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An electrochemical immunosensor for detecting progesterone in milk from dairy cows

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ABSTRACT

In this study, an electrochemical immunosensor for milk progesterone produced by dairy cows was developed. Using the immunosensor, milk progesterone levels in healthy estrus dairy cows was found to range from 1 to 6 ng/mL 20 days after estrus. There were high levels of progesterone in the milk from cows with prolonged luteal phase and luteal cysts, which ranged from 15 to 28 and 19 to 29 ng/mL, respectively. Cows with inactive ovaries also showed low milk progesterone levels of 1-8 ng/mL, but they had lower plasma follicle-stimulating hormone (FSH) levels than the healthy estrous cycle dairy cows.

Key words: dairy cows; electrochemical immunosensor; progesterone; reproductive status

Introduction

The reproductive performance of cows is an important economic factor in the dairy industry. Recently milk yields of dairy cows have improved, but reproductive performance has declined (COMIN et al., 2005; DARWASH et al., 1999). High milk producing cows usually exhibit poorer conception rates compared to lower-yielding cows (RABIEE et al., 2002). Normal estrus of dairy cows at postpartum is considered to indicate healthy reproductive performance. Several diseases, such as prolonged luteal phase, inactive ovaries and luteal cysts, frequently cause postpartum dairy cow reproductive disorders. In the dairy industry, progesterone measurements have been used for pregnancy diagnoses, to monitor postpartum ovarian activity, to assess the reproductive status of a cow, and to detect some reproductive diseases (GORZECKA et al., 2011). Because of the regularity

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of the changes in progesterone levels in milk samples from dairy cows, milk samples are usually used to assess reproductive performance (MANN et al., 2005). Traditional immunoassay methods for milk progesterone analysis include radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (GORZECKA et al., 2011; RABIEE et al., 2002; LAMMING and DARWASH, 1998), but these methods have the limitations of being time consuming, generating radioactive waste and requiring skilled analysts. Accordingly, they cannot be widely used in the dairy industry. Recently, electrochemical immunosensors have been widely employed in the detection of proteins, nucleic acids, hormones and pesticides. Along with the high specificity and sensitivity of immunoassay methods, electrochemical immunosensors have important features, such as a low detection limit, rapid analysis, ease of handling, low cost and low endogenous background using different types of electrodes (AHMADI et al., 2014). Several technologies, such as gold nanoparticles and paramagnetic microbeads, have been widely used in electrochemical immunosensors to improve detection limits (VIDAL et al., 2012; VOLPE et al., 2013). To take advantage of the easy handling and low costs, a traditional electrochemical immunosensor was developed for estimation of progesterone in dairy milk, and the characteristics of this immunosensor were analyzed. The immunosensor thus developed was also used to analyze milk progesterone levels in cases of prolonged luteal phase, the inactive ovaries and luteal cysts in dairy cows. The progesterone levels detected by the immunosensor were compared to the results from a commercial ELISA kit-based analysis.

Materials and methods

Apparatus and reagents. A CHI660A electrochemical workstation (Shanghai Jiao Tong University, China) with a three-electrode setup was used for electrochemical measurements. The screen-printed electrode (SPE) was prepared using graphite ink for the working and auxiliary electrodes, and silver/silver chloride ink for the reference electrode, which had a working electrode diameter of 3 mm (Nanjing McKesson Electronics Co., Nanjing, China). Mouse anti-progesterone monoclonal antibody (mAb) and progesterone complete antigen (11a-OH-P4-HS-OVA, P4-OVA) were produced in our laboratory. The mAb was conjugated to horseradish peroxidase (HRP) and was prepared as described by WILSON and NAKANE (1978). The commercial ELISA kit for milk progesterone was purchased from Nanjing Jiancheng Bio (China).

