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Determination of plasma kisspeptin concentrations during reproductive cycle and different phases of pregnancy in crossbred cows using bovine specific enzyme immunoassay

Mohan Mondal [a,b,*], Kishore Kumar Baruah [b], Bukkaraya Samudram Prakash [c]

[a] Animal Physiology & Reproduction Laboratory, ICAR-National Dairy Research Institute, Kalyani 741235, India
[b] Animal Endocrinology Laboratory, ICAR-National Research Centre on Mithun, Jharnapani, Medziphema, Nagaland 797 106, India
[c] Assistant Director General (Animal Nutrition & Physiology), ICAR, Krishi Bhavan, New Delhi, India

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ABSTRACT

Kisspeptin, a decapeptide and potent secretagogue of GnRH has been emerged recently as a master player in the regulation of reproduction in animals. Determination of kisspeptin in peripheral circulation is, therefore, very important for studying the control of its secretion and its role on reproduction in bovine species, the information on which is not available during any physiological state in this species, may probably be due to non-availability of simple assay procedure to measure the hormone. Therefore, the objective of this study was to develop and validate a simple and sufficiently sensitive enzyme immunoassay (EIA) for kisspeptin determination in bovine plasma using the biotin–streptavidin amplification system and second antibody coating technique. Biotin was coupled to kisspeptin and used to bridge between streptavidin-peroxidase and the immobilized kisspeptin antiserum in the competitive assay. The EIA was conducted directly in 100 μl of unknown bovine plasma. Kisspeptin standards ranging from 0.01 to 25.6 ng/100 μl/well were prepared in hormone-free plasma. The lowest detection limit was 0.1 ng/ml plasma. Plasma volumes for the EIA, viz., 50, 100 and 200 μl did not influence the shape of standard curve even though a drop in OD_{450} was seen with higher plasma volumes. A parallelism test was carried out to compare the endogenous bovine kisspeptin with kisspeptin standard used. It showed good parallelism with the kisspeptin standard curve. For the biological validation of the assay, plasma kisspeptin was measured in blood samples collected from six non-lactating cyclic cows during entire estrous cycle and from 18 pregnant cows during different stages of pregnancy. The mean plasma kisspeptin concentration during different days of the estrous cycle was different ($P < 0.001$). Three peaks of kisspeptin were recorded, one on a day before appearance of preovulatory LH surge, second at day 6 and third one at day 18 of the estrous cycle. Plasma kisspeptin concentrations increased ($P < 0.001$) from first through last trimester of pregnancy. Kisspeptin concentrations were also measured in different follicular, luteal and placental tissues. Follicular and placental kisspeptin levels increased ($P < 0.01$) during follicular development and with the advancement of pregnancy, respectively. On the other hand, luteal concentrations of kisspeptin decreased ($P < 0.01$) with its developmental process. In conclusion, a simple, sufficiently sensitive and direct EIA procedure has been developed for the first time to determine plasma kisspeptin levels in bovine. A wide range of kisspeptin concentrations can be detected during different physiological stages in bovine using this kisspeptin–EIA procedure.

1. Introduction

Kisspeptin (a product of the KiSS1 gene) has been emerged recently as a master player in the regulation of reproduction in animals, mainly through controlling centrally the GnRH secretion (Seminara et al., 2003; Smith et al., 2006). Kisspeptin acts through its receptor, G-protein coupled receptor 54 (GPR54), also called KiSS1R. The initial product of the KiSS1 gene is a 145-amino-acid peptide, which is cleaved into shorter, biologically active peptides such as kisspeptin-54, kisspeptin-14, kisspeptin-13, and kisspeptin-10, where each number corresponds to the number of amino acids and with kisspeptin-10 representing the common C-terminal decapeptide sequence shared by all (Kotani et al., 2001).

Recently reported experimental evidences clearly indicates the involvement of kisspeptin in the process of animal reproduction,

* Corresponding author at: Animal Physiology & Reproduction Laboratory, ICAR-National Dairy Research Institute, Kalyani 741235, India.
E-mail address: drmmondal@gmail.com (M. Mondal).

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including positive and negative feedback of sex steroids on gonadotrophins secretion (Terasawa et al., 2010), generation of the preovulatory GnRH/LH surge that trigger and guide the tempo of sexual maturation at puberty (Lehman et al., 2010), metabolic regulation of fertility (Roa et al., 2008), photoperiodic control of reproduction in seasonal breeders like sheep (Chalivoix et al., 2010), onset of puberty (Amstalden et al., 2010; Clarkson et al., 2010) and, sex and species-specific differences of kisspeptin neurons in signaling hypothalamic GnRH cells (Homma et al., 2009; Kauffman et al., 2009). Determination of kisspeptin in peripheral circulation is, therefore, very important for studying the control of its secretion and its role on reproduction in bovine species. To the best of our knowledge, the information on secretion patterns of kisspeptin and its role on reproduction are not available during any physiological state in this species. Lack of information in this line may probably be due to non-availability of simple assay procedure to measure the hormone. Hence, we decided to develop and validate an efficient and sensitive enzyme immunoassay for kisspeptin in bovine plasma and apply the procedure for determining kisspeptin profiles during reproductive cyclicity and different stages of pregnancy in bovine species.

