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·*Original Article*·

Effects of 17beta-estradiol on distribution of primordial germ cell migration in male chicks

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Abstract

Aim: To assess whether exogenous estradiol has any effect on migration of primordial germ cells (PGCs) in the chick. **Methods:** Fertilized eggs were treated with 17beta-estradiol (E_2) (80 μg/egg) at stage X (day 0 of incubation), stages 8–10 (incubation 30 h) and 13–15 (incubation 55 h). Controls received vehicle (emulsion) only. Changes in PGC number were measured on different days according to developmental stages. **Results:** In male right gonads, but not in female left gonads, at stages 28–30 (incubation 132 h) significant decreases in the mean number of PGCs aggregating were observed compared with the controls ($P < 0.05$) while the total PGC number in the right and left gonads at each stage did not change ($P > 0.05$). **Conclusion:** The present study provides evidence that E_2 has significant effects on the localization of PGCs in male right, but not female left, gonads of chicken embryos at stages 28–30, compared with controls. *(Asian J Androl 2008 Mar; 10: 243–248)*

Keywords: 17beta-estradiol; primordial germ cells; male chick

1 Introduction

Estrogens play a key role in the gonadal differentiation of birds. During embryogenesis, 17beta-estradiol (E_2), the most potent endogenous estrogen, acts as a morphogen for the development of female characteristics [1]. Traditionally, estrogens have been considered to be primarily female hormones. However, estrogens also play an important role in male development [1]. Estrogen secretion in boars can influence the development of the somatic cells within the testis that ultimately contribute to testicular size and sperm production [2]. The estrogen receptors α and β are expressed throughout the male reproductive tract, including in germ cells in a number of species [3]. In Hess *et al.* [3], when the estrogen receptor α was knocked out in mice, the males became infertile, primarily because of malfunction of the efferent ductules of the epididymis, with consequent gross disruption of the seminiferous tubules.

Experimental data also suggest that the chicken estrogens could be involved in gonadal development [4]. However, expression of estradiol receptor mRNA was not detected in gonads of either male or female chickens until the seventh day of incubation [5]. In chicken primordial germ cells (PGCs), migration has been reported to originate from the epiblast to reach the hypoblast of the area pellucida after several hours of incubation [5]. What controls the chicken PGC migration *in*

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vivo is still poorly understood. The present study investigated the effects of E_2, given at different stages of development, on the migration of chicken PGCs in gonads of male and female embryos from the initial stage until completion of their settlement in the gonadal primordium.

2 Materials and methods

2.1 Chicken eggs and preparation of 17beta-estradiol-solution

Fertilized White Leghorn (*Gallus domesticus*) eggs weighing 63.0 g ± 2.0 g (mean ± SD) were obtained from the Experimental Station of China Agricultural University. 17beta-estradiol (E_2) (Sigma, St. Louis, MO, USA) was dissolved in a peanut oil/lecithin mixture (9:1, wt/wt) and then the oil/lecithin mixture was emulsified in water (1:1.5, vol/vol) at a final concentration of 80 μg/0.1 mL [6]. The standard injection volume chosen were 100 μL/egg or embryo and a control 100 μL of the vehicle (emulsion) were similarly injected.

The dose of E_2 was similar to the dose used previously in White Leghorn eggs [7], to ensure that significant effects could be demonstrated with E_2 treatment.

2.2 Experimental design

Developmental stages of chicken embryos were expressed according to the normal tables of Eyal-Giladi and Kochav (before incubation in Roman numerals), or Hamburger and Hamilton (after incubation in Arabic numerals). Group 1: Treatment of eggs with E_2 or vehicle control at stage X (day 0 of incubation), assessment of PGCs in male and female anlages at stages 8–10 (killed at 30 h of incubation), 13–15 (killed at 55 h of incubation) and 28–30 (killed at 132 h of incubation). Group 2: Treatment of embryos at stages 8–10 with E_2 or vehicle control, assessment of PGCs in male and female anlages at stages 13–15 and 28–30. Group 3: Treatment of embryos at

Table 1. The number of primordial germ cells of chicken embryos following injection of estradiol. Results are expressed as mean ± SD. The controls received vehicle alone. Each group of embryos in experiments is 10. $^*P < 0.05$, compared with vehicle-injected.

