—Original Article—

Seasonal Changes in Luteal Progesterone Concentration and mRNA Expressions of Progesterone Synthesis-related Proteins in the Corpus Luteum of Mares

Keisuke KOZAI¹⁾, Takuo HOJO¹⁾, Masashi TAKAHASHI²⁾, Tomas J ACOSTA¹⁾, Yasuo NAMBO³⁾ and Kiyoshi OKUDA¹⁾

¹⁾Laboratory of Reproductive Physiology, Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan

²⁾National Agricultural Research Center for Kyushu Okinawa Region, Kumamoto 861-1192, Japan

³⁾Hidaka Training and Research Center, Japan Racing Association, Urakawa 057-0171, Japan

Abstract. Although circulating progesterone (P_4) levels tend to change with the season, little is known about the seasonal changes of P_4 synthesis-related proteins in the corpus luteum (CL) of mares. To examine these changes, seventy-four ovaries containing a CL were collected from Anglo-Norman mares at a local abattoir in Kumamoto, Japan ($\sim N32^\circ$), five times during one year. The stages of the CLs were classified as early, mid and regressed by macroscopic observation of the CL and follicles. The mid CL, which had the highest P_4 concentration, was used to evaluate the seasonal changes in P_4 synthesis. The luteal P_4 concentration and mRNA expression of luteinizing hormone receptor (*LHCGR*) were lowest during early winter and highest during late winter. The mRNA expressions of steroidogenic acute regulatory protein (*StAR*), P450 cholesterol side-chain cleavage enzyme (*P450scc*) and 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase (*3\beta*-HSD) were lowest during early winter and increased during late winter. These results suggest that P_4 synthesis in the CL is affected by the seasonal changes in the mRNA expressions of P_4 synthesis-related proteins in mares.

Key words: Corpus luteum, Mares, Progesterone, Reproductive seasonality, Steroidogenesis

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Mares are seasonal breeders in which day length (DL) is one of the main environmental factors controlling reproductive seasonality [1–3]. The reproductive activity of mares reaches a peak during summer, then decreases during autumn and eventually stops through winter as DL decreases. January 1 in the Northern Hemisphere and August 1 in the Southern Hemisphere are used as the official birth dates for many breeds, especially for racing breeds [4]; therefore, the exposure of mares to artificial light has been used to hasten the reproductive season. Thus, studying reproductive seasonality in mares is relevant for the equine industry.

In mammals, the corpus luteum (CL) is an endocrine organ producing progesterone (P_4), which is essential for the establishment and maintenance of pregnancy. Although several studies have examined seasonal changes in circulating P_4 concentration in mares, the reported results are not consistent, i.e., the circulating P_4 concentration has been reported to decrease [5–7], increase [8] or remain unchanged [9] from the breeding to non-breeding season. Therefore, the effects of season on P_4 synthesis in the CL of mares remain unclear.

Luteal P_4 production is regulated by several P_4 synthesis-related proteins [10]. Luteinizing hormone (LH) secreted by the anterior pituitary is the main regulator of luteal function. LH acts through its

specific cell surface receptor (LHCGR) and stimulates P_4 production by the CL in ewes [11], rats, sows [12], cows [13] and mares [14]. Steroidogenic acute regulatory protein (StAR) transfers cholesterol from the outer to inner mitochondrial membrane, allowing enzymatic cleavage of the side chain of cholesterol to pregnenolone by P450 cholesterol side-chain cleavage (P450scc). Pregnenolone is then transported to the smooth endoplasmic reticulum, which is usually closely associated with mitochondria, where 3β -hydroxysteroid dehydrogenase/ $\Delta 5$ - $\Delta 4$ isomerase (3β -HSD) converts pregnenolone to P_4 .

In the present study, we examined the seasonal changes in luteal P_4 concentration and mRNA expressions of P_4 synthesis-related proteins in the CL of mares.

