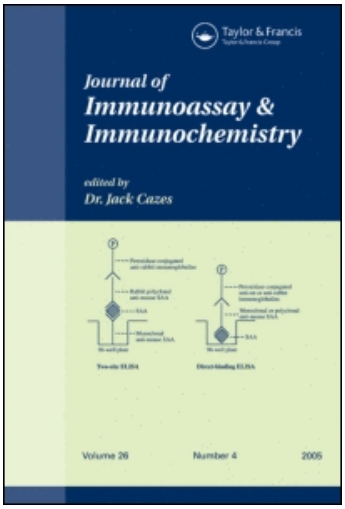

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Development and Validation of a Sensitive Radioimmunoassay Procedure for Estimating FSH in Mithun (*Bos frontalis*) Plasma

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Development and Validation of a Sensitive Radioimmunoassay Procedure for Estimating FSH in Mithun (*Bos frontalis*) Plasma

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Abstract: The present study was designed to develop and validate a simple and sensitive radioimmunoassay (RIA) procedure to estimate FSH in mithun (*Bos frontalis*) plasma. The assay was carried out in 100 μL of mithun plasma. The bovine FSH standards (10 to 5000 pg/100 μL/tube) in hormone-free plasma were used in the assay. The sensitivity of the assay was 20 pg/100 μL/tube, which corresponded to 0.20 ng/mL plasma. The 50% relative binding sensitivity of the assay was 80 pg/100 μL/tube, which corresponded to 0.80 ng/mL plasma. The intra- and inter-assay coefficients of variation were 4.6% and 12.4%, respectively.

The biological validation of the assay was carried out in plasma samples that were collected during different stages of the estrous cycle. In the entire estrous cycle, plasma FSH concentration ($p < 0.01$) attained two peaks (on day 3 to 4 before estrus 5.1 ± 0.3 ng/mL and on the day of estrus 6.9 ± 0.2 ng/mL). FSH concentration remained at basal level (1.3 ± 0.1 to 1.6 ± 0.2 ng/mL) during day 4 to 16 of the estrous cycle. The concentration of plasma FSH was found to be significantly ($p < 0.05$) higher (4.9 ± 0.3 to 6.8 ± 0.5 ng/mL) until 48 h following the estrus onset.

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In conclusion, the RIA procedure that was developed in the current study is sufficiently reliable and sensitive to estimate different physiological levels of FSH in mithun plasma.

Keywords: RIA, FSH, Standardisation and validation, Estrous cycle, Mithun

INTRODUCTION

In mammals, FSH plays a crucial role in follicular development and ovulation. The process of ovulation is associated with various morphological, chemical, and physiological changes in the ovulatory follicle. All these changes occur in the ovulatory follicle in response to the preovulatory LH and FSH surges.[1] The onset of estrus and preovulatory surges of LH and FSH are simultaneous events in cattle.[2,3] The action of FSH during preovulatory follicular development is the induction of LH receptors on granulosa cells.[4] In bovine, FSH is the initiator of ovarian activities, as it directly promotes the growth of the ovarian follicle. Before each wave of follicular emergence, a rise in plasma FSH above the basal level occurs and, as follicles develop, FSH concentration declines, which plays a key role in the emergence of follicular wave and subsequent follicle selection.[5,6] Therefore, the determination of peripheral FSH levels is important to study the follicular development during different phases of estrous cycle.

Mithun (*Bos frontalis*) is a unique domesticated free-range bovine species which is found in the northeastern hilly region of India and, in many pockets of southeast Asia. This species is believed to have originated more than 8000 years ago and is considered to be the domesticated form of wild gaur. This unique ungulate plays an important role in the economic, social, and cultural life of its rearers.[7] Due to the typical geographic distribution, this species has long remained unknown to researchers. Although, during the last two decades, many scientific studies have been carried out on various aspects of this species, authentic scientific studies on reproductive biology in this species are scanty at present.

To conduct the advanced researches in the field of estrous cycle endocrinology and follicular development, a reliable assay procedure to measure peripheral FSH level was urgently required. Therefore, the current study was designed to develop a simple and sensitive radioimmunoassay (RIA) protocol to estimate FSH in mithun plasma.

