Article

Accuracy of a rapid enzyme-linked immunosorbent assay to measure progesterone in mares

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Abstract — The aim of this study was to validate an enzyme-linked immunosorbent assay (ELISA) for the measurement of progesterone (P4) in mares. Specifically, the objectives were as follows: 1) to determine the specificity and sensitivity of the ELISA test for determination of P4, 2) to measure the potential agreement between the 2 people performing the test, and 3) to evaluate the effect of time on the outcome. Ten mares were sampled on the day before ovulation (D-1), and on days 1 (D1), 3 (D3), and 5 (D5) following ovulation, during the reproductive season. While mares were cycling regularly, estrus was induced by the injection of 5 mg of prostaglandin (PGF2) and monitored starting on the 4th day by daily transrectal palpation and ultrasonography to determine the time of ovulation. Blood was collected and all samples (n = 96) were assayed for P4 by a semiquantitative ELISA, by chemiluminescent immunoassay, and by radioimmunoassay (RIA). Based on the RIA, values of P4 on D-1, D1, D3, and D5 were significantly different ($P < 0.0001$) with mean and standard deviation(s) of 0.004, s = 0.52; 2.05, s = 2.58; 8.37, s = 4.17; and 12.76, s = 4.00 ng/mL respectively. The sensitivity and specificity of the semiquantitative assay were 94% and 95%, respectively for the lowest values of P4 (< 1.0 ng/mL). The value of kappa was 0.90 between 2 individuals performing the test. In conclusion, these results suggest that the semiquantitative test may be used reliably and economically to evaluate P4 levels in equine plasma in the clinical situation.

Résumé — Exactitude d'un titrage immunoenzymentique rapide utilisant un antigène adsorbé pour mesurer la progestérone chez les juments. Le but de cette étude était de valider un titrage immunoenzymentique utilisant un antigène adsorbé (ELISA) pour mesurer la progestérone (P4) chez des juments. Plus spécifiquement, les objectifs étaient les suivants : 1) préciser la spécificité et la sensibilité du test ELISA pour déterminer la P4, 2) mesurer la concordance potentielle entre les 2 personnes qui effectuent le test et 3) évaluer l'effet du temps sur les résultats. Au cours de la saison de reproduction, dix juments ont été échantillonnées le jour précédent l'ovulation (J-1) et aux jours 1 (J 1), 3 (J 3) et 5 (J 5) suivant l'ovulation. Alors que le cycles ovariens se déroulait normalement, l'œstrus était provoqué par l'injection de 5 mg de prostaglandine (PGF2) et suivi à partir du 4^{ième} jour par palpations transrectales et échographies quotidiennes pour déterminer le moment de l'ovulation. La P4 de tous les échantillons de sang récoltés (n = 96) a été déterminée par ELISA semi-quantitatif, par essai immunologique chimioluminescent et par essai radioimmunologique (ERI). Selon les résultats obtenus par ERI, les valeurs de P4 aux J-1, J 1, J 3 et J 5 étaient significativement différentes ($P < 0{,}0001$) avec moyennes et écarts-types respectifs de 0,004, s = 0,52; 2,05, s = 2,58; 8,37, s = 4,17 et 12,76, s = 4,00 ng/ml. La sensibilité et la spécificité des dosages semi-quantitatifs étaient respectivement de 94 % et 95 % pour les valeurs les plus basses de P4 (< 1,0 ng/ml). La valeur de kappa était de 0,90 entre les 2 individus réalisant le test. En conclusion, ces résultats suggèrent que le test semi-quantitatif peut être utilisé de façon fiable et économique pour évaluer les niveaux de P4 dans le plasma équin en situation clinique.

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Introduction

The average length of the estrous cycle in mares during the breeding season is 21 to 22 d. The estrous cycle of the mare consists of a 4- to 7-day period of estrus (sexual receptivity), with ovulation occurring 24 to 48 h before the end of estrus, followed by 14 to 15 d of diestrus (not receptive to the stallion). Plasma levels of progesterone (P4) increase significantly within the first 12 h of ovulation (1) and reach > 2.0 ng/mL 48 h after ovulation (2,3). Contrary to P4 concentration measured in plasma, the intrafollicular concentration of P4 increases in the dominant follicle about 2 d before ovulation (4). Increased secretion of P4 into plasma occurs earlier in mares than in any other species and continues to rise until approximately day 6 of the estrous cycle, when it reaches a plateau (> 10 ng/mL) (5). Plasma concentrations then decrease rapidly, beginning approximately 14 d after the end of estrus.

