SEASONAL CHANGES IN LUTEAL FUNCTION AND REGULATORY MECHANISMS OF FUNCTIONAL LUTEOLYSIS IN THE MARE

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Keisuke KOZAI

Graduate School of Environmental and Life Science

(Doctor's course)

OKAYAMA UNIVERSITY

PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Environmental and Life Science (Doctor's course), Okayama University, Japan, from April 2012 to March 2015, under the supervision of Professor Kiyoshi OKUDA.

This dissertation has not been submitted previously in whole or in part to a council, university or any other professional institution for a degree, diploma or other professional qualifications.

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ABSTRACT

The corpus luteum (CL) is a mammalian endocrine organ which is transiently formed in the ovary after ovulation. Progesterone (P_4) produced by the CL is essential for the establishment and maintenance of pregnancy. In the absence of pregnancy, the CL regresses to allow the subsequent ovulation to occur. It is clarified in several mammalian species that the luteal function is regulated not only by pituitary hormones but also by local regulatory factors. However, little is known about the regulatory mechanisms of luteal function in mares. The mare is a seasonal breeder. Therefore, it is important to consider whether luteal function changes seasonally for precisely understanding the regulatory mechanism of luteal function. In the present study, we evaluated the seasonal changes in luteal P_4 concentration and mRNA expressions of P_4 synthesis-related proteins in the CL, and investigated the mechanism of functional luteolysis to elucidate the regulatory mechanism of luteal regression in mares.

(i) Seventy-four ovaries containing a CL were collected from Anglo-Norman mares at a local abattoir in Kumamoto, Japan (～N32°), five times during one year. The CLs were classified as being in the early, mid and regressed luteal phases 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 *LHCGR* were lowest during early winter (December) and highest during late winter (January-February). The mRNA expressions of *StAR*, *P450scc* and *3β-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.

(ii) In mares, aldo-keto reductase (AKR) 1C23 has 20α-hydroxysteroid dehydrogenase (20α-HSD) activity which catabolizes P_4 into its biologically inactive form, 20α-hydroxyprogesterone (20α-OHP). To clarify whether AKR1C23 is associated with functional luteolysis in mares, we investigated the expression of AKR1C23 in the CL in different luteal phases using CLs collected during the breeding season. The luteal P_4 concentration and levels of *3β-HSD* mRNA were higher in the mid luteal phase than in the late and regressed luteal phases ($P<0.05$), but the level of 3β-HSD protein was higher in the late luteal phase than in the regressed luteal phase ($P<0.05$). The luteal 20α-OHP concentration and the level of *AKR1C23* mRNA were higher in the late luteal phase than in the early and mid luteal phases ($P<0.05$), and the level of AKR1C23 protein was also highest in the late luteal phase. Taken together, these findings suggest that metabolism of P_4 by AKR1C23 is one of the processes contributing to functional

luteolysis in mares.

In conclusion, the findings in CHAPTER 2 suggest that the suitable timing of collecting the CL should be considered depending on a purpose of research because luteal function changes seasonally, and the results in CHAPTER 3 show that conversion of P_4 into 20α-OHP by AKR1C23 is one of the processes contributing to functional luteolysis in mares.

CHAPTER 1

GENERAL MATERIALS AND METHODS

P_4 and 20α-OHP extraction

P_4 and 20α-OHP were extracted from luteal tissues as described previously [1]. Briefly, 2 ml of ethanol was added to the minced luteal tissue samples (approximately 0.2 g). The tissues were then homogenized 5 times for 10 sec on ice by a tissue homogenizer (Physcotron; NITI-ON, Chiba, Japan; NS-50) and 3 times for 1 min on ice by an ultrasonic homogenizer (VCX400 Vibra-Cell; Sonics & Materials, Inc., CT, USA). 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 enzyme immunoassay (EIA) buffer (7.12 g Na_2HPO_4-$2H_2O$, 8.5 g NaCl in 1,000 ml ultra pure water, pH 7.2).

P_4 and 20α-OHP determination

The concentrations of P_4 in the tissue extracts were determined directly by EIA as described previously [2]. The P_4 standard curve ranged from 0.391 to 100 ng/ml, and the ED50 of the assay was 3.5 ng/ml. The intra- and interassay coefficients of variation were on average 5.4% and 7.0%, respectively.

The concentrations of 20α-OHP were determined directly from the tissue extracts by EIA. The 20α-OHP EIA was identical to the EIA for P_4. Standards of samples (20 μl) were incubated with 50 μl polyclonal antibody (raised in a rabbit against 20α-OH-Progesterone-3-CMO-BSA; Cosmo Bio Co., Tokyo, Japan) solution (1:800,000) and 50 μl 20α-OH-Progesterone-3-CMO-HRP (1:400,000; Cosmo Bio Co.) for 16 h at 4 C. The standard curve ranged from 0.0976 to 50 ng/ml, and the ED50 of the assay was 2.5 ng/ml. The intra- and interassay coefficients of variation were on average 6.8% and 8.6%, respectively. The cross-reactivity of the antibody were 100% for 20α-OHP, 1.22% for P_4, 0.99% for pregnenolone, 0.35% for testosterone.

RNA isolation and cDNA synthesis

Total RNA was prepared from luteal tissues using TRIzol Reagent according to the manufacturer's directions (no. 15596-026; Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse transcribed using a ThermoScript RT-PCR System (no. 11146-016; Invitrogen).

