



Exploring the impact of exogenous Kisspeptin-10 on ovarian follicular development and estrus behaviour in Lakhimi cows of Assam

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ABSTRACT

The present study was carried out to assess the influence of exogenous intravenous kisspeptin-10 on the follicular dynamics, hormonal profile, and expression pattern of KiSS1 and KiSS1R of Lakhimi cows of Assam during the estrus period. Cows were examined every alternate day during the estrous cycle using a linear array ultrasound scanner and profiled in an ovarian map. The follicular wave patterns were mostly (66.7%) 2-wave cycle and 33.3% with 3-wave cycle in both treated and control group of cows. Kisspeptin-10 induced an early emergence of follicular waves with more follicular count and accelerated growth of dominant follicle in both 2 and 3 waves in treated group compared to the control. Significant augmentation of serum concentration of progesterone (10th day), LH (day 1) and estradiol (on the day before estrus) in treated cows was observed, however the hormonal dynamics were similar to other mammals. Administration of Kisspeptin-10 has elicited a significant rise in the serum kisspeptin level with gradual increase of the transcripts encoding *Kiss1* and *Kiss1R* genes (highest on 3rd day of estrous cycle) and declined gradually till day 11. Both the transcripts encoding *Kiss1* and *Kiss1R* genes were gradually increased from day 17 and were higher two days before the beginning of the next estrous cycle. Intravenous administration of exogenous Kisspeptin-10 can be promising approach to augment the ovarian follicular dynamics in regards to early onset of wave, follicular numbers and diameter, to explore the optimum fertility in indigenous cows, Lakhimi.

Keywords: Follicular dynamics, Kisspeptin, KiSS1, Lakhimi cow, Ovarian follicle

The indigenous cattle of North-Eastern India, are well known for their high endurance, resistance, adaptability, and heritability. Lakhimi is one such popular indigenous cattle breed of Assam and other nearby north-eastern states of India, constituting 7.9 million out of 8.4 million indigenous cattle. Successful reproduction is the indispensable phenomenon for the upliftment of cattle husbandry and to preserve the germplasm with valuable traits. Deviation in reproductive rhythm can cause issues like low conception, poor performance, and infertility. Ovarian function is characterized by a follicular wave along with an interplay of hormones, genes, environment, nutrition, breed, and individuality. Kisspeptin is a small peptide secreted from hypothalamic KP neurons, product of the *KiSS-1* gene, and receptor KISS1R, functions through HPG axis which influences the regulation of ovarian steroids (Masumi *et al.* 2022, Sivalingam *et al.* 2022). It has exposed a new potential to interpret the regulatory mechanisms

of ovarian function like, follicle development, oocyte maturation and ovulation. Expression of KPs and KPRs has also been detected in extra hypothalamic sites like brain, placenta, ovary, etc (Bowe *et al.* 2019). KP-signalling has been found to be critical for onset of puberty, metabolic regulation of fertility, oocyte survival, maturation and ovulation (Abbara *et al.* 2020 and Ruohonen *et al.* 2022). The application of exogenous kisspeptin for the assessment of follicular dynamics and therapeutics has been reported by Khan *et al.* (2019) and Burke *et al.* (2022). Determination of kisspeptin in peripheral circulation is important to assess its secretion and role in reproduction in bovine species. A perusal of available literature indicated that no study has been done to study the effects of intravenous kisspeptin-10 administration on follicular development, ovarian steroid profile, and ovulation in Lakhimi cows and to characterize the roles of the Kisspeptin-Kiss1R system during the entire estrous cycles both in spontaneous estrus and kisspeptin-induced estrus in Lakhimi cow of Assam, which helped design this study.

MATERIALS AND METHODS

The study was conducted at the Experimental Animal Shed, NRL (Nuclear research laboratory), Department

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of Veterinary Physiology, Department of Veterinary Microbiology and Animal Biotechnology Department, College of Veterinary Science, AAU, Khanapara, Assam India. The experiment was approved by the Institutional Ethical Committee Approval 770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/569.

Experimental animals and their management: For the study, six adult healthy cyclic cows were selected. Cows were housed together in well-ventilated sheds with *ad lib.* fresh and clean water throughout the study period. Lakhimi cows were selected for drug effect studies which were initially studied as a Control group and later treated with kisspeptin injection. Kisspeptin-10 was injected intravenously @1.3 µg/kg body weight on the day of estrus.

Ultrasound scanning of the ovary: All cows were scanned by using a real-time, B-mode, ultrasound scanner (M turbo C, Fujifilm Sonosite Inc., Bothell, USA) equipped with a 7.5-9.0 MHz linear rectal transducer as per the procedure mentioned by Pierson *et al.* (1984) in cows. The ovaries of each cow were examined every day throughout estrous cycle as described by Tom *et al.* (1998). The number of follicles recruited (as evidenced by small 3-4 mm size follicles on the day of emergence) and the characteristics of the dominant follicles (DFs) during the first follicular wave (Wave 1) and ovulatory wave were compared. As in the follicular study, the luteal structure was mapped during each examination and the developmental parameters, viz. the maximum diameter, the day at which it attained the maximum diameter and the day of initiation of constant regression, the position of the antral follicle, luteal dimensions were recorded in different waves. Each antral follicle (≥ 3 mm in diameter) in each section was drawn

on an ovarian map. Small, medium, large, and ovulatory follicles (Fig. 1) were identified with diameters of 3-6 mm, above 6-9 mm, above 9 mm, respectively.

