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Author(s)	Matsuura, Yukiko; Suzuki, Masatsugu; Hayakawa, Daisuke; Asano, Makoto; Sasaki, Motoki; Kitamura, Nobuo; Yamada, Junzo; Tsubota, Toshio; Ohtaishi, Noriyuki
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Immunohistochemical localization of steroidogenic enzymes in corpus luteum of wild sika deer during early mating season

Yukiko Matsuura¹⁾, Masatsugu Suzuki¹⁾, Daisuke Hayakawa²⁾, Makoto Asano³⁾, Motoki Sasaki²⁾, Nobuo Kitamura²⁾, Junzo Yamada²⁾, Toshio Tsubota³⁾ and Noriyuki Ohtaishi¹⁾

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Abstract

We analyzed the localization of steroidogenic enzymes (P450 scc, 3 β HSD, P450 arom and P450 c17) in the corpora lutea of two Hokkaido sika deer (*Cervus nippon yesoensis*) during the early mating season. Two corpora lutea were found in each female and the timing of formation of the corpora lutea seemed different. P450 scc, and 3 β HSD, positive luteal cells were found in both corpora lutea. The existence of two functional corpora lutea from the early mating season through pregnancy suggests that progesterone secreted by two or more corpora lutea is necessary for maintenance of pregnancy in sika deer.

Key Words : corpus luteum, progesterone, steroidogenic enzyme.

Sika deer, *Cervus nippon*, is a seasonal breeder, with mating in autumn, and fawning in early summer. Although they basically deliver a single fawn, two (or more) functional corpora lutea (CLs) are found in the ovaries in sika deer^{14,16)} as in the red deer (*Cervus elaphus*)^{1,7)}. The occurrence rate of surplus CLs attained to 77.8-80.7%^{14,15)}. During preg-

nancy, there was neither histological difference nor ability to synthesize steroid hormones between CLs, but only a difference in size was detected (unpublished data). To elucidate the function of steroid synthesis of these CLs in the early mating season, we performed immunohistochemical staining to detect the expression of steroidogenic enzymes

¹⁾Laboratory of Wildlife Biology, Department of Environmental Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

²⁾Laboratory of Anatomy, Department of Basic Veterinary Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan

³⁾United Graduate School of Veterinary Science, Gifu University, Gifu 501-1193, Japan

*Corresponding author : Yukiko Matsuura

Laboratory of Wildlife Biology, Graduate School of Veterinary Medicine, Hokkaido University, N18-W9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan

+81-11-706-5210 (tel)

+81-11-706-5569 (fax)

koyum@seagreen.ocn.ne.jp

in CLs.

Two female deer were shot for research by hunters on October 26 and 27, 2003, in Nishi-Okoppe Village in northern Hokkaido (142° 56' E, 44° 56' N). The ages of each female were estimated at over two years according to tooth replacement using criteria for sika deer¹²⁾. The body weights of these females were 93.2kg and 95.0kg.

The ovaries were fixed and preserved in 10% formalin. After fixation, the specimens were dehydrated in an ethanol series and embedded in paraffin. Thin sections, 5- μ m thick, were mounted on silane-coated glass slides (Matsunami, Tokyo, Japan). The sections were deparaffinized with xylene and treated with a 10-min microwave oven process in phosphate-buffered saline (PBS) to enhance immunoreactivity. Then, the sections were incubated for 10 min in methanol containing 3% H₂O₂ to block endogenous peroxidase activity. The specimens were then washed in 0.01M PBS for 10min and treated with normal goat serum in PBS (1 : 50) for 30min, to reduce the background. Sections were treated with one of the following primary antibodies (1 : 500 or 1 : 1,000) overnight at 4 °C : polyclonal antiserum raised in rabbits against bovine adrenal cholesterol side-chain cleavage cytochrome P450 (P450scc)³⁾, polyclonal antiserum raised in rabbits against human placental 3 β -hydroxysteroid dehydrogenase (3 β HSD)⁴⁾, polyclonal antiserum raised in rabbits against human placental aromatase cytochrome P450 (P450arom)⁹⁾ or polyclonal antiserum raised in rabbits against porcine testicular 17 α -hydroxylase cytochrome P450 (P450 c17)⁶⁾. The antibodies of 3 β HSD and P450arom were kindly supplied by Dr. Mason and Dr. Harada, respectively, and the antibodies of P450scc and P450 c17 were kindly supplied by Dr. Kominami. Immunolocalization was demonstrated by the avidin-biotinylated

peroxidase complex (ABC) method using a VECTASTAIN *Elite* ABC kit (Vector, Burlingame, CA, U.S.A.). After a final washing in PBS, the sections were colorized with diaminobenzidine solution. Control sections were treated with Diluent Buffer solution instead of the primary antiserum. The sections were also stained with hematoxylin and eosin.

