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Physiological Effects of Diethylstilbestrol Exposure on the Development of the Chicken Oviduct

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ABSTRACT

Estrogen has dramatic effects on the development and function of the reproductive tract in mammals. Although diethylstilbestrol (DES) triggers the development of reproductive organs in immature animals, continued exposure to DES induces dysfunction of the female reproductive tract in mice. To investigate the effects of neonatal estrogen exposure on the reproductive tract of female chickens, we implanted DES pellets into the abdominal region of immature female chicks and then examined the effects of DES on the oviducts of both immature chicks and sexually mature chickens (30 weeks old). DES induced mass growth and differentiation of the oviduct in immature chicks. The chick oviduct increased by 2.7- and 29-fold in length and weight, respectively, following primary DES stimulation. In secondary DES stimulation, the length and weight of the chick oviduct increased by 4.5- and 74-fold, respectively. Additionally, DES treatments caused abnormal development of the infundibulum and magnum in hen oviducts. Furthermore, infundibulum abnormality gave rise to unusual ovulation of follicles and resulted in infertility and dysfunction of the magnum, such as less production of egg white proteins. Our results indicate that DES exposure during early developmental stages in chickens has detrimental effects on the development and maintenance of the female reproductive tract after sexual maturation.

(**Key words :** Chicken, Diethylstilbestrol, Estrogen, Oviduct)

INTRODUCTION

Steroid hormones are major modulators of the development of the female reproductive system. Estrogen, the primary female sex hormone, is mainly secreted by granulosa cells of preovulatory follicles under the regulation of gonadotrophin in humans and mice (McNatty et al., 1979; Voronina et al., 2007). Estrogen also regulates the proliferation and differentiation of germ cells, induces the development of female reproductive organs, including the uterus and oviduct, and influences pregnancy in mammals (De Pol et al., 2001; Hewitt et al., 2005; Jefferson et al., 2006).

In chickens, estrogen regulates the ovulatory cycle and production of egg white proteins. The peak of estradiol concentration in laying hens is related to ovulation and ovoposition (Lague et al., 1975), and estrogen treatment increases the expression of egg white proteins such as ovalbumin (Oka and Schimke, 1969). Estrogen also influences the sexual differentiation of unspecialized gonads during chicken embryo development, and estrogen receptors are expressed in the medulla of both gonads and the germinal epithelium. However, the role of estrogen in the sexual differentiation process is considered a neutral factor and not an origin of sex differences (Gasc, 1980). However, administration of estrogen affects the development of immature chick oviducts (Kohler et al., 1969). Not only does estrogen trigger cell proliferation and differentiation of the chick oviduct, but also prevents cellular apoptosis. For example, oviduct weight

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increases by up to 1.5 g following estrogen stimulation during the 2 weeks after birth (Palmiter and Wrenn, 1971). Epithelial cells in the chicken oviduct differentiate into tubular gland cells, goblet cells, and ciliated cells in response to estrogen (Kohler et al., 1969). Also, the removal of estrogen treatment results in sharply increased cell death in the oviduct and increased expression of genes related to apoptosis, such as the caspase gene family (Monroe et al., 2000; Monroe et al., 2002).

Estrogen analogs have been used to examine the functions of estrogen in the mammalian reproductive tract and for medical applications. One estrogen analog is diethylstilbestrol (DES), which is a synthetic non-steroidal compound. Because DES has activity similar to estrogen, exposure to DES in immature chicks induces mass growth and differentiation of the chick oviduct, and rapidly increases the expression of egg white genes such as ovalbumin and lysozyme (Oka and Schimke, 1969). Furthermore, DES was medically utilized to prevent consecutive miscarriages from the late 1940s through the 1970s (Newbold, 2004). However, the medical application of DES was based on incomplete scientific information. Because the nature and function of DES are not wholly the same as those of natural estrogen, DES treatment unexpectedly caused harmful effects on the development of the female reproductive system and pregnant women. Perinatal exposure to DES induced structural malformation of the reproductive tract and paraovarian cysts of mesonephric origin (Newbold, 2004). As exposure to DES changes the genetic status of the developing uterus, it induces abnormal cytodifferentiation in the uterus (Huang et al., 2005).

