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IN OVO EXPOSURE TO NONYLPHENOL AND BISPHENOL A RESULTED IN DOSE-INDEPENDENT FEMINIZATION OF MALE GONADS IN JAPANESE QUAIL (*COTURNIX JAPONICA*) EMBRYOS

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Abstract—Sex reversal effects of nonylphenol and bisphenol A on the gonads in F_1(AWE × WE) Japanese quail (*Coturnix japonica*) embryos were investigated using an in vivo screening model developed previously. The F_1 (AWE × WE) Japanese quail are a useful avian model because sex differentiation is confirmed by the plumage color before hatching, ruled by a criss-cross inheritance. The nonylphenol at 200, 2,000, 20,000, and 200,000 ng/egg and bisphenol A at 20, 200, 2,000, and 20,000 ng/egg were injected into the egg white just before incubation. At 16 d of incubation, embryos were subjected to a complete necropsy, and their gonads were both grossly observed and examined histopathologically and morphometrically. Grossly, genetic sex was confirmed because plumage color coincided completely with the external sex phenotype of the gonads in all embryos. Histopathologically, feminization of the male gonad, called ovotestis, developed in the left testis in all nonylphenol- and bisphenol A-treated groups. The incidence of the lesion in all treated groups was significantly higher than that in the control group, whereas there were no dose-dependent changes in the incidence and area of the ovotestis in both nonylphenol- and bisphenol A-treated groups. The present study revealed that nonylphenol and bisphenol A have a dose-independent potential of ovotestis induction in the Japanese quail embryo. Environ. Toxicol. Chem. 2012;31:1091–1097. © 2012 SETAC

Keywords—Bisphenol A Feminization Japanese quail embryo Nonylphenol Ovotestis

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are broadly defined as chemicals that can interfere with hormone action. A number of EDCs might possess estrogenic or other endocrine activity in wild animals and humans [1]. However, assessment systems to evaluate the possible adverse effects of EDCs in a variety of wildlife species remain to be developed. Birds are the top predators in aquatic and terrestrial environments, and one of the important wildlife species exposed to environmental pollutants with endocrine disrupting potential [2,3]. In addition, the unique sexual differentiation of birds has been considered to make them sensitive to the effects of EDCs with estrogenic activity [1]. The estrogen-dependent sexual differentiation of birds and the ease of chemical retention in the egg suggest a significant risk of avian embryos for EDCs with estrogenic activity. The use of Japanese quail (*Coturnix japonica*) is a cost-effective method for endocrine-disruptor testing, being recommended as one of the species in the avian reproduction test (Organisation for Economic Co-operation and Development test guideline 206) [4,5]. Therefore, we have developed and proposed a modified avian one-generation reproduction study using Japanese quail to assess the endocrine disrupting potential of chemicals [6,7]. However, avian screening models using eggs or embryos, which are convenient to obtain preliminary information on the endocrine disrupting potential of chemicals to birds, are likely to be required.

A new avian in vivo screening model called the sex reversal test was developed previously using embryos of the unique strain F_1(AWE × WE) of Japanese quail (*C. japonica*) [8]. In the sex reversal test, 17 β-estradiol (E_2) induced a dose-dependent feminization detected as ovotestis of the male left gonad, whereas methyltestosterone induced no effects in male and female gonads in Japanese quail embryos. Consequently, the sex reversal test could evaluate estrogenic, not androgenic, endocrine-disrupting effects of chemicals. Additionally, reproducibility of the sex reversal test was demonstrated by a subsequent study using E_2, diethylstilbestrol (DES), and ethinyl estradiol (EE_2) as positive control chemicals [9], resulting in sex reversal effects; that is, the proportions of ovarian tissue area in the ovotestis of E_2, DES, and EE_2 were closely correlated with the treatment dose levels.

Nonylphenol is a product of industrial synthesis formed during the alkylation process of phenols, and bisphenol A is a chemical used worldwide in manufacturing polycarbonate plastics. It is well known that both chemicals are found in the environment, including river water. Consequently, birds may be highly exposed to nonylphenol and bisphenol A through parts of the food chain such as insects and aquatic organisms. Both nonylphenol and bisphenol A are well known to be the most probable candidates for estrogenic EDCs [10], which seem to affect reproductive functions through the estrogen–estrogen receptor (ER) signaling pathway [5]. The present study is undertaken to investigate sex reversal effects of nonylphenol and bisphenol A on male gonads in F_1(AWE × WE) Japanese quail embryos by using the sex reversal test.

