Comparative Evaluation of Sex Reversal Effects of Natural and Synthetic Estrogens in Sex Reversal Test Using \( F_1 \) \((AWE \times WE)\) Japanese Quail Embryos

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Sex reversal effects of 17 beta-estradiol (E\(_2\)), diethylstilbestrol (DES) and ethynylestradiol (EE\(_2\)) on male gonads in \( F_1 \) \((AWE \times WE)\) Japanese quail \((Corturnix japonica)\) embryos were comparatively evaluated in a newly developed \textit{in vivo} screening model called as the sex reversal test. Male and female offspring of \( F_1 \) \((AWE \times WE)\) Japanese quail exhibit exactly wild and albino plumage colors, respectively, ruled by a criss-cross inheritance. The natural and synthetic estrogens were injected into egg white just before the incubation. At 16 days of incubation, embryos were subjected by a complete necropsy and their gonads were grossly observed and examined histopathologically and morphometrically. Grossly, genetic sex confirmed by plumage colors coincided completely with external sex phenotype of the gonads in all embryos of the control group and E\(_2\) and DES-treated groups. However, several male embryos with wild plumage in the EE\(_2\) 2000 ng group possessed an ovary-like gonad in the left side and a vestigial right gonad. Histopathologically, E\(_2\), DES and EE\(_2\) exposures induced a dose-dependent sex reversal effect, i.e. ovotestis development, in the left testis. The left testes showing an ovary-like morphology in the EE\(_2\) 2000 ng group consisted of the most of area replaced with ovarian tissue and the small area of remaining testicular cords. The incidence and morphometric analysis of the ovotestis revealed that the order of potency of sex reversal effect in Japanese quail embryos was EE\(_2\) > DES > E\(_2\). E\(_2\), DES and EE\(_2\) exposures induced no noticeable changes in the ovaries of any embryos. The present study suggests that the sex reversal test using \( F_1 \) \((AWE \times WE)\) Japanese quail embryo is possible to evaluate feminization effects of endocrine disrupting chemicals with estrogenic activities in avian male embryos.

\textbf{Key words} : endocrine disrupting effects, estrogens, Japanese quail embryo, ovotestis, screening model

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\textbf{Introduction}

Endocrine disrupting chemicals (EDCs) are any chemicals known or suspected to cause adverse endocrine effects in organisms or their progeny and have been considered to possess estrogenic or other endocrine activity in wildlife animals and humans \((\text{Damstra et al., 2002})\). 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 important wildlife species exposed by environmental pollutants with endocrine disrupting potential (Rattner et al., 1984; Ankley et al., 1998). There are aspects of sexual differentiation in birds that may make them uniquely sensitive to the effects of EDCs with estrogenic activity (Damstra et al., 2002). It is well known that avian embryos have a significant risk for 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.

Previously we developed a new avian in vivo screening model called as the sex reversal test using embryos of the unique strain, F1 (AWE×WE), of Japanese quail (Corturnix japonica) (Shibuya et al., 2004). In the sex reversal test, 17 beta-estradiol (E2) 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. Thus we concluded that the sex reversal test would be possible to evaluate estrogenic, not androgenic, endocrine disrupting effects of chemicals.

The present study is undertaken to investigate comparatively sex reversal effects as estrogenic endocrine disrupting effects of E2, diethylstilbestrol (DES) and ethynylestradiol (EE2) on male gonads in F1 (AWE×WE) Japanese quail embryos using the sex reversal test.

**Materials and Methods**

**Chemicals**

17 beta-estradiol (E2, molecular weight : 272.4) was purchased from Sigma Chemical Co. (MO, U.S.A.). Diethylstilbestrol (DES, molecular weight : 268.36) was purchased from ICN Biochemicals Inc. (OH, U.S.A.). Ethynylestradiol (EE2, molecular weight : 296.41) 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. A mating between male AWE quail with albino plumage color and female WE quail with wild plumage color produces inheritably male F1 quail with wild plumage color and female F1 quail with albino plumage color ruled by a criss-cross inheritance as described previously (Shibuya et al., 2004). 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 500 F1 (AWE×WE) Japanese quail (Corturnix japonica) eggs were purchased from the Laboratory Animal Research Station of the Nippon Institute for Biological Science and 418 eggs were used in the present study. Before the experiments, all eggs were observed externally and candel to check abnormalities and fine cracks.
Abnormal eggs that were cracked, broken or abnormal externally were excluded from the study.