Treatment stages and sex	Developmental stages				
	8–10 (Per embryo)	13–15 (‰ Cells per embryo)	28–30 (Per left anlage)	28–30 (Per right anlage)	28–30 (Per embryo)
Group 1 (X)					
Male					
Treatment	103.0 ± 20.7	0.11 ± 0.06	1 167.0 ± 159.6	125.6 ± 29.9*	1 292.6 ± 184.9
Control	131.0 ± 32.3	0.17 ± 0.05	1 147.3 ± 116.3	516.2 ± 58.3	1 663.6 ± 170.4
Female					
Treatment	93.0 ± 19.9	0.13 ± 0.08	1 120.8 ± 160.5	386.0 ± 50.7	1 509.4 ± 211.3
Control	114.8 ± 28.5	0.13 ± 0.05	995.9 ± 127.2	342.7 ± 37.7	1 338.6 ± 162.3
Group 2 (8–10)					
Male					
Treatment		0.12 ± 0.06	1 587.3 ± 102.6	166.5 ± 35.5*	1 747.9 ± 139.3
Control		0.13 ± 0.03	1 389.4 ± 162.2	429.3 ± 59.6	1 743.5 ± 211.3
Female					
Treatment		0.09 ± 0.05	1 201.8 ± 130.4	417.0 ± 48.5	1 628.4 ± 172.4
Control		0.15 ± 0.04	1 030.9 ± 104.8	346.7 ± 41.6	1 327.5 ± 146.6
Group 3 (13–15)					
Male					
Treatment			1 357.4 ± 138.6	128.3 ± 47.3*	1 420.6 ± 168.1
Control			1 326.5 ± 99.8	482.7 ± 77.4	1 758.5 ± 151.8
Female					
Treatment			1 170.8 ± 194.8	406.5 ± 73.3	1 513.4 ± 253.2
Control			1 474.0 ± 125.9	531.7 ± 81.6	1 941.0 ± 210.4

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Table 2. The percentage distribution of primordial germ cells (PGCs) in the gonads at stages 28–30. Results are analyzed with Fisher's exact test. Percentage values represent numbers of right or left gonad PGCs compared with the total PGCs. $^cP < 0.01$, compared with vehicle-injected.

	Male gonads		Female gonads	
	Left	Right	Left	Right
Group 1				
Treatment	90.3	9.7^c	74.3	25.7
Control	68.9	31.1	74.4	25.6
Group 2				
Treatment	90.8	9.2^c	73.8	26.2
Control	79.7	20.3	77.6	22.4
Group 3				
Treatment	95.5	4.5^c	77.4	22.6
Control	75.4	24.6	75.9	24.1

stages 13–15 with E_2 or vehicle control, assessment of PGCs in male and female anlages at stages 28–30.

All embryos were sexed by polymerase chain reaction (PCR) amplification of a W-specific *XhoI* repeat from a genomic DNA template. The group size treated with E_2 or vehicle must be sufficient for assessment of PGCs at different stages. The embryo number of random investigation for every result (Tables 1 and 2) is 10.

2.3 Estradiol treatments and incubation

At stage X, shell windowing was as descried by Speksnijder and Ivarie [8]. The eggs were swabbed with 70% ethanol and placed horizontally with respect to their long axis for approximately 2 h. Using a dental drill, a window of approximately 5 mm in diameter was ground through the shell near the top of the air cell of the still horizontally placed egg. Thereafter, the newly ground hole was swabbed with 70% ethanol and the shell membrane was cut cleanly around the window. The underlying blastoderm was located by turning the egg under illumination using a fiber optic light source (Nikon SMZ-10, Tokyo, Japan). Injection was into the yolk at the vicinity of the blastoderm with a 27-gauge syringe; the windows were then sealed with a coverslip and paraffin wax, and the sealed eggs were further incubated at 38ºC and 70% relative humidity until the end of stages 8–10, or 13–15 or 28–30, at which time the embryos were removed and killed.

E_2 was similarly injected into the yolk at stages 8–10 and 13–15, as above. However, the embryo becomes distinguishable at these stages, and moves easily in the shell, and not need place horizontally some time for cutting a hole.

2.4 Primordial germ cell collection and staining

2.4.1 Primordial germ cell collection from the germinal crescent

At 30 h of incubation (stages 8–10), the germinal crescent PGCs were dissected according to Speksnijder and Ivarie [8]. A filter paper ring was placed around each developing embryo, and the vitelline membrane was cut around the outside of the ring. The ring with the adhering germinal crescent was removed from the yolk and placed ventral side up in sterile phosphate buffered saline (PBS) containing 5.6 mmol/L D-glucose (PBS-G). Yolk was removed from the embryo by microdissection and gentle rinsing with PBS-G, and the separated germinal crescent was dissociated in 0.25% (wt/vol) trypsin solution supplemented with 0.05% (wt/vol) EDTA by gentle pipetting. After the inactivation of trypsin-EDTA with MEM (minimum essential medium) containing 10% fetal bovine serum, the cells were harvested by centrifugation at 300 × *g* for 5 min at normal temperature.