MATERIALS AND METHODS

Chemicals

The nonylphenol (1-hydroxy-4-nonylbenzen, molecular weight: 220.35) and bisphenol A (4,4′-isopropylidenediphenol, molecular weight: 228.29) were purchased from Kanto Chemical Co.

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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 under specific pathogen-free conditions. A mating between male AWE quail with albino plumage color (AL*A/AL*A) and female WE quail with wild plumage color (AL*N/W) produced inheritably male F_1 quail with wild plumage color (AL*N/AL*A) and female F_1 quail with albino plumage color (AL*A/W) ruled by a criss-cross inheritance as described previously [8,9] (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

A total of 500 F_1(AWE × WE) Japanese quail (*C. japonica*) eggs were purchased from the Laboratory Animal Research Station of the Nippon Institute for Biological Science, and 450 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.

Study design

A total of 450 eggs were allocated into 10 groups. In the nonylphenol experiment, doses of 0 (control), 200, 2,000, 20,000, and 200,000 ng were selected, and each group consisted of 50 eggs. In the bisphenol A experiment, doses of 0 (control), 20, 200, 2,000, and 20,000 ng were selected, and each group consisted of 40 eggs. The dose levels of nonylphenol and bisphenol A were selected to cover a broad range of concentrations with a factor of 10× based on the following results. Relative binding affinity of nonylphenol and bisphenol A to quail ER α and β has been reported to be very low (1/15–1/64) compared with that of DES [11,12]. Ovotestis of the 16-d quail embryos was detected at 20 ng and higher doses of DES, and also detected at 200 ng and higher doses of E_2 [8]. Because the main aim of this study was to investigate the effects of these chemicals on male gonads in Japanese quail embryos, the concentrations of chemicals employed in this study are considered to be quite higher than those existing in the environment.

Each egg was treated with a single injection of 20 μl corn oil containing the appropriate dose of nonylphenol or bisphenol A, and each egg in the control group was treated with a single injection of 20 μl corn oil just before 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 [8]. After injection, the eggshell was sealed with paraffin wax, and the eggs were incubated in an incubator controlled at 38.6°C, 65% relative humidity, and egg-turning once per hour. At incubation day 7, all eggs were candled to determine fertility, and eggs containing no developed embryo were dissected to confirm whether the eggs were unfertilized or