**Study Design**

Eggs were allocated into 10 groups, which consisted of the control, E₂ 20, 200, and 2000 ng, DES 20, 200, and 2000 ng, and EE₂ 20, 200, and 2000 ng groups. Each egg was treated with a single injection of 20 μl of corn oil containing each dose of E₂, DES, and EE₂, 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 (Berg et al., 2001). 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 once/h of egg-turning cycle. At incubation day-7, 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 early embryo death. At incubation day-16, 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. The reasons that the embryos were necropsied at incubation day-16 were as follows: the first; sexual differentiation of the gonads has been finished, the second; the gonads became the sufficient size for histopathological examination, and the third; the identification of chicks was difficult after the hatch. After 3 days of fixation, 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 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 Inc., 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).

**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 Dunnnett’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. Coefficient (r) of correlation between the proportions of ovarian tissue area in the testis area and doses of E_2, DES, and EE_2 were estimated.

**Results**

*Fertility and Viability*

Fertility and viability in the control group and E_2, DES, and EE_2-treated groups were shown in Table 1. Fertility of the eggs in the DES 2000 ng group was significantly higher (p<0.05) than that in the control group. No significant differences of the fertility compared with the control group were detected in any E_2 or EE_2-treated groups or other DES-treated groups. Viabilities of the embryos at 16 days of incubation in all E_2 and EE_2-treated groups were not significantly different from that in the control group. Viability in the DES 20 ng group was significantly (p<0.05) lower than that in the control group, whereas viabilities in the DES 200 and 2000 ng group were not significantly different from that in the control group.

*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, all E_2 and DES-treated groups, and the EE_2 20 and 200 ng groups (Table 2). However, six out of 14 male embryos with wild plumage color in the EE_2 2000 ng group possessed an ovary-like left gonad and a vestigial right gonad, indicating that the sex of these embryos was reversed (Fig. 1). Sex ratios ( % of male) in all E_2 and DES-treated groups and the EE_2 20 and 200 ng groups were not significantly different from that in

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fertility (%)</th>
<th>Viability (%) of incubation day-16 embryos</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.6 (28/44)</td>
<td>92.9 (26/28)</td>
</tr>
<tr>
<td>E_2 20 ng</td>
<td>71.1 (32/45)</td>
<td>90.6 (29/32)</td>
</tr>
<tr>
<td>E_2 200 ng</td>
<td>66.7 (30/45)</td>
<td>83.3 (25/30)</td>
</tr>
<tr>
<td>E_2 2000 ng</td>
<td>70.5 (31/44)</td>
<td>83.9 (26/31)</td>
</tr>
<tr>
<td>DES 20 ng</td>
<td>75.0 (30/40)</td>
<td>70.0 (21/30)</td>
</tr>
<tr>
<td>DES 200 ng</td>
<td>72.5 (29/40)</td>
<td>79.3 (23/29)</td>
</tr>
<tr>
<td>DES 2000 ng</td>
<td>87.5 (35/40)</td>
<td>85.7 (30/35)</td>
</tr>
<tr>
<td>EE_2 20 ng</td>
<td>77.5 (31/40)</td>
<td>80.6 (25/31)</td>
</tr>
<tr>
<td>EE_2 200 ng</td>
<td>70.0 (28/40)</td>
<td>92.9 (26/28)</td>
</tr>
<tr>
<td>EE_2 2000 ng</td>
<td>77.5 (31/40)</td>
<td>77.4 (24/31)</td>
</tr>
</tbody>
</table>

* 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.
the control groups. However, the percent of male in the EE₂ 2000 ng group was significantly ($p < 0.05$) lower than that in the control group and consequently the conformability of the EE₂ 2000 ng was also significantly ($p < 0.05$) lower than that in the control group (Table 2).

**Pathology**

Grossly, genetically female embryos with albino plumage color possessed the externally normal ovary in the control group, and all E₂, DES and EE₂-treated groups. In genetically male embryos with wild plumage color, morphology of the ovotestis was
not distinguished externally from that in the normal testis in the E₂ 200 and 2000 ng groups, all DES-treated groups and the EE₂ 20 and 200 ng group. In the EE₂ 2000 ng group, 6 genetically male embryos with wild plumage color possessed an ovary-like left gonad and a vestigial right gonad (Fig. 1). Atrophy of the right testis was observed in 9 out of 12 (75.0%) males of the DES 200 ng group, 19 out of 20 (95.0%) males of the DES 2000 ng group, 10 out of 11 (90.9%) males of the EE₂ 200 ng group, and 14 out of 14 (100%) males of the EE₂ 2000 ng group, and the incidences of these groups were significantly higher (p < 0.01) than that of the control group.

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. The normal ovary of the incubation day-16 embryo showed the germinial 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. The ovotestis, feminization of the testis, was detected only in the left testis and possessed a various volume of the cortical area consisting of oocyte-like germ cells similar to the secondary sex cords and was covered with a roughened single layer of the cuboidal epithelial cells like a ovary and the inner portion consisted of normal testicular cords (Fig. 2 a). In the ovotestis of the EE₂ 2000 ng group, which showed externally an ovary-like feature, the most of testicular area was replaced with secondary sex cord-like tissues of the ovary, whereas small area of the testicular cords remained in the medulla (Fig. 2 b). The right vestigial or atrophic testes showed a decrease in size but normal morphology. There were no noticeable changes in the ovaries of quail embryos in any E₂, DES, and EE₂-treated groups.