In addition to the effect of day of the cycle, there are differences in circulating P4 concentrations during the estrous cycle that can be explained by a mare effect (6), circadian and seasonal changes (7), and the number of ovulations (8). Differences in P4 levels among mares are significant, irrespective of the number of cycles (5). The effect of circadian rhythms on P4 secretion has been studied and the results showed that P4 concentrations tended to be higher for samples taken in the afternoon than for those taken in the morning (9). This rhythmic variation of P4 concentrations in peripheral plasma could be explained by P4's pulsatile release (9). Mares with double ovulations have a higher plasma P4 concentration in diestrus than those with a single ovulation, but they do not differ significantly (5,10). Following synchronous multiple ovulations, plasma P4 increases more rapidly during the first 5 d after ovulation than following asynchronous multiple ovulations (5). An influence of season on circulating P4 concentration has been reported (2,5). The morphology of the corpus luteum (CL) (fluid-filled CL) does not affect the peripheral P4 concentrations (2).

Clinically, estimation of plasma P4 levels in mares is relevant, and the most reliable measure is obtained by radioimmunoassay (RIA) (8,11,12). Radioimmunoassay is very sensitive and specific, but the technique is time consuming and involves the use of radioactive material. Quantitative enzyme-linked immunosorbent assays (ELISAs) have been developed to measure P4 in different species and their correlation with RIA is high (13). Use of a semiqualitative ELISA for rapid assessment of P4 would be advantageous in equine reproductive management. The assay involves enzyme-induced color changes that do not require the use of radioactive materials and elaborate laboratory equipment. Rapid determination of the presence or absence of a functional CL in different clinical situations, such as mares with irregular estrous cycle in transitional season, silent estrus, an inconclusive finding after genital examination for confirmation of ovulation, high risk pregnancy, and embryo transfer, would help to maximize reproductive efficiency. The test could be an attractive alternative to traditional ultrasonography or transrectal palpation, especially in intractable or small-sized mares like American miniature horses.

The objective of the current study was to determine the specificity, sensitivity, and positive and negative predictive values of a commercial semiquantitative ELISA kit for P4 determination and the presence of a functional CL in mares. The RIAs were used as the gold standard (GSTD).

Materials and methods

Animal

Ten standardbred mares from the University herd, in good condition (550 kg of body weight) and with an average age of 9 y, were monitored daily from May 1 to October 1, 2004. Mares were maintained on 16 h of artificial light between December 2003 and April 2004. Estrus was induced by the injection of 5 mg of prostaglandin (PG) (Lutalyse; UpJohn, Orangeville, Ontario) and the animals were monitored, starting on day 4 post-PG injection, by daily transrectal palpation and ultrasonography to determine the day of ovulation. The number and size of follicles and the size and quality of the CL were recorded. The mares were sampled on the day before ovulation (D-1), and on days 1 (D1), 3 (D3), and 5 (D5) following ovulation during the reproductive season (May 1 to August 2004). Blood was collected into heparinized tubes following jugular venipuncture and centrifuged; the plasma was then stored at −20°C until being assayed. In all samples, P4 levels were determined by 3 techniques: RIA, ELISA, and chemiluminescent assay (Immulite; Diagnostic Products Corporation (DPC), Los Angeles, California, USA). Ninety-six blood samples were analyzed in a double blind experiment.

Progesterone determination

Radioimmunoassay

First, P4 concentrations were determined by a nonextraction RIA, using I^{125}–P4 (Amersham Biosciences, Oakville, Ontario) as tracer and danazol (Sanofi-Withrop, Markham, Ontario) as the steroid displacer (14). The sensitivity of the assay was 0.2 ng/mL. All samples were run in a single assay with an intra-assay coefficient of variation of 9%.

Chemiluminescent immunoassay

The P4 values of the 96 samples were determined by using the sequential solid-phase competitive chemiluminescent-labeled immunoassay with an analytical sensitivity of 0.2 ng/mL. The assay was carried out using a commercially available kit with polyclonal rabbit anti-progestereone (Diagnostic Products Corporation) using the automated chemiluminescent immunoassay system (ImmuliteTM 2000, Diagnostic Products Corporation) according to the manufacturer's protocol. Rabbit anti-progesterone antibodies were captured by mouse anti-rabbit IgG coupled to paramagnetic particles. There is an inverse relationship between the amount of progesterone in the mare sample and the amount of relative light units detected.