EXPERIMENTAL

Chemicals and Antibodies

The radioisotope, ^{125}I, that used in the assay was procured from Amersham Corp., Arlington Heights, IL, USA. The non-radioactive chemicals that were used in the assay were either procured from Sigma-Aldrich Chem.,

St. Louis, USA or from local reputed firms. The bovine FSH (bFSH), (USDA-bFSH-1-2, for standards and USDA-b-1-2, for radioiodination) that were used in the assay were procured through the USDA Animal Hormone Program, Beltsville, MD, USA. The rabbit anti-oFSH antiserum (AFP-C5288113) and anti-rabbit gamma globulin sheep serum that were used in the assay were obtained from Dr. A. F. Parlow through the program of the National Institute of Diabetes and Digestive and Kidney Diseases, Harbor-UCLA Medical Center, Torrance, CA, USA.

Radioiodination of FSH

The bFSH was radioiodinated by a modified method described previously.[8] Briefly, 5 mg bFSH were dissolved in 20 mL 0.3M phosphate buffer saline (pH 7.5) and was reacted with 800 mCi ^{125}I and 0.8 mg Chloramines-T for 5 min. This reaction was stopped by addition of 1 mg sodium metabisulfite, chromatographed on Sephadex G100, and eluted with 0.05 M phosphate buffer saline (pH 7.5). The peak fraction of the radioiodinated bFSH was diluted to 20,000 cpm/100 μL in the assay buffer (50 mM $NaPO_4$, 0.15 M NaCl, 0.02% Thimerosal, 0.1% BSA, and 0.1% sodium azide, pH 7.5).

Preparation of FSH Free Plasma

To prepare the FSH free plasma, the blood samples were collected on day 4 after parturition, when plasma FSH concentration was expected to be low. The plasma samples were treated according to the following steps to obtain the FSH free plasma.

In a beaker, 50 mL plasma, 10 g activated charcoal, and 1 g Dextran T-70 were taken. The content was stirred overnight at 4°C with the help of a magnetic stirrer. On the next day, the supernatant was collected after centrifugation at 3000 × g for 30 min. The entire procedure was repeated thrice.

Finally, the supernatant was collected and passed through 1.0, 0.45, and 0.22 μm filters. The filtered plasma was stored in small aliquots (2 mL) at −20°C after the test for hormone free plasma.

Test for Charcoal-Treated FSH Free Plasma

The charcoal-treated plasma was tested further to check for possible contamination of FSH. Two sets of 6 borosilicate glass tubes, containing either 100 μL charcoal-treated plasma or 100 μL assay buffer, and 100 μL FSH antiserum (rabbit anti-oFSH antiserum, dilution 1:80,000) and 100 μL bFSH tracer (20,000 cpm) were run in an assay according to the procedure that is described in the assay procedure section. The mean counts in the tubes that contained charcoal-treated plasma and assay buffer were not found to be different. The result confirmed that the charcoal-treated plasma was free from FSH.

Assay Procedure

The unknown plasma samples (100 μL), in duplicate, were pipetted into borosilicate glass tubes, followed by the addition of 100 μL FSH antiserum (dilution 1:80,000) and 100 μL bFSH tracer (20,000 cpm). The tubes were then incubated for 24 h at 4°C for the immune reaction. After incubation, to accomplish the precipitation of antibody-bound FSH, 100 μL anti-rabbit gamma globulin sheep serum (dilution 1:10) was added to the tubes and they were incubated further at room temperature for 2 h. Tubes were then washed with distilled water and centrifuged (3200 × g) at 4°C for 10 min. The supernatants were discarded and radioactivity in each pellet was determined with the help of a gamma counter (LKB Wallac 1277 automatic gamma counter, Wallac). Along with the unknown samples, the FSH standards (ranging from 10 to 5000 pg/100 μL/tube), prepared in charcoal-treated FSH-free plasma, were run in duplicate in the assay.

