Journal of Poultry Science, 40 : 247–253, 2003

Effects of Estrone Sulfate Administration on Reproductive Functions in Male Japanese Quail

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Estrone sulfate (E_1S) is predominantly present in the maternal circulation of pregnant animals and is excreted into the feces and urine. Hence, it is estimated that E_1S is partly present in the environment, which may be discharged from farms. The present study was undertaken to examine whether E_1S affected the endocrine function in animals. Male Japanese quails were intramuscularly treated 1mg of E_1S everyday for 4 days. Blood samples were obtained just before the treatment and at the 4 days after the onset of treatment to measure the concentrations of testosterone, E_1S and triacylglyceride. Apoptotic cell death was detected in the paraffin sections of testis. The weight of testis was significantly reduced in the E_1S treated groups compared with that of untreated group. Treatment of E_1S to the quail significantly increased plasma E_1S and triacylglyceride concentrations and significantly reduced plasma testosterone concentration. Some Sertoli cells in the E_1S treated group showed apoptotic cell death, whereas there is few apoptotic cells in the untreated group. These results indicate that E_1S has ability to induce apoptosis in the testicular cells and reduction of testicular weight and testosterone concentration, possibly due to the unconjugation of E_1S. It is also suggested that E_1S may be one of risk factors for endocrine disrupter in wild life.

Key words : apoptotic cell death, estrone sulfate, Japanese quail, testes, testosterone

Introduction

The estrone conjugated to sulfate form, estrone sulfate (E_1S), which is predominantly present in the maternal circulation of pregnant animal including cows, and E_1S concentration in the blood of cows starts to increase between days 70 and 100 of gestation to reach a plateau of about 15–30 nmol/L between days 265 and parturition (Hoffmann *et al.,* 1997 ; Hoffmann and Schuler, 2002). The higher concentrations of E_1S were also detected in feces and urine during a late period of gestation in cows and sows (Ohtaki *et al.,* 1999 ; Hoffmann *et al.,* 2001 ; Isobe and Nakao, 2002). Thus, it is estimated that E_1S which may be discharged from farms, is partly present in the

Received : April 7, 2003, Accepted : May 27, 2003
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[1] This work was partly supported by Grant-in-Aid for Scientific Research from Hiroshima University (M.S.) and by Satake Memorial Foundation (N.I.)

environment.

The steroid hormones are considered to be one of the environmental problems due to their endocrine disruption potential. Many researchers showed that estrone, estradiol 17β and estriol were detected in effluents of sewage treatment plants and surface water (Desbrow *et al.,* 1998 ; Ternes *et al.,* 1999 ; Kuch and Ballschmiter, 2001). They may interfere with the normal function of endocrine system, thus affecting the functions of reproduction and development in aquatic life (Jobling *et al.,* 1998). In fact, in British rivers, vitellogenesis and feminization in male fish have been observed (Desbrow *et al.,* 1998 ; Jobling *et al.,* 1998). E_1S has been considered as inactive estrogen, however it is reported that the E_1S is hydrolyzed by steroid sulfatase to exert its biologic function, and the steroid sulfatase is expressed liver, brain, oviduct, endometrium, ovary and testis (Park *et al.,* 2000 ; Clemens *et al.,* 2000 ; Yanaihara *et al.,* 2001 ; Miki *et al.,* 2002). However, to our knowledge, there is little information about the effects of E_1S on the reproductive function in male animals.

In the recent study, administration of estrogen like substance, diethylstilbestrol (DES), to the male Japanese quail drastically decreased the size of testes (Maeda and Yoshimura, 2002). Estrogen receptor is present in the testes (Makinen *et al.,* 2001). These results indicate that this animal model is utilized to check whether some estrogen like substances bind to the estrogen receptor and have biological activities or not. Therefore, if the size of testes in male quail is decreased by administration of E_1S, we can consider that the E_1S is hydrolyzed to estrone that binds to the estrogen receptor before or after converting to estradiol. Moreover, the production of triacylglyceride (TG) is induced by the action of estrogen in the laying hen (Hermier *et al.,* 1989). Hence, TG can be the one of indicators of estrogenic substance.

The present study was undertaken to examine whether the injections of E_1S has effects on the levels of testosterone and triacylglycerol in plasma and weight of testis in the male Japanese quails.