Two CLs were detected in each female. According to the hematoxylin and eosin-stained sections, distinct clots of blood remained in one of the CLs in each female. Hence, the CL appeared much darker than the other one macroscopically (Fig. 1), while a difference between the plural CLs was not detected during pregnancy¹¹⁾. Furthermore, the size of the luteal cells in the CLs with the clots of blood (Fig. 2, a and c) appeared to be smaller than that in the others (Fig. 2, b and d). Because the cells derived from granulosa cells grow in size as luteal develop¹¹⁾, the CLs with the clots of blood were considered to be formed after other CLs had formed, and were likely in the early stage of development. Therefore, the CL with the clots of blood will be called a newly formed CL, (NFCL) and the other one will be called a former formed CL (FFCL), for remainder of this paper.

P450scc-positive luteal cells were found in both CLs, NFCL and FFCL, in each female (Fig. 2, a and b). 3 β HSD-positive luteal cells were also found in both CLs (Fig. 2, c and d), but only the luteal cells located in the periphery of the CL reacted in the NFCL in each female (Fig. 2, c). P450 arom and P450 c17 were negatively immunostained. There were small and flat luteal cells and large luteal cells, both of which reacted similarly. These two kinds of cells were probably derived from thecal cells and granulosa cells, respectively¹³⁾. All control sections were negatively stained.

Our results suggest that both CLs, NFCL

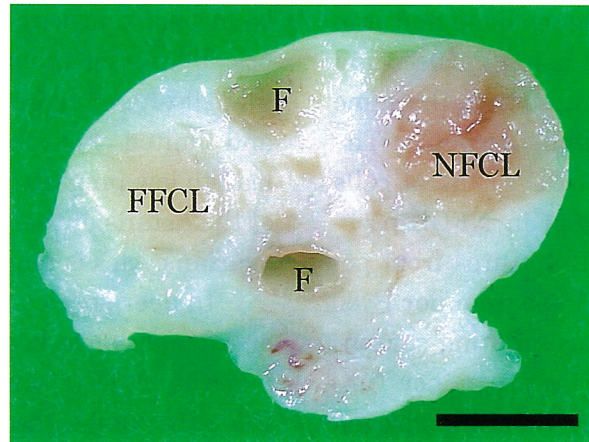


Fig. 1 Macroscopic view of an ovary of a sika deer collected on October 26 (10% formalin fixed). Bar, 5mm. NFCL; newly formed corpus luteum, FFCL; former formed corpus luteum, F; follicle. NFCL appeared darker than FFCL because clot of blood remained in NFCL.

