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Validation of a direct time-resolved fluoroimmunoassay for progesterone in milk from dairy and beef cows

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ABSTRACT

The aim of this study was to validate a direct time-resolved fluoroimmunoassay (TR-FIA) for quantifying progesterone concentrations in milk during the bovine oestrous cycle. Holstein–Friesian and suckled and non-suckled Japanese Black cows were used to demonstrate the relationship between milk and plasma progesterone concentrations and to monitor progesterone profiles in milk and plasma during the oestrous cycle. The minimum detection level of the assay was 1.53 ng/mL. Progesterone concentrations in milk and plasma changed in a similar manner throughout the oestrous cycle in dairy and beef cows, and milk and plasma progesterone profiles were significantly correlated ($P < 0.001$). The study confirmed that a direct TR-FIA can be used to monitor the oestrous cycle in cattle and to quantify progesterone concentrations in whole milk.

Introduction

Milk progesterone concentrations are used to monitor oestrous cycles and for early pregnancy diagnosis in bovine reproductive management (Petersson et al., 2006; Simersky et al., 2007). These are usually determined by radioimmunoassay (RIA) or enzyme immunoassay (EIA). The main disadvantage of RIA is the risk of exposure to radioisotopes, while EIA has been suggested to be less sensitive than RIA (Marcus and Hackett, 1986).

Time-resolved fluoroimmunoassay (TR-FIA) has a better sensitivity than RIA (Fiet et al., 2001; Small and Davis, 2002) and does not require the use of special facilities to deal with radioisotopes. Lanthanoid-labelled hormones are chemically stable for a longer period than radioactively-labelled compounds (Iwasawa et al., 1994; Noguchi et al., 2007) or enzyme-labelled compounds. The correlation between concentrations determined by TR-FIA and those measured by RIA has been reported to be very high (Meurman et al., 1982; Bacigalupo et al., 1990) and TR-FIA has been used for the measurement of various physiologically active substances in blood and other body fluids (Elliott et al., 1995; Takahashi et al., 2002, 2004). In human medicine, plasma progesterone and oestradiol-17β concentrations have been determined using commercial TR-FIA kits. Although Ius et al. (1993) used TR-FIA to measure progesterone concentrations in bovine milk, the progesterone profile during the oestrous cycle has not been measured.

The use of milk is convenient because herd personnel can take daily samples. Daily samples for progesterone assays would help practitioners to diagnose reproductive disorders more accurately, leading to improved reproductive performance in the herd. Milk samples are useful for the reproductive management of both dairy and beef cattle. Milk sampling from beef cows is not difficult in herds where intensive beef farming is performed, such as with the Japanese Black cows in Japan. However, information regarding milk progesterone profiles during the oestrous cycle in beef cows is scarce. Mann et al. (2005) reported the use of detailed milk progesterone analysis to assess reproductive function in suckled beef cows, but there are no reports on milk progesterone analysis in cyclic non-suckled beef cows.

The objectives of this study were to validate a TR-FIA for measuring whole milk progesterone concentrations in cattle and to compare milk progesterone concentration profiles during the oestrous cycle in dairy and suckled and non-suckled beef cows.

Materials and methods

Validation of direct TR-FIA for progesterone concentrations in bovine milk

Progesterone-free whole milk (approximately 1 L) was collected from an ovariectomised Jersey cow (6.7 year-old; five parities; 90 days postpartum) and used to prepare standard solutions for TR-FIA.

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The minimum detection level of the assay (assay sensitivity) was defined as the concentration resulting in a response measuring two standard deviations below the dose response at 1.0 ng/mL. Intra- and inter-assay coefficients of variation (CV) were calculated by measuring the same control milk samples six times simultaneously in one assay (for intra-assay CV), or in six assays at different times (for inter-assay CV).

Accuracy was evaluated using the recovery test by adding different concentrations of progesterone (1, 2, 4, 8, 16 and 32 ng/mL; 4-pregnen-3,20-dione, Merck) to each pooled milk sample. The pooled milk sample was composed of bulk tank milk from a dairy herd and milk from a recently-ovariectomised cow in a ratio of 3:2. The progesterone concentration of the original pooled milk sample was 4.7 ng/mL. Assuming the dilution factor to be negligible, the recovery efficiency was calculated as follows:

$$P_4B\ (ng/mL)/[P_4\ added\ (ng/mL) + P_4A\ (ng/mL)] \times 100$$

where P_4A is the progesterone concentration before the addition of progesterone and P_4B is the progesterone concentration after the addition of progesterone. The final results were expressed by averaging the recovery percentages obtained with 1, 2, 4, 8, 16 and 32 ng/mL of added progesterone.