Table 1. Macromorphological criteria of the ovary for classifying the luteal phase in mares

Tissue	Criteria	Early luteal phase (Corpus hemorrhagicum)	Mid luteal phase	Late luteal phase	Regressed luteal phase
CL	Appearance	A large central cavity is filled with blood clot	CL is irregular, mushroom shaped or gourd shaped	CL is irregular, mushroom shaped or gourd shaped	CL has a small and oblong structure
	Color	Dark red	Flesh colored	Buff colored	Straw colored
Follicle	Diameter of the largest follicle (mm)		15–20	30–35	>35

Table 2. Primers used in real-time PCR

Gene	Primer	Sequence	Accession No.	Product size
LHCGR	Forward	5'-TTGCCACATCATCCTATTCTC-3'	AY464091	122 bp
	Reverse	5'-TTCTTTTGTTGGCAAGTTTCT-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'-TGGCATCCTGACACACAACT-3'	D89666	132 bp
	Reverse	5'-AACTGTCCTTGGATGCTTGG-3'		
AKR1C23	Forward	5'-CATGAAAGTCCTAGATGGCCTAAAC-3'	AY955082	148 bp
	Reverse	5'-CACTATCCACACACAGGGCTTC-3'		
GAPDH	Forward	5'-CGACCACTTTGTCAAGCTCA-3'	NM_001163856	135 bp
	Reverse	5'-TCCTTCTCTTGCTGGGTGAT-3'		
β-actin	Forward	5'-ATGGGCCAGAAGGACTCATA-3'	NM_001081838	113 bp
	Reverse	5'-TTCTCCATGTCGTCCCAGTT-3'		

CHAPTER 2

SEASONAL CHANGES IN LUTEAL PROGESTERONE CONCENTRATION AND mRNA EXPRESSIONS OF PROGESTERONE SYNTHESIS-RELATED PROTEINS IN THE CORPUS LUTEUM OF MARES

Introduction

Mares are seasonal breeders in which day-length (DL) is one of the main environmental factors controlling the reproductive seasonality [3-5]. 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 date for many breeds, especially for racing breeds [6], therefore, the exposure of mares to the 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. circulating P_4 concentration has been reported to decrease [7-9], increase [10], or remain unchanged [11] 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 [12]. Luteinizing hormone (LH) secreted by the anterior pituitary is the main regulator of the luteal function. LH acts through its specific cell surface receptor (LHCGR) and stimulates P_4 production by the CL in ewes [13], rats, sows [14], cows [15] and mares [16]. Steroidogenic acute regulatory protein (StAR) transfers cholesterol from the outer to the 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/Δ5-Δ4 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 CLs

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 (～N32°) 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 nonpregnant 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 (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 activities in the ovaries [17]. 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 phases (n = 3-6/phase) by macroscopic observation of the CL and follicles (Table 1) as described previously [18, 19]. Following determination of the phases, CL tissues were immediately separated from 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.

Real-time PCR

Gene expression was measured by real-time PCR using a MyiQ (Bio-Rad Laboratories, Melville, NY, USA) and SYBR® *Premix Ex Taq*™ 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 [21] as shown in Table 2, 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, β2-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 the SYBR® *Premix Ex Taq*™ 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.

Statistic analysis

All experimental data are shown as means ± 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 as a multiple comparison test.

Results

The P_4 concentration (Figure 1) and mRNA expression of *LHCGR* (Figure 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 (Figure 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 (Figure 2C; $P<0.05$). The mRNA expression of *3β-HSD* was higher during spring than during autumn and early winter (Figure 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.

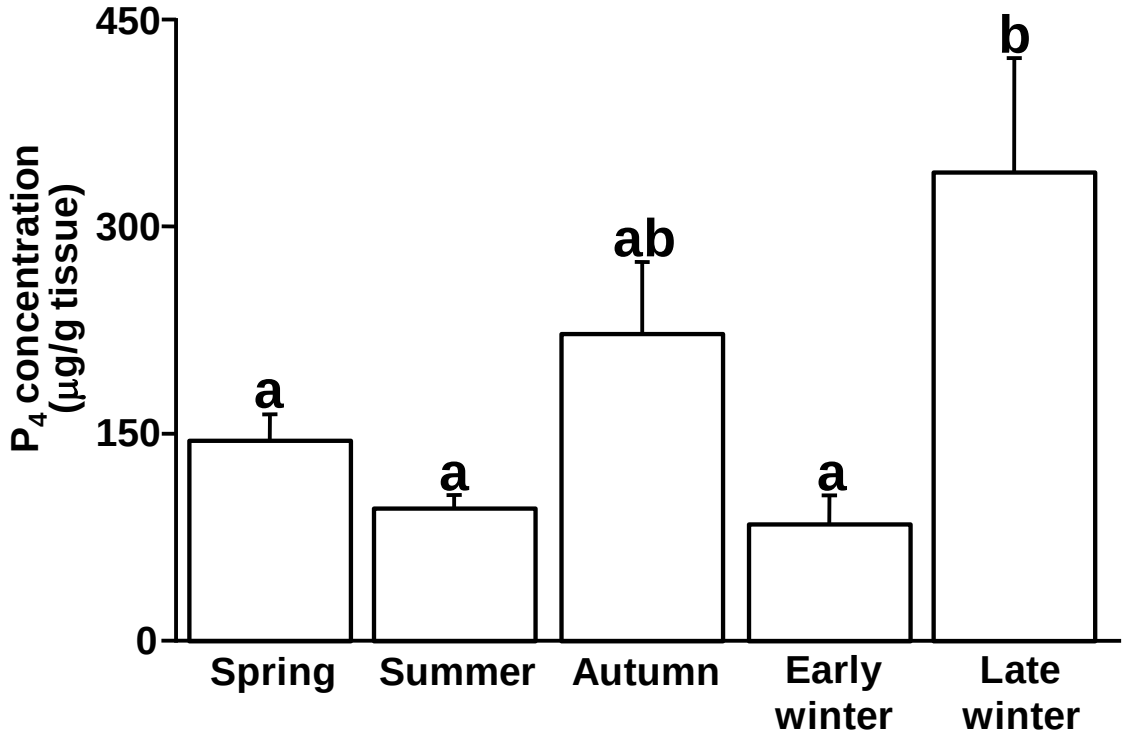

Figure 1. Effect of season on luteal P_4 concentration. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison test.