2. Materials and methods

2.1. Experimental animals

A total of six non-lactating cows that were in regular cyclicity and 18 pregnant cows of different stages of pregnancy viz., early (<10 weeks; $n = 6$), mid (>10 to <20 weeks; $n = 6$) and late (>20 to 36 weeks; $n = 6$) stages of pregnancy, weighing 321–370 kg and 3.5–5.3 years were selected for the experiment. The animals selected for the study were free from any anatomical and reproductive disorders and were not suffering from any health problems. These animals were fed *ad libitum* with mixture of locally available green grasses and tree leaves. Besides, all cows were fed with a concentrate mixture (92.7% organic matter, 19.0% crude protein, 6.6% ether extract, 4.9% crude fiber, 62.1% nitrogen free extract, 69.9% total carbohydrate, and 9.6% acid detergent fiber) at the rate of 2.0 kg per day per animal. The temperature and relative humidity recorded during the experiment were 27–32 °C and 69–93%, respectively.

2.2. Blood sampling

Blood samples (3 ml) were collected in heparinised tubes (20 IU heparin per ml of blood) by jugular venipuncture from six cycling non-lactating cows for 30 days to estimate plasma kisspeptin and luteinizing hormone (LH) for an entire reproductive cycle. Blood samples (3 ml) were also collected from 18 pregnant cows of early, mid and late stage of pregnancy ($n = 6$ each) for estimation of plasma kisspeptin. Blood samples were centrifuged immediately and plasma thus obtained was stored at −20 °C until assayed for hormones. All experimental protocols and animal care were approved by the Institute Research Council (IRC).

2.3. Preparation of tissue homogenate

Ovaries containing corpus luteum (CL) of different stages of estrous cycle (early, mid-cycle and mature CL), preovulatory (15–16 mm in size), growing (8–10 mm) and antrum (5–6 mm) follicles, uterus of cyclic cow, placenta of early, mid and late pregnancy were collected. All tissues (10 g each) were minced to small pieces and homogenized them in 10 ml of PBS with a glass homogenizer on ice. The resulting suspension was subjected to two freeze–thaw cycles to further break the cell membranes. After that, the homogenates were centrifuged for 10 min at 6000 × *g*. The supernatant were then separated and stored at −20 °C for hormone assay.

2.4. Analysis of the samples

Plasma LH was estimated in the samples collected throughout the entire reproductive cycle by a simple, sensitive enzyme immunoassay as detailed by Mondal et al. (2005a,b). The samples were analyzed for progesterone by radioimmunoassay for cyclicity monitoring and determining the day of estrus (Mondal et al., 2005a). Kisspeptin profile was analyzed by enzyme immunoassay procedure using the second antibody coating technique and the biotin–streptavidin amplification system as detailed below.

2.5. Enzyme immunoassay of kisspeptin

2.5.1. Preparation of affinity purified goat IgG to rabbit IgG

The affinity purified goat IgG to rabbit IgG was developed following the procedure of Anandlaxmi and Prakash (2001). Briefly, about 40 ml plasma from goats immunized against rabbit IgG containing 20 IU heparin/ml of blood was vortexed with rabbit IgG agarose and loaded onto a small column. First, non-specific proteins were eluted with PBS (0.5 M, 0.15 M NaCl, pH 7.2) buffer. Proteins bound specifically were eluted with 15 ml of 0.1 M glycine–HCl (pH 2.0). All steps were performed at room temperature. The eluted fractions (3 ml each) were collected in vials containing 0.2 ml of 1 M Tris–HCl (pH 8.0). The eluted IgG was dialyzed overnight against PBS and the protein content determined by measuring the absorbance spectrophotometrically at 260 nm and 280 nm, and extrapolated from a normograph.

2.5.2. Preparation of hormone free plasma

For the preparation of kisspeptin free plasma, blood samples were collected from cow at day 7 postpartum in which kisspeptin concentration in the blood anticipated being bare minimum. Plasma was prepared after centrifugation of the blood and to remove any trace amount of kisspeptin in the plasma, the plasma was treated with charcoal and dextran mixture as follows:

Mixture of activated charcoal (14 g) and dextran T-70 (1.4 g) per 100 ml plasma were taken and the mixture was washed with distilled water by through mixing using a magnetic stirrer overnight at room temperature. After allowing the mixture to stand for 5 min, the supernatant was discarded and the mixture was again subjected to the washing process for three more times at 8 h intervals to remove all traces of suspended fine charcoal particles. Plasma was then added to the washed charcoal–dextran mixture and mixed thoroughly for 2 h at 4 °C. Then the mixture was centrifuged at 3000 rpm for 1 h at 4 °C and the supernatant was filtered to remove suspended particles. The filtrate was again centrifuged at 10,000 rpm for 1 h at 4 °C and the supernatant was re-filtered. This kisspeptin free plasma was then stored at −20 °C for future use.

2.5.3. Preparation of biotinyl-kisspeptin conjugate

An amount of 1 mg of metastin (45–54), a decapeptide with C-terminally amidated LRF-motif, also called kisspeptin-10 with the peptide sequence of H-YNWNSFGLRF-NH_2 (EMD Millipore Corporation, Calbiochem, 290 Concord Road, Billerica, MA 01821, United States of America; Cat. No. # 445888) was dissolved in 1 ml of PBS (50 mM $NaPO_4$, 0.15 M NaCl) and added to 5 mg of biotinamido caproate-N-hydroxysuccinimide ester (Biotin, Sigma, Germany) dissolved in 50 µl of dimethyl sulfoxide. The reagents were vortexed and incubated further for half an hour. For separation of the biotinyl kisspeptin from free botin and/or any unbound kisspeptin, the mixture was loaded on a small Sephadex G-25 gel