Materials and Methods

Collection of equine CL

Seventy-four Anglo-Norman mares of various ages and weighing approximately 1,000 kg, which were imported from Canada and then fattened in a ranch adjacent to an abattoir ($\sim N32^\circ$) in Kumamoto, Japan, for at least two years before exsanguination, were utilized in the present study. Mares were exposed to ambient light conditions. Seventy-four ovaries containing a CL were collected from randomly designated cyclic mares at the abattoir in accordance with protocols approved by the local institutional animal care and use committee during spring (April, DL: 12.8–13.3 h), summer (July, DL: 13.8–14.1 h), autumn (September–October, DL: 11.5–12.0 h), early winter

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Correspondence: K. Okuda (e-mail: kokuda@cc.okayama-u.ac.jp)

(December, DL: 10.0–10.1 h) and late winter (January–February, DL: 10.4–10.6 h). Ovulation rates, which were defined as the proportion of mares with ovaries containing a CL to the number of mares sacrificed, were 75.8% in spring, 95.4% in summer, 92.2% in autumn, 90.5% in early winter (before winter solstice) and 62.5% in late winter (after winter solstice). Mares possessing ovaries with a macroscopic abnormality including anovulatory hemorrhagic follicles were eliminated. The ovulation rate was high even in late winter. However, this is not surprising due to the fact that mares with a high body condition score (BCS) continued to ovulate throughout the winter or had significant follicular activity in the ovaries [15]. The mares used in the present study weighed approximately 1,000 kg and were expected to have a high BCS. After midsagittal sectioning, the CLs were classified as being in the early, mid and regressed luteal stages ($n=3-6/\text{stage}$) by macroscopic observation of the CL and follicles as described previously [16, 17]. Briefly, the early CL had a corpus hemorrhagicum; the mid CL had a solid, although trabeculated, appearance; and the regressed CL was straw colored and associated with follicles > 35 mm in diameter. Following determination of the stages, CL tissues were immediately separated from the ovaries, rinsed with cold sterile saline, frozen rapidly in liquid nitrogen and then stored at -80°C until being processed for P_4 and RNA extraction.

P₄ extraction

P_4 was extracted from luteal tissues as described previously [18]. Briefly, approximately 0.2 g of minced luteal tissue was added to 2 ml of ethanol. The tissues were then homogenized 5 times for 10 sec on ice by a tissue homogenizer (Phycostron; NITI-ON, Chiba, Japan; NS-50) and 3 times for 1 min on ice by an ultrasonic homogenizer. The suspension was incubated at room temperature for 1 h. After centrifugation (1,700 rpm for 30 min at 4°C), the supernatant was evaporated in a vacuum oven at 50°C , and the residue was dissolved in 2 ml of assay buffer for enzyme immunoassay (EIA).

P₄ determination

Concentrations of P_4 were determined directly from the tissue extracts with an EIA as described previously [19]. The standard curve ranged from 0.391 to 100 ng/ml, and the effective dose of the assay for 50% inhibition (ED_{50}) was 4.5 ng/ml. The intra- and interassay coefficients of variation were 5.6 and 6.9%, respectively.

Since the P_4 concentration in the luteal tissues reached the highest

levels at the mid luteal stage (data not shown), the mid CL was used to evaluate the seasonal changes in P_4 synthesis in mares.

Real-time PCR

Total RNA was extracted from luteal tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA; 15596-026) according to the manufacturer's instructions. One μg of each total RNA was reverse transcribed using a ThermoScriptTM RT-PCR System (Invitrogen; 11146-016). Gene expression was measured by real-time PCR using a MyiQ (Bio-Rad Laboratories, Melville, NY, USA) and SYBR[®] Premix Ex TaqTM II (RR081B, TaKaRa) starting with 2 ng of reverse-transcribed total RNA as described previously [20]. Briefly, for quantification of the mRNA expression levels, the primer length (20 or 21 bp) and GC contents of each primer (50–55%) were synthesized using an online software package as shown in Table 1, and the primer specificity was validated by BLAST. To determine the most stable internal control gene under our experimental conditions, three potential housekeeping genes were initially considered (β -actin, β 2-microglobulin and glyceraldehyde-3-phosphate dehydrogenase; GAPDH). GAPDH gene transcription was unaffected by our experimental conditions and was therefore selected as the internal control. The primers for LHCGR, StAR, P450scc, 3β -HSD and GAPDH generated specific 122-bp, 120-bp, 123-bp, 132-bp and 135-bp products, respectively. Each PCR yielded only a single amplification product. PCR was performed under the following conditions: 95°C for 3 min, followed by 45 cycles of 94°C for 15 sec, 55°C for 20 sec and 72°C for 15 sec. Use of SYBR[®] Premix Ex TaqTM II at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient $r > 0.99$). The expression of each gene was evaluated based on the GAPDH mRNA expression in the individual samples.