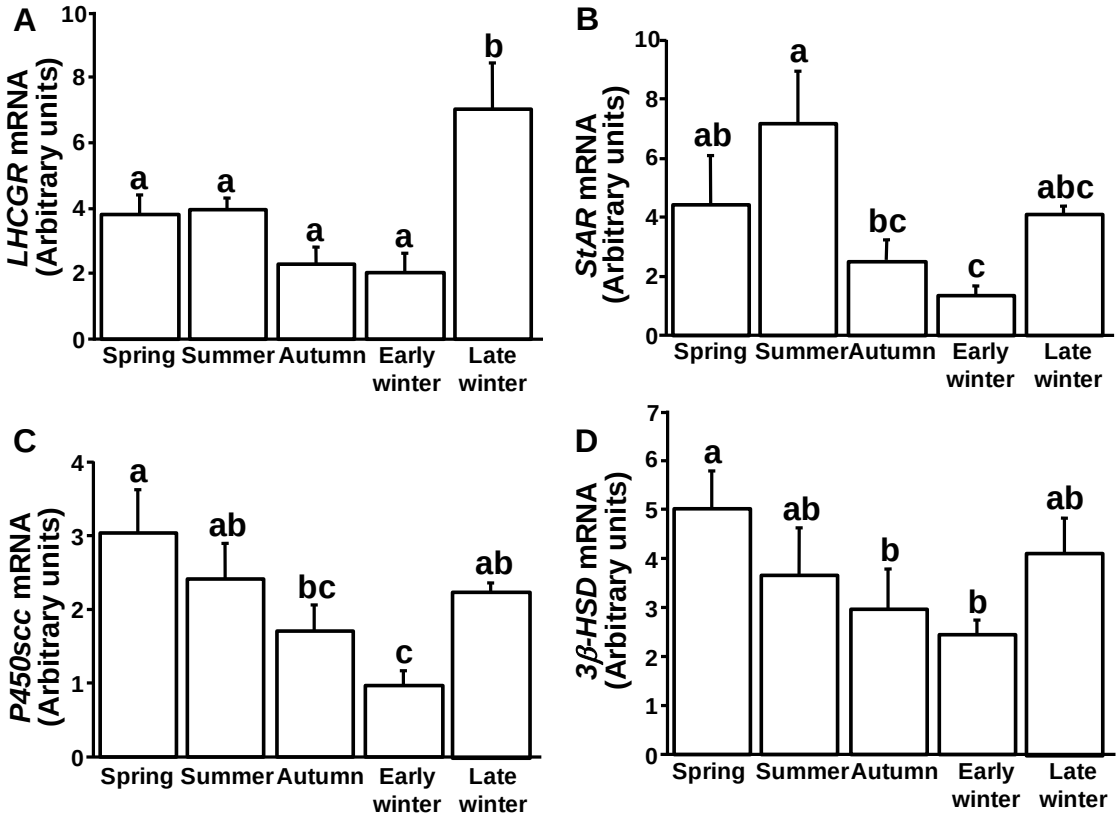

Figure 2. Effects of season on mRNA expressions of (A) *LHCGR*, (B) *StAR*, (C) *P450scc* and (D) *3β-HSD* in the mid CL. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison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

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 [12-16, 22, 23]. In mares, administration of GnRH, eLH or hCG increased circulating P_4 concentrations *in vivo* [24-27]. In addition, *in vitro* studies demonstrated that either eLH or hCG increased the P_4 production by equine luteal cells [16, 25]. Moreover, treatment of mares with an antiserum against equine gonadotropins during diestrous decreased the luteal size [28, 29], and treatment of mares with a GnRH antagonist during diestrous decreased the circulating P_4 concentrations [30, 31]. 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 [12, 22, 23]. In mares, circulating LH concentrations were high during summer and low during winter [32]. 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 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 [32] 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 F2α reduced circulating P_4 concentrations and the mRNA expression of *LHCGR* in the CL of ewes [33], cows [34] and mares [35]. Moreover, circulating and luteal P_4 concentrations were highly correlated with the number and affinity of LHCGR in the CL of mares [36]. 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 [32]. 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 [32]. 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 [32]. 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 [35, 37, 38]. 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.

Summary

Although circulating P_4 levels tend to change with the season, little is known about the seasonal changes of P_4 synthesis-related proteins in the 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 (～N32°), five times during one year. The CLs were classified as being in the early, mid and regressed luteal phases 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 *LHCGR* were lowest during early winter and highest during late winter. The mRNA expressions of *StAR*, *P450scc* and *3β-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.

CHAPTER 3

EXPRESSION OF ALDO-KETO REDUCTASE 1C23 IN THE EQUINE CORPUS LUTEUM IN DIFFERENT LUTEAL PHASES

Introduction

The corpus luteum (CL) is a mammalian endocrine organ that is formed in the ovary after ovulation. The CL produces progesterone (P_4), which is essential for the establishment and maintenance of pregnancy. In the absence of pregnancy, the CL regresses following the pulsatile release of uterine prostaglandin F2α (PGF). Regression of the CL is characterized by a decay in P_4 production (functional luteolysis) and the disappearance of luteal tissue (structural luteolysis) [39]. In mares, structural luteolysis has been suggested to be caused by apoptosis of luteal cells [40], while functional luteolysis is poorly understood.

P_4 is catabolized into its biologically inactive form, 20α-hydroxyprogesterone (20α-OHP), by 20α-hydroxysteroid dehydrogenase (20α-HSD). In rodents, ovarian 20α-HSD plays a role in functional luteolysis at term of pseudopregnancy as well as during the estrous cycle [41, 42]. Recently, it has also been demonstrated in the bovine CL that 20α-HSD is expressed throughout the estrous cycle and that 20α-HSD expression is remarkably higher in the late stage than in the other stages of the estrous cycle [43].

Aldo-keto reductases (AKRs) are multifunctional enzymes that act on a wide range of substrates including steroid hormones [44]. In mares, AKR1C23 has 20α-HSD activity and converts P_4 to 20α-OHP [45]. Although it is reported that AKR1C23 is expressed in the equine CL [45], the role of AKR1C23 in the equine CL remains unclear. In the present study, to clarify the possible mechanism of functional luteolysis in mares, we investigated the 20α-OHP concentrations and expression of AKR1C23 in the equine CL in the different luteal phases.