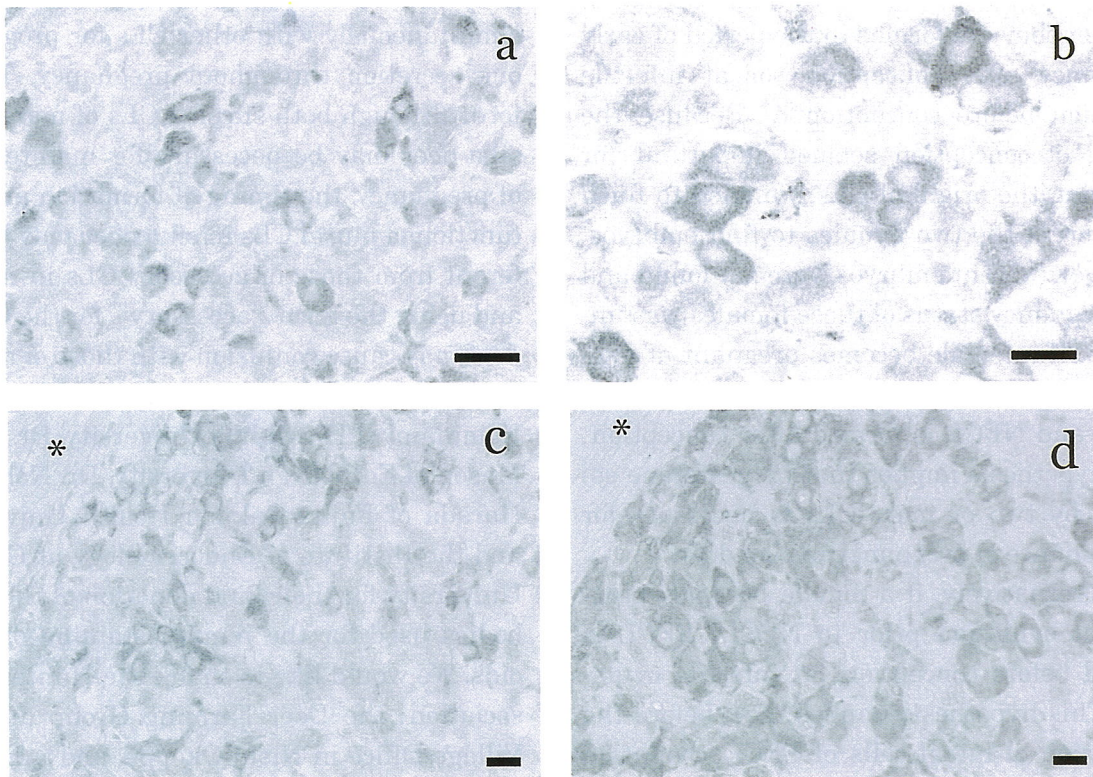


Fig. 2 Representative section of corpora lutea of sika deer. Bar, 20 μ m. (a) Newly formed corpus luteum (NFCL). Luteal cells showed positive immunostaining for P450scc. (b) Former formed corpus luteum (FFCL). Luteal cells showed positive immunostaining for P450scc. (c) NFCL. Only luteal cells located in the periphery of the corpus luteum showed positive immunostaining for 3 β HSD. *: residue of theca folliculi. (d) FFCL. Luteal cells showed positive immunostaining for 3 β HSD. *: residue of theca folliculi. Size of luteal cells in NFCL (a and c) appeared to be smaller than that in FFCL (b and d).

and FFCL, synthesize pregnenolone and progesterone from cholesterol using P450_{scc} and 3 β HSD in the early mating season, the same as during pregnancy (unpublished data). The negative staining for 3 β HSD of luteal cells located in the central region in NFCL probably indicated that the CL matures from periphery to center. Neither P450_{arom} nor P450_{c17} were stained in the early mating season, but they were detected during pregnancy (unpublished data). Thus, secretions of androgen and estrogen from CLs were suppressed in the early mating season.

Thus, the two CLs were formed at different times in the early mating season and both CLs obviously synthesized progesterone through pregnancy. In deer species, the origin of plural CLs has been discussed regarding whether they are formed in the period of early pregnancy after conception⁸⁾ or at infertile ovulation before conception¹⁶⁾. Because the timing of conception seemed important for clarifying the origin of CLs, we tried to flush the uteri of the two females to find embryos. However, evident embryos were not found and the pregnancy status of these females was unknown. If a female becomes pregnant at ovulation prior to FFCL, FFCL is the CL of pregnancy and NFCL is formed after conception. This hypothesis implies that progesterone secreted by two or more CLs is necessary for maintenance of pregnancy in sika deer.

Alternatively, if a female becomes pregnant at ovulation prior to NFCL, FFCL is formed before conception, at infertile ovulation. This hypothesis implies that, before the regression of luteal cells of FFCL, ovulation and progesterone secretion occurs, and, FFCL may synthesize progesterone simultaneously with NFCL to achieve and maintain pregnancy. However, progesterone concentration declined to the nadir when ovulation occurred in deer species^{1,10)}. These phenomena seem to

be inconsistent with each other. There are the possibilities of conversion from progesterone into 20 α -hydroxyprogesterone, like in rats⁵⁾, and limited cholesterol supply, both of which lead to low progesterone concentration. Study of the details of the CL control system during ovarian cycles is required.

In deer species, two CLs observed within one female have been classified only by size, and the smaller one is called the accessory CL⁷⁾. In mares, accessory CLs begin to develop from 40 days of gestation and secrete progesterone, instead of the primary CL which develops from the ovulation that led to the pregnancy²⁾. Accessory CLs in mares function as the transitional source of progesterone secretion between the primary CL and placenta, while surplus CLs in sika deer work simultaneously with other CLs for progesterone secretion throughout pregnancy. Therefore, although both surplus CLs of mares and sika deer may be necessary for maintenance of pregnancy, the timing of formation and the function of plural CLs in sika deer may be different from those of accessory CLs in mares, and using the term "accessory" for the CLs of deer may cause confusion with those of mares.

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