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Distribution of ERα in the Bovine Dominant Follicle and Corpus Luteum

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Summary

The objective of this study was to describe the distribution of estrogen receptor alpha (ERα) in the first wave dominant follicle, preovulatory dominant follicle and corpus luteum in Japanese Black cows by means of immunohistochemistry. Cows (n = 9) were randomly divided into 3 groups in accordance with the day of ovariectomy performed on Day 7 (n = 3), Day 10 (n = 3) and Day 18 (n = 3) (Day 0 = estrus). Ovarian dynamics were observed twice daily using ultrasonography. Distribution of ERα was analyzed using a light microscope equipped with a digital camera. The immunohistological staining intensity in cells and the percentage of staining cells which represented the total ERα expression were analyzed. In general, the ERα expression in theca interna of DF in all groups (n = 9) was higher than that in mural GC, antral GC or TE. This was shown by the high percentage of positive nuclei and high staining intensity. Within the same follicular cells, ERα expression in mural GC on Day 18 was lower than that in mural GC on Day 7 ($P < 0.05$) and no significant difference was found between the other cells. In the luteal cells and stromal cells, no significant difference was found within the same cells on Day 7, Day 10 and Day 18, except in the luteal cells on Day 10 where significantly higher expressions were found than those in the luteal cells on Day 18 ($P < 0.05$). In general, the ERα expression in luteal cells was higher than that in the stromal cells of the corpus luteum ($P < 0.05$).

Key words: corpus luteum, dominant follicle, estrogen receptor alpha, immunohistochemistry, Japanese Black cow

Introduction

Two different ER subtypes exist, namely estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ). The presence of ERs in the ovary has already been demonstrated in monkeys [7], sheep [14], humans [3, 13], rats [10], cows [1, 11] and pigs [4]. Estrogen receptors play important roles in the maintenance of fertility. The ERα knockout mice are acyclic, infertile and possess hyperemic ovaries devoid of corpora lutea. Folliculogenesis is arrested at the antral stage with large follicles becoming cystic and haemorrhagic. The female ERβ knockout mice have small ovaries and

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some arrested follicular development [8]. The bovine ERβ mRNA expression in the granulosa cells decreases with increasing follicular size [2]. By contrast, the mRNA expression of ERα in theca interna tissue increases continuously during final growth of the bovine follicles, whereas it does not increase in the granulosa cells [1]. A relatively high expression of ERα is found in the thecal cells in comparison with that in granulosa cells [11]. In the ovary of beagle bitches, the ERα mRNA levels increase from proestrus to diestrus and positively correlate with plasma progesterone levels, whereas ERβ mRNA levels increase from mid anestrus to proestrus and are positively correlated with plasma estradiol-17β levels [6]. A low level of ERα is observed in the bovine corpus luteum [2]. ERα is not consistently detected in the luteal tissues of rhesus monkeys [5]. However, a little information exists concerning the specific distribution of ERα in dominant follicular cells or luteal cells during the bovine estrous cycle.

The aim of this study was to determine the distribution of ERα in the first wave dominant follicle and preovulatory dominant follicle, and in the corpus luteum by means of immunohistochemistry.

Materials and Methods

Animals

The animals used in this study were 9 Japanese Black cows (12.4±1.5 years old). They were clinically healthy, showing sound body condition scores (BCS: 3.0-4.0 out of 5) and having calved 9.0±1.8 times. They were randomly divided to 3 groups in accordance with the day of ovariectomy, which was performed on Day 7 (n = 3), Day 10 (n = 3) and Day 18 (n = 3) (Day 0 = estrus). Day 7 is considered the stage at which dominant follicle (DF) emerges and the corpus luteum (CL) develops; Day 10 is marked by early regressing DF and static CL; and Day 18 is the point for preovulatory DF and regressing CL. During the experiment, animals were kept in a stanchion barn in the Animal Hospital of Kagoshima University. Ovulation was induced with $PGF_{2\alpha}$ (Pronalgon F, Pharmacia, Japan) injected intramuscularly twice a day at 07:00 (15 mg) and 19:00 (10 mg) during the mid-luteal phase.