Enzyme-linked immunosorbent assay

The semiquantitative ELISA measures the P4 concentration in a drop of plasma or serum. The quantity of P4 present is indicated by a change in color, which is compared with progesterone standards. The kit includes 32 antibody-coated microwells, 3 ready-to-use standards [low (1 ng/mL), intermediate (5 ng/mL), and high (10 ng/mL)], a ready-to-use conjugate, a substrate, and a

Table 1. Sensitivity, specificity, positive predictive value (PPV), and negative predictive values (NPV) of the semiquantitative enzyme-linked immunosorbent assay (ELISA) for the low progesterone P4 values (< 1.0 ng/mL and ≥ 1.0 ng/mL)

		RIA[a]		
		Negative	Positive	Total
ELISA	Negative	59	2	61
	Positive	3	32	34
	Total	62	34	95

specificity : 59/62 = 95.2%
sensitivity : 32/34 = 94.1%
PPV : 32/35 = 91.4%
NPV : 59/61 = 96.7%

[a] Radioimmunoassay

buffer solution. First, samples and standards are placed in the wells. Then, a P4, conjugated to alkaline phosphatise, is added to each well. The wells are incubated at room temperature for a predetermined period of time while being covered to avoid negative effects from the light. After this incubation period, the wells are washed and the drops of a substrate-chromogen are added to the wells. The intensity of the resulting color will be inversely proportional to the P4 concentration of the samples. The tests were set up and performed on the same day by 2 observers. Sample identity was unknown to all observers at the time of testing.

Statistical analysis

In order to determine the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the semiquantitative ELISA, the categorical scale (< 1 ng/mL; ≥ 1 ng/mL to < 10.0 ng/mL; ≥ 10.0 ng/mL) was converted to a binary scale. Contingency tables were constructed for each person to calculate the semiquantitative ELISA sensitivity, specificity, PPV, NPV, and accuracy, based on the status of each animal at the time the blood sample was collected, and the RIA results. Agreement among personnel was evaluated by using kappa values.

Results

The semiquantitative ELISA had PPV and NPV values of 91% and 97%, respectively, for the 1st range (< 1.0 ng/mL and ≥ 1.0 ng/mL) category, based on 96 samples (Table 1). The sensitivity of the ELISA, a measure of the ability of the assay to correctly detect a P4 level lower than 1.0 ng/mL was 94%, leaving only a 6% chance that a mare would be erroneously classed as having low P4 levels. The specificity of the test, a measure of the ability of the test to correctly detect a P4 level higher than 1.0 ng/mL, was 95%. For the 2nd range (< 1.0 ng/mL or ≥ 10.0 ng/mL, ≥ 1.0 ng/mL and < 10.0 ng/mL) and the 3rd range (≥ 10.0 ng/mL and < 10.0 ng/mL) categories, the sensitivity, specificity, PPV, and NPV were 90%, 85%, 73%, 95%, and 75%, 100%, 100%, 89%, respectively. The analysis was also done with 4 categories (< 1 ng/mL; ≥ 1 ng/mL and < 5 ng/mL; ≥ 5 ng/mL and < 10.0 ng/mL; ≥ 10.0 ng/mL), where there was a reduction in the sensitivity and specificity, with most errors occurring in intermediate P4 values.

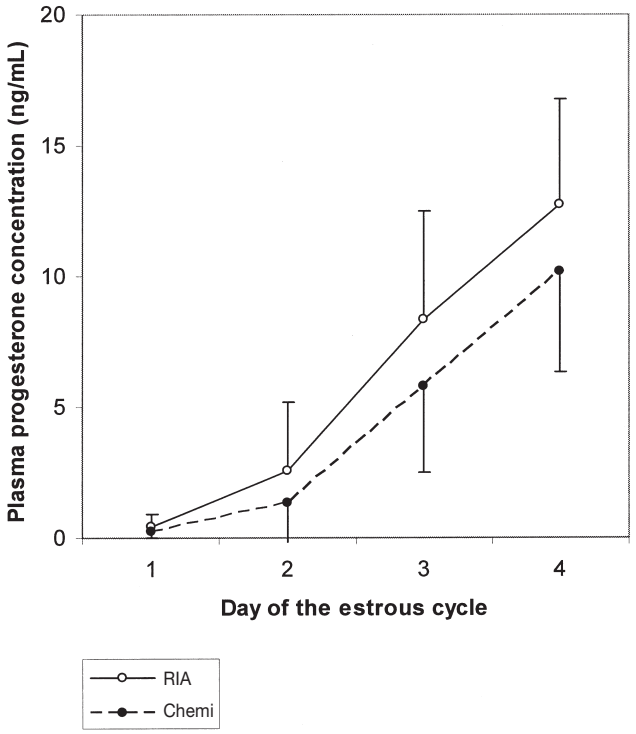

Figure 1. Variation in progesterone (P4) concentrations between radioimmunoassay and chemiluminescent immunoassay during the first 4 days of the estrous cycle in the mare.

