

Intraovarian Immunolocalization of Steroidogenic Enzymes in a Hokkaido Brown Bear, *Ursus arctos yesoensis* during the Mating Season

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ABSTRACT. Immunolocalization for four steroidogenic enzymes was performed on an ovary taken from a Hokkaido brown bear during the mating season. This specimen is considered to be in the follicular phase because of the presence of large follicles. In large follicles, cholesterol side-chain cleavage (P450_{scc}) and 3 β -hydroxysteroid dehydrogenase (3 β HSD) were immunolocalized in theca interna cells and granulosa cells. 17 α -hydroxylase/C17-C20 lyase cytochrome P450 (P450_{c17}) was immunolocalized in theca interna cells but not in granulosa cells. Aromatase cytochrome P450 (P450_{arom}) was immunolocalized only in granulosa cells. In medium follicles, however, P450_{scc} and 3 β HSD were immunolocalized only in theca interna cells, and the immunoreactivity of P450_{arom} was detected in neither theca interna cells nor granulosa cells. Immunoreactivities of P450_{scc}, 3 β HSD and P450_{c17} but not P450_{arom} were detected in interstitial cells. This study suggests that estrogen biosynthesis takes place through interrelation between theca cells and granulosa cells and is explained by the so-called two-cell mechanism. Furthermore, the granulosa cells in large follicles have the capability for pregnenolone and progesterone biosynthesis, and the interstitial cell in the bear ovary is also a steroidogenic site. — **KEY WORDS:** bear, immunocytochemistry, ovary.

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The Hokkaido brown bear, *Ursus arctos yesoensis*, is one of the large terrestrial carnivores inhabiting the island of Hokkaido in Japan, and is herbivorous or omnivorous rather than carnivorous in its real food habits [7]. Captive brown bears have a mating season from late April to early July [10], and in the males, peak serum testosterone concentrations precede the mating season and active spermatogenesis is maintained longer than the mating period [11]. Ovarian steroidogenic features in female brown bears during the mating season have never been clarified.

Estrogen and progesterone are key steroids for reproductive cycles and are predominantly released during the follicular and luteal phases, respectively. During the follicular phase, steroidogenesis occurs in relation to cell differentiation of the two cell types, theca and granulosa cells. In mammalian species studied until now, it is a general understanding that the theca cells produce androgen, whereas the granulosa cells produce estrogen. The immunolocalization of the steroidogenic enzymes is useful to learn the status of steroidogenesis in a given tissue, however, to our knowledge, there is no immunocytochemical study for detecting steroidogenic enzymes in the Ursidae ovary.

The objective of this study was to immunolocalize 4 steroidogenic enzymes during the follicular phase in the ovary of Hokkaido brown bears. An adult female bear of 24-year-old and weighing 115 kg was bitten to death by other bears at Noboribetsu Bear Park (Noboribetsu, Japan) on May 28, 1994. The ovaries were immediately taken and fixed in 4% formaldehyde solution, followed by dehydration with ethanol and embedding in paraffin wax. Each paraffin section (5 μ m) was placed on poly-L-lysine coated glass slides and was deparaffinized with xylene. The sections

were immunostained with antisera of cholesterol side-chain cleavage cytochrome P450 (P450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β HSD), 17 α -hydroxylase/C17-C20 lyase cytochrome P450 (P450_{c17}) and aromatase cytochrome P450 (P450_{arom}) by an avidin-biotin-peroxidase complex method described by Tsubota *et al.* [12] with some modifications. Briefly, the sections were put into methanol with 0.3% H₂O₂ for 10 min to block endogenous peroxidase activity and were incubated by phosphate buffer saline (PBS) with the addition of 10% normal goat serum for 10 min to reduce background staining, followed by treatment with primary antisera (1:500 or 1,000) raised in rabbits against bovine adrenocortical mitochondrial P450_{scc} [9], human placental 3 β HSD [1], guinea-pig adrenal microsomal P450_{c17} [5] and human placental P450_{arom} [4]. Thereafter, the sections were incubated with a second antibody, goat anti-rabbit IgG conjugated with biotin and peroxidase with avidin using a rabbit ExtrAvidin™ staining kit (Sigma, U.S.A.), followed by coloring with 30 mg 3,3'-diaminobenzidine (Wako, Japan) solution in 150 ml/0.05 M Tris-HCl buffer, pH 7.6 plus 30 μ l H₂O₂. Finally, the reacted sections were counterstained with hematoxylin solution. The control was treated with normal rabbit serum in place of the primary antisera.

Based on macroscopical observation, the pair of ovaries possessed 6 medium follicles (between 6 and 2 mm in diameter) in the left and 6 medium follicles and 2 large follicles (larger than 6 mm in diameter) in the right. No corpus luteum was observed in either of the ovaries. In these medium and large follicles, a small number of pyknotic granulosa cells (Fig. 1a) and a slight loosening and shrinkage of the granulosa layers (Figs. 1b-h) were observed histologically.