Immunosensor protocol. The procedure used to prepare the electrochemical immunosensor was described previously (ZHANG et al., 2013), but it was improved with some modifications. Briefly, the SPE was prepared in a solution of 1 mM $K_3Fe(CN)_6$ with an applied potential ranging from -0.6 to 0.2 volt (V) at a scan rate of 50 mV/sec. Then the SPE was rinsed several times with deionized water and dried. Ten μL of P4-OVA (4 ng/

mL, prepared in PBS) was placed on the carbon area of the SPE and it was immobilized for 45 min at 37 °C. The immunosensors were washed with PBS to remove the weakly adsorbed P4-OVA, and then they were blocked by the application of 50 μL 1% BSA in PBS (pH 7.4) for 1 h at 37 °C. After washing with PBS, the SPE were dried and stored at 4 °C prior to use.

Assay procedure. To analyze milk progesterone levels, dairy milk was prepared by glucose coated absorbite to develop hormone-free milk (GAO et al., 2009). Then, hormone-free milk with 100, 50, 25, 10, 2.5, 1.25, 0.625, 0.31, 0.16 and 0 ng/mL progesterone was prepared as standard milk samples. Five μl of HRP-labelled mAb (2.5 μg/mL, prepared in PBS) and 5 μL milk samples were premixed and the mixture was added to the work area of the electrodes. After 40 min incubation at 37 °C, the SPE was washed with PBS and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (0.4 mM TMB in phosphate citrate buffer, pH 5.5, with 1 mM H_2O_2) was added. After 10 min, the concentration of progesterone in each electrode was measured using chronoamperometry.

Evaluation of clinical specimens using the immunosensor. In this study, milk progesterone levels were analyzed from healthy dairy cows in estrus or pregnancy, and milk progesterone concentrations from dairy cows with prolonged luteal phase, inactive ovaries or luteal cysts were also evaluated by the immunosensor. Milk samples from eight healthy estrus dairy cows were collected at 0, 5, 10, 15 and 20 days after the first estrus performance of postpartum. Milk samples from seven pregnant dairy cows were obtained at 10, 15, 20, 25 and 30 days after artificial insemination, and a final diagnosis was made by rectum palpation. Milk from six dairy cows with prolonged luteal phase, inactive ovaries or luteal cysts was also collected and evaluated. Milk samples were simultaneously analyzed by an immunosensor and a commercial ELISA kit for progesterone measurements. In this study, plasma samples were also collected at the same time as the milk samples, and plasma progesterone (P4), luteinizing hormon (LH), estradiol (E_2) and follicle-stimulating hormone (FSH) were analyzed using a commercial ELISA kit (Nanjing Jiancheng Bio, China). All experimental animals were treated according to the International Guiding Principles for Biomedical Research Involving Animals (DEMERS et al., 2006).

Results

Characterization of the progesterone immunosensor: Progesterone standard solutions were analyzed using the electrochemical immunosensor. The logarithm concentration (Log c) of progesterone was used on the abscissa, and the logarithm current of the immunosensor (Log i) was used as an ordinate (Fig. 1). It was found that the electrochemical immunosensor had a linear detection range of 0.31-50 ng/mL with a detection limit of 0.16 ng/mL. The precision of the progesterone electrochemical immunosensor was evaluated

using the intra- and inter-assay coefficients of variation (CVs). Our experimental results suggested that the intra-assay CVs were 12.6%, 6.3% and 4.2% (n = 4) for 0.5, 10 and 40 ng/mL progesterone, respectively, while the inter-assay CVs were 8.6%, 8.4% and 5.2% (n = 4) for 0.5, 10 and 40 ng/mL progesterone, respectively. Thus, the precision and reproducibility of the progesterone immunosensor were both acceptable. These findings indicated that the specificity of the immunosensor was excellent. The P4-OVA solution can be stored as a stock solution for at least 2 years at -20 °C without any noticeable loss of performance. When P4-OVA is diluted and coated on the electrode, the stability drops significantly to ~4 days.