Materials and Methods

Collection of equine CLs

Fifty-eight 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 (～N32°) in Kumamoto, Japan, for at least two years before exsanguination,

were utilized in the present study. Mares were exposed to ambient light conditions. Fifty-eight ovaries containing a CL were collected from randomly designated cyclic nonpregnant mares at the abattoir in accordance with protocols approved by the local institutional animal care and use committee from April until the end of August. Mares possessing ovaries with a macroscopic abnormality including anovulatory hemorrhagic follicles were eliminated. After midsagittal sectioning, the CLs were classified as being in the early, mid, late and regressed luteal phases (n = 3-8/phase) by macroscopic observation of the CL and follicles (Table 1) as described previously [18, 19]. Following determination of the phases, CL tissues were immediately separated from ovaries, rinsed with cold sterile saline, frozen rapidly in liquid nitrogen and then stored at -80 C until being processed for P_4, 20α-OHP, RNA and protein extraction.

Real-time PCR

Gene expression was determined by real-time PCR using a MyiQ system (no. 170-9740, Bio-Rad Laboratories) and SYBR® *Premix Ex Taq*™ II (RR081B, TaKaRa, Shiga, Japan) starting with 2 ng of reverse-transcribed total RNA as described previously [20]. Briefly, for quantification of the mRNA expression levels, the primer length (20-25 bp) and GC contents of each primer (50-60%) were synthesized (Table 2) and were chosen using an online software package [21] as shown in Table 2, 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 (β2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin), and geNorm analysis was performed using the geNorm software (version 3.5). The M values of B2M, GAPDH and β-actin were 0.784, 0.505 and 0.501, respectively. Therefore, β-actin was identified as the most stable gene. The primers for 3β-HSD, AKR1C23 and β-actin generated specific 132-bp, 148-bp and 113-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 Taq*™ II at elevated temperatures resulted in reliable and sensitive quantification of the RT-PCR products with high linearity (Pearson correlation coefficient $r > 0.99$). To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of the target gene by the quantity of β-actin in each sample.

Western blot analysis

Each protein in the luteal tissues was assessed by Western blot analysis. The luteal tissues were homogenized on ice in homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete [protease inhibitor cocktail; no. 11 697 498 001; Roche Diagnostics, Mannheim, Germany], pH 7.4) by the tissue homogenizer, followed by filtration with a metal wire mesh (150 μm). For protein analysis, tissue homogenates were centrifuged at 600×g for 10 min at 4 C. The supernatants were then centrifuged at 9,000×g for 30 min at 4 C, and the resulting supernatants were used as cytoplasmic fraction. Protein concentrations were determined by the BCA method [46] using BSA as a standard.

The protein samples (50 μg protein) were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [no. 31607-94; Nacalai Tesque, Kyoto, Japan], 10% glycerol, 1% β-mercaptoethanol [no. 137-06862; Wako Pure Chemical Industries, Osaka, Japan], pH 6.8), heated at 95 C for 10 min and subjected to 15% SDS-PAGE for 1 h at 250 V. Separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (RPN303F; GE Healthcare, Milwaukee, WI, USA) for 40 min at 200 mA using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (no. 170-3940, Bio-Rad Laboratories). The membranes were incubated in blocking buffer (5% nonfat dry milk in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5] with 0.1% Tween 20 [TBST]) for 1 h at room temperature and then incubated separately with specific primary antibodies to 3β-HSD (40 kDa; 1:6,000; ab75710; Abcam plc., Cambridge, UK), AKR1C1 (37 kDa; 1:5,000; LS-B6269; LifeSpan BioSciences, Inc., Seattle, WA, USA) and β-actin (42 kDa; 1:4,000; A2228; Sigma-Aldrich, St. Louis, MO, USA) in TBST overnight at 4 C. After primary antibody incubation, the membranes were washed in TBST for 10 min 3 times at room temperature. After washing, the membranes were incubated with secondary antibody (anti-mouse, HRP-linked whole antibody produced in sheep [NA931; Amersham Biosciences, San Francisco, CA, USA]; 1:6,000 for 3β-HSD, 1:10,000 for AKR1C1, 1:40,000 for β-actin) in TBST for 1 h at room temperature, washed in TBST for 10 min 3 times at room temperature and then incubated with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500; Merck KGaA, Darmstadt, Germany). Images were captured using a ChemiDoc™ XRS+ System (#170-8265; Bio-Rad Laboratories). The intensity of the immunological reaction was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The results are expressed as mean ± SEM values obtained from 3-8 luteal

tissues/phase. The statistical significance of differences in luteal P_4 and 20α-OHP concentrations, the levels of *3β-HSD* and *AKR1C23* mRNA, and 3β-HSD and AKR1C23 protein levels was analyzed by a one-way analysis of variance (ANOVA). The comparisons between phases were performed using the Tukey-Kramer multiple comparison test. P values < 0.05 were considered significant.

Results

Luteal P_4 and 20α-OHP concentration in the different luteal phases

The luteal P_4 concentration was higher in the mid luteal phase than in the late and regressed luteal phases (Figure 3a: $P<0.05$). The luteal 20α-OHP concentration was higher in the late luteal phase than in any other luteal phases (Figure 3b: $P<0.05$).

Expressions of 3β-HSD mRNA and protein in the equine CL in the different luteal phases

Specific transcripts for 3β-HSD were detected in the equine CL throughout the luteal phase. The level of *3β-HSD* mRNA was higher in the early and mid luteal phases than in the late and regressed luteal phases (Figure 4a: $P<0.05$). 3β-HSD protein was expressed in the equine CL throughout the luteal phase. The level of 3β-HSD protein was higher in the late luteal phase than in the regressed luteal phase (Figure 4b: $P<0.05$).

Expressions of AKR1C23 mRNA and protein in the equine CL in the different luteal phases

Specific transcripts for AKR1C23 were detected in the equine CL throughout the luteal phase. The level of *AKR1C23* mRNA was higher in the late luteal phase than in the early and mid luteal phases (Figure 5a: $P<0.05$). AKR1C23 protein was expressed in the equine CL throughout the luteal phase. The level of AKR1C23 protein was higher in the late luteal phase than in the early luteal phase (Figure 5b: $P<0.05$).