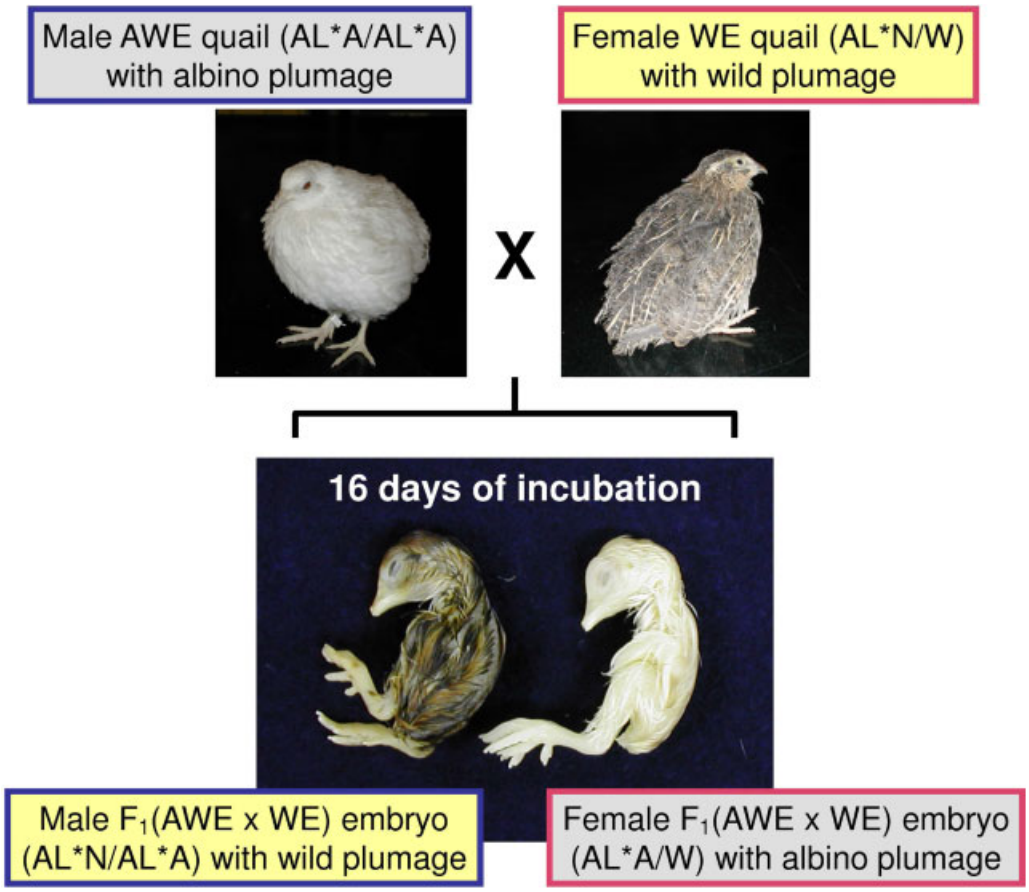

Fig. 1. Schema of a criss-cross inheritance. A mating between male AWE quail and female WE quail produces male F_1 quail with wild plumage (AL*N/AL*A phenotype) and female F_1 quail with albino plumage (AL*A/W phenotype).

experienced an early embryo death. At incubation day 16, all eggs were dissected and viability and plumage types of the embryos were determined. The viable embryos were completely necropsied, observed grossly, and fixed in 10% neutral buffered formalin. After 3 d of fixation, the gonads of the embryos were observed in detail under a dissecting microscope. The gonads were collected, embedded in paraffin wax, sectioned, and stained with hematoxylin-eosin for histopathological examination.

Proportions of the ovarian tissue area to the testis area were calculated using the following method: microphotographs of the testis with or without ovarian tissue were taken by a Digitalnet camera (DN100, Nikon) and processed to stock as PICT files using a graphic software (Photoshop, Adobe); the areas of ovarian tissue in the testis were extracted, and the whole testis area was also extracted; and the proportions of the ovarian tissue area to the testis area and the proportions of the ovarian tissue to the ovotestis area were calculated by a graphic analysis system (ATTO Corp.).

Statistical analysis

Quantitative data were initially analyzed by 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 a significant difference was seen between groups, multiple comparisons were performed by 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 a significant difference was seen between groups, ranking comparison was performed by Dunnett's multiple comparison test (two-tailed, significance level: 5% and 1%). Data of incidences were analyzed by Fisher's exact probability test. Values of $p < 0.05$ were considered significant. Coefficient (r) of correlation between proportions of ovarian tissue area to the testis area and doses of nonylphenol and bisphenol A were estimated.

RESULTS

Fertility and viability

Fertility and viability in the control groups and nonylphenol- and bisphenol A-treated groups are shown in Table 1. Fertility of the eggs in any nonylphenol- and bisphenol A-treated groups was not significantly different from that in the corresponding control groups, and no dose-related changes of fertility were noted in any nonylphenol- and bisphenol A-treated groups. Viability of the embryos in any nonylphenol- and bisphenol A-treated groups was not significantly different from that in the corresponding control groups, and no dose-related changes of viability were noted in any nonylphenol- and bisphenol A-treated groups.

Table 1. Fertility and viability of embryos treated with nonylphenol (NP) and bisphenol A (BPA)

Group	Fertility (%)[a]	Viability (%)[b]
NP 0 ng (control)	72.0 (36/50)[c]	86.1 (31/36)[d]
NP 200 ng	70.0 (35/50)	82.9 (29/35)
NP 2,000 ng	80.0 (40/50)	82.5 (33/40)
NP 20,000 ng	80.0 (40/50)	77.5 (31/40)
NP 200,000 ng	82.0 (41/50)	85.4 (35/41)
BPA 0 ng (control)	90.0 (36/40)	83.3 (30/36)
BPA 20 ng	92.5 (37/40)	89.2 (33/37)
BPA 200 ng	90.0 (36/40)	86.1 (31/36)
BPA 2,000 ng	90.0 (36/40)	86.1 (31/36)
BPA 20,000 ng	92.5 (37/40)	91.9 (34/37)

[a] Embryos at 7 days of incubation.
[b] Embryos at 16 days of incubation.
[c] Numbers of fertile eggs/numbers of eggs set.
[d] Numbers of viable embryos/numbers of fertile eggs.