Materials and Methods

Animals and treatment of E_1S

Eight male Japanese quail whose ages were about 4 months were used. Four quails were intramuscularly treated 1 mg of E_1S (Sigma Chemical Co., St Louis, MO, USA) in 1 m*l* of ethanol everyday for 4 days. Other 4 quails for control were injected with 1 m*l* of ethanol without E_1S. Blood samples (1 m*l*) were obtained from blood vessel under wing just before the treatment and the 4 days after the onset of treatment. The blood was immediately centrifuged (1700 x g, 15 min). Plasma for the supernatant was collected and stored at -30℃ in plastic tubes until assay. Animals were fed in accordance with the regulations of Hiroshima University.

Assay for estrone sulfate

Plasma concentration of E_1S was measured by the competitive ELISA method as described by Isobe and Nakao (2002). Briefly, plasma samples were diluted 20 times with assay buffer and then applied 50μl directly to the wells that have previously coated by 4μg/m*l* goat anti rabbit IgG antibody (ICN Biomedicals Inc. Aurora, OH, USA).

Anti-estrone rabbit antibody (Kambegawa Institute, Tokyo, Japan) and horse radish peroxidase labeled estrone (Kambegawa Institute) were added to the wells and cultured for 2 h. After washing 3 times with PBS, o-phenylenediamine for substrate was added and absorbance at 492 nm was recorded. The intra-assay and inter-assay coefficient of variation (CV) were 7.8–9.6% and 7.2–14.4%, respectively. The recovery rates ranged between 84.6 and 98.5%.

Assay for testosterone

Quantification of testosterone by HPLC-UV was based on the procedures reported by our previous study (Shimada *et al.,* 2002). Briefly, 1 m*l* of plasma sample was mixed with 0.5 m*l* of 0.3 N NaOH. Testosterone was extracted from the plasma by 5-min mixing with 10 m*l* dichloromethane (Nacalai tesque, Kyoto, Japan). After centrifugation, the 10 m*l* of dichloromethane fraction was collected into a disposal tube and the solvent from this fraction was removed by vacuum extraction for 120 min at 5℃. Samples were reconstituted in 100 μl of 50% (v/v) methanol solution.

The samples were separated using a reverse-phase CAPCELL PAK column (2.0× 100 mm ; Shiseido, Tokyo, Japan). The solvent delivery system contained 50% (v/v) methanol solution. The detection of testosterone was performed at 225 nm using a UV detector, and peak heights were measured using a computer integrator. The standard curve of testosterone for the determination of concentration was linear, from zero to 800 ng/m*l*. The intra-assay CV in medium with 100 ng/m*l* of testosterone was 6.4%. Recovery rate of 50 ng of testosterone added to 0.5 m*l* of the sample (100 ng/m*l*) was 94.2±4.7%.

Assay for triacylglyceride

The concentration of triacylglyceride was determined in the plasma by a commercially available enzymatic serum triglyceride assay (Triglyceride G-test Wako ; Wako, Osaka, Japan).

TUNEL procedure

After the 4 days-treatment of E_1S, quails were sacrificed. Immediately, the testes were fixed with 10% formalin and processed for paraffin sections. The sections (6-μm thick) were air-dried on slides treated with 3-aminopropyltriethoxysilane (Sigma).

After being deparaffined with xylene, the sections were rehydrated through a series of ethanol and distilled water solution and were washed with PBS. Nuclei in the tissue were stripped from protein by incubation with 20 μg/m*l* proteinase K (Sigma) in 10 mM Tris-HCl, pH 7.4, at 37℃ for 20 min. Apoptosis was detected by the terminal deoxynucleotidyl transferase (Tdt)-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labeling (TUNEL) method. An *in situ* cell death detection kit with horse radish peroxidase (POD) (Roche Diagnostics K. K., Tokyo, Japan) was used for this TUNEL procedure. After washing with PBS for 5 min 2 times, the slides were incubated with TUNEL reaction mixture in a humidified chamber at 37℃ for 60 min, followed by rinsing with PBS for 5 min 2 times. The slides were incubated with converter anti-fluorescein antibody conjugated with peroxidase diluted to 1 : 1 with PBS containing 0.05% BSA, for 30 min in a humidified chamber. After washing with PBS for 5 min 2 times, immunoreactions were detected by incubating with substrate

solution consisting of 2 mg 3′ 3 diaminobenzidin-4HCl and 10 μl 5% H_2O_2 in 10 ml 0.05 M Tris-HCl (pH 7.5). The slides were rinsed with water and counter-stained briefly with hematoxylin. After washing with running water, the slides were dehydrated, cleared and covered. Control sections were incubated with TUNEL reaction mixture without enzyme (terminal deoxynucleotidyl transferase).

