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Expression of angiogenic factors and luteinizing hormone receptors in the corpus luteum of mares induced to ovulate with deslorelin acetate

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ABSTRACT

The effects of deslorelin acetate use in inducing ovulation need to be clarified to improve the results of equine embryo transfer. The mRNA abundance for angiogenic factors and LH receptor (LHR) in corpus luteum (CL) was studied in mares with natural (control group [CG]) and induced ovulation with deslorelin acetate (treatment group [TG]; follicles: ≥35 mm). Transrectal ultrasonography was used to verify the ovulation day, and on Days 4, 8, and 12 after ovulation (Day 0), CL samples were obtained through ultrasound-guided biopsy. The messenger RNA expression of vascular endothelial growth factor (*VEGF*), basic fibroblast growth factor (*bFGF*), and *LHR* genes were analyzed by real-time polymerase chain reaction. A positive correlation was observed between VEGF and LHR ($P < 0.00001$, $r = 0.78$), and it was possible to detect higher LHR expression in the TG than in the CG on Day 4 ($P < 0.05$). Moreover, this expression was higher on Days 4 and 8 than on Day 12 in the TG. Basic fibroblast growth factor was also expressed in luteal tissue on all days for both groups; however, these differences were not significant. In conclusion, deslorelin acetate was effective for the induction of ovulation in mares, resulting in higher expression of LHR, especially on the fourth day after ovulation. In addition, VEGF expression was influenced by induced ovulation, with a lower level on Day 12, which is expected in nonpregnant mares.

1. Introduction

Assisted biotechnology in animal reproduction, especially embryo transfer (ET), has been used frequently worldwide. In 2013, Brazil reported the collection and transfer of 19,680 equine embryos, followed by Argentina; these are the only countries that currently report significant equine ET activities [1].

To improve results in terms of successful pregnancies, studies on embryo recipient mares are very important, including CL quality, vascularization, and progesterone production. Recipient quality and selection are the major factors that can be successfully manipulated to increase pregnancy rates after ET [2].

The GnRH agonist deslorelin acetate has been useful to hasten and predict ovulation in mares within 2 days of treatment without suppressing subsequent follicular development [3]. The ovulation is due to LH secretion from the adenohypophysis, induced by the GnRH agonist, which works in the ovary through specific receptors to promote follicular maturation and ovulation [4]. Although this

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ovulation inducer has been used in cyclic [5] and transitional mares [6], few studies have focused on the influence of this treatment on subsequent CL formation and function.

Luteinizing hormone is the main gonadotropin enrolled in the CL formation [7], and the number of LH receptors (LHRs) in the CL is correlated with progesterone production [8]. However, an appropriate supply of blood is necessary for the CL to function, with angiogenesis being a crucial process at the time of CL formation [9]. The new vascularization depends on endothelial cell proliferation and migration from the remaining vessels in the ruptured follicle [10]. The CL vascularization is associated with the increase in blood flow that occurs as the CL grows and as the progesterone secretion increases [11]. The vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are the main proangiogenic cytokines acting during CL formation [12,13].

The following investigation was designed to characterize messenger RNA (mRNA) abundance for VEGF, bFGF, and LHR in the CL of mares after ovulation that has been induced with deslorelin acetate.

2. Materials and methods

2.1. Experimental design

Cyclic mares (n = 5), aged between 3 and 14 years and weighing 380 to 450 kg, were used in this study. The experiment was conducted during the months of January through April, in the Southern Hemisphere (08°01′ S, 34°58′ W). All mares were healthy, and the animals were handled in an ethically appropriate manner. The research was approved by the Ethical Committee of the Federal Rural University of Pernambuco (license no. 063/2014/CEUA).

The mares were tested daily for signs of estrus using a stallion. At the first sign of estrus, the mares were scanned daily by transrectal ultrasound until the dominant follicle reached 35 mm. Then, the mares were treated (treatment group [TG]) with deslorelin acetate (750 μg, intramuscular) or maintained without treatment (control group [CG]). In both groups, the follicles were scanned daily to determine the day of ovulation. All mares were used twice, once in each group, using two consecutive cycles.