In addition to the above mentioned tubes, another three sets of tubes, in duplicate, were also run in the assay. These were a) non-specific binding tubes: contained 100 μL charcoal-treated FSH-free plasma, 100 μL assay buffer, 100 μL bFSH tracer, and 100 μL anti-rabbit gamma globulin sheep serum to determine non-specific binding in the assay; b) maximum binding tubes: contained 100 μL charcoal-treated FSH-free plasma, 100 μL FSH antiserum, 100 μL bFSH tracer, and 100 μL anti-rabbit gamma globulin sheep serum to determine the maximum binding of tracer with antiserum in the assay and; c) total count tubes: contained 100 μL bFSH tracer, 500 μL FSH antiserum, and 500 μL anti-rabbit gamma globulin sheep serum to determine the total counts of added tracer in the assay. All of these tubes were incubated for 24 h at 4°C, followed by the rest of the procedure that is described above for unknown samples and FSH standards.

Calculation of Percent Binding and Intra- and Inter-Assay Coefficients of Variation

The percent binding (relative binding) was calculated with the following formula:

$$\text{Percent binding} = \frac{\text{Maximum binding count} - \text{Nonspecific binding count}}{\text{Count of std or unknown} - \text{Nonspecific binding count}}$$

To calculate the intra- and inter-assay coefficients of variation (CV), the plasma samples of known FSH concentration (low 0.9 ng/mL and high 8.4 ng/mL) were run in duplicate in three places in each assay. The CV was calculated for within assay (intra-assay CV) and between assays (inter-assay CV) with the following formula:

$$\text{CV}(\%) = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Biological Validation of the Assay

The RIA protocol for estimation of FSH in mithun plasma that was developed in the current study was biologically validated with six healthy adult mithun cows during the estrous cycle. Jugular vein blood samples were collected in heparinised (20 IU/mL blood) polystyrene tubes daily form these animals during the two consecutive estrous cycles. Besides, from all the animals, since estrus onset, the jugular vein blood samples were collected in heparinised tubes, with the help of an indwelling jugular catheter, every 4 h for 72 h. The samples were kept on ice immediately after collection, plasma was separated within 1 h by centrifugation (1200 × g) for 20 min at 4°C, and stored at −20°C until analysed for FSH. All the experimental protocols and animal care met the Institute Animal Care Use Committee regulations during the investigation.

Data Analysis

All the statistical analyses were performed using the SPSS software package (SPSS 10.0.1., SPSS Inc., USA). To determine the significant variation in percent binding among different concentrations of FSH standard, the data were analysed by means of ANOVA. The model included concentration of FSH standard as a source of variation. The variations in plasma FSH concentration during the estrous cycle and during the 72 h following estrus onset were analysed by means of repeated measure ANOVA. The model included day of the estrous cycle and hour of collection, respectively, as sources of variation. The Student Newman Keuls (SNK) test was used to isolate means, when the effect was found to be significant.

RESULTS

Standardisation of RIA Protocol for Estimating FSH in Mithun Plasma

Titration of FSH Antiserum

The FSH antiserum was serially diluted from 1:20,000 to 1:640,000 to determine the optimum dilution of FSH antiserum to be used in the assay. The antibody titre of 1:80,000 was found to be most suitable and achieved a count of approximately 2400 in the maximum binding tubes.

Assay Validation

To investigate the possible influence of plasma volume on the assay sensitivity, FSH standards (10 to 5000 pg/100 μL/tube) in different volumes of charcoal-treated hormone-free plasma (0, 20, 50, and 100 μL) were run in

an assay. No difference in the absolute binding sensitivity between 20 and 100 μL plasma standards was observed and the sensitivity was found marginally lower than that of the buffer standards. However, the absolute binding sensitivity was found lowest in 50 μL plasma standards, in comparison to the standards in 20 and 100 μL plasma volumes and buffer (Figure 1). It was observed that the count was increased when standards were prepared in 100 μL plasma (Figure 2). Considering all of the above mentioned observations, the standards were subsequently prepared in charcoal-treated hormone-free plasma and run along with the unknown samples in the assay. The counts in the non-specific binding tubes, using different volumes of plasma, were found to be low and varied from 92 to 108. Therefore, in the assay, 100 μL of unknown plasma samples and standards per tube were run in duplicate to increase the sensitivity of the assay. The lowest FSH detection limit was significantly ($p < 0.01$) different from zero concentration and was observed to be 20 pg/100 μL/tube, which corresponded to 0.20 ng/mL plasma. The 50% relative binding sensitivity was found to be 80 pg/100 μL/tube, which corresponded to 0.80 ng/mL plasma.