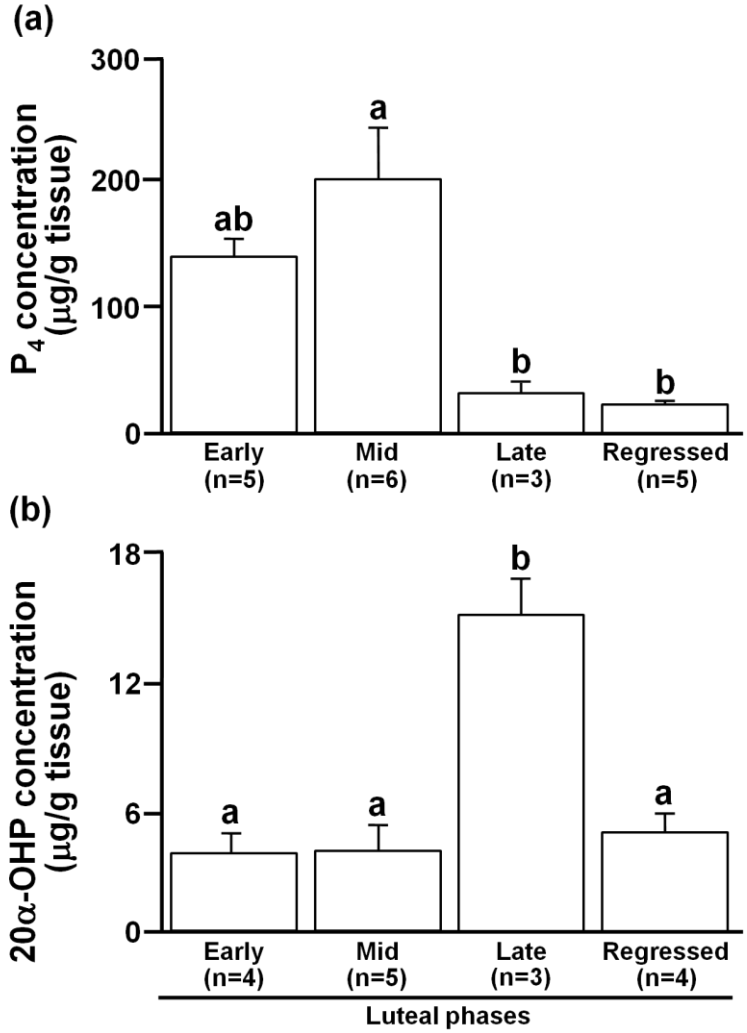

Figure 3. Luteal P_4 (a) and 20α-OHP (b) concentrations in the different luteal phases. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

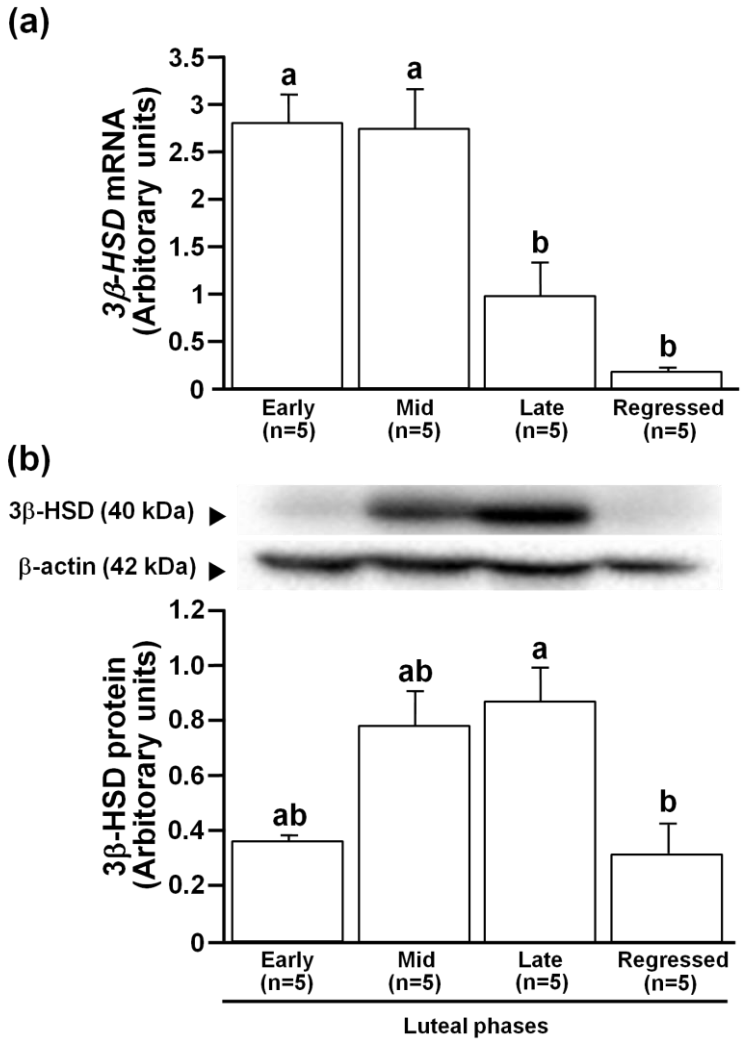

Figure 4. Expressions of 3β-HSD mRNA (a) and protein (b) in the equine CL in the different luteal phases. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