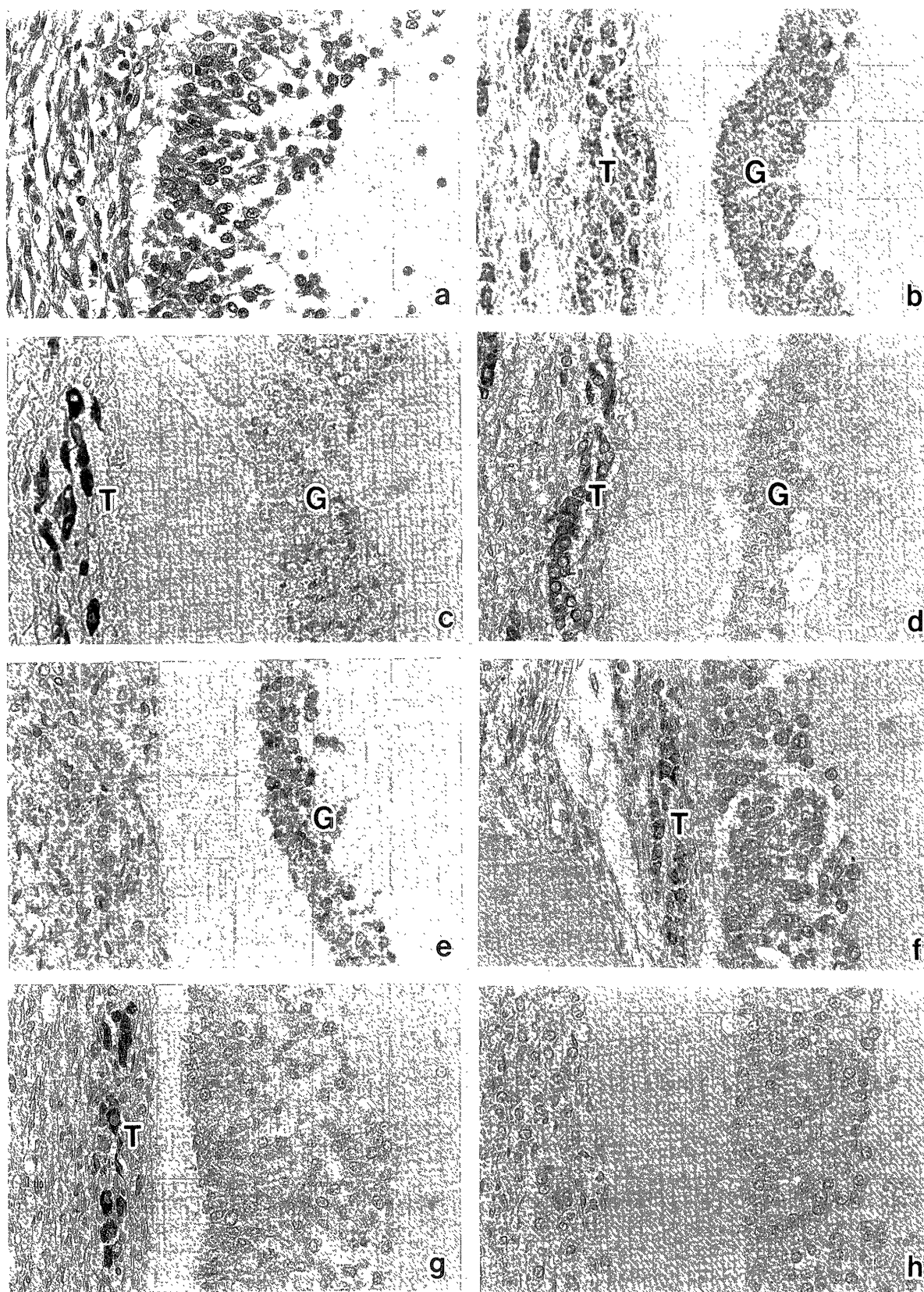


Fig. 1. Sections of the follicles taken from a Hokkaido brown bear during the mating season. (a) Hematoxylin and eosin staining. A small number of pyknotic granulosa cells, and a slight loosening and shrinkage of the granulosa layers are observed. (b) Immunostaining for P450scc in a large follicle. Both theca interna cells (T) and granulosa cells (G) possess immunoreactivity. (c) Immunostaining for 3βHSD in a large follicle. Both theca interna cells (T) and granulosa cells (G) possess immunoreactivity.