Statistical analysis

All experimental data are shown as means $\pm$ SEM. The statistical significance of differences in luteal P_4 concentration and mRNA expressions of LHCGR, StAR, P450scc and 3β -HSD were assessed by analysis of variance (ANOVA) followed by a Fisher protected least-significant difference procedure (PLSD) as a multiple comparison test.

Table 1. Primers used in real-time PCR

Gene	Primer	Sequence	Accession No.	Product size
LHCGR	Forward	5'-TTGCCACATCATCCTATTCTC-3'	AY464091	122 bp
	Reverse	5'-TTCTTTTGTGGCAAGTTTCT-3'		
StAR	Forward	5'-CGAGCAGAAAGGTGTCATCA-3'	NM_001081800	120 bp
	Reverse	5'-CATCCCTTGAGGTCAATGCT-3'		
P450scc	Forward	5'-GCCTCCTAGCAAGCAACAAG-3'	NM_001082521	123 bp
	Reverse	5'-TGCGTGCTATCTCGTACAGG-3'		
3β -HSD	Forward	5'-TGGCATCCTGACACAACT-3'	D89666	132 bp
	Reverse	5'-AACTGTCCTTGGAATGCTTGG-3'		
GAPDH	Forward	5'-CGACCACTTTGTCAAGCTCA-3'	NM_001163856	135 bp
	Reverse	5'-TCCTTCTCTTGCTGGGTGAT-3'		

Results

The P_4 concentration (Fig. 1) and mRNA expression of *LHCGR* (Fig. 2A) were highest during late winter (both $P < 0.05$). The mRNA expression of *StAR* was higher during spring than during early winter and was higher during summer than during autumn and early winter (Fig. 2B; $P < 0.05$). The mRNA expression of *P450scc* was higher during spring than during autumn and early winter and was higher during summer and late winter than during early winter (Fig. 2C; $P < 0.05$). The mRNA expression of 3β -HSD was higher during spring than during autumn and early winter (Fig. 2D; $P < 0.05$). The mRNA expressions of *StAR*, *P450scc* and 3β -HSD decreased from summer to early winter and then increased to late winter in the same fashion.

Discussion

The preceding data demonstrate that both the luteal P_4 concentration and mRNA expressions of P_4 synthesis-related proteins in the CL changed seasonally.

In most mammals, LH is a major luteotropic hormone that stimulates P_4 production by the CL [10–14, 21, 22]. In mares, administration of GnRH, eLH or hCG increased circulating P_4 concentrations *in vivo* [23–26]. In addition, *in vitro* studies demonstrated that either eLH or hCG increased the P_4 production by equine luteal cells [14, 24]. Moreover, treatment of mares with an antiserum against equine gonadotropins during diestrus decreased the luteal size [27, 28], and treatment of mares with a GnRH antagonist during diestrus

Fig. 1. Effect of season on luteal P_4 concentration. All experimental data are shown as means $\pm$ SEM. Different letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison test.

decreased the circulating P_4 concentrations [29, 30]. These findings indicate that LH stimulates the production of P_4 by the CL and support luteal function in mares, as shown in other species [10, 21, 22]. In mares, circulating LH concentrations were high during summer and low during winter [31]. Therefore, we expected that the P_4 concentration in the luteal tissue would be high during summer and low during winter. In the present study, however, P_4 concentrations in the luteal tissues were low during summer and

