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≪**Research Note**≫

Effects of Ethynylestradiol Injection into Maternal Japanese Quail (*Coturnix japonica*) on Male Reproductive Function of the F1 Generation.

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This study examined whether administration of ethynylestradiol to laying quail affects male reproductive function in the F1 generation. Regularly laying Japanese quail were daily-injected with 50μl of corn oil or 1μg of ethynylestradiol dissolved in 50μl of corn oil for 5 days. Fertilized eggs were then collected 2, 4 and 6 d after the first injection (groups C1, C3 and C5 for oil treatment ; E1, E3 and E5 for ethynylestradiol treatment). The chicks that hatched from these eggs were raised and their reproductive functions (age at sexual maturity, copulation frequency, testicular weight, semen or sperm quality and fertility) were examined. Reproductive function did not differ significantly between ethynylestradiol-treated groups and corresponding controls (C1 to E1, C3 to E3, C5 to E5) and a among treatment days within control or treatment (C1—C5 or E1—E5). These results suggest that injection of ethynylestradiol in laying birds under the conditions used in this study does not significantly disrupt male reproductive function in the F 1 generation.

Key words : ethynylestradiol, Japanese quail, F1 generation, male reproductive function

Introduction

In the male quail, an active spermatogenesis and the maturation of sperm in the epididymis and ductus deferens are essential for normal genital function. Moreover, as a normal male copulates and ejaculates sperm with seminal fluid and the female produces fertilized eggs for a certain period, an anatomical analysis of these organs, check of semen quality, inspection of copulation action, and test of fertility after mating are necessary to evaluate the reproductive ability of males.

Previously, we demonstrated that administration of diethylstilbestrol (DES, 0.1 or 1mg on a daily basis for one week) to male Japanese quail caused a disruption in reproductive function by affecting spermatogenesis and influencing sperm motility (Maeda and Yoshimura, 2002). Moreover, it has been reported that endocrine disrupters cause reproductive abnormalities in a number of animal species (Perrin *et al.*, 1995 ; Khan *et al.*, 1998 ; Atanassova *et al.*, 1999 ; Berg *et al.*, 1999 ; Stoker *et al.*,

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2000 ; Masuda and Koyanagi, 2001, 2002 ; McKinnell *et al.*, 2001 ; Williams *et al.*, 2001 ; Yoshimura *et al.*, 2000 ; Yoshimura and Kawai, 2002). It is assumed that the majorities of endocrine disruptors exert estrogenic effects, and may cause reproductive disorders in the next generation. However, no study has examined the influence that endocrine disrupters exert on the reproductive functions of the F1 generation.

Berg *et al.* (2001) reported that a series of abnormalities was observed after injection of ethynylestradiol (2 or 20 ng/g egg) into the yolk on day 3 of incubation. In this study, ethynylestradiol was used as a typical estrogenic chemical causing endocrine disruption. The aim of this study was to determine whether administration of ethynylestradiol to laying quail affects male reproductive functions in the F1 generation.

Materials and Methods

Animals

Matured male and female quail provided by Tokaiyuki Co. (Toyohashi, Japan) and kept in cages under a light regimen of 14 L : 10 D with feed and water available *ad libitum,* were used in this study. The females were daily injected intramuscularly with 50 μl of corn oil (Wako Pure Chem., Lot SEE7703, Osaka) or 1 μg of ethynylestradiol (Wako Pure Chem., Lot DWP3541, Osaka) dissolved in 50 μl of corn oil for 5 days. The dosage of ethynylestradiol was referred to the current report (Berg *et al.*, 2001). Their eggs were collected 2, 4 and 6 d after the first injection, and therefore had been exposed to the injected materials for 1, 3 and 5 d, respectively. They were hatched using an electric incubator (Showa Incubator Co., Tokyo) at 37.5℃. The hatchings from the eggs collected at 2 d, 4 d and 6 d were referred to as C1, C3 and C5 in the oil-injected group, and E1, E3 and E5 in the ethynylestradiol-injected group. They were kept in a brooder (37℃) for 3 weeks and provided with feed and water *ad libitum.* They were maintained under a light regimen as follows : continuous lighting until 7-d-old, 20L : 4D until 2 weeks old, 16L : 8D until 4 weeks old and then 14L : 10D thereafter.

Analysis of reproductive functions of F1 males

The numbers of F1 male birds used for the analysis were 9, 6, and 8 in C1, C3 and C5, and 10, 9 and 6 in E1, E3 and E5, respectively. The age at sexual maturity was judged as follows. The cloaca of each bird was observed every day from four weeks of age. The sexual maturity was determined by secretion of a foam-like fluid from the cloaca which took on a red and swollen appearance.

Each matured male (58-d-old) was cohabited with a normal female for one hour, and the copulation frequency was scored. Then, the male was kept with the female for one day and apart thereafter. The eggs were collected for 14 days after two days later copulation. They were incubated for seven days using an electric incubator at 37.8℃, and the fertility at one and two weeks was examined.