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 groups and all nonylphenol- and bisphenol A-treated groups (Table 2). Sex ratios (percentage of male) in any nonylphenol- and bisphenol A-treated groups were not significantly different from those in the corresponding control groups, and no dose-related changes of sex ratios occurred in any nonylphenol- and bisphenol A-treated groups (Table 2).

Gross observation of gonads

Grossly, genetic male embryos with wild plumage color in all nonylphenol- and bisphenol A-treated groups showed normal external appearances of the testis, which were not different morphologically from those in the control groups. Genetic female embryos with albino plumage color in all nonylphenol-treated groups possessed normal ovaries. The ovary in all bisphenol A-treated groups also showed normal appearances, except for one female embryo in each of the bisphenol A 20, 2,000, and 20,000 ng groups, which had small ovary-like tissues observed on the right side, where no gonad is normally present in female birds (Fig. 2). No noticeable changes were grossly observed in other organs and tissues.

Histopathology of gonads

Histologically, the testis of the incubation day 16 embryo in the control group was characterized by densely packed testicular cords containing the germ cells, surrounded by the smooth germinal epithelium, which consisted of a few flat epithelial cells and tunica albuginea (Fig. 3a). The ovary of the incubation day 16 embryo in the control group showed the germinal epithelium consisting of a single layer of the cuboidal or columnar cells, secondary sex cords, and the medullary cords such as denser superficial medulla and reticular deeper medulla (Fig. 3b). The ovotestis, feminization of the testis, was detected only in the left testis of embryos in the nonylphenol- and

Table 2. Conformability in sex difference between plumage color and gross gonad appearance of embryos treated with nonylphenol (NP) and bisphenol A (BPA)

	Plumage			Gonad			Conformability
Groups	Male	Female	% of male	Male	Female	% of male	(%)
NP 0 ng (control)	15[a]	16	48.4	15	16	48.4	100.0
NP 200 ng	14	15	48.3	14	15	48.3	100.0
NP 2,000 ng	17	16	51.5	17	16	51.5	100.0
NP 20,000 ng	16	15	51.6	16	15	51.6	100.0
NP 200,000 ng	23	12	65.7	23	12	65.7	100.0
BPA 0 ng (control)	14	16	46.7	14	16	46.7	100.0
BPA 20 ng	17	16	51.5	17	16	51.5	100.0
BPA 200 ng	21	10	67.7	21	10	67.7	100.0
BPA 2,000 ng	15	15	48.4	15	15	48.4	100.0
BPA 20,000 ng	22	16	64.7	22	16	64.7	100.0

[a] Numbers of embryos examined.