Ultrasonography and Progesterone Assay

The dynamics of the ovaries were monitored twice daily at 07:00 and 19:00 with ultrasonography before and after treatment with the $PGF_{2\alpha}$ injection until the day of ovariectomy. A real time B-mode ultrasound scanner (EUB-405, Hitachi-Medical Co., Tokyo) equipped with a 7.5 MHz transducer (EUP-033J, Hitachi-Medical Co., Tokyo) was used. Sequential identification of individual follicles more than 2 mm in diameter was carried out. Appropriate images of follicles were arrested on screen and maximum diameters of follicles were measured using the built in caliper system. The images of follicles were hard-printed. Ultrasonic images of the ovaries were recorded on a follicular map for retrospective analysis. Blood from the jugular vein was collected daily before $PGF_{2\alpha}$ treatment and twice daily following $PGF_{2\alpha}$ treatment at the same time with the ultrasonography. Plasma was collected by centrifugation of the blood samples at 4℃, at 3000 rpm for 20 min. The plasma samples were stored at -20℃. Double antibody RIA was used to determine the concentrations of progesterone (P_4) using antisera to progesterone (GDN # 337) [12]. The intra and interassay coefficients for progesterone were 4.2 and 8.0%, respectively. Ovariectomy was performed by flank laparotomy under epidural anesthesia in the morning on Day 7, Day 10 or Day 18 of the estrous cycle.

Processing of Ovaries

Following ovariectomy, ovaries were immediately placed in physiological saline. Thereafter, ultrasonic examinations were carried out to confirm the location of DF and CL, which had been identified by previous ultrasonic examinations before ovariectomy. After conformation, DF and CL were dissected free from extraneous tissue under the stereomicroscope. The follicular walls of DF and CL were immediately put into 10% phosphate-buffered formalin for 24 h. Follicular tissues were processed for histology and embedded in paraffin wax.

Immunohistochemistry for ERα

The follicular and luteal tissues were cut to a thickness of 5 μm and mounted on 3-aminopropyl-triethoxysilane-coated slides. In order to determine the ERα expressions, each section was deparaffinized, rehydrated, and washed with PBS for 5 min three times. These steps were followed by heating the sections with microwave irradiation (500 watt) in 0.1 M citrate buffer pH 6.0 (preheated 5 min with microwave irradiation) for 5 min. Then, sections were washed with PBS for 5 min three times and endogenous peroxidase activities were reduced by dipping sections into 3% H_2O_2 in methanol for 10 min. A blocking non-specific binding protein with normal horse serum (Vector Laboratories Inc., Burlingame, CA) to all sections were performed in a dark and humidified chamber for 30 min at room temperature (RT). After that, the sections were incubated overnight at 4℃ in a humidified chamber with antibody ERα from a mouse (C-311, Santa Cruz, CA) 8 μg/ml. The negative control tissue sections from uteri were incubated with normal mouse IgG (Santa Cruz, CA) 5 μg/ml. Then, a second antibody (Biotinylated IgG, Vector Laboratories Inc., Burlingame, CA) was added to the tissue sections for 60 min in a humidified chamber at RT. The sections were then washed with PBS for 5 min three times, followed by incubation with a horseradish peroxodase avidin-biotin complex (Vectastain Elite, Vector Laboratories, Burlingame, CA) for 60 min in a humidified chamber at RT. Color development was performed with 3' 3-diaminobenzidine (Roche Diagnostics, Mannheim, Germany) for 30 sec. All sections were counterstained with Gill's hematoxylin for 5 sec. Stained cells (brown staining) were observed using a light microscope (Eclipse E800, Nikon, Tokyo) equipped with a digital camera (Digital Camera DXM1200, Nikon, Tokyo) at x40 magnification for luteal cells and at x100 magnification for follicular cells.