The semiquantitative ELISA had an overall accuracy of 87.5% and 83.3% for the 2 observers, respectively. Reduced accuracy was observed when the ELISA was compared with the chemiluminescent immunoassay, resulting in 69% accuracy for both observers. The chemiluminescent immunoassay and the RIA showed similar profiles with a strong statistical correlation ($P < 0.0001$), especially for the low concentrations of P4. However, P4 levels obtained by the chemiluminescent immunoassay were systematically lower (Figure 1), with a greater variation in the upper P4 values ($P < 0.0005$). In addition, the kappa statistic test was used to measure agreement between the 2 observers and to express the proportion of potential agreement beyond chance alone. Overall agreement between the 2 observers resulted in a kappa of 0.90 based on the 96 samples.

Based on the RIA (gold standard), there was an effect ($P < 0.001$) of day of the cycle on plasma P4 concentrations. Mean plasma concentration on (D-1) was 0.40, $s = 0.52$ ng/mL, with the majority of the results below the sensitivity of the test (0.2 ng/mL). The P4 concentration increased rapidly to 2.05, $s = 2.58$; 8.37, $s = 4.17$; and 12.76, $s = 4.00$ ng/mL by D1, D3, and D5, respectively, after the ovulation (Figure 1). There was no statistical significant effect of month ($P > 0.05$) or day by month interaction ($P > 0.05$) for P4 concentration in the population. However, there was a tendency toward a slight decrease in circulating P4 concentrations between the beginning and the end of the reproductive season.

Discussion

The sensitivity (94%) and specificity (95%) of the 1st range category of the semiquantitative ELISA were similar to those

reported previously (15); however, the cutoff point of 2.0 ng/mL was arbitrarily determined after analysis of the results. For the 2nd and 3rd range categories, the results were similar, except for the PPV of the 2nd range and the sensitivity of the 3rd range, which decreased to 73% and 75%, respectively. As indicated by the present data, most errors were made with intermediate P4 concentrations, which is consistent with published reports of the descriptive analysis for a similar commercially available test (15). With a PPV of 73% in the middle range values, 73% of the samples with a middle range value of P4 were diagnosed correctly. The same observation was made when the 4 category analysis was performed The fact that P4 measurements obtained by the chemiluminescent immunoassay were systematically lower and with a greater variation of the upper values of P4 could explain the loss of accuracy of the ELISA when compared with the chemiluminescent immunoassay. The systematic lower levels of P4 measured by the chemiluminescent immunoassay contrast with results obtained with the quantitative ELISA, where values were usually greater than those with RIA (13,16). The better correlation between ELISA and RIA increases the confidence in the present results.

The mean plasma concentrations on the different days of sampling were similar to those previously reported (2,5,15) with a rapid increase between D0 and D5. The rapid increase in P4 concentration after ovulation was in contrast with the cow, where the CL secretes P4 over 1.0 ng/mL only by day 4 or 5 after ovulation, with a maximum concentration around day 10 of the cycle (17). Mares with multiple ovulations had similar levels of P4 to those presenting a single ovulation ($P > 0.05$). Nagy et al (5) did not report an overall significant effect of the morphology of the CL (with or without a fluid center) or multiple ovulations on plasma P4 concentrations during the estrous cycle of mares. However, the mean plasma P4 values for synchronous and asynchronous multiple ovulations were significantly different during the first 5 d of the cycle. For mares with asynchronous multiple ovulations, P4 levels increase more rapidly than for those with synchronous multiple ovulations, between D2 and D5. The stage of the reproductive season may affect the P4 secretion by the CL. The present authors observed a tendency toward a decrease of circulating P4 level between the beginning and the end of the reproductive season. This phenomenon was also observed by others (2,5); a significant influence of season occurred, indicated by a progressive decline of P4 concentrations in diestrus between the beginning and the end of the reproductive season. The small number of mares in the present study could explain the nonsignificance of our results.

Equine reproductive management relies on the ability of veterinary clinicians to accurately predict the stage of the estrous cycle of the mare, based on transrectal palpation, ultrasonography, and vaginoscopy, but difficulties may arise in interpreting the results of these diagnostic procedures. Determination of P4 levels by a rapid immunoassay diagnostic test could contribute to solving this reproductive problem in mares. In the present study, the ELISA proved to be a reliable and practical test to evaluate P4 levels in equine plasma in clinical situations. The accuracy of this assay would be beneficial for breeding management decision-making, particularly for irregularly cycling mares, mares with inconclusive findings after genital examination, and in intractable mares. However, the assay could be used as an additional diagnostic tool to increase or maximize reproductive efficiency in breeding mares. CVJ

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