex reversal test

Introduction

Endocrine disrupting chemicals (EDCs), which are any chemicals known or suspected to cause adverse endocrine effects in organisms or their progeny, have been considered to possess estrogenic or other endocrine activity in wildlife animals and humans. Many environmental contaminants are known or suspected to interfere with hormonal function in animals[1]. Therefore, it has been necessary to develop assessment systems to evaluate any adverse effects of EDCs in a variety of wildlife species. Birds are top predators in both aquatic and terrestrial environments and one of the most important wildlife species exposed by environmental pollutants with endocrine disrupting potential[2,3]. In facts, several species of colonial fish-eating birds, such as herring gulls, nesting in the Great Lakes basin have exhibited chronic impairment of reproduction, in which eggshell thinning is caused by high levels of DDT and its metabolites[4]. Birds are fundamentally different from mammals in the control of their sexual differentiation and reproduction system[5]. There are aspects of sexual differentiation in birds that may make them uniquely sensitive to the effects of EDCs with estrogenic activity1. It is well known that avian embryos have a significant risk with regard to EDCs with estrogenic activity because of retention in the egg and estrogen-dependency of sex expression. Consequently, separate testing for assessing impact of chemicals with endocrine disrupting potential to birds is required.

There are several guidelines for identifying toxic effects in the reproduction system in birds[6–10], whereas these guidelines are insufficient to detect mimic adverse endocrine disrupting effects of EDCs in birds. On the other hand, there are no screening models, which are a rapid and cost-effective tool, for detecting and characterizing endocrine effects of environmental contaminants in birds. Previously, it has been described that the injection of estrogen or related female hormones into the eggs of hen during the early stages of incubation caused transformation of the left testis into an

Received: 4 October 2004, Accepted: 10 November 2004
Mailing address: Kazumoto Shibuya, Nippon Institute for Biological Science, 9–2221–1 Shinmachi, Ome, Tokyo 198–0024, Japan
TEL: 81-428-33-1042 FAX: 81-428-33-1080
E-mail: kazumoto.shibuya@nibs.or.jp