The incidences of ovotestis in the control, E₂ 20, 200 and 2000 ng, DES 20, 200 and 2000 ng, and EE₂ 20, 200 and 2000 ng groups were 0.0, 0.0, 53.3, 90.9, 61.5, 66.7, 100.0, 86.7, 100.0, and 100.0%, respectively (Table 3). Significant differences (p < 0.01) of the incidence of ovotestis from the control group were detected in the E₂ 200 and 2000 ng groups, and all DES and EE₂-treated groups.

**Morphometric Analysis of Ovotestis**

Proportions of the ovarian tissue area in the testis area of all embryos in the control group, E₂ 20, 200, 2000, DES 20, 200, 2000, and EE₂ 20, 200, 2000 ng groups were 0.0 ± 0.0, 0.0 ± 0.0, 9.1 ± 9.0, 26.1 ± 22.1, 9.0 ± 8.5, 12.9 ± 11.0, 49.6 ± 25.8, 19.9 ± 18.6, 40.1 ± 8.0, and 73.1 ± 22.9%, respectively (Fig. 3). The proportions in the E₂ 200 and 2000 ng groups, all DES and EE₂-treated groups were significantly higher (p < 0.01) than that in the control group. Proportions of the ovarian tissue area in the testis area of embryos with ovotestis in the E₂ 20, 200, 2000, DES 20, 200, 2000, and EE₂ 20, 200, 2000 ng groups were 0.0 ± 0.0, 17.0 ± 3.0, 31.4 ± 20.4, 14.7 ± 5.5, 19.4 ± 6.9, 49.6 ± 25.8, 22.9 ± 18.1, 40.1 ± 8.0, and 73.1 ± 22.9%, respectively (Fig. 4), showing similar tendency to the proportion of the ovarian tissue area in the testis area. The coefficient values (r) of correlation between the proportions of ovarian tissue area in the testis and doses of E₂, DES, and EE₂ were 0.618, 0.728, and 0.761, respectively (Fig. 5).
Fig. 2. Histopathology of the ovotestes in male F$_1$ (AWE×WE) Japanese quail embryos at 16 day of incubation. a: An ovotestis in the DES 200 ng group showing a feminized cortical area consisting of oocyte-like germ cells covered with a roughened single layer of the cuboidal epithelial cells and the medullary testicular cords. b: An ovotestis in the EE$_2$ 2000 ng group showing the most of testicular area being replaced with secondary sex cord-like tissues of the ovary. Note a small area of the testicular cords remaining in the medulla. Hematoxylin and eosin stain, ×75.

Table 3. Incidence of Ovotestis in Male Embryos Treated with 17 Beta-estradiol (E$_2$), Diethylstilbestrol (DES) and Ethynylestradiol (EE$_2$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovotestis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 (0/17)$^a$</td>
</tr>
<tr>
<td>E$_2$ 20 ng</td>
<td>0.0 (0/15)</td>
</tr>
<tr>
<td>E$_2$ 200 ng</td>
<td>53.3 (8/15)$^b$</td>
</tr>
<tr>
<td>E$_2$ 2000 ng</td>
<td>90.9 (10/11)$^b$</td>
</tr>
<tr>
<td>DES 20 ng</td>
<td>61.5 (8/13)$^b$</td>
</tr>
<tr>
<td>DES 200 ng</td>
<td>66.7 (8/12)$^b$</td>
</tr>
<tr>
<td>DES 2000 ng</td>
<td>100.0 (20/20)$^b$</td>
</tr>
<tr>
<td>EE$_2$ 20 ng</td>
<td>86.7 (13/15)$^b$</td>
</tr>
<tr>
<td>EE$_2$ 200 ng</td>
<td>100.0 (11/11)$^b$</td>
</tr>
<tr>
<td>EE$_2$ 2000 ng</td>
<td>100.0 (14/14)$^b$</td>
</tr>
</tbody>
</table>

$^a$: Numbers of male embryos with ovotestis/numbers of male embryos examined.