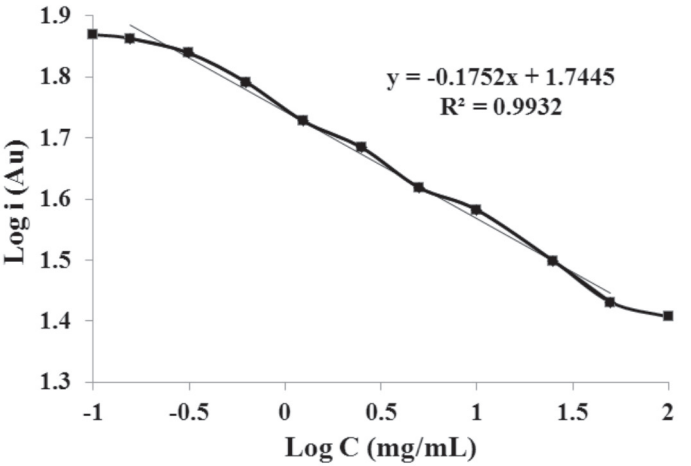

Fig. 1. Progesterone calibration curves for the electrochemical immunosensor. The logarithm of progesterone concentration (Log C) was used as abscissa, and the logarithm current of the immunosensor (Log i) was used as ordinates. The valve of Log 0 (ng/mL) in X axis was sign by -1. The linear range of the assay was 0.31-50 ng/mL, with a regression value of 0.9932.

Evaluation of clinical specimens with the immunosensor: Considering that the estrous cycle for a dairy cow is 21 days, the milk progesterone levels of dairy cows from 0 to 20 days after estrus performance were analyzed (Table 1). There was a change in milk progesterone levels, which reached a peak level of 21.55 ng/mL at 15 days. There was no significant difference in milk progesterone levels analyzed by ELISA and the electrochemical immunosensor. Consequently, the immunosensor that was developed in this study showed ideal characteristics for milk progesterone analysis. Progesterone levels were lower in the plasma than in the milk samples, but they did show a positive correlation with milk levels. Plasma E_2 in healthy estrus dairy cows was detected at a higher level at 0 and 20 days. The concentrations of plasma LH and FSH showed no significant changes during the estrous cycle.

Table 1. Analysis of levels of milk progesterone and plasma hormones of dairy cows in the estrous cycle

	Days after the first estrus postpartum				
Hormones	0 d	5 d	10 d	15 d	20 d
MP4-I (ng/mL)	1.57 ± 1.02	8.18 ± 1.61	16.67 ± 3.20	21.55 ± 3.11	2.33 ± 1.38
MP4-E (ng/mL)	1.40 ± 0.81	8.10 ± 1.89	15.25 ± 2.82	20.96 ± 2.76	2.17 ± 1.16
P4 (ng/mL)	1.17 ± 0.64	3.10 ± 0.96	6.13 ± 0.99	7.27 ± 1.61	2.19 ± 1.03
E_2 (pg/mL)	7.58 ± 1.17	5.80 ± 1.63	4.89 ± 0.91	5.12 ± 0.89	7.86 ± 1.04
FSH (mIU/mL)	17.63 ± 0.83	17.57 ± 0.59	17.91 ± 0.54	17.68 ± 0.61	18.01 ± 0.36
LH (mIU/mL)	35.67 ± 2.15	28.69 ± 2.23	38.21 ± 2.52	31.19 ± 1.82	32.67 ± 2.46

Values represent means ± SEM. MP4-I: milk progesterone was analyzed by an immunosensor. MP4-E: milk progesterone was analyzed by a commercial ELISA kit. Plasma P4, E_2, LH and FSH were analyzed using a commercial ELISA kit.

Following artificial insemination, the concentration of milk progesterone in pregnant dairy cows was found to be elevated (Table 2). Milk progesterone concentrations were more than 14 ng/mL after day 20 post-artificial insemination. Plasma progesterone levels also showed an elevated change, and the concentration of progesterone was greater than 7 ng/mL at 20 days after artificial insemination. Plasma E2 levels in pregnant cows peaked at day 20, whereas plasma LH and FSH levels showed no significant changes following artificial insemination during the study period.

