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PATHFAST, a novel chemiluminescent enzyme immunoassay for measuring estradiol in equine whole blood and serum

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Abstract. A novel chemiluminescent enzyme immunoassay system, PATHFAST, for the measurement of estradiol in horses was evaluated. The concentrations of estradiol in the whole blood and serum of mares were measured using PATHFAST and the estradiol concentrations measured by PATHFAST were compared with those measured by a time-resolved fluoro-immunoassay (FIA). To monitor physiological changes, serum estradiol concentrations in mares were measured using PATHFAST throughout the gestation period. The serum estradiol concentrations correlated highly with those in whole blood samples. The serum concentrations of estradiol measured by PATHFAST also correlated well with FIA. Circulating estradiol increased during mid-gestation and high levels of serum estradiol were maintained in late gestation, followed by an abrupt decline to term. These results demonstrate the utility of PATHFAST in equine clinics as an accurate diagnostic tool for the rapid assay of estradiol within 26 min using unextracted whole blood.
Key words: Chemiluminescent enzyme immunoassay, Estradiol, Horses, PATHFAST, Time-resolved fluoro-immunoassay
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Estradiol-17β is secreted by the granulosa cells of the mature antral follicle in mares [1–6]. It induces estrus and prepares the uterus for fertilization and implantation by promoting endometrial growth, development of the uterine gland, and secretion of uterine and vaginal mucus [6]. The physiological function of estradiol is to induce embryo implantation in the uterus and maintain uterine conditions for the growing fetus during pregnancy [7–12]. Therefore, measurement of estradiol is useful for detecting follicular and placental function in cyclic and pregnant mares. Circulating estradiol has routinely been measured by radioimmunoassay (RIA), enzyme immunoassay (EIA) or time-resolved fluoroimmunoassay (FIA) using extracted plasma or serum samples in equine clinics. Although these assays have sufficient sensitivity, accuracy, and simplicity, a period of one or two days is required to get their results. Therefore, there is a need for a rapid measurement technique that can be perfomed at equine clinics for early diagnosis.

The chemiluminescent enzyme immunoassay system (PATHFAST) was developed as a small, automated, bench top type analyzer that uses a chemiluminescent enzyme immunoassay that can measure protein and steroid hormones from whole blood without extraction within 26 min for use in human reproductive medicine [13–16]. This new assay method uses an alkaline phosphatase-conjugated antigen and the Magtration technology as the tracer and the separation method for bound and free hormones.

The purpose of this study was to evaluate the validity of PATHFAST, as an accurate diagnostic tool for a rapid measurement of circulating estradiol in mares using whole blood and serum samples without extraction in equine clinic.

The correlation of estradiol concentrations in whole blood and serum samples from pregnant mares as measured by PATHFAST is shown in Fig. 1. There was a significant positive correlation between the concentrations in whole blood and serum samples ($r = 0.9341$, $P < 0.0001$, $n = 50$).

The pattern of circulating estradiol in the serum of two pregnant mares as measured by PATHFAST and FIA is shown in Fig. 2 as a sequential pattern throughout gestation. In panel A (Fig. 2), serum concentrations of estradiol measured by PATHFAST and FIA began to increase from four months after copulation, peaked at eight months after copulation, and abruptly declined to the term. In panel B (Fig. 2), the serum estradiol concentrations measured by PATHFAST and FIA also began to increase from five months after copulation, peaked at eight and nine months after copulation followed by an abrupt decline to the term. In panel A, the values measured by PATHFAST tended to be high compared with those measured by FIA during the increasing phase between four and eight months after copulation. This was also true of panel B. The concentrations of estradiol in

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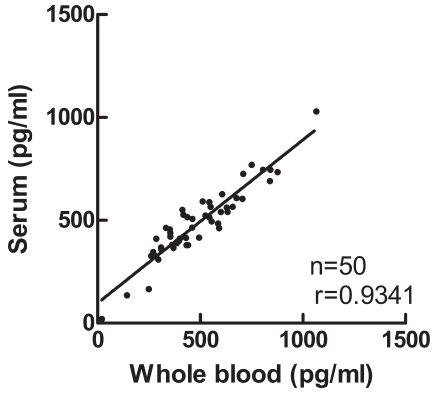

Fig. 1. Correlation plots of estradiol concentrations in the whole blood and serum of mares, generated using chemiluminescent enzyme immunoassay (PATHFAST).

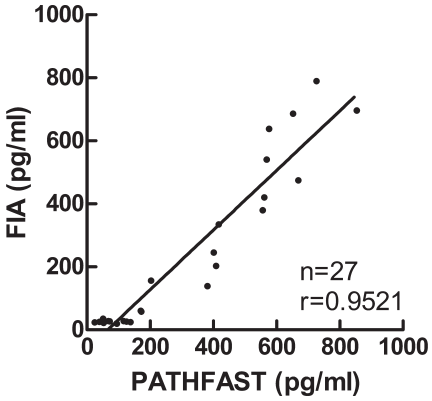

Fig. 3. Correlation plots of estradiol concentration in serum of pregnant mares, generated using chemiluminescent enzyme immunoassay (PATHFAST) and time-resolved fluoroimmunoassay (FIA).