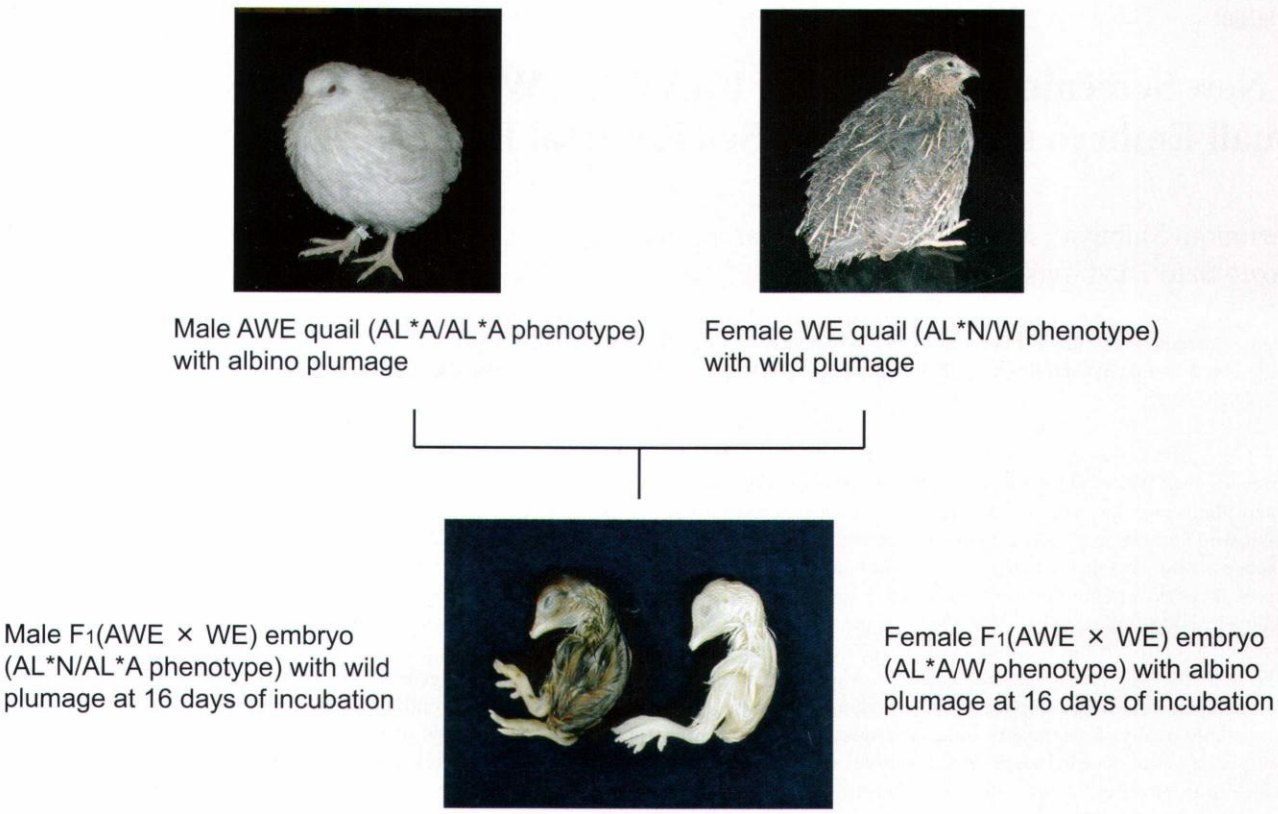

Fig. 1. Schema of a Criss-Cross inheritance. A mating between male AWE quail and female WE quail produces male F_1 quail with AL*N/AL*A phenotype exhibiting wild plumage and female F_1 quail with AL*A/W phenotype exhibiting albino plumage.

ovotestis and persistence of the left oviduct in genetic males[5]. In the experiment using California gull eggs, DDT injection into the yolk before incubation resulted in feminization of the testis accompanied by the development of a left oviduct and shell gland, development of a right oviduct, reduced size of the right testis in male embryos[11]. Feminization of the gonad in genetic males of Japanese quail resulted from early treatment of the eggs by diethylstolbestrol[12–14] and ethynylestradiol[14]. The plastic monomer bisphenol A induced feminization of the left testis (ovotestis) in male chicken embryo when the compound was injected into the yolk of eggs early during incubation[14].

The objectives of the present study was to develop a screening model for detecting endocrine disrupting effects using embryos of F_1(AWE × WE) Japanese quail (*Corturnix japonica*) and to determine whether the screening model can be used to evaluate estrogenic or androgenic endocrine disrupting chemicals.

Materials and Methods

Chemicals

17 beta-estradiol (E_2, CAS No. 50-28-2, molecular weight: 272.4) was purchased from Sigma Chemical Co. (MO, USA). Methyltestosterone (MT, CAS No. 58-18-4, molecular weight: 302.46) and corn oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Parent strains of Japanese quail

The parent strains, AWE and WE, of Japanese quail have been maintained in the Laboratory Animal Research Station of the Nippon Institute for Biological Science (Yamanashi, Japan) under the specific pathogen free condition. In birds, the female is the heterogametic sex (ZW) and the male is the homogametic sex (ZZ)[15]. The WE strain of Japanese quail possesses wild plumage. The genetic phenotype of female WE quail is AL*N/W, in which wild plumage color gene (N), a dominant character, and female sex chromosome (W) are heterozygous. The AWE strain exhibits albino plumage, which has been developed from the WE strain. The genetic phenotype of male AWE quail is AL*A/AL*A, in which albino plumage color gene (A), a recessive character, is homozygous. A mating between male AWE quail and female WE quail produces male F_1 quail with AL*N/AL*A phenotype exhibiting wild plumage and female F_1 quail with AL*A/W phenotype exhibiting albino plumage ruled by a criss-cross inheritance (Fig. 1). 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).

Eggs

Total of 550 F_1(AWE × WE) Japanese quail (*Corturnix japonica*) eggs were purchased from the Laboratory Animal

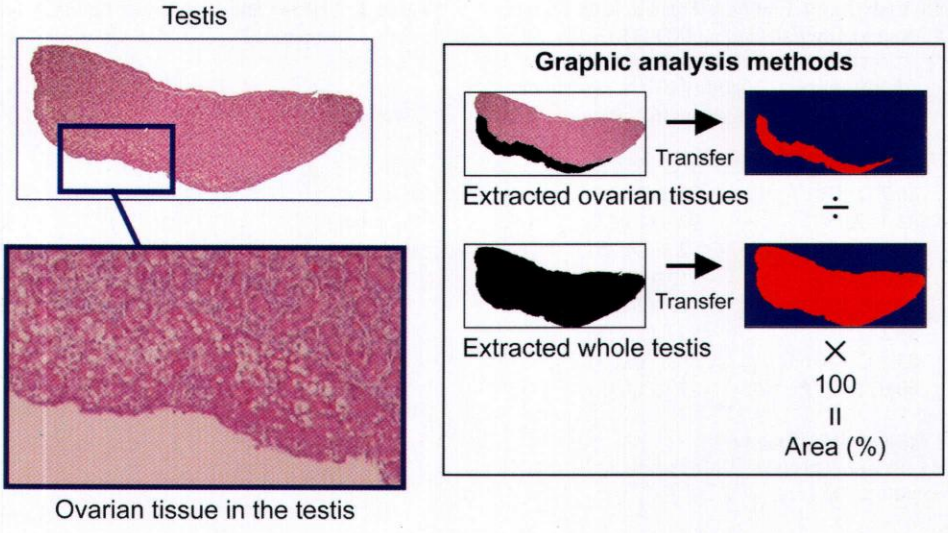

Fig. 2. Schema of the method of graphic analyses. Area of the ovarian tissue in the testis and area of the whole testis were extracted and then proportion of ovarian tissue in the testis was calculated using a graphic analysis system (ATTO Corp.).