In chickens, DES has been used to elucidate the mechanisms of oviduct development influenced by estrogen, to culture tubular gland cells from the chicken oviduct *in vitro,* and to study regulatory mechanisms of the chicken *ovalbumin* gene (Dougherty and Sanders, 2005). However, detrimental effects of DES on the chicken female reproductive system have not been clearly revealed. In this study, we evaluated the effects of DES on the development and function of the oviduct in neonatal (1-week-old) chicks and sexually matured chickens. We found that neonatal exposure to DES stimulated the growth and differentiation of neonatal chick oviducts; however, DES induced dysfunction of the adult chicken oviduct after sexual maturation and caused hen infertility.

MATERIALS AND METHODS

1. Animals

White leghorn chickens (*gallus gallus dometicus*) were raised and cared for at the University Animal Farm, College of Agriculture and Life Sciences, Seoul National University, Korea. All procedures for chicken management, estrogen treatment, and surgeries were performed according to standard protocols of the Laboratory of Animal Genetic Engineering, Seoul National University. Suitable animal management and use in this research were also carried out.

2. DNA preparation and PCR sexing

The sex of chicks was discerned by PCR amplification of a non-repetitive DNA sequence on the W chromosome, as previously described (Lee et al., 2009). Feather pulp and blood samples were collected from 4-day-old chicks and genomic DNA was isolated with a PUREGENE DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN). For PCR sexing, W-specific primers were used: W chromosome-specific forward primer 5'-CTA TGC CTA CCA CAT TCC TAT TTG C-3', W chromosome-specific reverse primer 5'-ACG TGG ACT TCA GAC CAT CTT CT-3'. Briefly, the total volume of the PCR reaction was 25 μl and contained 100 ng of genomic DNA, 10X PCR buffer (Biosesang, Seongnam, Korea), 2 μl of dNTPs (10 mM each), 2 pmol each of 5'-CTA TGC CTA CCA CAT TCC TAT TTG C-3' and the W chromosome-specific reverse primer 5'-ACG TGG ACT TCA GAC CAT CTT CT-3', and 0.5 units of Taq polymerase (Biosesang). The thermo-cycling conditions were as follows: 5 min at 94^{o}C; followed by 35 cycles of 1 min at 94^{o}C, 1 min at 64^{o}C, and 30 s at 72^{o}C; and finally 5 min at 72^{o}C.

3. DES treatment

For making the DES pellets, 100mg of DES powder (Sigma, St. Louis, MO) were put into the mold of laboratory press and then the mold was kept for 5 minutes under 5 tons of pressure. The DES treatment was divided into two steps (McKnight, 1978). Selected 1-week-old female chicks were stimulated with one DES pellet (15 mg/pellet) over 10 days and then the

DES pellet was withdrawn for 10 days. Primary-treated chicks were restimulated with two DES pellets over 10 days. After stimulation, some DES-treated chicks were sacrificed to analyze oviduct development in neonatal chicks and others were maintained until they were 30 weeks old to investigate the effects of DES treatment on hen oviducts.

4. Western blot analyses

To detect the chicken ovalbumin protein, 2 μl of egg white and 10 μg of total protein were extracted from the magnum of control and DES-treated chickens using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's guidelines and then electrophoresed on a 15% polyacrylamide gel and transferred to a Polyvinylidene Fluoride (PVDF) membrane (Millipore, Billerica, MA). Ovalbumin was detected with a mouse anti-chicken egg albumin (ovalbumin) antibody (Sigma) and rabbit anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA).

5. Statistical analyses

A *t*-test was used for statistical analyses; a significant difference between control and DES-treated samples was determined when the *P*-value was less than 0.01. Statistical analyses were conducted by student t-test in R program.

RESULTS

1. PCR sexing analyses

The 374-bp PCR product was only obtained from the genomic DNA of female chicks. Using PCR sexing, we selected 32 female chicks for the study. Three chicks each were used for the primary and secondary DES stimulation treatments. Three chicks were used for an analysis of hen sexual activity after DES treatment. Three or five chicks were used in each experiment as controls.