Data Analysis

The expression of ERα was analyzed following the description by Broeck et al. [2]. Intensity of immunohistological staining was graded on 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining) scale within the 10 fields of observation and a proportional score given representing the relative amount of cells which had expressed the receptor (Table 1). The staining

Table 1. Immunohistochemical grading scores

Staining intensity score	Proportional score
0: no staining	0: no positive nuclei
1: weak staining	1: < 25% positive nuclei
2: moderate staining	2: 25-50% positive nuclei
3: strong staining	3: 50-75% positive nuclei
	4: > 75% positive nuclei

The staining intensity was first put into a weighted average and was summated with a proportional score for representing the total ERα expression.

intensity was first put into a weighted average and was summated with the proportional score to represent the total ERα expression. All data are reported as the mean$\pm$S.D. Mean values for the total score representing the total ERα, diameter of dominant follicle or corpus luteum and plasma progesterone levels were analyzed statistically by means of Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Ovarian dynamics of cows during the experiment are shown (Figure 1). Diameters of DF and CL, and plasma P_4 levels increased for all cows until Day 7. In the case of the cows which underwent ovariectomy on Day 10, the diameter of DF started to decrease from Day 8, while the CL diameter and plasma P_4 levels were static. In the remaining cows, which underwent ovariectomy on Day 18, two follicular waves were observed prior to Day 18. The diameter of the preovulatory DF increased until the day of ovariectomy and the diameter of CL decreased. It was concomitant with progressively decreasing plasma P_4 levels. Mean diameters of DF at the last observation before ovariectomy on Day 7, Day 10 and Day 18 were 13.4$\pm$2.4 mm, 12.4$\pm$0.2 mm, 11.7$\pm$0.8 mm, respectively. Mean diameters of CL at the last observation before ovariectomy on Day 7, Day 10 and Day 18 were 20.7 $\pm$1.9 mm, 19.1$\pm$1.7 mm, 21.7$\pm$0.8 mm, respectively. No significant difference in mean diameters