Research Station of the Nippon Institute for Biological Science and 480 eggs were used in the present study. Before the experiments, all eggs were observed externally and candled to check abnormalities and fine cracks. Abnormal eggs that were cracked, broken or abnormal externally were excluded from the study.

Experiments

The eggs were allocated into 9 groups for experiment 1 (Exp-1) and 7 groups for experiment 2 (Exp-2), in which each group consisted of 30 eggs. Exp-1 was performed as a dose-finding study and consisted of the control, E_2 20, 200, 2000 and 20000 ng, and MT 20, 200, 2000, and 20000 ng groups. Exp-2 was performed to confirm results of each test substance in the F_1(AWE × WE) embryos and consisted of the control, E_2 20, 200, 2000 and 20000 ng, and MT 20, 200, 2000, and 20000 ng groups. Each egg was treated with a single injection of 20 μL of corn oil containing each dose of E_2 and MT, and each egg in the control group was treated with a single injection of 20 μL of corn oil just before the incubation.

The compounds were injected into the egg white through a small hole punched with a sterilized disposable 25-gauge needle at the blunt end of the egg, using a sterilized disposable 27-gauge needle attached to a sterilized Hamilton syringe as described previously[16]. After injection, the eggshell was sealed with paraffin wax and the eggs were incubated and controlled at 38.6°C, 65% relative humidity, and once/h of an egg-turning cycle. At 7-day of incubation, all eggs were candled for determining fertility and embryo viability, and then eggs possessing no developed embryo were dissected to confirm whether the eggs were unfertilized or an early embryo death. At 16 days of incubation, all eggs were dissected and then viability and plumage types of embryos were determined. The viable embryos were necropsied, observed grossly and fixed in 10% neutral buffered formalin. After 3-day fixation, gonads of the embryos were observed in detail under a dissecting microscope. The gonads were sampled, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H & E) for histopathological examination.

Proportions of the ovarian tissue area in the testis area were calculated by the following method. Briefly, microphotographs of the testis with or without ovarian tissue were taken by a Digitalnet camera (DN100, Nikon Corp., Tokyo, Japan) and processed to stock as PICT files using a graphic software (Photoshop, Adobe System Incorp., Tokyo, Japan). Areas of ovarian tissue in the testis were extracted and the whole testis area was also extracted. Proportions of the ovarian tissue area in the testis area were calculated by a graphic analysis system (ATTO Corp., Tokyo, Japan) (Fig. 2).

Statistical analysis

Quantitative data were initially analyzed 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.

Table 1. Fertility and Viability of Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-1)

Groups	Fertility (%)	Viability (%) of incubation day-16 embryos
Control	86.7 (26/30)[a]	96.2 (25/26)[b]
E_2 20 ng	86.7 (26/30)	96.2 (25/26)
E_2 200 ng	83.3 (25/30)	92.0 (23/25)
E_2 2000 ng	86.7 (26/30)	92.3 (24/26)
E_2 20000 ng	66.7 (20/30)	95.0 (19/20)
MT 20 ng	83.3 (25/30)	96.0 (24/25)
MT 200 ng	83.3 (25/30)	92.0 (23/25)
MT 2000 ng	83.3 (25/30)	88.0 (22/25)
MT 20000 ng	60.0 (18/30)[c]	77.8 (14/18)

a: Numbers of fertile eggs/numbers of eggs set.
b: Numbers of viable embryos/numbers of fertile eggs.
c: $p < 0.05$ from the control group.

Table 2. Fertility and Viability of Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-2)

Groups	Fertility (%)	Viability (%) of incubation day-16 embryos
Control	86.7 (26/30)[a]	96.2 (25/26)[b]
E_2 20 ng	93.3 (28/30)	92.9 (26/28)
E_2 200 ng	83.3 (25/30)	100.0 (25/25)
E_2 2000 ng	86.7 (26/30)	88.5 (23/26)
MT 20 ng	90.0 (27/30)	96.3 (26/27)
MT 200 ng	76.7 (23/30)	87.0 (20/23)
MT 2000 ng	80.0 (24/30)	91.7 (22/24)

a: Numbers of fertile eggs/numbers of eggs set.
b: Numbers of viable embryos/numbers of fertile eggs.

Table 3. Conformability in Sex Difference between Plumage Color and Gross Gonad Appearance of Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-1)

	Plumage			Gonad			Conformability
Groups	Male	Female	% of male	Male	Female	% of male	(%)
Control	16[a]	9	64.0	16	9	64.0	100.0
E_2 20 ng	13	12	52.0	13	12	52.0	100.0
E_2 200 ng	13	10	56.5	13	10	56.5	100.0
E_2 2000 ng	11	13	45.8	11	13	45.8	100.0
E_2 20000 ng	12	7	63.2	12	7	63.2	100.0
MT 20 ng	14	10	58.3	14	10	58.3	100.0
MT 200 ng	13	10	56.5	13	10	56.5	100.0
MT 2000 ng	11	11	50.0	11	11	50.0	100.0
MT 20000 ng	8	6	57.1	8	6	57.1	100.0

a: Numbers of embryos examined.