Test for Homology Between bFSH and Endogenous FSH in Mithun Plasma

The homology between bFSH and endogenous FSH in mithun plasma was checked by conducting the parallelism test. The mithun plasma with a high level of endogenous FSH (6.2 ng/mL) was serially diluted (contained

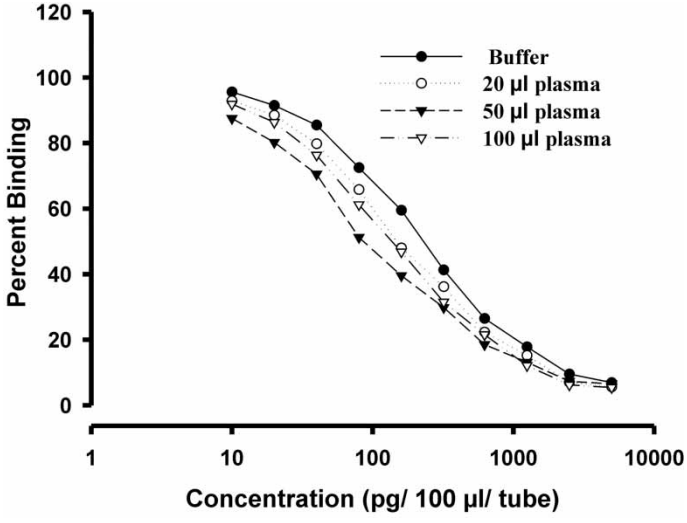

Figure 1. Influence of different plasma volumes (10, 50, and 100 μL of mithun plasma) on percent binding in the FSH standard curve.

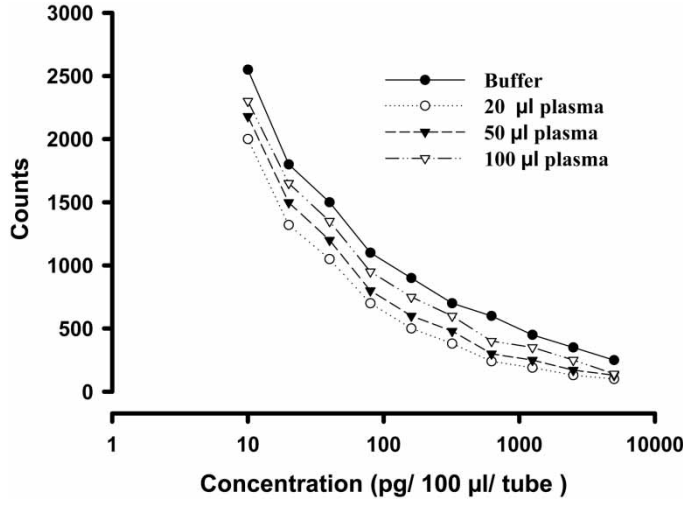

Figure 2. Influence of different plasma volumes (10, 50, and 100 µL of mithun plasma) on count displacement in the FSH standard curve.

100, 50, 25, 12.5, and 6.25 µL of mithun plasma volume) and run along with the bFSH standards in buffer in an assay. The test revealed a parallel drop in the relative percent binding when plotted with increasing plasma volumes and increasing standard concentrations (Figure 3). The result confirmed that the endogenous FSH in mithun plasma behaves immunochemically similar to the bFSH standard.

Intra- and Inter-Assay Coefficients of Variation

The intra- and inter-assay coefficients of variation that were determined, using plasma samples of known FSH concentration 0.9 and 8.4 ng/mL, were found to be 4.6% and 12.4%, respectively.