$^b$: p < 0.01 from the control group.
Discussion

In the present study, viability of the F₁ (AWE×WE) quail embryos at 16 days of incubation in the control group was 92.9%, which was within the range of normal viability (percentage in fertile eggs), 85 to 98%, in Japanese quail documented in the testing guidelines of Avian Reproduction Test (OECD Testing Guideline 206, 1984; EPA OPPTS 850.2300, 1996). On the other hand, viabilities of the E₂ 200 and 2000 ng, DES 20 and 200 ng, and EE₂ 20 and 2000 ng groups showed less than 85% and the
viability of the DES 20 ng group was significantly lower than that in the control group. In addition, the fertility of the DES 2000 ng group was significantly higher than that in the control group. It seems to be accidental because there were no dose-dependencies of them in these groups. Our previous study revealed high viabilities (more than 21%) of the F₁ (AWE × WE) quail embryos in the no treated group and treated with E₂ at a dose as low as 20000 ng/egg and with methyltestosterone at a dose as low as 2000 ng/egg (Shibuya et al., 2004). It is likely that quality of the F₁ (AWE × WE) quail can be applied in avian in vivo screening models such as the sex reversal test. The injection into the egg white has been described to be one of routs in in ovo exposures of chemicals (Rissman et al., 1984), including other routs such as egg dipping (Scheib and Reyss-Brion, 1979; Perrin et al., 1995) and injection into the egg yolk (Gildersleeve et al., 1985; Berg et al., 1999; Halldin et al., 1999).

Fig. 5. Correlations between the proportions of ovarian tissue area in the testis and treatment doses of 17 beta-estradiol (E₂), diethylstilbestrol (DES) and ethynylestradiol (EE₂). The coefficient values (r) of E₂, DES and EE₂-treatments are 0.618, 0.728, and 0.761, respectively.
Genetic sex difference, i.e. plumage color, of all embryos in the control group, all E₂ and DES-treated groups, and the EE₂ 20 and 200 ng groups coincided completely with morphological sex phenotype of the gonads. However, morphology of the left gonad of 6 male embryos in the EE₂ 2000 ng group was not identical with genetic sex. These embryos with wild plumage color exhibiting male possessed an ovary-like left gonad and a vestigial right gonad, indicating that the sex of these embryos would be reversed. Histologically, the most of tissue of the ovary-like gonads in the EE₂ 2000 ng group consisted of the ovarian tissues, but small area of the testicular cords remained in the medulla. These results suggest that EE₂ treatment at 2000 ng/egg resulted in strong sex reversal effects in Japanese quail embryos at 16-days of incubation. 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 (Fridolfsson and Ellgren, 1999). The system of our sex reversal test using the F₁ (AWE×WE) quail embryos is easy to distinguish genetic sex, being suitable to evaluate sex reversal effects of chemicals.

Natural estrogen and synthetic estrogens have been described to induce feminization of the male embryos in Japanese quail and chicken (Romanoff, 1960). Feminization of genetically male embryos of Japanese quail has induced by DES and EE₂ in the routes of egg dipping (Scheib and Reyss-Brion, 1979; Perrin et al., 1995) and injection into the egg yolk (Berg et al., 1999). Development of the ovotestis in male embryos was observed at 0.7 ng/g egg for EE₂ and at 2 ng/g egg for DES (Berg et al., 1999), indicating that feminization activity of DES was weaker than EE₂. However, there have been no comparative evaluations of the potential of feminization effects among E₂, DES and EE₂ in the Japanese quail embryos. In the present study, the treatment of E₂ at 20 ng/egg (approximately 2 ng/g egg) resulted in no development of the ovotestis, although the treatment of DES and EE₂ at 20 ng/egg (approximately 2 ng/g egg) induced the ovotestis, suggesting that the sex reversal potential of DES and EE₂ is stronger than E₂. On the other hand, the incidence of the ovotestis and the proportion of ovarian tissue area in the testis or ovotestis in the EE₂-treated groups were higher than those in the DES-treated groups at each dose. These results indicate that the sex reversal potential of EE₂ is stronger than that of DES. The coefficient values (r) of correlation between the proportions of ovarian tissue area in the testis and the treatment doses of E₂, DES and EE₂ showed high levels, suggesting that feminization effects of E₂, DES and EE₂ are dose-dependent and the order of coefficient value (r) was EE₂ > DES > E₂. Thus it can be reasoned that the order of potency of sex reversal effect in Japanese quail embryos is EE₂ > DES > E₂. Consequently, it seems remarkable that the sex reversal test using the F₁ (AWE×WE) quail embryos can be applied to evaluate comparatively chemicals with sex reversal potential such as feminization of the male gonad.

It has been described that several environmental contaminants such as DDT, methoxychlor, and bisphenol A, called as EDCs, resulted in the feminization of the gonads of male embryos in birds (Fry and Toone, 1981; Berg et al., 2001; Damstra et al., 2002). However, avian in vivo screening that evaluated comparatively sex reversal potential of various EDCs with estrogenic activity has not been provided. The newly
developed sex reversal test using F₁ (AWE×WE) Japanese quail embryos may be useful to evaluate comparatively estrogenic endocrine disrupting effects of EDCs in birds. Sequential studies using various EDCs remains to provide information on sex reversal effects in avian embryos.

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References