Table 4. Conformability in Sex Difference between Plumage Color and Gross Gonad Appearance of Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-2)

	Plumage			Gonad			Conformability
Groups	Male	Female	% of male	Male	Female	% of male	(%)
Control	13[a]	12	52.0	13	12	52.0	100.0
E_2 20 ng	9	17	34.6	9	17	34.6	100.0
E_2 200 ng	10	15	40.0	10	15	40.0	100.0
E_2 2000 ng	13	10	56.5	13	10	56.5	100.0
MT 20 ng	13	13	50.0	13	13	50.0	100.0
MT 200 ng	11	9	55.0	11	9	55.0	100.0
MT 2000 ng	13	9	59.1	13	9	59.1	100.0

a: Numbers of embryos examined.

Results

Fertility

Fertilities of the eggs in the control group, and all E_2 and MT-treated groups in Exp-1 were shown in Table 1. Fertility of the eggs in the MT 20000 ng group was significantly lower ($p < 0.05$) than that in the control group but no significant differences of the fertility were detected in any E_2-treated or other MT-treated groups, although the E_2 20000 ng group showed low fertility. Fertilities of the eggs in the control group, and all E_2 and MT-treated groups in Exp-2 were shown in Table 2. There were no significant differences of the fertility between the control group and any E_2-treated groups or between the control group and any MT-treated groups.