The birds (80-d-old) were killed by decapitation. The collection of semen and the analysis of sperm density and motility were done according to our previous reports (Maeda and Yoshimura, 2002 ; Maeda, 2002). The white blood cells in semen were enumerated using a hemocytometer by diluting the semen five-fold with a PBS solution supplemented with 2% (v/v) formalin to confirm whether the inflammation has

happened in the sexual glands. The number of cells per milliliter of semen was scored. Then, the testes were excised and weighed to obtain the tissue weight per 100 g of body weight (BW).

Statistical analysis

All percentage data on sperm motility and fertility were subjected to an arcsin transformation prior to statistical analysis. The significance of differences among treatment days (C1—C3 and E1—E3) was examined by one-way ANOVA, followed by Fisher's protected least significant difference post-hoc test. The significance of differences between ethynylestradiol treatments and corresponding controls (C1 to E1, C3 to E3 and C5 to E5) was examined by Student's t-test using StatView software (Abacus Concepts Inc, Berkeley, CA). Significance was taken at $P<0.05$.

Results

The age at sexual maturity and the copulation frequency of F1 males did not differ significantly between ethynylestradiol-treated groups and corresponding controls (C1 to E1, C3 to E3, C5 to E5) or among treatment days within control or ethynylestradiol-treated groups (C1—C5 or E1—E5) (Figs. 1 and 2). No significant differences were

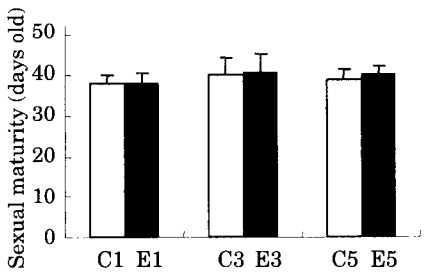

Fig. 1. Effects of maternal administration of ethynylestradiol on the sexual maturity of F1 males. Each bar represents the mean±S.D. of the age at sexual maturity. C1 (n=9), C3 (n=6), C5 (n=8), E1 (n=10), E3 (n=9) and E5 (n=6) : F1 birds derived from eggs laid by quail injected daily with 50μl of corn oil or 1μg of ethynylestradiol, which were collected 2 d, 4 d and 6 d after the first injection (C1, C3 and C5, respectively, in oil group ; E1, E3 and E5, respectively, in ethynylestradiol group).

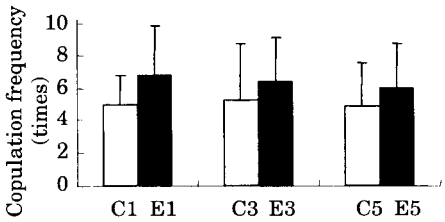

Fig. 2. Effects of maternal administration of ethynylestradiol on the copulation frequency of F1 males. Each bar represents the mean ±S.D. See Fig. 1 for further explanation.

also observed in the testicular weight (Fig. 3), semen or sperm quality (Fig. 4) and fertility (Fig. 5) of F1 males between treatment and corresponding control groups or among treatment days.

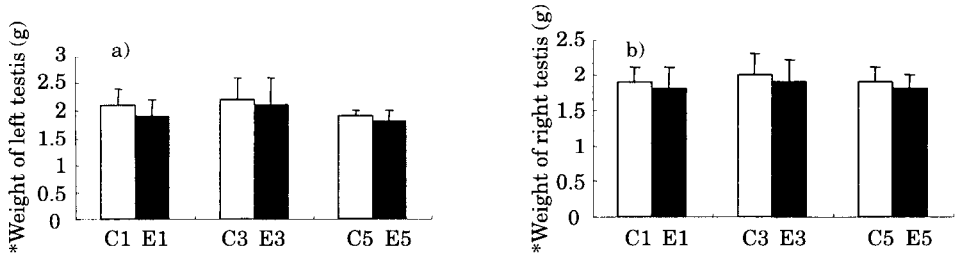

Fig. 3. Effects of maternal administration of ethynylestradiol on testicular weight (right and left) in F1 males. Each bar represents the mean±S.D. See Fig. 1 for further explanation. *weight per 100 g body weight.