Biological Validation

The representative variations in plasma FSH concentration on different days of the estrous cycle are depicted in Figure 4. It was observed that FSH concentration attained two peaks ($p < 0.01$) in the entire cycle. The concentration was gradually increased from day 5 before estrus and attained the first peak on day 3 to 4 before estrus (5.1 ± 0.3 ng/mL). The second FSH peak was observed on the day of estrus onset (6.9 ± 0.2 ng/mL) and the concentration was decreased gradually thereafter. The FSH concentration ($p < 0.01$) remained at the basal level (1.3 ± 0.1 to 1.6 ± 0.2 ng/mL) during day 4 to 16 of the cycle. The secretion pattern of plasma FSH around estrus in mithun

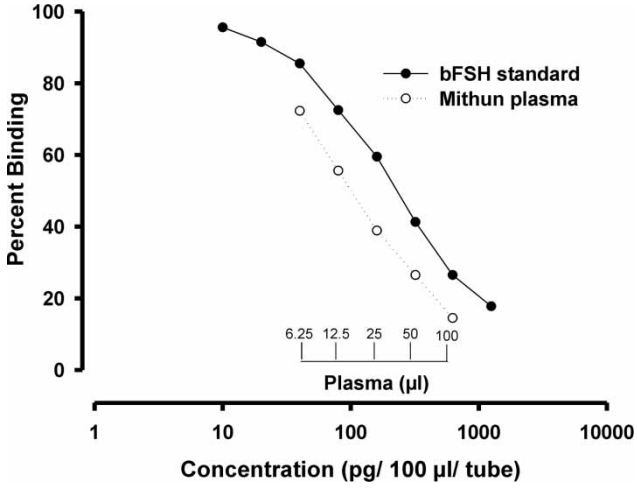

Figure 3. Parallelism between the curves of serially diluted mithun plasma containing high endogenous FSH (6.2 ng/mL) and bovine FSH (bFSH) standards prepared in the assay buffer.

is depicted in Figure 5. The concentration of plasma FSH was found to be significantly ($p < 0.05$) higher till 48 h following the estrus onset and it decreased thereafter. The average FSH concentrations (4.9 ± 0.3 to 6.8 ± 0.5 ng/mL) during this phase were found to be considerably higher than the basal FSH concentration that was observed during day 4 to day 16 of the estrous cycle.

DISCUSSION

To the best of our knowledge, the FSH-RIA protocol that is described in this manuscript is the first report of standardization and validation of a sensitive RIA procedure for estimating FSH in mithun plasma. A wide range of plasma FSH concentrations (0.8 to 9.8 ng/mL) could be detected during different stages of the estrous cycle with this RIA protocol. The study revealed that the FSH-RIA protocol that is standardized in the current study is sufficiently sensitive and reliable for estimating FSH in mithun plasma.

The test of homology between bFSH and endogenous FSH in mithun plasma revealed that the mithun FSH behaves immunochemically similar to the bFSH. The sensitivity of the present FSH-RIA protocol is comparable to or higher than the previously described RIA method for estimating FSH in cattle.[9–11] However, the RIA methods for FSH estimation with comparatively higher sensitivity have been demonstrated in previous reports.[12–14] The intra- and inter-assay CVs of the present FSH-RIA are comparable to the previous reports in cattle.[9,11,14,15]