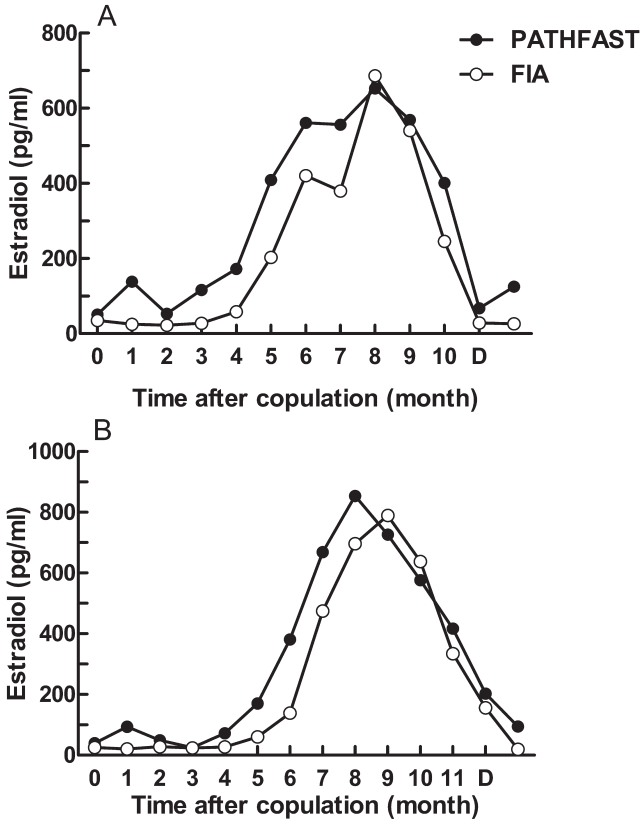

Fig. 2. Serum estradiol concentration of two pregnant mares, as measured by chemiluminescent enzyme immunoassay (PATHFAST) (●) and time-resolved fluoroimmunoassay (FIA) (○). "0" and "D" represent the days of copulation and delivery, respectively.

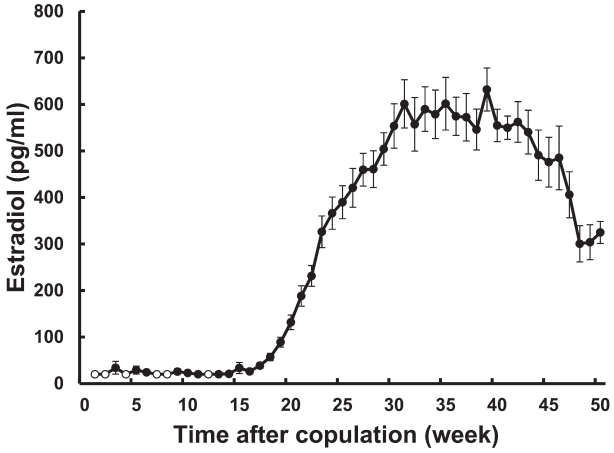

Fig. 4. Changes in circulating estradiol in mares during pregnancy, as measured by chemiluminescent enzyme immunoassay (PATHFAST). Results are expressed as means ± SEM (n = 11–52). Values less than 20 pg/ml are denoted as white circles.

serum samples as measured by PATHFAST were compared with those measured by FIA (Fig. 3). The results obtained by PATHFAST showed a significant correlation with those measured by FIA ($r = 0.9521$, $P < 0.0001$, $n = 27$).

To monitor physiological changes, the concentrations of estradiol in serum samples obtained throughout the gestation period were measured by PATHFAST (Fig. 4). Circulating estradiol began to increase in 15 weeks after copulation, and linearly increased until 31 weeks after copulation. High levels of serum estradiol were maintained between 30 and 43 weeks after copulation, followed by an abrupt decline (Fig. 4).

The present study evaluated the validity of the rapid measurement of equine estradiol using the new practical assay system, PATHFAST.

In the present study, estradiol concentrations in whole blood and serum of mares showed excellent positive correlation ($r = 0.9341$, $P < 0.0001$, $n = 50$), indicating that estradiol concentrations can be measured in whole blood as well as in serum samples using PATHFAST. Comparison of PATHFAST and FIA revealed a significant positive correlation ($r = 0.9521$, $P < 0.0001$, $n = 27$). In the present study, serum concentrations of estradiol as measured by PATHFAST

showed a parallel pattern to those measured by FIA, even though the values measured by PATHFAST tended to be higher than those measured by FIA during mid-gestation. Although the exact reason for this is not presently clear, the difference could be attributed to the differing cross reactivity of the anti-estradiol antibodies used in PATHFAST and FIA. In the present study, the physiological changes in circulating estradiol levels during the gestation in mares were further examined using PATHFAST. The pattern of serum estradiol levels during gestation in mares was similar to that observed in our previous results obtained using RIA [17, 18], indicating that PATHFAST correctly monitored the physiological changes in circulating estradiol during the gestation.