2. Effects of DES treatment on chicken oviducts

To emulate oviduct development of chickens that had reached puberty, 1-week-old chicks were stimulated with DES. Chicks were given 10 days for primary stimulation and then secondary stimulation was conducted during the 10 days after the first DES pellet had been removed for 10 days. After primary stimulation, the wet weight of 17-day-old chick oviducts increased 29-fold (32.7 ± 0.23 mg vs. 961 ± 60.7 mg) and the length increased 2.7-fold (3.07 ± 0.40 cm vs. 8.43 ± 1.01 cm; Table 1). After secondary stimulation, the wet weight of 37-day-old chick oviducts increased about 74-fold (49.6 ± 5.1 mg vs. 3665 ± 469 mg) and the length increased 4.5-fold (3.73 ± 0.42 cm vs. 16.8 ± 3.2 cm; Table 1). Oviducts from DES-treated chicks were morphologically differentiated into the infundibulum, magnum, isthmus, and shell gland (Fig. 1).

Table 1. Weight and length of chick oviducts from DES-stimulated and non-stimulated chicks

	Wet weight of oviduct (mg)	Length of oviduct (cm)	§No. of hens producing eggs/ Total no. of hens
No treatment (control 1†)	32.7 ± 0.23[a]	3.07 ± 0.40[a]	N/A
Primary DES treatment	961 ± 60.7[b]	8.43 ± 1.01[b]	N/A
No treatment (control 2‡)	49.6 ± 5.1[c]	3.73 ± 0.42[c]	5/5
Secondary DES treatment	3665 ± 469[d]	16.8 ± 3.2[d]	0/3

* Mean ± standard error

† 17-day-old chicks

‡ 37-day-old chicks

§ The number of hens producing eggs was determined after sexual maturation of control and DES-treated hens.

[a,b] Different letters between control 1 and primary DES treatment indicate significant differences ($p<0.01$). Data were analyzed using a student *t*-test.

[c,d] Different letters between control 2 and secondary DES treatment indicate significant differences ($p<0.01$). Data were analyzed using a student *t*-test.

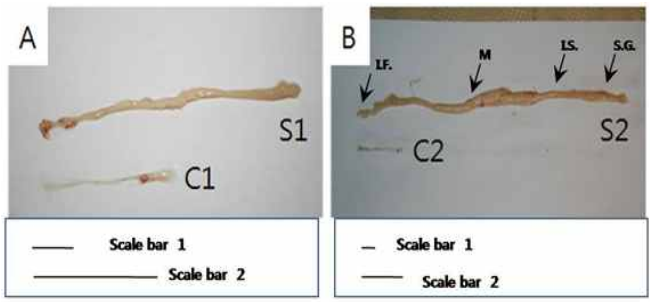

Fig. 1. Oviduct length in DES-treated and non-treated chickens. Scale bars 1 and 2 in panels A and B indicate 1 cm and 3 cm, respectively. The oviduct length of control 1 (C1) chickens in panel A was similar to that of control 2 (C2) chickens in panel B. Legend: C1, control one (17-day-old chicks); C2, control two (37-day-old chicks); S1, primary DES stimulation (17-day-old chicks); S2, secondary DES stimulation (37-day-old chicks); I.F., Infundibulum; M, magnum; I.S., isthmus; S.G., shell gland.

DES-treated chicks had lower body weight than of non-treated chicks (Fig. 2). As illustrated in Fig. 2, the body weight of primary and secondary DES-treated chicks decreased about 1.46-and 1.76-fold, respectively, compared to that of non-treated chicks. This result indicates that the growth effect of DES treatment is mainly seen in oviduct development, not overall body weight.

3. Evaluation of reproductive ability in DES-treated hens

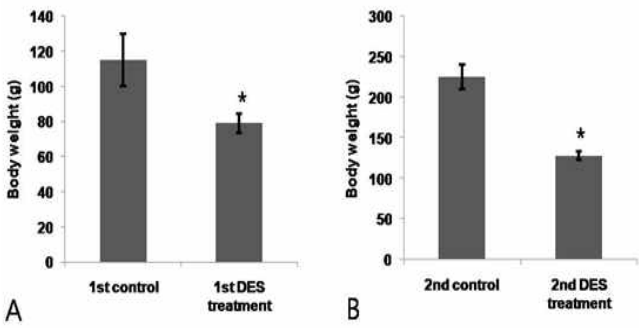

Fig. 2. Body weight in control and DES-stimulated chicks. Statistical significance was determined using a Student's *t*-test. Asterisk denotes $P < 0.01$. The body weight of DES-stimulated chicks was significantly lower than that of control chicks. (A) Control and primary-stimulated chicks (17 days old). (B) Control and secondary-stimulated chicks (37 days old).