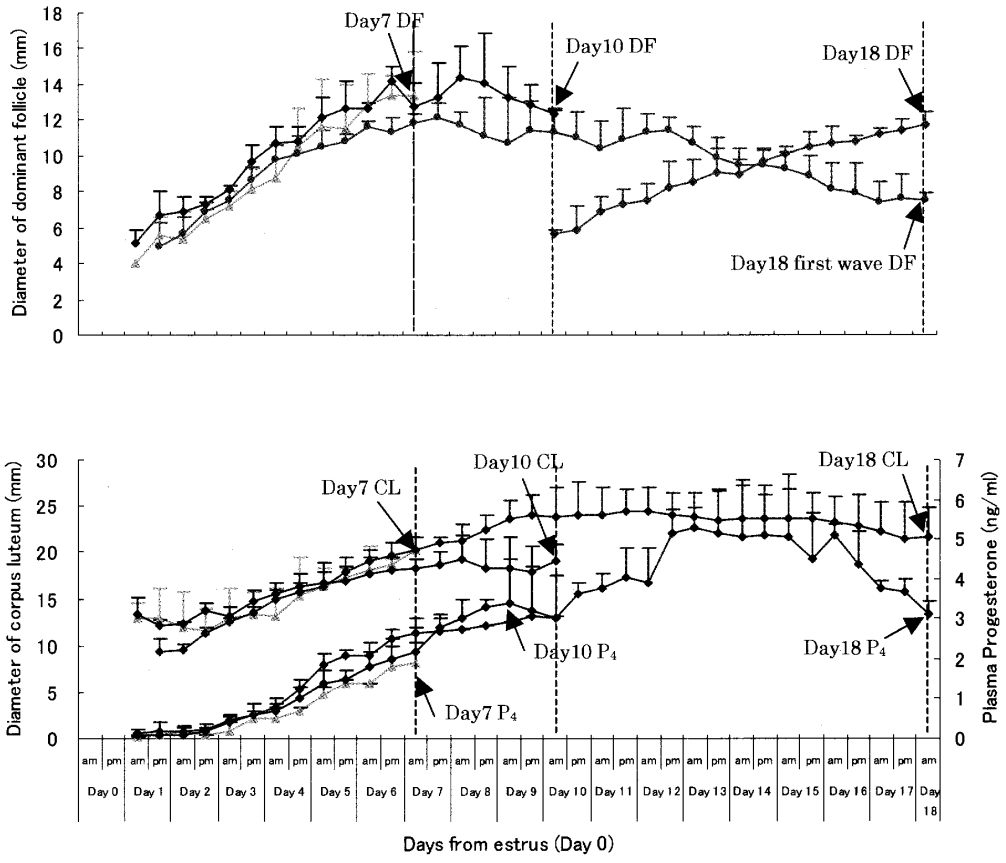

Figure 1. Diameter (mean$\pm$SD) of DF and CL before ovariectomy on Day 7 (n = 3), Day 10 (n = 3) and Day 18 (n = 3) and plasma progesterone level. No significant difference was found in diameter within DF or CL at the last observation before ovariectomy. Plasma P_4 levels on Day 7 were lower than plasma P_4 on Day 10 or Day 18. No significant difference was found in plasma P_4 levels between Day 10 and Day 18. DF = dominant follicle; CL = corpus luteum; P_4 = plasma progesterone.

was found within DF or CL at the last observation before ovariectomy. Plasma P_4 levels on Day 7 were lower than plasma P_4 levels on Day 10 or those on Day 18. Plasma P_4 levels on Day 7 were similar to Day 18. No significant difference was observed in plasma P_4 levels between Day 7 and Day 18.

The ERα expression in theca interna (TI, 2.5±0.3) of DF was higher than that in mural GC (mGC, 1.7±0.5), antral GC (aGC, 1.1±0.1) or theca externa (TE, 1.4±0.3) in all groups (n = 9) (Figure 2). The higher percentage of positive nuclei and higher staining intensity were observed (Figure 4a). Within the same follicular cells, ERα expression on Day 18 was lower than that in mGC on Day 7 ($P < 0.05$). There was no significant difference in TI, TE and aGC cells.

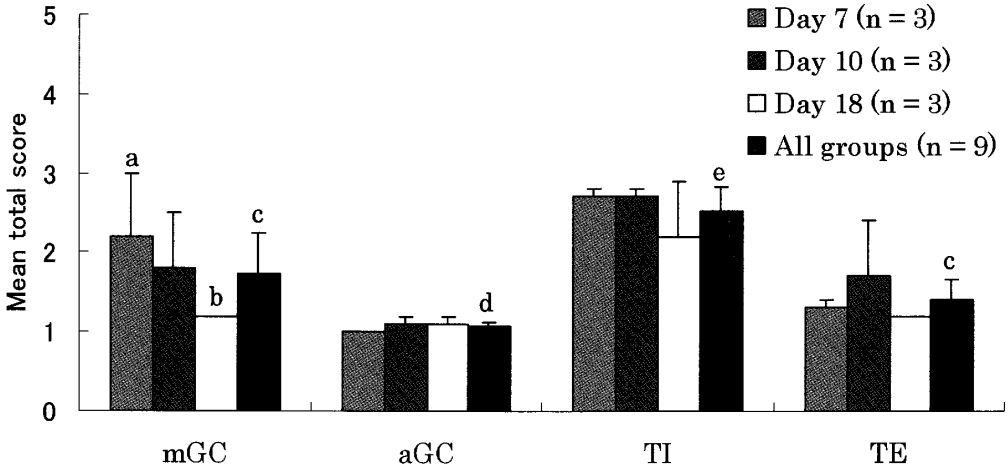

Figure 2. Mean staining scores (±SD) for ERα in bovine follicular cells. No significant difference in the same follicular cells on Day 7, Day 10 or Day 18, except mean staining score in mGC on Day 7 was significantly higher than that in mGC on Day 18 ($^{a,b}P < 0.05$). Mean total scores for ERα in TI were significantly higher than those in mGC and TE ($^{c,e}P < 0.05$) and were different statistically than those in aGC ($^{d,e}P < 0.05$). mGC = mural granulosa cells, aGC = antral granulosa cells, TI = theca interna, TE = theca externa.