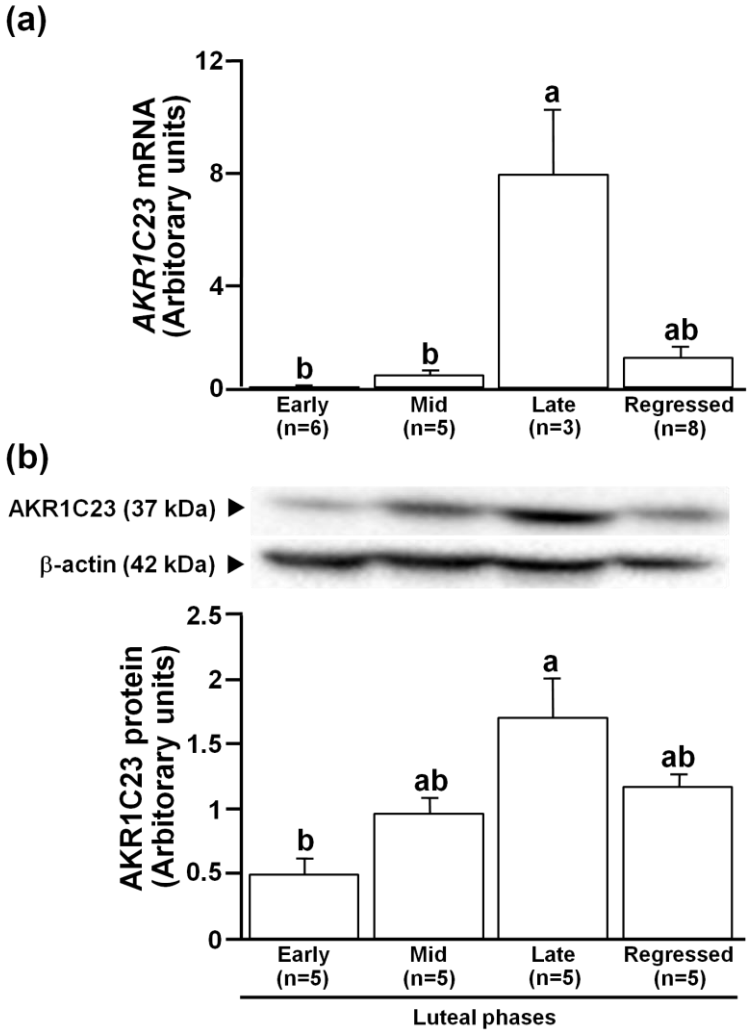

Figure 5. Expressions of AKR1C23 mRNA (a) and protein (b) in the equine CL in the different luteal phases. All experimental data are shown as means ± SEM. Different letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by the Tukey-Kramer multiple comparison test.

Discussion

In the present study, P_4 concentrations in the luteal tissues were high in the mid luteal phase and low in the late and regressed luteal phases. This result is consistent with previous reports that showed changes in circulating P_4 concentration throughout the luteal phase in mares [38, 47], indicating that functional luteolysis occurs in the late luteal phase. As in the case of the P_4 concentrations in the luteal tissues, the mRNA expression of *3β-HSD*, which converts pregnenolone into P_4, was higher in the early and mid luteal phases than in the late and regressed luteal phases. However, unexpectedly, the level of 3β-HSD protein was highest in the late luteal phase. In cattle [48, 49] and sheep [50, 51], the level of mRNA for *3β-HSD* decreased in parallel with circulating P_4 concentrations during PGF-induced luteolysis, but 3β-HSD protein expression or activity in the CL did not change. Based on the above findings, 3β-HSD activity in the CL may not play a role in functional luteolysis in mares.

The 20α-OHP concentrations in the luteal tissues were higher in the late luteal phase than in any other luteal phases. This finding strongly suggests that P_4 is converted into 20α-OHP in the late luteal tissues. In rodents, 20α-HSD expressed in luteal cells catabolizes P_4 into 20α-OHP at the termination of pregnancy to allow parturition to occur [52]. In mares, AKR1C23 has 20α-HSD activity; i.e., it converts P_4 into 20α-OHP [45]. In the present study, the mRNA and protein expressions of AKR1C23 were highest in the late luteal phase when the 20α-OHP concentration in the luteal tissues reached a peak. These results are consistent with previous observations in the bovine CL [43]. Together, our findings suggest that AKR1C23 converts P_4 into 20α-OHP in the late luteal phase, resulting in a decrease in the luteal P_4 concentration (Figure 3A). In mares, PGF secreted from the uterus in the late luteal phase induces luteolysis, i.e., a decrease in circulating P_4 concentration [26]. In addition, Fas ligand (FASL) expressed in the late CL reduces P_4 production and stimulates PGF production by equine luteal cells [16]. In rodents, PGF has been demonstrated to induce 20α-HSD expression in the CL, which is accompanied by a significant reduction in the circulating levels of P_4 and a rise in the levels of 20α-OHP [52]. Based on the above findings, AKR1C23 expression in the equine CL may be induced by PGF and FASL, leading to functional luteolysis. Further study is needed to clarify the mechanism of the regulation of AKR1C23 expression in the equine CL.

The overall findings in the present study strongly suggest that conversion of P_4 into 20α-OHP by AKR1C23 is one of the processes contributing to functional luteolysis in mares.

Summary

Regression of the corpus luteum (CL) is characterized by a decay in progesterone (P_4) production (functional luteolysis) and disappearance of luteal tissues (structural luteolysis). In mares, structural luteolysis is thought to be caused by apoptosis of luteal cells, but functional luteolysis is poorly understood. 20α-hydroxysteroid dehydrogenase (20α-HSD) catabolizes P_4 into its biologically inactive form, 20α-hydroxyprogesterone (20α-OHP). In mares, aldo-keto reductase (AKR) 1C23, which is a member of the AKR superfamily, has 20α-HSD activity. To clarify whether AKR1C23 is associated with functional luteolysis in mares, we investigated the expression of AKR1C23 in the CL in different luteal phases. The luteal P_4 concentration and levels of 3β-hydroxysteroid dehydrogenase (3β-HSD) mRNA were higher in the mid luteal phase than in the late and regressed luteal phases ($P<0.05$), but the level of 3β-HSD protein was higher in the late luteal phase than in the regressed luteal phase ($P<0.05$). The luteal 20α-OHP concentration and the level of *AKR1C23* mRNA were higher in the late luteal phase than in the early and mid luteal phases ($P<0.05$), and the level of AKR1C23 protein was also highest in the late luteal phase. Taken together, these findings suggest that metabolism of P_4 by AKR1C23 is one of the processes contributing to functional luteolysis in mares.

CONCLUSION

The present study investigated the seasonal changes in luteal function and the regulatory mechanisms of functional luteolysis in mares. The first series of experiments in chapter 2 showed 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 since both the luteal P_4 concentration and mRNA expressions of P_4 synthesis-related proteins in the CL change seasonally in mares. The second series of experiments in chapter 3 showed that conversion of P_4 into 20α-OHP by AKR1C23 is one of the processes contributing to functional luteolysis since luteal P_4 concentrations are low, and luteal 20α-OHP concentrations and AKR1C23 expressions are high in the late luteal phase in mares.