Table 2. Analysis of levels of milk progesterone and plasma hormones related to pregnancy in dairy cows

	Days of pregnancy				
Hormones	10 d	15 d	20 d	25 d	30 d
MP4-I (ng/mL)	17.41 ± 2.49	21.76 ± 3.69	22.93 ± 4.69	25.45 ± 3.56	25.56 ± 3.46
MP4-E (ng/mL)	17.11 ± 2.76	19.47 ± 4.67	21.31 ± 3.72	22.31 ± 3.61	24.79 ± 2.72
P4 (ng/mL)	6.30 ± 1.16	8.01 ± 1.67	9.09 ± 1.43	9.90 ± 1.65	9.54 ± 1.61
E_2 (pg/mL)	4.66 ± 0.96	6.09 ± 1.01	8.02 ± 1.67	7.70 ± 1.43	6.17 ± 0.98
FSH (mIU/mL)	17.50 ± 0.69	17.89 ± 0.79	17.62 ± 0.85	17.64 ± 0.65	18.03 ± 0.39
LH (mIU/mL)	38.91 ± 2.38	33.06 ± 3.35	40.28 ± 2.23	35.63 ± 5.00	39.91 ± 4.26

Values represent means ± SEM. MP4-I: milk progesterone was analyzed by the immunosensor. MP4-E: milk progesterone was analyzed by a commercial ELISA kit. Plasma P4, E_2, LH and FSH were analyzed using a commercial ELISA kit.

In this study, milk progesterone levels in dairy cows with prolonged luteal phase, inactive ovaries and luteal cysts were also analyzed. Dairy cows with prolonged luteal phase and luteal cysts had high levels of milk progesterone, ranging from 15 to 28 and 19

to 29 ng/mL, respectively (Table 3). Corpus luteum cyst dairy cows had a significantly higher level of plasma LH than cows with prolonged luteal phase. Dairy cows with inactive ovaries showed a consistently lower level of progesterone in milk (1-8 ng/mL) and plasma FSH.

Table 3. Analysis of levels of milk progesterone and plasma hormones associated with reproductive disorders in dairy cows

Hormones	Prolonged luteal phase	Inactive ovaries	Luteal cysts	Pregnancy (20d)	Estrous cycle (20d)
MP4-I (ng/mL)	21.12 ± 4.82^{a}	4.20 ± 2.49^{b}	24.34 ± 3.58^{a}	22.93 ± 4.69^{a}	2.33 ± 1.38^{b}
MP4-E (ng/mL)	20.09 ± 4.61^{a}	4.20 ± 2.56^{b}	23.59 ± 3.75^{a}	21.31 ± 4.72^{a}	2.07 ± 1.66^{b}
P4 (ng/mL)	7.91 ± 2.00^{a}	1.75 ± 0.39^{b}	8.36 ± 2.39^{A}	9.09 ± 1.43^{a}	2.19 ± 1.03^{b}
E_2 (pg/mL)	6.35 ± 1.17^{ab}	6.15 ± 1.16^{a}	6.45 ± 1.25^{ab}	8.02 ± 1.67^{c}	7.86 ± 1.05^{bc}
FSH (mIU/mL)	17.44 ± 0.87^{a}	15.53 ± 0.71^{b}	17.38 ± 0.63^{a}	17.62 ± 0.85^{a}	18.01 ± 0.36^{a}
LH (mIU/mL)	32.50 ± 3.13^{a}	31.08 ± 3.73^{a}	40.79 ± 2.82^{b}	40.28 ± 2.23^{b}	32.67 ± 2.46^{a}

Values represent means ± SEM. values within a row that do not share a common superscript are significantly different ($P<0.05$). MP4-I: milk progesterone was analyzed by the immunosensor. MP4-E: milk progesterone was analyzed by a commercial ELISA kit. Plasma P4, E_2, LH and FSH were analyzed using a commercial ELISA kit.