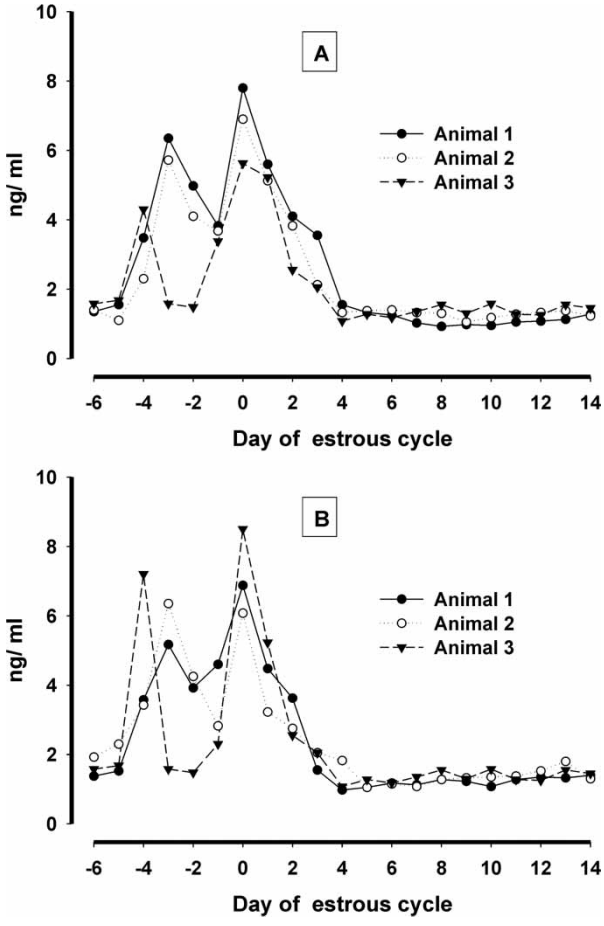

Figure 4. Representative variations in plasma FSH concentration on different days in the mithun estrous cycle; Day 0 indicates the day of estrus onset; Panel-A: Cycle 1; Panel-B: Cycle 2.

In the estrous cycle, the plasma FSH concentration attained two peaks. The first peak was observed on day 3 to 4 before the onset of estrus, followed by the second peak on the day of estrus. It has been shown, previously, in ruminant species, that the surges of FSH are rhythmic and periodic during the estrous cycle and an increase in plasma FSH concentration is observed around the day of follicular wave emergence.[5,10,16,17] In the present investigation, when repeated samples were collected at 4 h intervals following estrus onset, the FSH concentration was found to be significantly higher until 48 h, and then it decreased towards the basal level thereafter. In bovine, the onset of estrus and preovulatory surges of LH and FSH are simultaneous events.[2,3] These preovulatory LH and FSH surges are responsible for

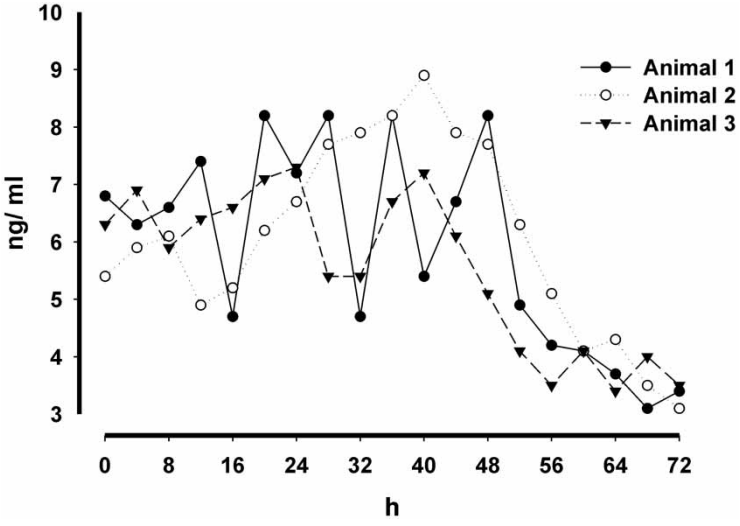

Figure 5. Representative variations in the plasma FSH concentration during 72 h following the estrus onset in mithun; 0 h indicates the onset of estrus.

various morphological, chemical, and physiological changes in the ovulatory follicle. These changes cause the final maturation and rupture of the ovulatory follicle to release a fertilisable ovum.[1] In mithun, the sustained increase in FSH level following the estrus onset is likely to be associated with the final maturation of the ovulatory follicle and subsequent ovulation.

In conclusion, the RIA procedure that was developed in the current study is sufficiently reliable and sensitive to estimate FSH in mithun plasma. A wide range of FSH concentrations can be detected during different physiological stages in mithun using this FSH-RIA procedure.

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