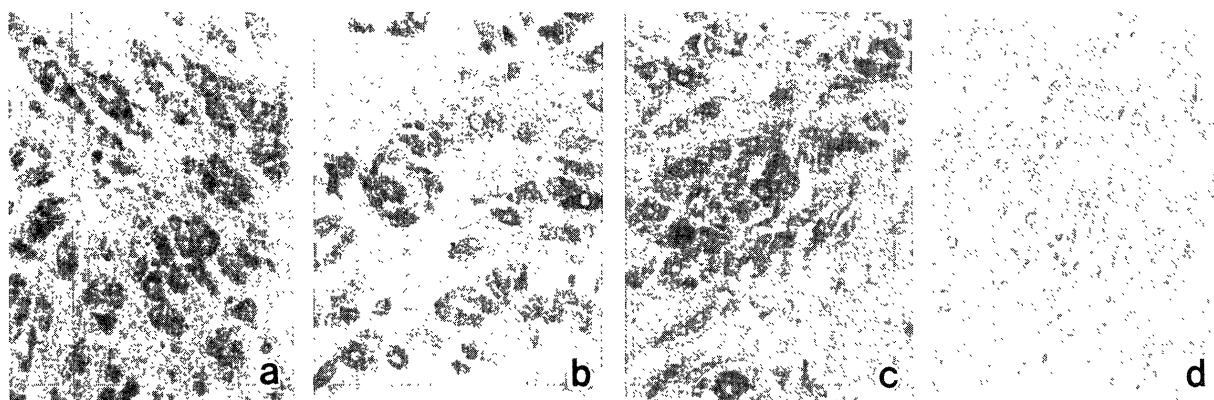


Fig. 2. Immunostaining for 4 kinds of steroidogenic enzymes in the interstitial cells of the bear ovary. Immunoreactivity for (a) P450scc, (b) 3β HSD, (c) P450c17 is detected but (d) P450arom is not ($\times 300$).

In the large follicles, P450scc and 3β HSD were immunolocalized in theca interna cells and granulosa cells (Figs. 1b, c). P450c17 was also immunolocalized in theca interna cells but not in granulosa cells (Fig. 1d). P450arom was immunolocalized only in granulosa cells (Fig. 1e). In the medium follicles, however, P450scc and 3β HSD were immunolocalized only in theca interna cells (Figs. 1f, g), and immunoreactivity of P450arom was not observed in either the theca interna cells or the granulosa cells. The immunoreactivities of P450scc, 3β HSD and P450c17 were detected in interstitial cells (Figs. 2a-c) but P450arom was not immunostained in these cells (Fig. 2d). In the other cells, no significant immunoreactivity was observed in the ovaries. The control exhibited no immunoreactivity on all the whole sections (Fig. 1h).

This is the first report on the immunolocalization of steroidogenic enzymes in a bear ovary during the mating season. The pair of the ovaries were obtained in late May and possessed several medium and large follicles but not corpus luteum. These results suggest that these ovaries were in the follicular phase. The follicles observed in this study, however, showed a small number of pyknotic granulosa cells and a slight loosening and shrinkage of the granulosa layers, as has been reported previously [2], hence these follicles were presumably in atresia.

Intrafollicular steroids are known to be synthesized by coordination between theca interna cells and granulosa cells. Theca interna cells are a source of androgen which will be aromatized to estrogen in granulosa cells by the so-called the "two-cell mechanism" [3]. In the present immunocytochemical study, P450arom was immunolocalized in granulosa cells while P450c17 was immunolocalized in theca interna cells. These results are in agreement with the biosynthetic site of estrogen and androgen explained by the "two cell mechanism".

In our study, the distribution of P450scc was similar to that in bovine follicles. Rodgers *et al.* [8] reported that, using cattle, the immunofluorescent staining against P450scc was observed in the theca interna and less intensely in the granulosa cells, and that the staining of the granulosa cells was more intense in larger follicles. Our results of P450scc localization may indicate that the granulosa cells in the large follicles of the bear ovary have the capability for pregnenolone biosynthesis.

Generally, 3β HSD in theca interna cells has a high and constant activity throughout follicular development, and its activity in granulosa cells increases with follicular development [6]. In this study, immunoreactivity of 3β HSD was only observed in the theca interna cells of medium follicles, but its immunoreactivity was also observed in the granulosa cells of large follicles. These results may indicate that granulosa cells also have the capability of progesterone biosynthesis and 3β HSD activity in granulosa cells may increase with follicular development in the bear ovary.

In some mammalian species including humans, interstitial cells biosynthesize several kinds of steroid hormones [6]. The presence of P450scc, 3β HSD and P450c17 in interstitial cells in the bear ovary suggests the steroidogenic activity of these cells during the follicular phase.

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(d) Immunostaining for P450c17 in a large follicle. Theca interna cells (T) possess immunoreactivity but granulosa cells (G) do not. (e) Immunostaining for P450arom in a large follicle. Immunoreactivity is observed only in granulosa cells (G). (f) Immunostaining for P450scc in a medium follicle. Immunoreactivity is observed only in theca interna cells (T). (g) Immunostaining for 3β HSD in a medium follicle. Immunoreactivity is observed only in theca interna cells (T). (h) Control incubated with normal rabbit serum in place of primary antisera ($\times 300$).

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