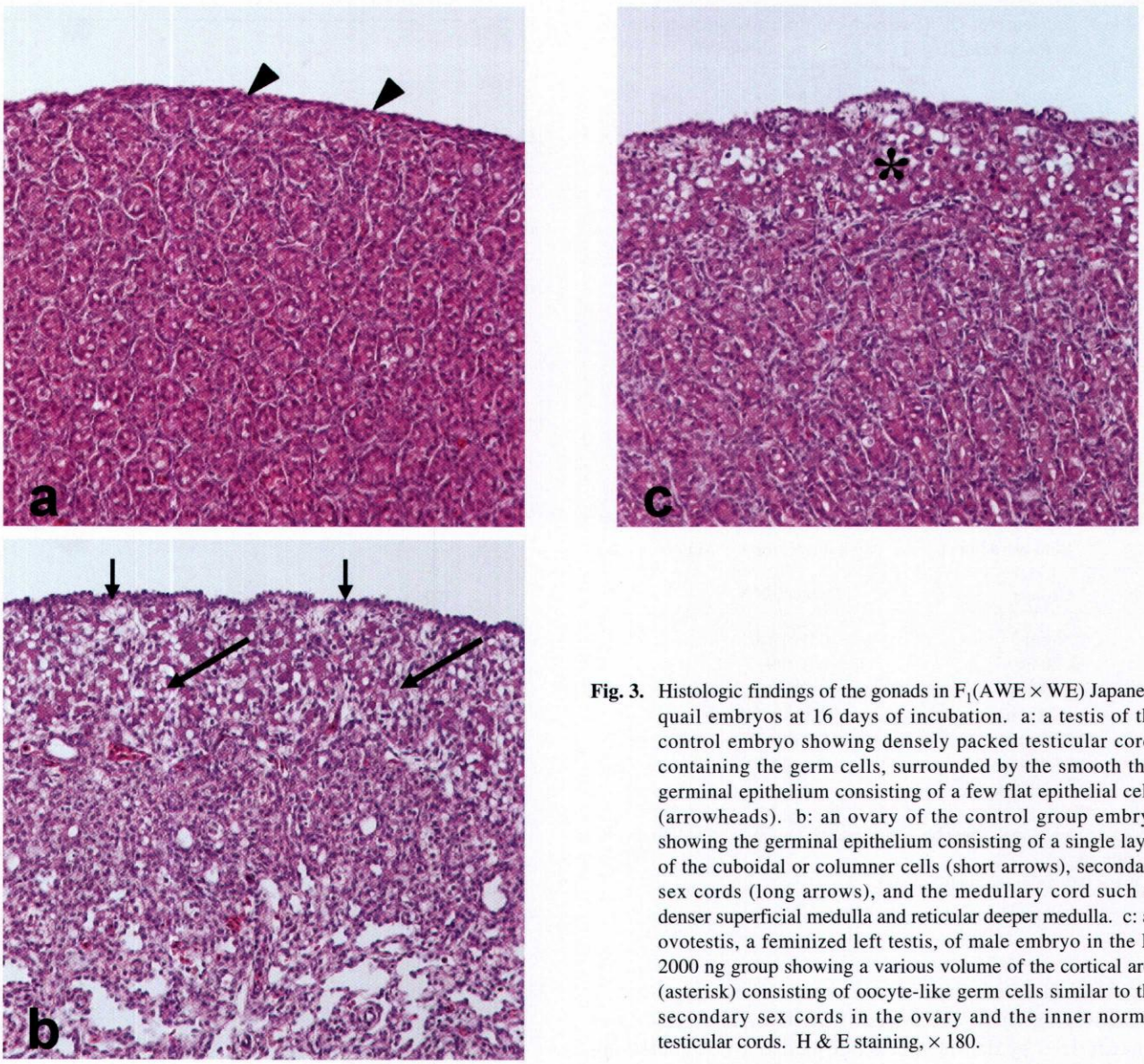

Fig. 3. Histologic findings of the gonads in F_1(AWE × WE) Japanese quail embryos at 16 days of incubation. a: a testis of the control embryo showing densely packed testicular cords containing the germ cells, surrounded by the smooth thin germinal epithelium consisting of a few flat epithelial cells (arrowheads). b: an ovary of the control group embryo showing the germinal epithelium consisting of a single layer of the cuboidal or columner cells (short arrows), secondary sex cords (long arrows), and the medullary cord such as denser superficial medulla and reticular deeper medulla. c: an ovotestis, a feminized left testis, of male embryo in the E_2 2000 ng group showing a various volume of the cortical area (asterisk) consisting of oocyte-like germ cells similar to the secondary sex cords in the ovary and the inner normal testicular cords. H & E staining, × 180.

Viability of embryos

Viabilities of the embryos at 16 days of incubation in the control group, and all E_2 and MT-treated groups in Exp-1 were shown in Table 1. Viabilities of the embryos at 16 days of incubation in the control group, and all E_2 and MT-treated groups in Exp-2 were shown in Table 2. There were no significant differences in the viability between the control group and any E_2-treated groups or between the control group and any MT-treated groups in Exp-1 and Exp-2.

Conformability in sex difference

Genetic sex difference exhibiting plumage color of the embryos coincided completely with morphological sex characteristics of the gonads in the control group, and all E_2 and MT-treated groups in Exp-1 (Table 3). Similarly, complete conformability between genetic sex difference exhibiting plumage color and morphological sex characteristics of the gonads in the control group, and all E_2 and MT-treated groups was observed in Exp-2 (Table 4). In addition, sex ratio (% of male) in all E_2 and MT-treated groups were not significantly different from that in the control groups in both Exp-1 and Exp-2 (Tables 3 and 4).

Pathology

Grossly, no abnormal findings in the ovary were observed in the control group, any E_2 and MT-treated groups in both Exp-1 and Exp-2. In the left male gonad, the morphology of the ovotestis was not differentiated externally from that in the normal testis. Atrophy of the right testis was observed in 7 out of 12 males in the E_2 20000 ng group and the incidence was significantly higher ($p < 0.01$) than that of the control group in Exp-1. Atrophy of the right testis was also observed in 2 out of 13 males in the E_2 2000 ng group but there was no significant difference between the control and E_2 2000 ng groups in Exp-2. In the other E_2-treated groups and all MT-treated groups in both Exp-1 and

Table 5. Incidence of Ovotestis in Male Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-1)

Groups	Ovotestis (%)
Control	0.0 (0/16)[a]
E_2 20 ng	0.0 (0/13)
E_2 200 ng	46.2 (6/13)[b]
E_2 2000 ng	90.9 (10/11)[b]
E_2 20000 ng	100.0 (12/12)[b]
MT 20 ng	0.0 (0/14)
MT 200 ng	0.0 (0/13)
MT 2000 ng	0.0 (0/11)
MT 20000 ng	0.0 (0/8)

a: Numbers of male embryos with ovotestis /numbers of male embryos examined.
b: $p < 0.01$ from the control group.

Table 6. Incidence of Ovotestis in Male Embryos Treated with 17 Beta-estradiol (E_2) and Methyltestosterone (MT) (Exp-2)

Groups	Ovotestis (%)
Control	0.0 (0/13)[a]
E_2 20 ng	0.0 (0/9)
E_2 200 ng	10.0 (1/10)
E_2 2000 ng	92.3 (12/13)[b]
MT 20 ng	0.0 (0/14)
MT 200 ng	0.0 (0/13)
MT 2000 ng	0.0 (0/11)

a: Numbers of male embryos with ovotestis /numbers of male embryos examined.
b: $p < 0.01$ from the control group.