2.4.2 Primordial germ cell collection from gonad

After 132 h of incubation when the embryos were at stages 28–30, the eggs were removed, rinsed with PBS-G to remove the yolk, and the abdomen of the embryos was carefully dissected under a stereoscope and the gonads were collected with sharp tweezers. Gonadal tissues were dissociated by gentle pipetting in 0.25% (wt/vol) trypsin solution supplemented with 0.05%(wt/vol) EDTA. The cells were isolated from the gonadal tissues by centrigugation at 300 × *g* [9].

2.4.3 Primordial germ cell staining of the germinal crescent and gonad

The primordial germ cells obtained from the germinal crescent and gonadal regions were then identified by staining with Periodic Acid Schiff reaction (PAS reaction) [10]. The number of PGCs (PAS-positive cells) was counted under a light microscope.

2.4.4 Collection of blood and identification of primordial germ cells

After 55 h of incubation, 2 μL of blood was collected from individual embryos through the vitelline artery or the heart using a sharply cut glass tip of approximately 50 μm in diameter. When the blood was collected, the

embryos were mainly at developmental stage 14, but some were at stage 13 or 15.

Primordial germ cells could easily be distinguished from the blood cells and counted because of their remarkably large size, large spherical nuclei, and the presence of refractive lipids in the cytoplasm under a phase contrast inverted microscope [11].

2.5 Sexing of embryos

After PGC collection, a small piece of the tissue obtained from the embryo was digested in 50 μL of buffer composed of 10 mmol/L Tris, 1 mmol/L EDTA, 5% sodium dodecyl sulfate (SDS), and 10 μg/mL Proteinase K (pH 7.5) for 2 h at 38.5ºC. Then the sample was centrifuged at 15 000 × *g* for 5 min, and 5 μL of the supernatant was used for PCR reaction. PCR reactions were carried out using the W-linked (female-specific) *Xho1* repeat sequence as described by Smith *et al.* [12]. Amplification of chicken *β-actin* was used as a control. The *Xho1* oligonucleotide primers were: *Xho1*/*1*, 5'-ATC TAC CAC TTT TCT CAC GG-3' and *Xho1*/*2*, 5'-TTC AGA GTG ATA ACG CAT GG-3'. The actin primers were: *Actin*/*1*, 5'-TGG ATG ATG ATA TTG CTG C-3'; *Actin*/*2*, 5'-ATC TTC TCC ATA TCA TCC C-3'. PCR reactions were carried out in 20 μL of buffer (10 mmol/L Tris–HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$) containing 2 μL DNA, 200 mmol/L dNTPs, 1 U Taq DNA polymerase and 1 mmol/L each of the four primers. Cycling parameters were: 94ºC × 5 min, 30 cycles of (94ºC × 30 s; 56ºC × 30 s; 72ºC × 30 s), 72ºC × 5 min. PCR products were run on a 1.5 agarose gel in 1 × TAE buffer. Only female (ZW) embryos showed the 168 bp W-linked *Xho1* fragment.

2.6 Statistical analysis

The data are presented as means ± SD. Statistical analyses were undertaken using SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). Paired independent-sample *t*-tests were used to test for significant differences between vehicle-treated and E_2-treated embryos in PGC number at different stages of development. The percentage distribution of PGCs for significant difference was assessed using Fisher's exact test. Differences were regarded as significant at $P < 0.05$.

3 Results

3.1 Group1: E_2 treatment at stage X prior to incubation

When assessed at stages 8–10 no effect on total number and distribution of PGCs could be detected ($P > 0.05$, Table 1).

The number of PGCs per microlitre of blood counted at stages 13–15 ranged from 0.04 ‰–0.19 ‰ in males and 0.06 ‰–0.23 ‰ in females and was not different between sexes and to the vehicle control (Table 1).

Assessment at stages 28–30 revealed a significantly decreased number of PGCs in the male right anlage (125.6 ± 29.9) when compared with the vehicle control (516.2 ± 58.3) ($P < 0.05$, Table 1). However, the total number of PGCs was not different between sexes and when compared with the control.

3.2 Group 2: E_2 treatment at stages 8–10 (after 30 h of incubation)

Evaluation of stages 28–30 yielded a significantly ($P = 0.034$) decreased mean number of PGCs in the male right anlage (166.5 ± 35.5) when compared to the control (429.2 ± 59.6). The total number of PGCs was not affected and not different between sexes.