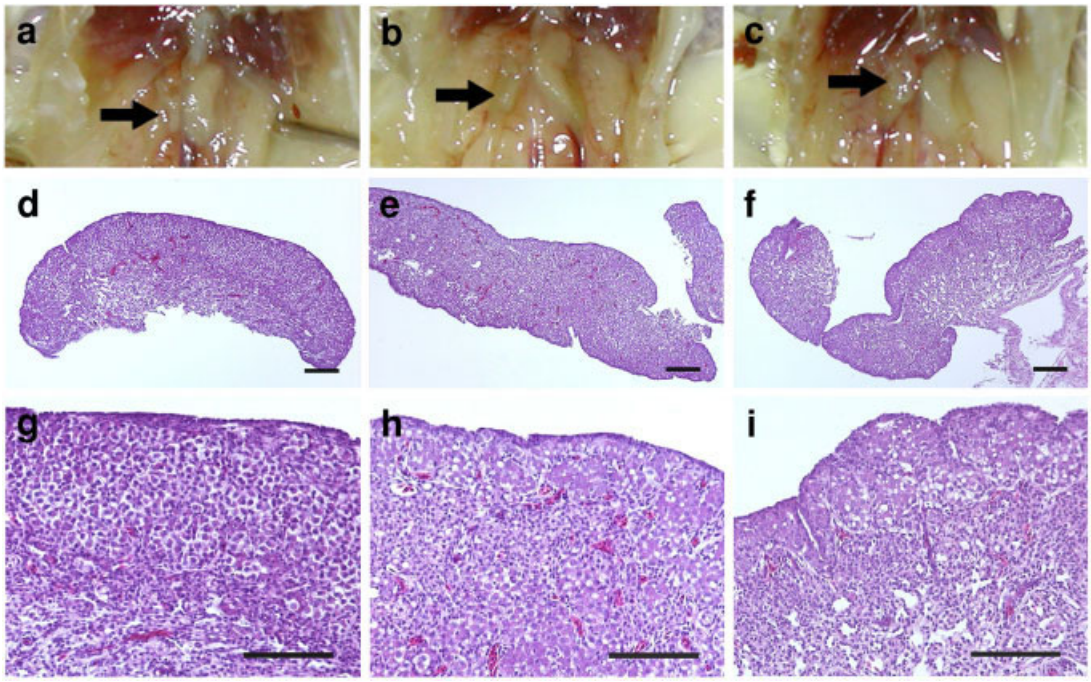

Fig. 2. Gross (**a, b, c**) and histologic (**d, e, f, g, h, i**) features of gonads in F_1(AWE × WE) quail embryos at the incubation day 16. Right ovaries of bisphenol A 20-ng group (**a, d, g**), 2,000 ng group (**b, e, h**), and 20,000-ng group (**c, f, i**) showing vestigial small tissues in the right portion grossly and normal histologic features. HE stain, bar: 200 μm (**d, e, f**), 50 μm (**g, h, i**).

bisphenol A-treated groups (Fig. 3c, d). A varied volume of the cortical area consisted of oocyte-like germ cells similar to the secondary sex cords and was covered with a roughened single layer of the cuboidal epithelial cells similar to the ovary, and the inner portion consisted of normal testicular cords (Fig. 3c, d). Qualitative differences in the morphology of the ovotestis were seen between the nonylphenol- and bisphenol A-treated groups. In female embryos, no noticeable changes occurred in the ovaries, which were present normally in the left side, of any nonylphenol- and bisphenol A-treated groups. Ovary-like tissues in the right side, which were detected in the bisphenol A 20, 2,000, and 20,000 ng groups, showed normal ovarian appearances (Fig. 2).

The incidences of ovotestis in the nonylphenol 0, 200, 2,000, 20,000, and 200,000 ng groups were 0.0, 28.6, 41.2, 31.3, and 34.8%, respectively, and significant differences ($p < 0.05$ or 0.01) of the incidence of ovotestis from the control group were detected in all nonylphenol-treated groups (Table 3). The incidences of ovotestis in the bisphenol A 0, 20, 200, 2,000, and 20,000 ng groups were 0.0, 41.2, 33.3, 40.0, and 45.5%, respectively and significant differences ($p < 0.05$ or 0.01) of the incidence of ovotestis from the control group were detected in all bisphenol A-treated groups (Table 3). No dose-related changes of the incidences of ovotestis occurred in any nonylphenol- and bisphenol A-treated groups.

Morphometric analysis of ovotestis

Proportions of the ovarian tissue area to the testis area in the nonylphenol 0, 200, 2,000, 20,000, and 200,000 ng groups were 0.0 ± 0.0, 4.2 ± 8.0, 4.9 ± 6.5, 6.2 ± 11.9, and 4.7 ± 7.1%, respectively (Fig. 4a). Proportions of the ovarian tissue area to the testis area in the bisphenol A 0, 20, 200, 2,000, and 20,000 ng groups were 0.0 ± 0.0, 4.2 ± 5.8, 4.1 ± 6.3, 9.9 ± 15.1, and 5.1 ± 6.9%, respectively (Fig. 4a). The proportions in all nonylphenol-treated groups and in all bisphenol A-treated groups were significantly higher ($p < 0.05$ or 0.01) than those in the corresponding control groups. No dose-related changes of the ovarian tissue area to the testis area were seen in any nonylphenol- and bisphenol A-treated groups. Proportions of the ovarian tissue area to the ovotestis area in the nonylphenol 200, 2,000, 20,000, and 200,000 ng groups were 14.7 ± 8.7, 11.8 ± 4.1, 19.7 ± 10.8, and 13.6 ± 4.6%, respectively (Fig. 4b). Proportions of the ovarian tissue area to the ovotestis area in the bisphenol A 20, 200, 2,000, and 20,000 ng groups were 10.2 ± 4.1, 12.3 ± 4.1, 24.7 ± 14.4, and 11.2 ± 5.8%, respectively (Fig. 4b). The proportions in all nonylphenol-treated groups and in all bisphenol A-treated groups were not significantly different among the corresponding groups. In addition, no dose-related changes of the ovarian tissue area to the ovotestis area were seen in any nonylphenol- and bisphenol A-treated groups. The coefficient (r) of correlation values between proportions of ovarian tissue area to the testis area and doses of nonylphenol and bisphenol A were -0.07146