Discussion

In the dairy industry, progesterone measurements have been used to diagnose pregnancy, to monitor ovarian activity postpartum, to assess the reproductive status of a cow and to detect some reproductive diseases. Milk samples are easy to obtain, they show reproducible changes in progesterone levels and have progesterone levels that show a positive correlation with blood progesterone levels. Therefore, they have been widely used for assessment of dairy cow reproduction (COMIN et al., 2005). For fast and quantitative detection of milk progesterone levels, an electrochemical immunosensor was developed for dairy milk that showed a linear detection range of 0.31-50 ng/mL, and had a detection limit of 0.16 ng/mL. Results could be obtained in less than 1 h, which was ideal for milk progesterone analysis. The stability of an electrode coated with P4-OVA was only 4 days, but because of the rapid development time, it was suitable for clinical specimen analysis. Milk progesterone levels were analyzed from healthy estrus and pregnant dairy cows using the immunosensor, and progesterone concentrations in the milk of dairy cows with prolonged luteal phase, inactive ovaries or luteal cysts were also evaluated using the immunosensor. To evaluate the accuracy of the immunosensor, a commercial ELISA kit was also used that showed good performance as an immunosensor for detecting milk progesterone. With the immunosensor, milk progesterone levels in healthy estrus dairy cows were found to range from 1 to 6 ng/mL 20 days after estrus performance, which

can be used to diagnose a pregnancy when there is more than 14 ng/mL progesterone 20 days after artificial insemination. BARNA et al. (2013), who analyzed milk progesterone levels 19-22 days after artificial insemination, reported that milk progesterone values >4 ng/mL had 75% accuracy in pregnancy assessments of dairy cows, whereas only 9.52% of pregnant dairy cows were detected when milk progesterone values <4 ng/mL were used. In this study, plasma E_2, FSH and LH are related to the development of ovarian follicles and the corpus luteum (HUNTER et al., 2004; MIHM and BLEACH, 2003). However, there were no significant changes in these parameters between pregnant and non-pregnant dairy cows. A prolonged luteal phase, inactive ovaries and luteal cysts were considered to be the most frequent diseases that affect reproduction and can cause the failure of artificial insemination. The milk progesterone levels in dairy cows with prolonged luteal phase and luteal cysts were 15-28 and 19-29 ng/mL, respectively, which were considered to be elevated. As a result, it was difficult to distinguish them on the basis of milk progesterone levels, but cows with luteal cysts had significantly higher levels of plasma LH than other cows. The measurement of plasma LH levels for diagnosis of luteal cysts is suggested. Compared to healthy estrous cycle dairy cows, cows with inactive ovaries also showed low milk progesterone concentrations (1-8 ng/mL). Compared to healthy cows, dairy cows with inactive ovaries showed low plasma FSH levels. These findings suggest that the association between milk progesterone and blood hormone levels could be used to assess the reproductive status of dairy cows.

In conclusion, an electrochemical immunosensor for detecting progesterone levels in dairy milk was developed. The sensor has a linear detection range of 0.31-50 ng/mL with a detection limit of 0.16 ng/mL, and results can be obtained in less than 1 h. Milk progesterone levels were analyzed using the immunosensor and a commercial ELISA kit; the immunosensor showed good performance for detecting milk progesterone levels. Using the immunosensor, milk progesterone levels in healthy estrus dairy cows were found to range from 1 to 6 ng/mL 20 days after estrus performance, while more than 14 ng/mL progesterone in pregnant dairy cows could be detected 20 days after artificial insemination. The peak milk progesterone levels in dairy cows with prolonged luteal phase or luteal cysts were 15-28 and 19-29 ng/mL, respectively. By contrast, cows with inactive ovaries showed low milk progesterone levels of 1-8 ng/mL.

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SAŽETAK

Ovim je istraživanjem razvijen elektrokemijski imunosenzor za otkrivanje progesterona u mlijeku dobivenom od mliječnih krava. Upotrebom imunosenzora u zdravih krava za proizvodnju mlijeka 20 dana nakon estrusa utvrđene su razine progesterona u rasponu od 1 do 6 ng/mL. U mlijeku krava s produljenom luteinskom fazom i luteinskim cistama utvrđena je visoka razina progesterona, u rasponu od 15 do 28 te od 19 do 29 ng/mL. Krave s neaktivnim jajnicima također su pokazale nisku razinu progesterona u mlijeku, od 1 do 8 ng/mL. Te su krave, u odnosu na zdrave krave za proizvodnju mlijeka u estrusnoj fazi ciklusa, očitovale nižu razinu folikulostimulacijskog hormona u plazmi (FSH).

Ključne riječi: mliječne krave; elektrokemijski imunosenzor; progesteron; reprodukcijski status