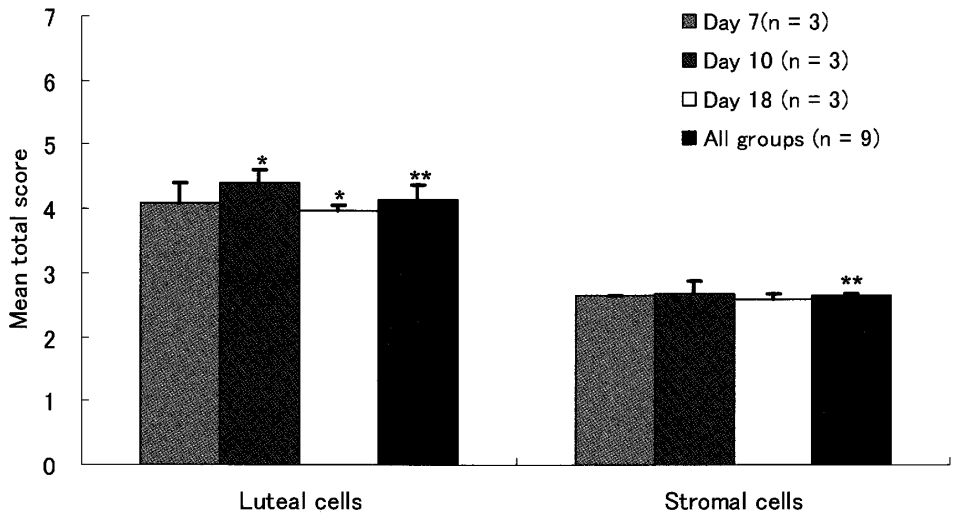

Figure 3. Mean staining scores (±SD) for ERα in bovine corpus luteum. No significant difference in the same cells on Day 7, Day 10 and Day 18. However, the mean staining score in luteal cells on Day 10 was significantly higher than that in luteal cells on Day 18 ($^{*}P < 0.05$). Mean total scores for ERα in luteal cells were statistically higher than those in stromal cells ($^{**}P < 0.01$).