Table 3. Incidence of ovotestis in male embryos treated with nonylphenol (NP) and bisphenol A (BPA)

Groups	Ovotestis (%)
NP 0 ng (control)	0.0 (0/15)[a]
NP 200 ng	28.6 (4/14)*
NP 2,000 ng	41.2 (7/17)**
NP 20,000 ng	31.3 (5/16)*
NP 200,000 ng	34.8 (8/23)*
BPA 0 ng (control)	0.0 (0/14)
BPA 20 ng	41.2 (7/17)**
BPA 200 ng	33.3 (7/21)*
BPA 2,000 ng	40.0 (6/15)*
BPA 20,000 ng	45.5 (10/22)**

[a] Numbers of male embryos with ovotestis/numbers of male embryos examined.
* $p < 0.05$ from the control group.
** $p < 0.01$ from the control group.

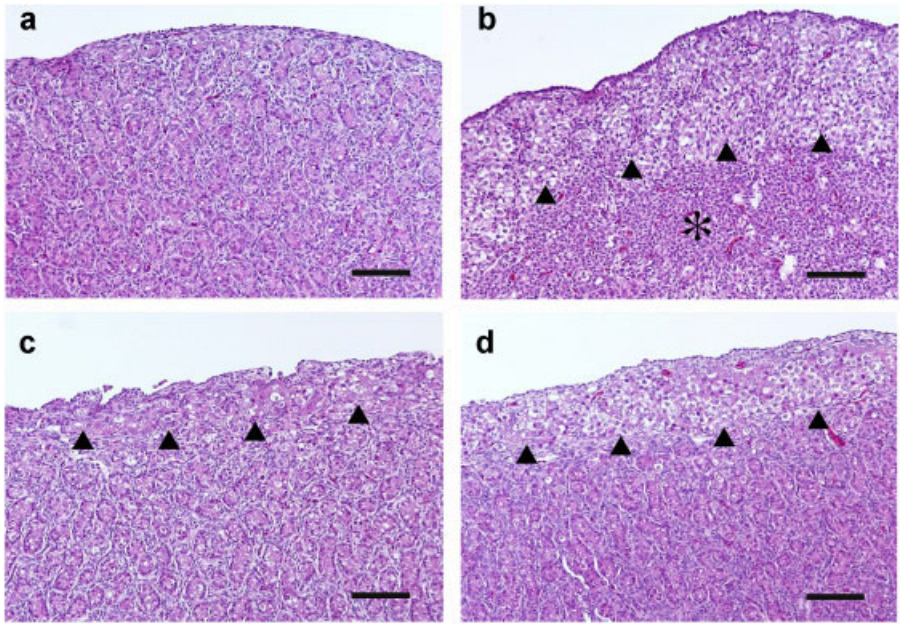

Fig. 3. Histologic features of gonads in F_1(AWE × WE) quail embryos at the incubation day 16. (**a**) A testis of nonylphenol 0 ng group showing normal appearance, consisting of densely packed testicular cords with a thin germinal epithelium. (**b**) An ovary of bisphenol A 0 ng group showing normal appearance, consisting of the cuboidal superficial epithelium, secondary sex cords containing immature oocytes (arrowheads), and medullary cords (asterisk). Ovotestes of nonylphenol 200,000-ng group (**c**) and of bisphenol A 200-ng group (**d**) showing various volumes of the cortical area consisting of oocyte-like germ cells (arrowheads) covering the testicular cords. HE stain, bar: 50 μm (**a, b, c, d**).

and -0.16717 in the nonylphenol and bisphenol A groups, respectively, showing few correlations.