After DES treatments, the treated chickens were raised until they were 30 weeks old. Three chickens were used to validate reproductive ability as hens after neonatal exposure to DES. Whereas the control group was able to produce eggs normally, DES-treated hens could not produce eggs (Table 1). To examine the cause of this reproductive abnormality, the DES-treated hens were sacrificed. Egg yolks that included matured oocytes, isthmus, and shell gland cells developed normally, but the infundibulum was abnormal (Fig. 3C and

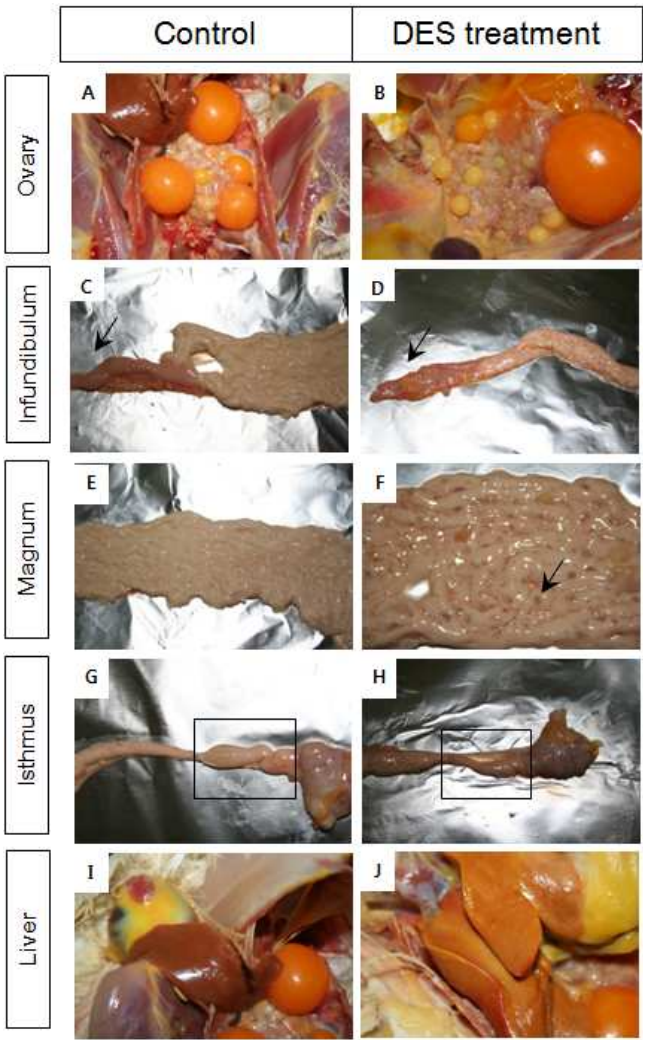

Fig. 3. Reproductive tract development in DES-treated and non-treated hens.
Hens were stimulated by DES when they were between 1 and 6 weeks old, and maintained until they were 30 weeks old. The arrows in panels C and D indicate a normal and abnormal infundibulum, respectively. The abnormal magnum structure of a DES-treated oviduct is indicted by a black arrow in panel F.

3D), which might be detrimental to the function of the infundibulum after ovulation. Matured egg yolk was ovulated into the abdominal cavity due to structural dysfunction of the infundibulum. Egg yolk in the abdominal cavity combined with the small intestine, resulted in change in liver color (Fig. 4). Furthermore, DES treatment caused abnormality of the oviductal magnum. The abnormal structures were observed in the magnum of DES-treated hen oviducts (Fig. 3F). Also, the production of egg white in the oviduct of DES-treated chickens was abnormal compared to the production of egg white in the magnum of control hens (Fig. 5A). Ovalbumin was detected in both the magnum of DES-treated and untreated chickens' oviducts (Fig. 5B). Collectively, neonatal DES exposure caused morphological and functional abnormalities in the infundibulum and magnum of hen oviducts.