2.2. Ultrasound-guided biopsy

The biopsies of luteal tissue were performed on Days 4, 8, and 12 after ovulation (Day 0). For each biopsy procedure, the mare was restrained in a palpation chute, and sedation and analgesia were induced by detomidine hydrochloride (0.02 mg/mL, 1 mL/500 kg).

The biopsy device (US Biopsy, Franklin, IN, USA) was a 48-cm long, automated, spring-loaded instrument with an inner trocar point plunger containing a 15 × 1.6-mm specimen notch surrounded by an outer 16-ga cutting needle [14]. This device was introduced through a needle guide mounted on a probe handle with an 8 MHz, transvaginal, ultrasound-guided, convex-array transducer (M5-VET; Mindray, China), which was used for placement of the biopsy needle within the ovary. When the ovary with the CL was positioned against the vaginal wall and transducer face, the needle was inserted into the CL, the trocar was advanced so that the specimen notch was within the CL, and the device was fired and withdrawn [15]. Immediately after the fragment was collected, the debris and ovarian stroma were removed, and the CL tissue was placed in a cryotube and plunged into liquid nitrogen for storage at −80 °C until analysis.

2.3. Gene expression

Total mRNA was extracted from each luteal sample using TRI Reagent (Sigma, St. Louis, MO, USA), following the manufacturer's directions. The RNA concentration and purity were verified by spectrophotometer. The RNA was converted into complementary DNA (cDNA) using reverse transcription (ImProm-II Reverse Transcription System; Promega, Madison, WI, USA). The reaction was performed with 1000 ng of extracted RNA and 1 μL of Oligo (dT)15 and 1 μL of random primer, incubated at 70 °C for 5 minutes and 4 °C for 5 minutes. After the addition of 4 μL of ImProm-II (5X) buffer, 2.4 μL of $MgCl_2$ (1.5 mM), 1 μL of dNTP (0.5 mM), and 1 μL of reverse transcriptase, ultrapure water was used to bring the total volume to 20 μL. The reaction was kept at 25 °C for 5 minutes, 42 °C for 60 minutes, and at 70 °C to inactivate the enzyme for 15 minutes. The resulting cDNA was measured in a spectrophotometer. Samples were standardized to a concentration of 100 ng/μL and stored at −80 °C.

The abundance of mRNA for *VEGF*, *bFGF*, and LHR genes was evaluated by amplification of gene fragments using specific primers (Table 1) in a real-time polymerase chain reaction (quantitative PCR [qPCR]). The *GAPDH* gene was also measured for use as an internal control and to normalize the threshold cycle of each sample.

The qPCR reactions were performed using the QuantiFast SYBR Green PCR (Qiagen, Dusseldorf, Germany) kit in a thermocycler (Rotor-Gene Q; Qiagen). All samples were in duplicates, and a negative control was included. The following was added to each tube: 2.0 μL of cDNA (100 ng/μL), 7.5 μL of SYBR Green (2x), 0.5 μL of forward and reverse primers (10 pM), and 5.0 μL of H_2O, in a 15 μL final volume. The amplification conditions were 5 minutes at 95 °C, 45 cycles of 15 seconds at 95 °C (denaturation), and 30 seconds at 60 °C (annealing and extension). To confirm the presence of a single PCR product, melting curve analysis was performed with each sample being allowed to continue to run from 60°C to 95 °C, increasing 0.5 °C each 5 seconds. The normalized threshold (ΔCt) for each sample was used to calculate the gene abundance using the expression $2^{-\Delta\Delta Ct}$ [16].

Table 1
Primer sequences used with equine luteal complementary DNA samples for real-time polymerase chain reaction analysis.

Gene	Sequence	GeneBank
VEGF	F: TTGCCTTGCTGCTCTACCTC R: GTCCACCAGGGTCTCGATTG	NM_001081821.1
bFGF	F: CAAAAACGGGGGCTTCTTCC R: TAACGGTTCGCACACACTCC	NM_001195221.1
LHR	F: TTGCCACATCATCCTATTCTC R: TTCTTTTGTTGGCAAGTTTCT	AY271258
GAPDH	F: ACCACTTTGGCATCGTGGAG R: GGGCCATCCACGGTCTTCTG	AF157626

Abbreviations: F, forward; R, reverse.