DISCUSSION

Recently, using highly conserved primers flanking the intron of the W-linked gene (CHD1W), polymerase chain reaction amplification, and agarose electrophoresis, 47 of 50 quail species (*Coturnix coturnix*) were successfully sexed [13], although the method did not provide complete results and was time consuming. However, the genetic sex difference of F_1(AWE × WE) quail embryos ruled by a criss-cross inheritance [8,9] could be determined by the birds' plumage color. In the present study, the genetic sex of all embryos in all nonylphenol- and bisphenol A-treated groups and their corresponding control groups coincided completely with the external sex phenotype of the gonads, being similar to the results in our previous studies [8,9]. Therefore, the present screening model using F_1(AWE × WE) quail embryos, which are easy to distinguish genetic sex externally, is suitable to evaluate the sex reversal effects of chemicals on birds.

In the present study, viabilities of the F_1(AWE × WE) quail embryos at 16 d of incubation in all nonylphenol- and bisphenol A-treated groups were not significantly different from those in the corresponding control groups and were unrelated to the dose levels. In addition, high viabilities (86.1% and 83.3% in each control group) of the F_1(AWE × WE) quail embryos in the present study were similar to those in the previous studies, which assessed E_2 and methyltestosterone [8] and E_2, DES, and EE_2 [9]. Doses of nonylphenol and bisphenol A selected in the present study may not have been lethal or toxic for quail embryos. The injection amount (20 μl/egg) into the egg white is likely to be appropriate as described previously [14]. Consequently, the present screening model was applied successfully to investigate in ovo effects of EDCs on Japanese quail embryos. Other routes of in ovo exposure to chemicals have been tried, such as egg dipping [15,16] and injection into the egg yolk [17–19].

Natural estrogen and synthetic estrogens have been described to induce feminization of the male embryos in Japanese quail and chicken [15,16,18,20]. Development of the ovotestis in male embryos was observed at 0.7 ng/g egg for EE_2 and at 2 ng/g egg for DES [18], indicating that the potential of feminization effects may be different among natural estrogen and synthetic estrogens, as well as other estrogenic endocrine disrupting chemicals. Our previous study using the present screening model with F_1(AWE × WE) quail embryos revealed that in ovo treatment of E_2, DES, and EE_2 resulted in dose-dependent feminization of the male gonads and confirmed that the potential of feminization effects on the Japanese quail embryos were different among E_2, DES, and EE_2 [9]. In the present study, however, the incidence of ovotestis and the proportion of ovarian tissue area in the testis and in the ovotestis were not related to the dose levels of both nonylphenol and bisphenol A, even though a 10-fold multiplication factor for the doses was selected. In addition, the coefficient values (r) of correlation between the proportions of ovarian tissue area in the ovotestis and the dose levels of nonylphenol and bisphenol A were -0.07146 and -0.16717, respectively, suggesting that dose levels of nonylphenol and bisphenol A did not correlate with the potential of feminization effects on the testis of Japanese quail embryos.

Two subtypes of ER, ERα, and ERβ, are expressed in many organs in birds and in mammals. The ERα mRNA shows a higher expression than ERβ mRNA in the gonads of Japanese quail embryos by reverse transcription polymerase chain reaction analysis [21–23]. Estrogen-induced disruption of reproductive organ development such as ovotestis formation in the right gonad could be mediated via ERα [22,24]. Several environmental estrogens, including industrial chemicals such as nonylphenol and bisphenol A, and phytoestrogens such as genistein and coumestrol, have been shown to interact differentially with ERα