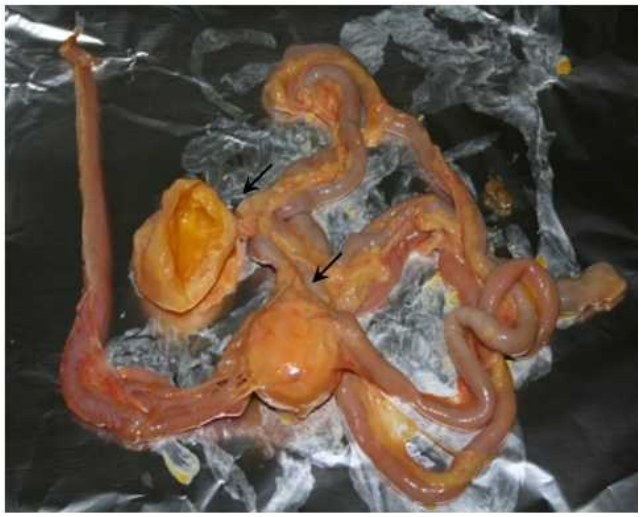

Fig. 4. Abdominal ovulation in DES-treated hens. Mature egg yolk was ovulated into the abdominal cavity due to abnormal development of the infundibulum. Egg yolk in the abdominal cavity combined with the small intestine. The combined region is indicated by a black arrow.

DISCUSSION

We demonstrated that subcutaneous implantation of DES over 10 days can stimulate the proliferation and differentiation of chick oviducts, as in previous reports (O'Malley, 1969; Palmiter and Wrenn, 1971). DES rapidly increased the wet weight and length of chick oviducts in early developmental stages. The weight of chick oviducts increased approximately 29- and 74-fold following primary and secondary stimulation, respectively. Likewise, chick oviducts were 2.7- and 4.5-fold longer following primary and secondary DES exposure, respectively. Collectively, the effects of DES on neonatal oviduct development in this study were similar to those in a previous report (Palmiter and Wrenn, 1971). However, the alteration of body weight was diametrically opposed to that of oviduct weight. The DES treatment reduced body weight compared to that of untreated chicks. Because the effect of DES stimulation was inordinately centered on oviduct growth and development, body weight may have been decreased by

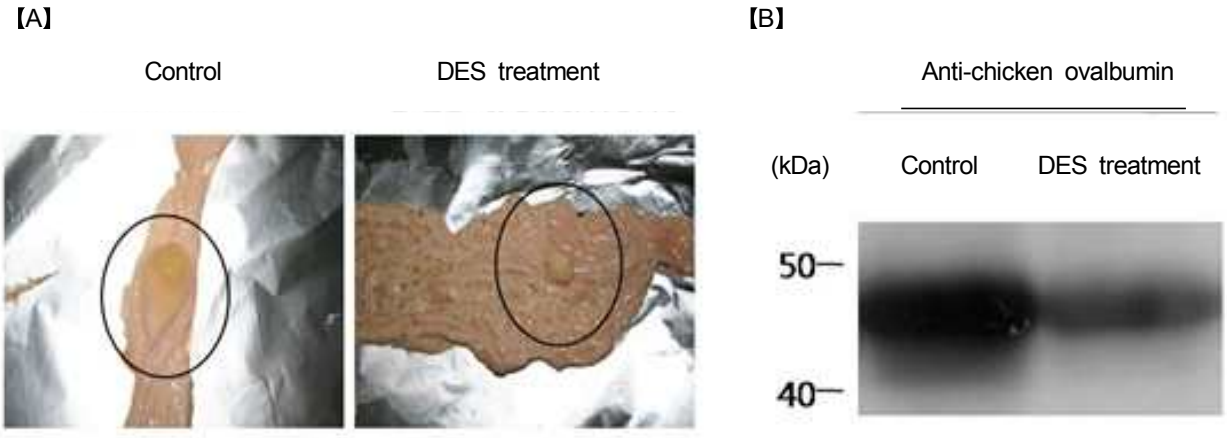

Fig. 5. Expression of ovalbumin in the magnum of control and DES-treated hens. Normal production of egg white appeared in the magnum of control chicks. However, DES treatment caused abnormal production of egg white in the magnum. [B] Ovalbumin expression was confirmed by Western blotting using an anti-chicken ovalbumin antibody. Ovalbumin was produced in the magnum of both the control and DES-treated hens.

the disproportionate growth of the oviduct.