The overall findings suggest that the timing of collecting the CL should be taken into account depending on a purpose of research because luteal function changes seasonally, and that AKR1C23 plays a role in functional luteolysis in mares.

REFERENCES

1. **Wijayagunawardane MPB, Miyamoto A, Cerbito WA, Acosta TJ, Takagi M, Sato K**. Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* 1998; 49: 607-618.
2. **Okuda K, Uenoyama Y, Fujita Y, Iga K, Sakamoto K, Kimura T**. Functional oxytocin receptors in bovine granulosa cells. *Biol Reprod* 1997; 56: 625-31.
3. **Kooistra LH, Ginther OJ**. Effect of photoperiod on reproductive activity and hair in mares. *Am J Vet Res* 1975; 36:1413-1419.
4. **Sharp DC, Ginther OJ**. Stimulation of follicular activity and estrous behavior in anestrous mares with light and temperature. *J Anim Sci* 1975; 41:1368-1372.
5. **Sharp DC, Kooistra L, Ginther OJ**. Effects of artificial light on the oestrous cycle of the mare. *J Reprod Fertil Suppl* 1975:241-246.
6. **Ginther OJ**. Reproductive seasonality. *In*: Reproductive Biology of the Mare: Basic and Applied Aspects. 2nd ed. Wisconsin: Equiservices; 1992: 105-134.
7. **King SS, Neumann KR, Nequin LG, Weedman BJ**. Time of onset and ovarian state prior to entry into winter anestrus. *J Equine Vet Sci* 1993; 13:512-515.
8. **King SS, Douglas BL, Roser JF, Silvia WJ, Jones KL**. Differential luteolytic function between the physiological breeding season, autumn transition and persistent winter cyclicity in the mare. *Anim Reprod Sci* 2010; 117:232-240.
9. **Nagy P, Huszenicza G, Reiczigel J, Juhasz J, Kulcsar M, Abavary K, Guillaume D**. Factors affecting plasma progesterone concentration and the retrospective determination of time of ovulation in cyclic mares. *Theriogenology* 2004; 61:203-214.
10. **Okolski A, Bielanski AB**. Seasonal fluctuations of the plasma progesterone levels in mares with similar ovarian morphology. *Int Cong Anim Reprod Artif Insem*; 1980; Madrid; 4:13-15.
11. **Townson DH, Pierson RA, Ginther OJ**. Characterization of plasma progesterone concentrations for two distinct luteal morphologies in mares. *Theriogenology* 1989; 32:197-204.
12. **Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW**. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 2000; 80:1-29.
13. **Rodgers RJ, O'Shea JD, Findlay JK**. Progesterone production *in vitro* by small and large ovine luteal cells. *J Reprod Fertil* 1983; 69:113-124.
14. **Tekpetey FR, Armstrong DT**. Steroidogenic response of rat and pig luteal cells to

estradiol-17β and catecholestrogens in vitro. *Biol Reprod* 1991; 45:498-505.

15. **Nishimura R, Shibaya M, Skarzynski DJ, Okuda K**. Progesterone stimulation by LH involves the phospholipase-C pathway in bovine luteal cells. *J Reprod Dev* 2004; 50:257-261.
16. **Galvao AM, Ramilo DW, Skarzynski DJ, Lukasik K, Tramontano A, Mollo A, Mateus LM, Ferreira-Dias GM**. Is FAS/Fas ligand system involved in equine corpus luteum functional regression? *Biol Reprod* 2010; 83:901-908.
17. **Gentry LR, Thompson DL Jr., Gentry GT Jr., Davis KA, Godke RA, Cartmill JA**. The relationship between body condition, leptin, and reproductive and hormonal characteristics of mares during the seasonal anovulatory period. *J Anim Sci* 2002; 80: 2695-2703.
18. **Ginther OJ**. Characteristic of the ovulatory season. *In*: Reproductive Biology of the Mare: Basic and Applied Aspects. 2nd ed. Wisconsin: Equiservices; 1992: 173-232.
19. **Ferreira-Dias G, Bravo PP, Mateus L, Redmer DA, Medeiros JA**. Microvascularization and angiogenic activity of equine corpora lutea throughout the estrous cycle. *Domest Anim Endocrinol* 2006; 30 (4): 247-259.
20. **Sakumoto R, Komatsu T, Kasuya E, Saito T, Okuda K**. Expression of mRNAs for interleukin-4, interleukin-6 and their receptors in porcine corpus luteum during the estrous cycle. *Domest Anim Endocrinol* 2006; 31:246-257.
21. **Rozen S, Skaletsky H**. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; 132: 365-86.
22. **Juengel JL, Niswender GD**. Molecular regulation of luteal progesterone synthesis in domestic ruminants. *J Reprod Feritil* 1999; 54 (Suppl): 193-205.
23. **Niswender GD**. Molecular control of luteal secretion of progesterone. *Reproduction* 2002; 123: 333-339.
24. **Johnson AL, Becker SE, Roma ML**. Effects of gonadotrophin-releasing hormone and prostaglandin F-2α on corpus luteum function and timing of the subsequent ovulation in the mare. *J Reprod Fertil* 1988; 83: 545-551.
25. **Kelly CM, Hoyer PB, Wise ME**. In-vitro and in-vivo responsiveness of the corpus luteum of the mare to gonadotrophin stimulation. *J Reprod Fertil* 1988; 84: 593-600.
26. **Ginther OJ**. Endocrinology of the ovulatory season. *In*: Reproductive Biology of the Mare: Basic and Applied Aspects. 2nd ed. Wisconsin: Equiservices; 1992: 233-290.
27. **Watson ED, Colston M, Broadley C**. LH and progesterone concentrations during diestrus in the mare and the effect of hCG. *Theriogenology* 1995; 43: 1325-1337.