Fig. 2. Effects of season on mRNA expressions of (A) *LHCGR*, (B) *StAR*, (C) *P450scc* and (D) 3β -HSD in the mid CL. All experimental data are shown as means $\pm$ SEM. Different letters indicate significant differences ($P < 0.05$) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison test.

early winter, and high during late winter. The luteal P_4 concentration was calculated as the P_4 content per one gram of tissue. Therefore, if the CL during summer is heavier than during late winter, total P_4 production from the CL may be higher during summer than during late winter. Thus, a discrepancy between the results of the present and previous studies [31] may be due to the difference in luteal tissue weight between seasons. Unfortunately, we did not weigh the luteal tissue in the present study. A further study is needed to clarify this point. In addition, since LH acts through LHCGR, which is present on the cell surface, it could be assumed that changes in the levels of LHCGR expression in luteal cells modify the stimulatory effect of LH on P_4 production by the CL. In the present study, the luteal P_4 concentration and mRNA expression of *LHCGR* in the CL showed similar seasonal changes and were highest during late winter. This finding suggests that an increase in the sensitivity of the CL to LH results in increasing P_4 concentrations in the luteal tissues during late winter. Administration of prostaglandin $F_{2\alpha}$ reduced circulating P_4 concentrations and the mRNA expression of *LHCGR* in the CL of ewes [32], cows [33] and mares [34]. Moreover, circulating and luteal P_4 concentrations were highly correlated with the number and affinity of LHCGR in the CL of mares [35]. The findings of the present and previous studies strongly suggest that P_4 production in the CL is closely related to LHCGR expression in mares. However, the mechanisms regulating LHCGR expression remain unknown. Further studies are needed to clarify the factors regulating LHCGR expression in the CL of mares.

We demonstrated in the present study for the first time the effects of seasons on the mRNA expressions of *StAR*, *P450scc* and 3β -*HSD* in the CL of mares. The mRNA expressions of *StAR* and 3β -*HSD* decreased from summer and spring to early winter, respectively, and then tended to increase during late winter, whereas the mRNA expression of *P450scc* decreased from spring to early winter, and then increased during late winter. In mares, circulating LH concentrations decreased from summer to winter and then remained at a low level until the following spring [31]. In the present study, the mRNA expressions of *StAR* and 3β -*HSD*, but not *P450scc*, showed seasonal changes similar to those of the circulating LH concentrations reported previously [31]. The mRNA expressions of *StAR* and 3β -*HSD* may be regulated by LH in mares. In addition, mRNA expressions of *StAR*, *P450scc* and 3β -*HSD* tended to increase during late winter when circulating LH concentrations remained low [31]. The slight increases in the mRNA expressions of *StAR*, *P450scc* and 3β -*HSD* might result from an increase in the sensitivity of the CL to LH due to an elevation in LHCGR expression, supporting the idea that LH stimulates the mRNA expressions of *StAR*, *P450scc* and 3β -*HSD* in the CL of mares. Furthermore, the changes in mRNA expressions of *StAR*, *P450scc* and 3β -*HSD* from autumn to early winter were coincident with those of P_4 concentrations in the luteal tissues. These findings confirmed that P_4 production by the CL is associated with the mRNA expressions of *StAR*, *P450scc* and 3β -*HSD*, as suggested earlier in mares [34, 36, 37]. Taken together, these findings suggest that LH regulates the mRNA expressions of *StAR*, *P450scc* and 3β -*HSD*, leading to the seasonal changes in luteal function in mares.

In conclusion, we demonstrated that both the luteal P_4 concentration and mRNA expressions of P_4 synthesis-related proteins in the CL changed seasonally and that there was a close relationship

between the luteal P_4 concentration and the mRNA expressions of P_4 synthesis-related proteins in the CL. The overall findings suggest that the capacity of the CL to synthesize P_4 is affected by the seasonal changes in the mRNA expressions of P_4 synthesis-related proteins in mares.

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