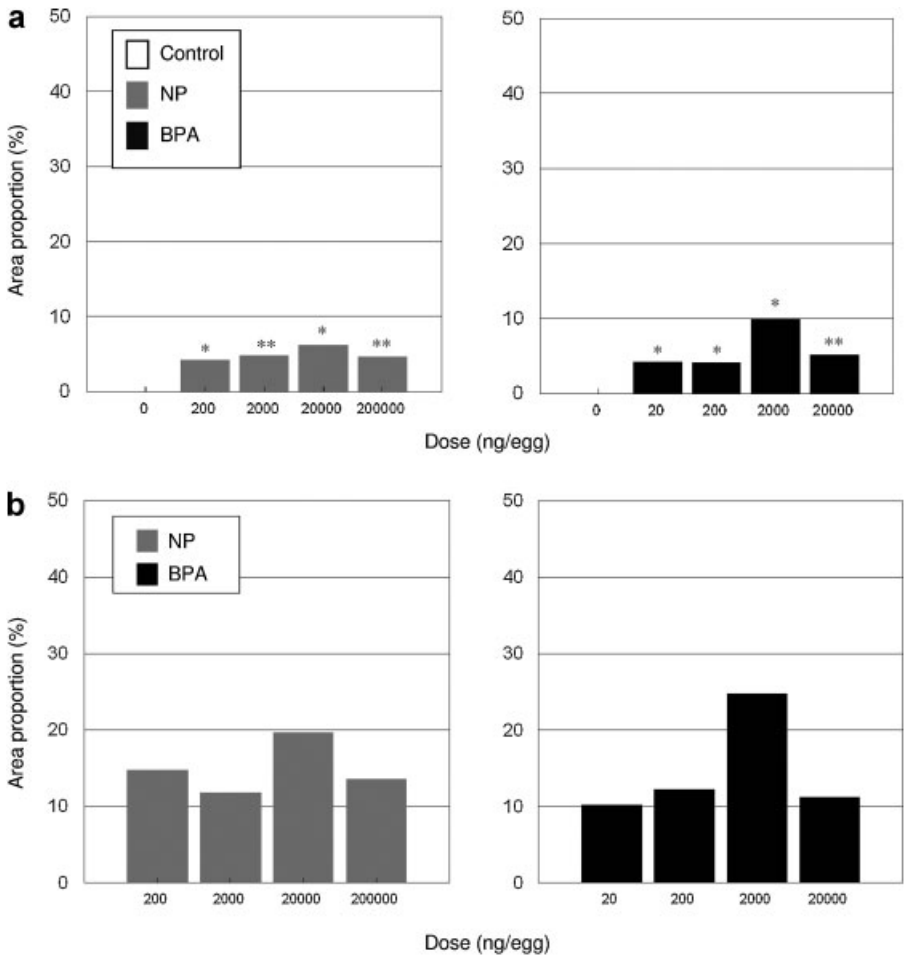

Fig. 4. (**a**) Area portion (%) of ovarian tissues in the testis of all male embryos treated with nonylphenol (NP) and bisphenol A (BPA). (**b**) Area portion (%) of ovarian tissues in the testis of male embryos with ovotestis treated with NP and BPA. $^{*}p < 0.05$; $^{**}p < 0.01$.

and ERβ [25,26]. Although in vitro binding assays using the ligand binding domain of quail ERα and ERβ revealed that nonylphenol had 6.00% and 4.30% and bisphenol A had 1.57% and 6.67% lower affinity for ERα and ERβ, respectively, than DES in Japanese quail [11], the ovotestis was detected in the lowest dose, 200 ng/egg and 20 ng/egg, nonylphenol and bisphenol A, respectively, and was not related to the dose levels.

The present study revealed that nonylphenol and bisphenol A have the dose-independent potential of ovotestis induction in the Japanese quail embryo. Clarifying the role of the ER signaling pathway or other possible compensatory routes of the dose-independent feminization effects of nonylphenol and bisphenol A remain to be investigated in future studies.

Three female embryos treated with bisphenol A at 20, 2,000, and 20,000 ng possessed aberrant small ovary-like tissues as the counterpart to the native left ovary. Histologically, those aberrant ovary-like tissues consisted of normal ovarian tissues, although the size was small. These results suggest that abnormal differentiation such as retention of the right gonad would be induced by the bisphenol A treatment because the right gonad of female birds regresses essentially during embryogenesis. In female embryo of Japanese quail, a slight asymmetry of size was seen in both gonads at 7 d of incubation, and subsequently the right gonad was almost completely regressed at 9 d of incubation [22]. Sequential studies are needed to provide information on the vestigial right ovary in female embryos of Japanese quail. In conclusion, the present study revealed that nonylphenol and bisphenol A have the dose-independent potential of ovotestis induction in the Japanese quail embryo.

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