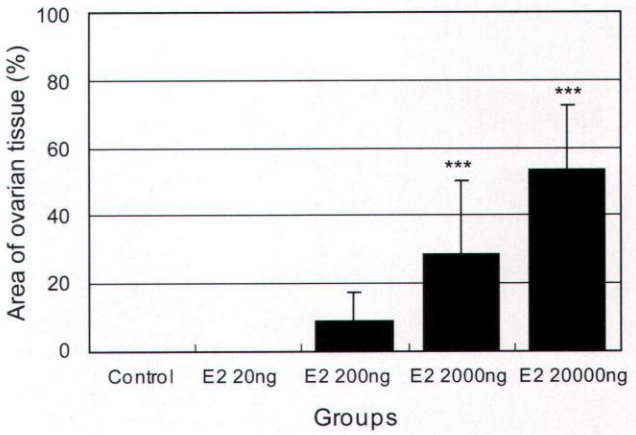

Fig. 4. Area (%) of ovarian tissues in the testis of male embryos treated with 17 beta-estradiol (E_2) (Exp-1). No ovotestis was detected in any methyltestosterone-treated groups.
***: $p < 0.001$.

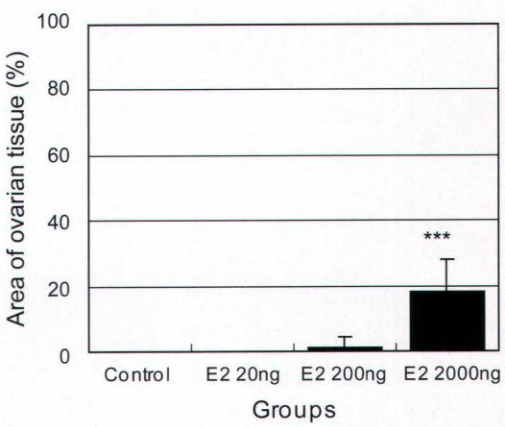

Fig. 5. Area (%) of ovarian tissues in the testis of male embryos treated with 17 beta-estradiol (E_2) (Exp-2). No ovotestis was detected in any methyltestosterone-treated groups.
***: $p < 0.001$.

Exp-2, no abnormal findings were observed in any testes.

Histologically, the testis of the control embryo was characterized by densely packed testicular cords containing the germ cells, surrounded by the smooth thin germinal epithelium, which consisted of a few flat epithelial cells (Fig. 3a). The ovary of a female embryo in the control group was characterized by the germinal epithelium consisting of a single layer of the cuboidal or columner cells, secondary sex cords which consisted of oval and cuboidal germ cells with plump eosinophilic cytoplasm, and the medullary cord such as denser superficial medulla and reticular deeper medulla (Fig. 3b). In the ovotestis, a feminized left testis, of a male embryo in the E_2 2000 ng group, a various volume of the cortical area consisted of oocyte-like germ cells similar to the secondary sex cords in the ovary, surrounded by a roughened single layer of the cuboidal epithelial cells, and the medullary portion consisted of normal testicular cords (Fig. 3c). There were no noticeable changes in the ovaries of quail embryos in any E_2 and MT-treated groups in both experiments.

Ovotestis development

The incidences of ovotestis in the control, E_2 20, 200, 2000 and 20000 ng groups in Exp-1 were 0.0, 0.0, 46.2, 90.9, and 100.0%, respectively (Table 5). The incidence of ovotestis in the E_2 200, 2000, and 20000 ng groups were significantly higher ($p < 0.01$) than that in the control group. No ovotestis was detected in any MT-treated groups in Exp-1. The incidences of ovotestis in the control, E_2 20, 200, and 2000 ng groups in Exp-2 were 0.0, 0.0, 10.0, and 92.3%, respectively (Table 6). The incidence of the ovotestis in the E_2 2000 ng group was significantly higher ($p < 0.01$) than that in the control group. No ovotestis was also detected in any MT-treated groups in Exp-2.

Proportions of the ovarian tissue area in the testis area in the control, E_2 20, 200, 2000 and 20000 ng groups in Exp-1 were 0.0 ± 0.0, 0.0 ± 0.0, 8.8 ± 8.7, 28.5 ± 21.5, 53.5 ± 19.0%, respectively (Fig. 4), and the proportions in the E_2 2000 and 20000 ng groups were significantly higher ($p < 0.001$) than that in the control group. Proportions of the

ovarian tissue area within the testis in the control, E_2 20, 200, and 2000 ng groups in Exp-2 were 0.0 ± 0.0, 0.0 ± 0.0, 1.0 ± 3.3, and 18.3 ± 9.9%, respectively (Fig. 5), and the proportion in the E_2 2000 ng groups was significantly higher ($p < 0.001$) than that in the control group.