The number of PGCs seen per microlitre of blood ranged from 0.07 ‰–0.19‰ in males and 0.05 ‰–0.16‰ in females it showed no effect of treatment and was not different between sexes and to the vehicle control (Table 1).

3.3 Group 3: E_2 treatment at stages 13–15 (after 55 h of incubation)

Following treatment with E_2 the mean number of PGCs in the male right gonad was significantly lower (128.0 ± 47.3) compared with the control (482.6 ± 77.4) while the total number of PGCs was not affected and not different between sexes.

3.4 The asymmetry of primordial germ cells distribution in the gonads at stages 28–30

In all groups, it was found that the percentages in the male right gonads produced a significant decrease when compared with to that of their controls (Table 2). Each of the embryos displayed a left-sided asymmetry with respect to PGC distribution.

4 Discussion

To increase the likelihood of an effect of E_2 treatment, E_2 was injected directly into the embryonic compartment and not as was done with xeno-biotics, as in many other

studies [13], into the air cell or the yolk sac.

Chicken PGCs have been reported to originate from the epiblast and to settle at the hypoblast of the pellucida area (germinal ridge). In doing so, the PGCs first enter the developing blood vessels at stages 10–12, circulate and migrate to the germinal ridge, where they develop into gonads. The PGCs then proliferate and differentiate to spermatogonia and oogonia. PGC proliferation and migration in birds has been reported as both passive and actively associated with chemo-attraction, extracellular matrix components and the vascular system [14], the chemical signals involved remains to be identified. Estrogens obviously play a key role in gonadal differentiation in birds; therefore, genetically female chicken embryos can develop testes and a male phenotype if estrogen synthesis is inhibited by treatment with an aromatase inhibitor before sexual differentiation [1]. Estrogens are also considered an essential component for normal testicular development and function in other animals. For example, involvement of estrogens has recently been implicated by aromatase and estrogen receptor detection in human testicular germ cells and ejaculated spermatozoa [15]. ER 1 and ER 2 are clearly present in germ, Leydig and Sertoli cells in prepubertal and sexually mature boars [16], and reducing endogenous estrogen production by inhibition of aromatase leads to increased proliferation of porcine Sertoli cells during the first 2 months of life.

Effects of E_2 are usually considered to be genomic following interaction with nuclear estrogen receptors α and β [17]. However, 'non-genomic' effects of estrogens have also been characterized in several cell types, including those from the reproductive system and possibly spermatozoa [17]. These effects are mediated via either membrane-bound receptors or interaction with other proteins and/or membrane lipids. The presence of estrogen receptors in avian PGCs was not reported before day 6 of incubation, whereas estrogen receptor-mRNA was detected with expression being restricted to the left gonad of both female and male embryos [5]. During the early phase of gonadal development (between days 5.5 and 7 of incubation), estrogen binding sites are present in the germinal epithelium and medulla of the left gonad and in the medulla of the right gonad of both sexes [5].

The present study indicated that treatment with E_2 leads to a significant decrease in the number of the PGCs localized in the male right gonads. However, the underlying mechanisms of action are still to be determined. Both genomic and non-genomic mechanisms might be considered, even in a synergistic manner.

In Swartz's study [18], testosterone was administrated to chick embryos at 33 h incubation in two forms: crystalline and dissolved in cottonseed oil. The PGC number decrease in gonadal area occurred in both groups at 5 days of incubation; however, in only the group receiving testosterone cypionate was the decrease found to be significant.

Meyer [10] found that the ratio of distribution of germ cells in the right and left gonads was uneven. The PGCs were initially localized evenly in both gonads, at least up to day 3, and the asymmetry began at stage 21 when approximately 65% of the PGCs colonized the left gonad [10]. This asymmetry might be utilized to determine the genetic sex of the embryo prior to histological sexual differentiation of gonads, occurring between 6 and 7 days [19]. In our study, each of the embryos displayed a left-sided asymmetry with respect to PGC distribution. E_2 treated groups exhibited a significant decrease in the percentage of the PGCs in the male right gonads at stages 28–30. Normal asymmetry in the distribution of the PGCs favoring the left side in the male chick was affected in treatment of the groups (Table 2).

In conclusion, the present study has provided evidence that E_2 has a significant and highly selective effect in decreasing the number of PGCs in the right gonad of male chicken embryos. The observation was made at stages 28–30. However, the underlying mechanisms of action including the time point of inhibition are unknown. These data are in agreement with earlier observations [1], showing that estrogen affect chicken sexual development, such as gonadal differentiation and differentiation of accessory sex structures, and suggest that E_2 is an important factor on PGC development and distribution in male chicken embryos.

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