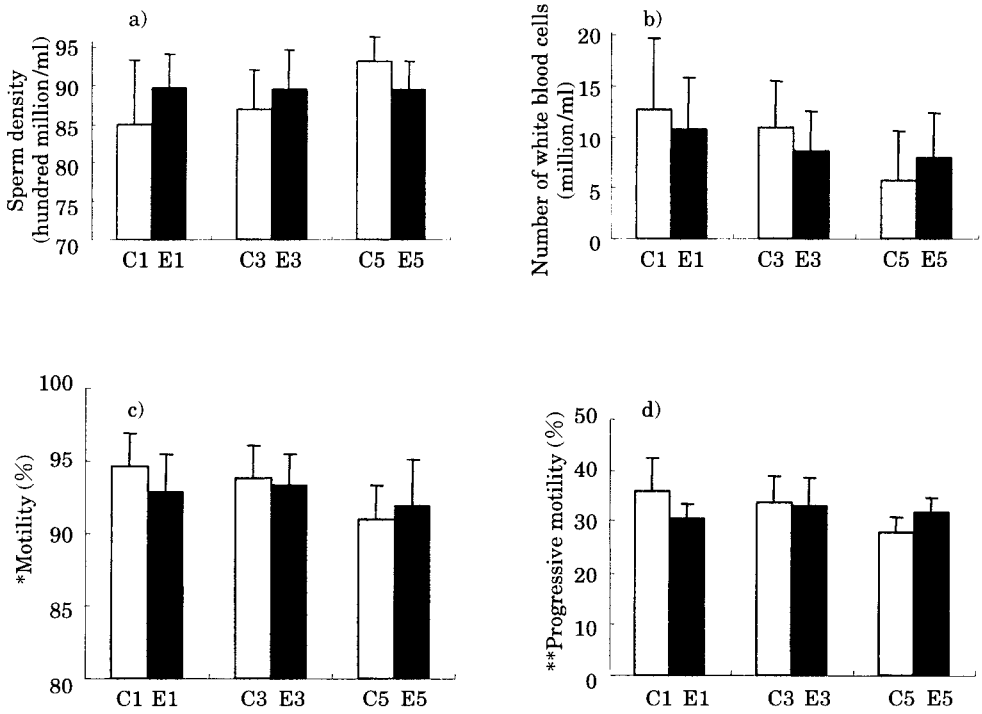

Fig. 4. Effects of maternal administration of ethynylestradiol on the semen or sperm quality (sperm density, number of white blood cells, motility and progressive motility) of F1 males. Each bar represents the mean±S.D. See Fig. 1 for further explanation. * Motility and **progress motility mean percentage of motile sperm with a path velocity of 12μm/s and percentage of motile sperm with a path velocity of 25μm/s and moving in a straight line 80% of the time, respectively.

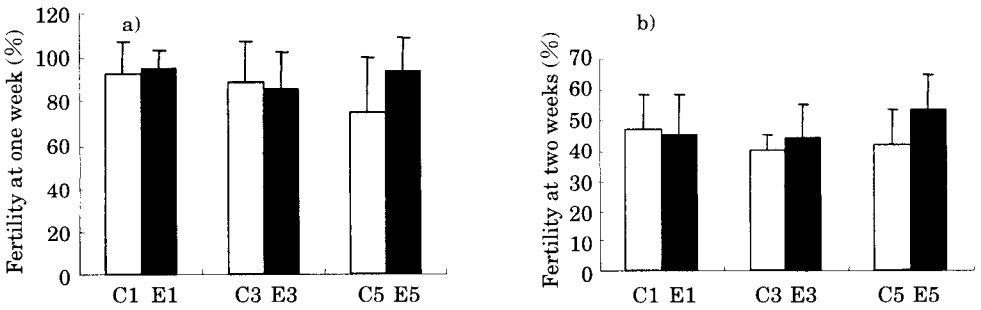

Fig. 5. Effects of maternal administration of ethynylestradiol on the fertility (one and two weeks) of F1 males. Each bar represents the mean±S.D. See Fig. 1 for further explanation.

Discussion

The exposure of eggs to estrogenic chemicals causes reproductive disorders in birds (Perrin *et al.*, 1995 ; Berg *et al.*, 1999 ; Masuda and Koyanagi, 2001, 2002 ; McKinnell *et al.*, 2001 ; Yoshimura and Kawai, 2002). Because the oocyte incorporates substances circulating in the blood, chemicals like endocrine disruptors taken up by laying hens may also enter the egg (Gildersleeve *et al.*, 1985). Thus an endocrine disruptor incorporated into the eggs may exert endocrine disruptive effects on the embryo or chick, namely the F1 generation.

Ethynylestradiol has been reported to cause noticeable abnormalities in reproductive organs leading to a loss of reproductive ability both in male and female quail when fertilized eggs were exposed in solution (Berg *et al.*, 1999). We have examined whether ethynylestradiol causes reproductive disorders in F1 males when injected in maternal quail. The age at sexual maturity, copulation frequency, testicular weight, semen or sperm quality and fertility of F1 males did not differ significantly between ethynylestradiol-treated groups and corresponding controls. Nor were differences in these measurements found among treatment days within control or ethynylestradiol-treated groups. These results suggest that ethynylestradiol injected in maternal birds in the amounts and under the conditions used in this study does not affect male reproductive functions including the function of sexual glands in the F1 generation.

The reason why the disruption to male reproductive function in the F1 generation was negligible in the present study is not clear. We assume that the amount of ethynylestradiol incorporated into the egg was too small to cause endocrine disruption, or the sensitivity of male quail to ethynylestradiol was low. Further study is required to clarify whether estrogenic chemicals cause reproductive disorders in the F1 generation when the dosage and period of administration are increased.

Acknowledgements

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