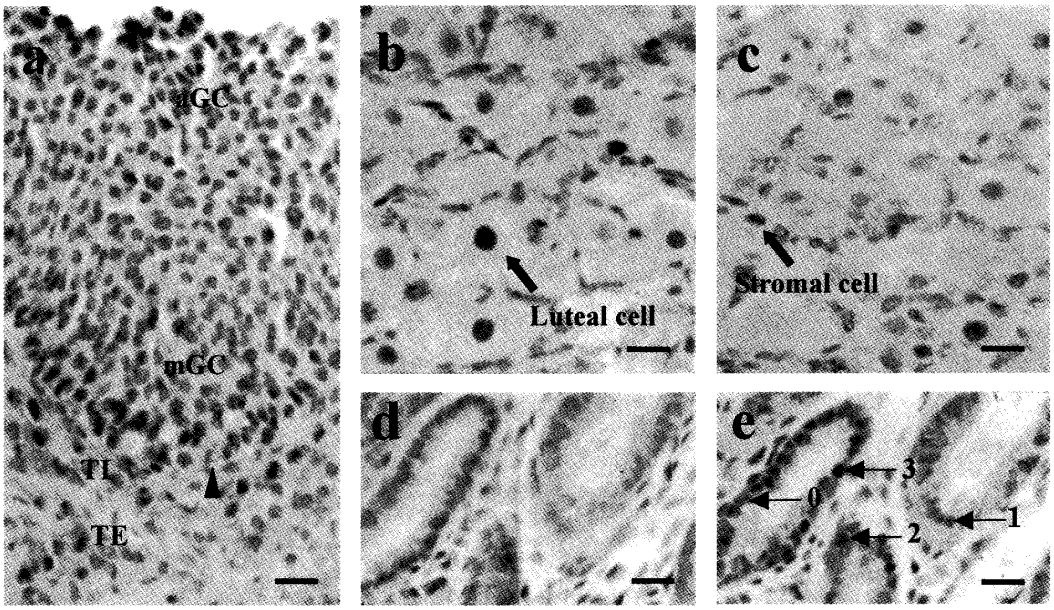

Figure 4. Localization of ERα in the bovine follicular and luteal cells fixed in 10% buffered formalin. (**a**) A representative photomicrograph of ERα expression in the dominant follicle on Day 7. ERα was detected regionalized in the mural granulosa cells and slightly in antral granulosa cells. The highest expression of ERα was detected in theca interna cells and in a few theca externa cells. A high percentage and staining intensity of nuclei were detected in the luteal cells on Day 10 (**b**) compared to the luteal cells on Day 18 (**c**). (**d**) The negative control from uteri showed no staining cells. (**e**) The positive control from uteri showed staining cells with various intensity scores: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining. aGC = antral granulosa cells; mGC = mural granulosa cells; TI = theca interna cells; TE = theca externa cells. Slides were counterstained with Gill's hematoxylin. Bars = 10 μm.

Expression of ERα in the corpus luteum is shown (Figure 3). In the luteal cells on Day 7, Day 10 and Day 18, the mean total scores were 4.1 ± 0.3, 4.4 ± 0.2 and 4.0 ± 0.1, respectively and in the stromal cells the mean total scores were 2.6 ± 0.0, 2.7 ± 0.2 and 2.6 ± 0.1, respectively. No significant difference was found within the same cells on Day 7, Day 10 and Day 18, except in the luteal cells on Day 10 (4.4 ± 0.2), where significantly higher expression was found than on Day 18 (4.0 ± 0.1) ($P < 0.05$). In all groups, the ERα expression in the luteal cells (4.2 ± 0.2) was higher than that in the stromal cells (2.6 ± 0.1) in the corpus luteum ($P < 0.05$). The higher percentage of positive nuclei and staining intensity of nuclei are shown in the luteal cells on Day 7 and Day 10 (Figure 4b, 4c).

Discussion

Expression of ERα was observed in the bovine dominant follicular cells throughout the estrous cycle with various degrees of staining intensity. Theca interna cells of developing DF on Day 7, slightly atretic DF on Day 10 or preovulatory DF on Day 18 showed a higher percentage and staining intensity for ERα than those in mGC, aGC and TE. This was consistent with a previous finding that mRNA expression of ERα in theca interna tissue increased continuously and significantly during the final follicle growth [1]. Expression of ERα was significantly higher in mGC than in aGC. Interestingly, expression of ERα was more regionalized in mGC and a significantly higher expression was found in mGC in developing DF on Day 7 than in mGC preovulatory DF on Day 18. These phenomena may be caused by the lower level of plasma P_4 in developing DF on Day 7 than in preovulatory DF on Day 18. It is known that P_4 strongly inhibits estrogen action [15].

Expression of ERα in the luteal cells of static CL on Day 10 was higher than that in the luteal cells of regressing CL on Day 18. Although no significant difference was found, ERα expression in theca cells of developing CL on Day 7 was slightly higher than that in the luteal cells of regressing CL on Day 18. These findings are consistent with previous research showing that the highest mRNA expression for ERα is detected in the CL during the early luteal phase, followed by a significant decrease during mid, late or regression phases [1]. Similar observations were found in regarding the ovine estrous cycle, where the weak expression of ERα is observed in large luteal cells on Day 6 of the estrous cycle. The weakest expression was found in the regressing CL, whereas intensive expression was found in CL collected on Day 9 of the estrous cycle [17]. In contrast, expression of ERα in the stromal cells showed no difference among all the cow groups. The existence of estrogen receptors in the large luteal cells is related to progesterone release. It is known that estradiol is produced locally in bovine CL throughout the estrous cycle [9]. Oestradiol stimulates progesterone release under in vitro and in vivo conditions, as an intraluteally acting luteotrophic signal [16]. It might be explained that ERα in the luteal cells has an important role in controlling CL function rather than ERα in the stromal cells of the corpus luteum.

The results indicate that ERα was present in the developing, early regressing and preovulatory dominant follicle especially in the theca interna and a few localized in the mural granulosa cells. In the corpus luteum ERα was highly expressed in the luteal cells and the expression became weak concomitant with the regressing corpus luteum.

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