28. **Pineda MH, Ginther OJ, McShan WH**. Regression of corpus luteum in mares treated with an antiserum against an equine pituitary fraction. *Amer J Vet Res* 1972; 33: 1767-1773.
29. **Pineda MH, Garcia MC, Ginther OJ**. Effect of antiserum against an equine pituitary fraction on corpus luteum and follicles in mares during diestrus. *Amer J Vet Res* 1973; 34: 181-183.
30. **Watson ED, Pedersen HG, Thomson SRM, Fraser HM**. Control of follicular development and luteal function in the mare: effects of a GnRH antagonist. *Theriogenology* 2000; 54: 599-609.
31. **Evans MJ, Alexander SL, Irvine CH, Kitson NE, Taylor TB**. Administration of a gonadotropin-releasing hormone antagonist to mares at different times during the luteal phase of the estrous cycle. *Anim Reprod Sci* 2011; 127: 188-196.
32. **Turner DD, Garcia MC, Ginther OJ**. Follicular and gonadotropic changes throughout the year in pony mares. *Am J Vet Res* 1979; 40:1694-1700.
33. **Guy MK, Juengel JL, Tandeski TR, Niswender GD**. Steady-state concentrations of mRNA encoding the receptor for luteinizing hormone during the estrous cycle and following prostaglandin F(2alpha) treatment of ewes. *Endocrine* 1995; 3:585-589.
34. **Tsai SJ, Kot K, Ginther OJ, Wiltbank MC**. Temporal gene expression in bovine corpora lutea after treatment with PGF2alpha based on serial biopsies in vivo. *Reproduction* 2001; 121:905-913.
35. **Beg MA, Gastal EL, Gastal MO, Ji S, Wiltbank MC, Ginther OJ**. Changes in steady-state concentrations of messenger ribonucleic acids in luteal tissue during prostaglandin F2alpha induced luteolysis in mares. *Anim Reprod Sci* 2005; 90:273-285.
36. **Roser JF, Evans JW**. Luteal luteinizing hormone receptors during the postovulatory period in the mare. *Biol Reprod* 1983; 29:499-510.
37. **Watson ED, Bae SE, Al-Zi'abi MO, Hogg CO, Armstrong DG**. Expression of mRNA encoding insulin-like growth factor binding protein-2 (IGFBP-2) during induced and natural regression of equine corpora lutea. *Theriogenology* 2005; 64: 1371-1380.
38. **Slough TL, Rispoli LA, Carnevale EM, Niswender GD, Bruemmer JE**. Temporal gene expression in equine corpora lutea based on serial biopsies in vivo. *J Anim Sci* 2010; 89:389-396.
39. **McCracken JA, Custer EE, Lamsa JC**. Luteolysis: a neuroendocrine-mediated event. *Physiol Rev* 1999; 79: 263-323.

40. **Al-Zi'abi MO, Fraser HM, Watson ED**. Cell death during natural and induced luteal regression in mares. *Reproduction* 2002; 123: 67-77.
41. **Ishida M, Choi JH, Hirabayashi K, Matsuwaki T, Suzuki M, Yamanouchi K, Horai R, Sudo K, Iwakura Y, Nishihara M**. Reproductive phenotypes in mice with targeted disruption of the 20α-hydroxysteroid dehydrogenase gene. *J Reprod Dev* 2007; 53: 499-508.
42. **Matsuda J, Noda K, Shiota K, Takahashi M.** Participation of ovarian 20 alpha-hydroxysteroid dehydrogenase in luteotrophic and luteolytic processes during rat pseudopregnancy. *J Reprod Fertil* 1990; 88: 467-474.
43. **Naidansuren P, Park CW, Kim SH, Nanjidsuren T, Park JJ, Yun SJ, Sim BW, Hwang S, Kang MH, Ryu BY, Hwang SY, Yoon JT, Yamanouchi K, Min KS**. Molecular characterization of bovine placental and ovarian 20α-hydroxysteroid dehydrogenase. *Reproduction* 2011; 142: 723-31.
44. **Jez JM, Bennett MJ, Schlegel BP, Lewis M, Penning TM**. Comparative anatomy of the aldo-keto reductase superfamily. *Biochem J* 1997; 326: 625-36.
45. **Brown KA, Boerboom D, Bouchard N, Doré M, Lussier JG, Sirois J**. Human chorionic gonadotropin-dependent induction of an equine aldo-keto reductase (AKR1C23) with 20α-hydroxysteroid dehydrogenase activity during follicular luteinization in vivo. *J Mol Endocrinol* 2006; 36: 449-61.
46. **Osnes T, Sandstad O, Skar V, Osnes M, Kierulf P**. Total protein in common duct bile measured by acetonitrile precipitation and a micro bicinchoninic acid (BCA) method. *Scand J Clin Lab Invest* 1993; 53: 757-63.
47. **Ginther OJ, Gastal EL, Gastal MO, Beg MA**. Regulation of circulating gonadotropins by the negative effects of ovarian hormones in mares. *Biol Reprod* 2005; 73: 315-23.
48. **Tian XC, Berndtson AK, Fortune JE**. Changes in levels of messenger ribonucleic acid for cytochrome P450 side-chain cleavage and 3 beta-hydroxysteroid dehydrogenase during prostaglandin F2 alpha-induced luteolysis in cattle. *Biol Reprod* 1994; 50: 349-56.
49. **Rodgers RJ, Vella CA, Young FM, Tian XC, Fortune JE**. Concentrations of cytochrome P450 cholesterol side-chain cleavage enzyme and 3 beta-hydroxysteroid dehydrogenase during prostaglandin F2 alpha-induced luteal regression in cattle. *Reprod Fertil Dev* 1995; 7: 1213-6.
50. **Hawkins DE, Belfiore CJ, Kile JP, Niswender GD**. Regulation of messenger ribonucleic acid encoding 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase in the ovine corpus luteum. *Biol Reprod* 1993; 48: 1185-90.

51. **Juengel JL, Meberg BM, McIntush EW, Smith MF, Niswender GD**. Concentration of mRNA encoding 3 beta-hydroxysteroid dehydrogenase/delta 5, delta 4 isomerase (3 beta-HSD) and 3 beta-HSD enzyme activity following treatment of ewes with prostaglandin F2 alpha. *Endocrine* 1998; 8: 45-50.
52. **Stocco CO, Zhong L, Sugimoto Y, Ichikawa A, Lau LF, Gibori G**. Prostaglandin F2alpha-induced expression of 20alpha-hydroxysteroid dehydrogenase involves the transcription factor NUR77. *J Biol Chem* 2000; 275: 37202-11.