Monitoring oestrous cycles by progesterone concentrations in milk and plasma in dairy and beef cows

To demonstrate the relationship between milk and plasma progesterone concentrations determined by TR-FIA, we used four cyclic Holstein–Friesian cows (mean age: 5.0 ± 0.8 years; mean bodyweight: 638 ± 86.5 kg; mean parity: 3.0 ± 0.8; mean days postpartum: 216.5 ± 79.2; mean daily milk yield: 35.5 ± 11.9 kg), and 11 Japanese Black cows (mean age: 4.6 ± 2.7 years; mean bodyweight: 445 ± 61.4 kg; mean parity: 3.1 ± 2.3). The total numbers of samples from Holstein–Friesian and Japanese Black cows were 75 and 143, respectively. Of the 11 beef animals, five were suckled (65 samples) and the other six were non-suckled cows (78 samples).

The four cyclic Holstein–Friesian cows and four cyclic (two suckled and two non-suckled) Japanese Black cows (mean age: 2.8 ± 0.9 years; mean bodyweight: 425 ± 54.5 kg; mean parity: 1.5 ± 0.6; mean days postpartum: 33.3 ± 20.0) were used to monitor progesterone profiles in milk and plasma during the entire oestrous cycle.

Blood and milk samples (5.0 mL of each) were taken in the morning every 2 days from April to August 2008. Blood samples were taken from the coccygeal vein into a heparinised vacuum tube. Plasma was separated by centrifugation at 2000 *g* for 15 min. Milk samples were taken from one quarter of the udder, just after a routine milking for dairy cows and in the early afternoon for beef cows. Plasma and milk samples were stored at −30 °C until hormone assays were performed.

All procedures were carried out in accordance with a protocol approved by the Animal Care and Use Committee of Iwate University (2008–27).

Hormone assays

Milk progesterone concentrations were measured using standard solutions and TR-FIA kit reagents (Wallac DELFIA progesterone reagent R066-101 kit, PerkinElmer Life and Analytical Sciences). A series of standard solutions (1, 5, 10, 30 and 50 ng/mL) were prepared by the addition of a known amount of progesterone (4-pregnen-3, 20-dione, Merck) to progesterone-free bovine milk. Ten microlitres of the standard solutions or of the milk samples were dispensed into a 96-well assay microplate (pre-coated with anti-rabbit IgG as secondary antibody), followed by the addition of 100 μL of anti-progesterone antibody (PerkinElmer Life and Analytical Sciences) and 100 μL of europium-labelled tracer to each well. The plate was then incubated with shaking for 2 h at 22 °C, washed three times with wash solution, supplemented with 200 μL of enhancement solution, and gently shaken for 5 min at 22 °C. Fluorescence was detected using a multi-plate reader (ARVO MX Wallac 1420 multilabel counter, PerkinElmer Life and Analytical Sciences).

Plasma progesterone concentrations were measured using a TR-FIA kit, according to the manufacturer's protocol, with some modifications as described previously (Takahashi et al., 2001). Briefly, 25 μL of a series of standard solutions (0, 0.33, 1.33, 4.0, 12.0 and 36.0 ng/mL) were prepared by the addition of a known amount of progesterone to charcoal-treated progesterone-free bovine serum, and 25 μL of plasma sample were dispensed into an anti-rabbit IgG pre-coated microplate. The rest of the procedures were as described above for milk progesterone. Minimum detection level of the assay was 0.65 ng/mL and the ED_{50} value of the standard curve was 1.47 ng/mL. Intra- and inter-assay CVs were 5.4% and 5.7%, respectively.

Milk progesterone concentrations were also measured using an EIA kit (KMK, Kawasaki Mitaka Pharmaceutical) with a modification of the series of standard solutions that were used for TR-FIA. The rest of the procedures were performed according to the manufacturer's instructions. The absorbance at 450 nm was measured using a multi-plate reader (ARVO MX Wallac 1420 multilabel counter). Minimum detection level of the assay was 2.72 ng/mL.