Statistical Analysis

Statistical analyses of all data from three or four replicates for comparison were carried out by analysis of one-way ANOVA followed by Duncan's multiple ranges test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA). Values were determined to be significant when $P < 0.05$.

Results and Discussion

The present study was undertaken to investigate whether the E_1S can cause some physiological changes or not using a male quail model. The weight of testis was significantly reduced in the E_1S treated groups compared with that of untreated group (Table 1). In the plasma obtained from the quail after treatment, E_1S concentration was significantly higher in the E_1S treatment group than untreated group (Table 1). According to Maeda and Yoshimura (2002), the administration of DES at 0.1 and 1 mg/day for a week significantly decreased the testicular weight. Since it has been reported that DES has the estrogenic function and estrogen receptor is expressed in testis (Petersen *et al.,* 1998), the present results suggest that E_1S cause the reactions in testis as estrogen do.

In the testis of control quail without E_1S treatment, many spermatozoon were observed in the lumen, and several of them were gathered partially (Fig. 1). In contrast, the treatment E_1S decreased the number of spermatozoon in the testis of quail (Fig. 1). When the sections were stained by TUNEL to detect the apoptotic cells, some Sertoli cells showed TUNEL positive in the E_1S treated group whereas there is few cells positive to TUNEL in the untreated group (Fig. 1). Additionally, the treatment with E_1S decreased the concentration of testosterone compared to quail of untreated group (Table 1). Williams *et al.* (2001) also reported a reduction of testosterone concentration in the rat treated with DES. These results indicate that E_1S has ability to induce apoptosis in the testicular cells containing testosterone producing cells, resulting in the

Table 1. Effects of E_1S treatment on the weight of testis, and plasma concentration of E_1S, testosterone and triacylglycerol in the male Japanese quail

E_1S treatment	Weight of testis (g)	Concentrations of		
		E_1S (ng/ml)	Testosterone (ng/ml)	Triacylglycerol (ng/ml)
−	1.4±0.1	0.4±0.1	5.5±0.2	92.7±10.3
+	1.0±0.2*	9.3±0.3*	1.7±0.7*	3676.5±1147.1*

* : Significant differences were observed between groups ($p < 0.05$)

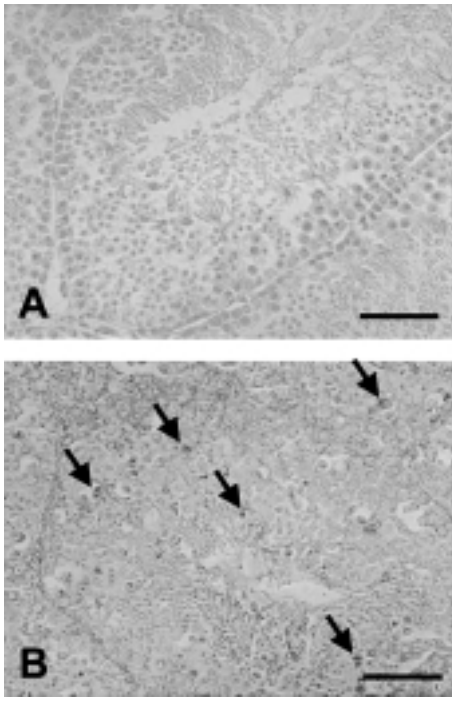

Fig. 1. In situ detection of DNA fragmentation in testis of the male Japanese quail without (A) or with (B) treatment of E_1S. Arrows mean TUNEL-positive cells. Bars=50μm

reduction of testicular weight and testosterone concentration. Moreover, it is suggested that injection of E_1S to the Japanese quail reduces the activity of spermatogenesis.

The laying hen has high concentration of triacylglycerol (TG) for growth of the egg yolk (Cross *et al.,* 1987). Production of TG is largely attributable to the level of estrogen secreted from follicular cells (Hermier *et al.,* 1989). In the present study, the administration of E_1S increase the TG concentration in the plasma of quail (Table 1), suggesting that E_1S must play roles in liver as estrogen.

It has been accepted that E_1S has no activity to bind to the estrogen receptor (Hobbirk, 1985). However, if E_1S is unconjugated by sulfatase and converted to estrone, it can bind to the estrogen receptor (Park *et al.,* 2000). In the present results shows some estrogenic reaction in the quail treated with E_1S, indicating that sulfatase could be present in some organs such as testis and liver. These present results indicate that E_1S which may be present partly in environment is one of a risk factor for endocrine disrupter in wild life.

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