Pregnancy with placentation is a peculiarity of mammals, including horses. Remarkable fetal growth occurs in the uterus during the late stages of pregnancy in all mammals. The most important factor involved includes the rapid elongation and cylindrical changes in the uterus to maintain fetal survival at the stage of rapid fetal growth during the late stages of pregnancy [9–12]. Progesterone, estrogen, prolactin and relaxin play key roles in the implantation of embryos, maintenance of pregnancy, and induction of parturition. Although the synergistic action of progesterone and estrogen is generally essential for mares, as in most mammals, for maintaining successful pregnancy, estrogen plays a dominant role in the maintenance of uterine elongation when the fetus grows rapidly in late pregnancy [9–12]. Therefore, monitoring circulating estradiol and progesterone is useful for detecting clinical signs of abortion, stillbirth, and perinatal fetal death during late pregnancy in mares [19–22].

Estradiol measurement is used to diagnose reproductive conditions at the equine clinic, and there is a need for a rapid measurement system in addition to the use of whole blood samples. In the present study, estradiol concentrations in both whole blood and serum samples could be measured by PATHFAST within 26 min without ether extraction. The only important point concerning the measurement of estradiol by PATHFAST for use in the equine clinic is the detection limit of the assay. The range of the basal level of circulating estradiol during the estrous cycle [1–5] and the developmental stage [23–27] in mares is less than 20 pg/ml, and the detection limit of estradiol by PATHFAST is 20–2000 pg/ml. Extraction of serum by ether or other chemicals [28] will be required for measuring the basal estradiol level in mares using this estradiol PATHFAST.

In conclusion, PATHFAST would be useful in the equine hospitals as an accurate diagnostic tool for the rapid assay of estradiol in pregnant mares.

Methods

Animals

One hundred twenty-one thoroughbred mares (2–22 years old) in Hokkaido, Japan housed under natural conditions were used for measuring the levels of circulating estradiol.

Sample preparation and experiments

For determining the correlation between the results for whole blood and serum, five pregnant mares, 8–19 years of age, were used. Whole blood samples were collected weekly from the jugular vein into commercially supplied plastic tubes containing heparin sodium as an anticoagulant during day 233 of pregnancy and the day of delivery. For serum sample collection, whole blood was drawn from the jugular vein of the same mares into plain blood collection tubes. Serum was separated by centrifugation at 1,700 × *g* for 10 min. The concentrations of estradiol in all samples were measured by PATHFAST.

For the experiments determining the correlation between the results of PATHFAST and time-resolved FIA, two pregnant mares, 4 and 15 years old, were used. Serum samples were collected monthly from the jugular vein into commercially supplied plastic tubes at the day of copulation and the day of delivery. The concentrations of estradiol in all samples were measured by PATHFAST and FIA.

For evaluating the pattern of circulating estradiol during gestation in mares, fifty-nine pregnant mares, ranging from 4–22 years of age, were used. Serum samples were collected during gestation from the jugular vein into commercially supplied plastic tubes. All mares delivered normally. The estradiol concentrations in all samples were measured by PATHFAST. All procedures were carried out in accordance with the guidelines established by the Tokyo University of Agriculture and Technology for the care and use of horses in research.

Hormone assay

PATHFAST: The concentrations of estradiol in whole blood and serum samples were determined using the PATHFAST analyzer with the PATHFAST reagent kit for estradiol as described previously [13–16]. In brief, estradiol measurement by PATHFAST was performed using a single reagent cartridge containing 100 µl of whole blood and serum samples without extraction. In the competitive assays, estradiol in the samples was inhibited from forming an immuno-complex by an alkaline phosphatase-conjugated antigen. Following immunoreaction for 5 min, separation of bound and free hormones was performed using the Magtration technology. After addition of the chemiluminescent substrate to the immune complex, the amount of chemiluminescence was measured. The assay results were obtained within 26 min. The assay range of PATHFAST was 20–2000 pg/ml. The intra -assay coefficients of variance were 7.0–12.4% for whole blood, and 6.3–12.9% for serum samples.

FIA: Serum estradiol concentrations were determined by a time-resolved fluoroimmunoassay using dissociation-enhanced FIA systems (PerkinElmer, Waltham, MA, USA) as described previously [19, 20]. The intra- and inter-assay coefficients of variation were 5.0% and 5.1%, respectively.

Statistics

Pearson's r was calculated to determine the correlation between variables using GraphPad Prism software version 5 for Windows (Graph Pad Software, San Diego, CA, USA). P value less than 0.05 was considered to be significant.

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