Discussion

In the present study, high fertility (86.7%) and viability (92.6%) of the F_1(AWE × WE) quail embryos were confirmed in the control groups of the duplicate experiments. Both OECD and US EPA test guidelines for the Avian Reproduction Test describe that normal viability (percentage of fertile eggs) is in the range of 85% to 98% in Japanese quail[7,9]. In addition, genetic sex appearance, i.e. plumage color, of all embryos in the control groups coincided completely with sex phenotype of the gonads in both experiments. In sex differentiation of birds, the male is homogametic (ZZ chromosomes) and the female is heterogametic (ZW chromosome)[16]. The genes present on the Z chromosome are generally not present on the W chromosome, leading to different patterns of gene expression in male and female birds known as sex-linkage[15]. Nevertheless, the absence of juvenile sexual dimorphism often makes it difficult or even impossible to determine a chick's sex on the basis of external morphology[17]. Recently, a W-linked (CHD1W) gene in all non-ratite birds has been discovered, but the gene also exists in a very similar copy in the Z chromosome (CHD1Z)[17]. Using highly conserved primers flanking the intron of the gene, PCR amplification and agarose electrophoresis, 47 out of 50 bird species were successfully sexing[17], whereas the method did not provide complete results and spent considerable cost and time. On the other hand, *in ovo* exposure of EDCs is the most important route in the birds[1–3,11] and avian eggs have been elucidated to be a good test system for evaluating effects of EDCs[5,12–14,18–22]. Maternal transfer of 17 beta-estradiol benzoate (EB) to egg yolk has been demonstrated in Japanese quail, in which female quails received EB in the routs of i.m. injection and subcutaneous implantation[23]. Thus, it seems remarkable that the system of our sex reversal test using the F_1(AWE × WE) quail embryos is suitable to be used as avian *in vivo* screening tests.

Natural estrogen and synthetic estrogens have been described to induce feminization of the male embryos in Japanese quail and chicken[5] and E_2 has been recommended for use in the study of detecting reproductive and developmental effects[10]. On the other hand, birds appear to be affected by an androgenic substance such as MT to a much lesser extent than they are affected by an estrogenic substance such as diethylstilbestrol (DES)[24]. In the present study, therefore, evaluation of any effects of both estrogenic and androgenic substances on Japanese quail embryos was necessary for developing the present new *in vivo* screening test. E_2 200 ng/egg and more doses induced the ovotestis in the left testis and there were dose-dependent increases in the incidence and relative ovarian tissue area (%) of the E_2-treated groups in our duplicate experiments. Normal value of the egg weight is approximately 10 g in the biological reference data[6] and in our institute (data not shown). The duplicate experiments suggest that the no observable adverse effect level (NOAEL) of E_2 in male F_1(AWE × WE) Japanese quail embryos is determined as less than 20 ng/g egg. These results provide the facts that E_2 has the potential to induce dose-dependent feminization of the left testis in Japanese quail embryos, coinciding with the previous study using synthetic estrogens such as DES and ethynylestradiol (EE_2)[14]. Significantly increased incidence of male embryos exhibiting an ovotestis was observed at 0.7 ng/g egg for EE_2 and at 2 ng/g egg for DES[14], indicating that feminization activity of E_2 may be weaker than EE_2 and DES. Markedly decreased fertilities of the E_2 20000 ng and MT 20000 ng groups suggest that these E_2 and MT doses may be a lethal toxic dose for quail embryos, although no noticeable effects on the testes or ovary were detected in any MT-treated groups, suggesting that the present sex reversal test can evaluate estrogenic disrupting activity of chemicals in Japanese quail embryos.

There is concern that estrogenic chemicals in the environment may be responsible for reproductive abnormalities affecting both wildlife and humans. In birds, estrogen is the differentiating hormone for both the gonads and behavior but is not involved in the differentiation of the gonads in mammals[1]. In avian embryos, the undifferentiated left gonad consists of a medulla surrounded by a germinal epithelium, and in males the medullary sex cords, containing the germ cells, develop into seminiferous tubules[5]. Nevertheless, excess estrogens stimulate the primordial germ cells in the cortex of the left gonad in a dose-dependent manner and consequently these cortical cells differentiate into primordial follicles, and the left gonad begins to resemble an ovary[10]. Because in birds an estrogen receptor gene is expressed in epithelium of the left, not the right, gonad of both sex, but the expression in the male left gonad is temporary and restricted to an early stage of development[25]. In the present study, it is revealed that the newly developed sex reversal test using F_1(AWE × WE) Japanese quail embryos has the potential to evaluate estrogenic endocrine disrupting effects in avian embryos and is cost-effective. Additional information on the dose and embryonic histological response to various synthetic estrogens and EDCs remains to accumulate for providing exposure information and comparison for field investigations on wild birds.

Acknowledgements: The present study was supported by Ministry of the environment, Japan. The authors are grateful to M. Yamazaki, M. Kaneko, H. Suzaki, and K. Gondaira for their technical assistance and K. Yamashita for his secretarial assistance.