Our current study also revealed that chick oviducts can be morphologically differentiated into the infundibulum, magnum, isthmus, and shell gland following DES stimulation. Chick oviducts induced by DES could be more clearly classified into four parts, including the infundibulum, magnum, isthmus, and shell gland, compared to those of control chicks. Previously, we observed ciliated epithelial cells in developing chick oviducts following DES treatment and characterized tubular gland cells with an anti-ovalbumin antibody in *in vitro* culture (Kim, 2006). This result indicated that the epithelia of the magnum stimulated by DES were cyto-differentiated into functional cell types, such as ciliated epithelial cells and tubular gland cells. Therefore, DES might play a pivotal role in the oviduct development of neonatal chickens equivalent in activity and nature to estrogen (Kohler et al., 1969).

We also found developmental disorders in the oviduct of matured hens after neonatal DES exposure. The maturated follicles of DES-treated hens ovulated into the abdominal cavity, not into the infundibulum of the oviduct. The accelerated growth of the oviduct following DES stimulation in neonatal chicks may result in abnormal development of the infundibulum. In addition, the production of outer thin albumen was not observed, and the abnormal structure existed in the magnum of DES-treated hens (Fig. 3D and 3F). Prenatal DES treatment reduces the expression of *ovalbumin* mRNA in response to estrogen (Teng and Teng, 1985). Abnormal production of egg white proteins may be related to desensitization of the chick oviduct response to natural estrogen following DES treatment. Alternately, abdominal ovulation caused by DES treatment may cause harmful effects on the production of egg white proteins in the oviduct. Collectively, our current results indicate that the detrimental effects of DES exposure in early developmental stages might hinder normal egg production in DES-treated hens.

Estrogen is a major steroid hormone in the reproductive system; it stimulates the proliferation and differentiation of the reproductive tract, controls reproductive ability, and prevents cellular apoptosis (De Pol et al., 2001; Dougherty and Sanders, 2005). The physiological behavior of estrogen is mainly mediated by its nuclear receptors, estrogen receptor α (ESR1) and β (ESR2). ESR1 and ESR2 drive physiological alterations through the regulation of various transcription factors and target genes (Dougherty and Sanders, 2005). In chickens, estrogen has various functions and acts as a stimulator of oviduct development. Estrogen is secreted by theca cells in large preovulatory follicles in chickens (Rodriguez-Maldonado et al., 1996), regulates expression of egg white proteins, and induces egg production in hens (Kohler et al., 1968). Although DES is an analog of estrogen, the effects of DES are quite different from those of estrogen. The physiological alterations caused by DES are mainly mediated by ESR1, rather than ESR2. In mice, the chronic effects induced by neonatal DES treatment are not exhibited in *Esr1* knockout mice (Couse and Korach, 2004). Additionally, DES changes the genetic pathways involved in the development of the epithelium of reproductive organs. DES increases the expression of wingless-type MMTV integration site family member 5A (*Wnt5A*) whereas it represses those of msh homeobox 2 (*Msx2*) and placental alkaline phosphatase (*Plap*) in the development of the murine uterine epithelium. *Wnt5A* is required for the normal development of uterine stroma, and *Msx2* and *Plap* are expressed only in normal uterine epithelia (Huang et al., 2005). Although we did not investigate the alteration of the genetic pathway following DES stimulation, one can postulate that differences in the genetic pathways stimulated by DES or estrogen may be related to the abnormal development of hen oviducts.

Collectively, we demonstrated that the effects of neonatal DES exposure on chick oviduct development are similar to those of estrogen exposure. However, neonatal exposure to DES reduces body weight and induces functional abnormality of hen oviducts related to egg production. DES caused abdominal ovulation due to structural abnormalities and induced abnormal morphology, such as abnormal structures in the infundibulum and magnum of the oviduct. Additionally, DES disturbed the production of the outer thin albumen in the magnum. Generally, the chicken oviduct is developed normally in the appropriate stage harmonized with the changes in other hormones. The biased and continuous stimuli such as neonatal DES treatment cause the physiological disruption of reproductive tract. In conclusion, the chronic effects of DES treatment are detrimental for the development of chicken oviducts and egg production. Because our study was focused on a phenomenological analysis of chicken oviduct development following DES treatment, we will elucidate the developmental mechanisms in

the oviduct following DES treatment in avian systems through future molecular-based analyses. Although DES treatment imitates the development of oviduct by combination of natural hormones in the proper developmental stage, the effect of neonatal DES treatment is not exactly the same as normal development through our research. At future study about the physiological effect of DES based on molecular biology, genetic alterations by DES treatment should be investigated compare to that of natural estrogen.

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