All of the samples in these assays were measured in duplicate, and those falling below the minimum detection levels of each assay were set to that limit as the highest possible progesterone concentration for that sample.

Statistical methods

Correlations between progesterone concentrations determined by TR-FIA and those determined by EIA, and between progesterone concentrations determined by TR-FIA in milk and plasma samples taken at the same time during the oestrous cycle were analyzed using Pearson's product-moment correlation coefficients. Kappa coefficients (κ) were used to determine the extent of agreement in terms of the presence of a functional corpus luteum, based on whole milk progesterone concentrations of $\geqslant$5 ng/mL measured by TR-FIA and EIA in whole milk, and were also calculated for the agreement between TR-FIA for whole milk and TR-FIA for plasma on the presence of a functional corpus luteum (progesterone concentrations of $\geqslant$5 ng/mL in milk, or $\geqslant$1 ng/mL in plasma). We considered κ values of 0.41–0.60 to represent moderate agreement, 0.61–0.80 to represent substantial agreement, and 0.81–1.00 to represent almost perfect agreement (Everitt, 1989). Unless otherwise stated, results were expressed as means ± SD and were considered statistically significant when $P < 0.05$.

Results

Validation of TR-FIA assay for bovine milk

The standard curve for TR-FIA for bovine whole milk is shown in Fig. 1. The minimum detection level of the assay was 1.53 ng/mL. Intra- and inter-assay CVs were 5.46% and 7.51%, respectively. The ED_{50} value of the standard curve was 6.0 ng/mL.

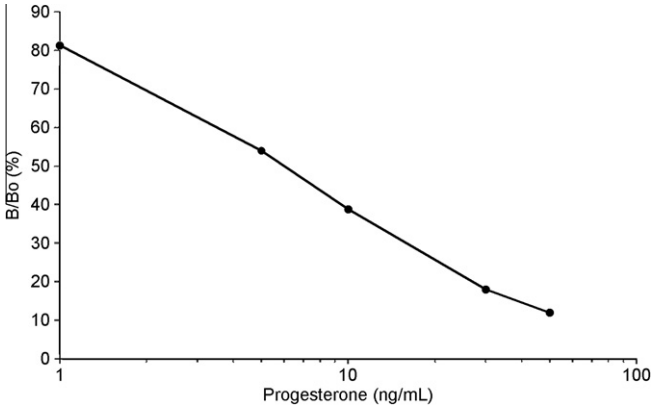

Fig. 1. Progesterone time-resolved fluoroimmunoassay (TR-FIA) standard curve for bovine milk.

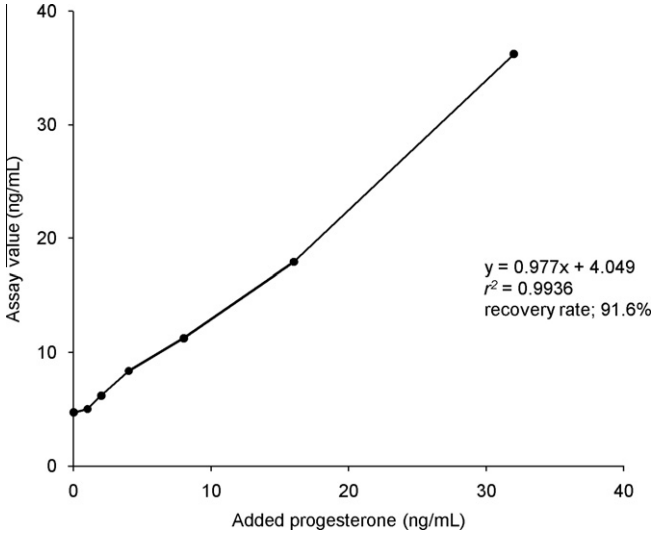

Fig. 2. Recovery test for progesterone from bovine milk: 1, 2, 4, 8, 16 or 32 ng/mL of progesterone was added to pooled milk. The progesterone concentration of pooled milk was 4.7 ng/mL. The average recovery rate was 91.58%. The linear regression equation was y (ng/mL) = 0.977x + 4.049 (where y is the measured value and x was the added amount of progesterone; r = 0.997).