References

1. Damstra T, Barlow S, Bergman A, Kavlock R, and van der Kraak G. International Program on Chemical Safety: Global Assessment of the State-of-the-Science of Endocrine Disruptors. World Health Organization, Geneva, Switzerland, 2002.
2. Ankley G, Mihaich E, Stahl D, Tilitt D, Colborn T, McMaster S, Miller R, Bantle J, Cambell P, Denslow N, Dickerson R, Folmar L, Fry M, Giesy J, Gray LE, Guiney P, Hutchinson T, Kennedy S, Kramer V, LeBlance G, Mayes M, Nimrod A, Patino R, Peterson R, Purdy R, Ringer R, Thomas P, Touart L, van der Kraak G, and Zacharewski T. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environ Toxicol Chem 1998; **17**: 68–87.
3. Rattner BA, Eroschenko VP, Fox GA, Fry DM, and Gorsline J. Avian endocrine responses to environmental pollutants. J Exp Zool 1984; **232**: 683–689.
4. Gilbertson M, Kubiak T Ludwig J, and Fox G. 1991. Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. J Toxicol Environ Health 1991; **33**: 455–520.
5. Romanoff AJ. editor. The Avian Embryo. New York: Macmillan, 1960.
6. OECD (Organization for Economic Cooperation and Development). OECD Guidelines for the Testing of Chemicals. Test Guideline 205: Avian Dietary Toxicity Test. Paris, France, 1984.
7. OECD (Organization for Economic Cooperation and Development). OECD Guidelines for the Testing of Chemicals. Test Guideline 206: Avian Reproduction Test. Paris, France, 1984.
8. OECD (Organization for Economic Cooperation and Development). OECD Guidelines for the Testing of Chemicals. Proposal for a New Test Guideline: Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite. Paris, France, 2000.
9. EPA (U.S. Environmental Protection Agency). Ecological Effects Test Guidelines. OPPTS 850.2300, Avian Reproduction Test. EPA 712-C-96-141. Washington DC, US Environmental Protection Agency, 1996.
10. EPA (U.S. Environmental Protection Agency). Revised Draft Detailed Review Paper for Avian Two-Generation Toxicity Test. EPA 68-W-01-023. Washington DC, US Environmental Protection Agency, 2003.
11. Fry DM and Toone CK. DDT-induced feminization of gull embryos. Science 1981; 213: 922–924.
12. Scheib D and Reyss-Brion M. Feminization of the quail by early diethylstilbestrol treatment: histoenzymological investigations on steroid dehydrogenases in the gonads. Arch Anat Microscp Morphol Exp 1979; **68**: 85–98.
13. Perrin FMR, Stacy S, Burgess AMC, and Mittwoch U. A quantitative investigation of gonadal feminization by diethylstilbestrol of genetically male embryos of the quail Coturnix coturnix japonica. J Reprod Fertil 1995; **103**: 223–226.
14. Berg C, Halldin K, and Brunstrom B. Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. Environ Toxicol Chem 2001; **20**: 2836–2840.
15. Stevens L. Sex chromosomes and sex determining mechanisms in birds. Sci Prog 1997; **80**: 197–216.
16. Shimada K. Sex determination and sex differentiation. Avian and Poultry Biol Rev 2002; **13**: 1–14.
17. Fridolfsson A-K and Ellegren H. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 1999; **30**: 116–121.
18. Berg C, Halldin K, Fridolfsson A-K, Brandt I, and Brunstrom B. The avian egg as a test system for endocrine disrupters: effects of diethylstilbestrol and ethynylestradiol on sex organ development. Sci Total Environ 1999; **223**: 57–66.
19. Halldin K, Berg C, Bergman A, Brandt I, and Brunstrom B. Distribution of bisphenol A and tetrabromobisphenol A in quail eggs, embryos and laying birds and studies on reproduction variables in adults following in ovo exposure. Dev Toxicol 2001; **75**: 597–603.
20. Gildersleeve RP, Tilson HA, and Mitchell CL. Injection of diethylstilbestrol on the first day of incubation affects morphology of sex glands and reproductive behavior of Japanese quail. Teratol 1985; **31**: 101–109.
21. Halldin K, Berg C, Btandt I, and Brunstrom B. Sexual behavior in Japanese quail as a test end point for endocrine distribution: effects if in ovo exposure to ethynylestradiol and diethylstilbestrol. Environ Health Perspect 1999; **107**: 861–866.
22. Halldin K, Holm L, Ridderstrale Y, and Brunstrom B. Reproductive impairment in Japanese quail (Coturnix japonica) after in ovo exposure to o,p'-DDT. Arch Toxicol 2003; **77**: 116–122.
23. Adkins-Regan E, Ottinger MA, and Park J. Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. J Exp Zool 1995; **271**: 466–470.
24. Brahmakshatriya RD, Snetsinger DC, and Waibel PE. Effects of estrogen and/or androgen on performance, egg shell characteristics and blood plasma changes in laying hens. Poult Sci 1969; **48**: 444–451.
25. Nakabayashi O, Kikuchi H, Kikuchi T, and Mizuno S. Differential expression of genes for aromatase and estrogen receptor during the gonadal development in chicken embryos. J Mol Endocrinol 1998; **20**: 193–202.