Blood samples and hormone assay: Blood samples (5 ml) were collected on every alternate day of estrous cycle in heparinized tubes. The concentration of progesterone was measured with a solid phase RIA kit (Progesterone C.T. RIA kit (Pkg: 100 T) Batch No 181008D, M/s Beckman Coulter) and the radioactivity was counted in a ^{125}I (STRATEC Germany) gamma counter. Estradiole and LH was estimated based on competitive enzyme immunoassay technique using Bovine Estradiol (Catalog No-CSB-E08173b, CUSABIO) and Serum LH (Catalog no. CSB-E12826B, CUSABIO) ELISA kit. Serum Kisspeptin was assayed by quantitative sandwich enzyme immunoassay technique using Bovine Kisspeptin-1 (KISS-1) ELISA kit (Biocodon Technology, USA). The blood samples were processed for characterization of the Kisspeptin-*KISS1/KISS1R* genes. RNA isolation was done by TRIzol Method (Amresco/Invitrogen) and cDNA was synthesized by reverse transcription process.

Analysis of results were done by using StepOne software v 2.1. The levels of various hormones were analyzed by Independent Sample T-test using SPSS (Statistical Package for Social Sciences) and Graph-pad Prism V-4.0 software. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Limited studies suggest that Kp may be effective in optimizing follicular dynamics during estrous cycles in indigenous cows (Lakhimi). In the present study to evaluate the effect of kisspeptin on ovarian follicular dynamics

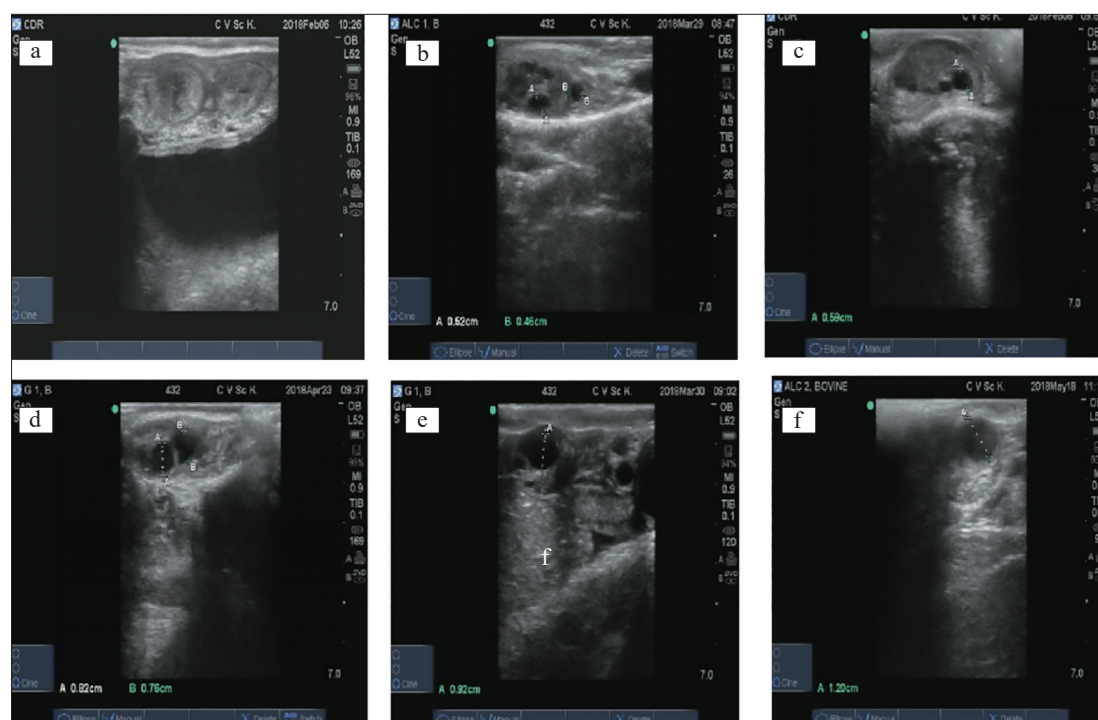


Fig.1. Ultrasonographic images showing the cross section of uterus (a) and small ovarian follicles (b), the medium-sized ovarian follicles (c,d), large-sized and ovulatory follicles (e,f).

and hormone in Lakhimi cows, exogenous Kisspeptin-10 @1.3 µg/kg body wt. was administered intravenously. Some previous studies have reported about the effectiveness of exogenous Kp administration, in cattle follicular dynamics (Leonardi *et al.* 2018, 2020, Macedo *et al.* 2021 and Burke *et al.* 2022).

The characteristic follicular wave patterns were recorded in both treated and untreated cows (Islam *et al.* 2020). Two types of follicular waves were either 2 or 3 wave cycle, with the dominance of two follicular waves (66%). These observations are in support with Islam *et al.* (2020). Cycles with three follicular (Gambini *et al.* 1998 in Zebu), four and five follicular waves (Viana *et al.* 2000 in Gir cows) were also reported. However, administration of exogenous kisspeptin did not show any change in the number of follicular waves in Lakhimi cows. Number of factors like time of CL regression, follicular growth rate, length of the luteal phase, follicular size, estradiol concentration, epigenetics, cellular adaptation (De Lima *et al.* 2020), peaks of FSH and IGF (Dogan *et al.* 2020) may have role in determining number of follicular waves during the estrous cycles. It is evident here that exogenous kisspeptin administration caused an early initiation, of follicular waves due to shortening of follicular wave lengths in Lakhimi cows, which is inconsistent with Pottapenjara *et al.* (2018), Rajin *et al.* (2022) and Khan *et al.* (2019). The significant role of Kisspeptin molecule in the regulation of GnRH/luteinizing hormone, accelerated follicular development, rupture of follicle, and ovulation were reported by various authors (Rajin *et al.* 2022 and Mishra *et al.* 2019). The follicular number increased in both 2 and 3 wave in the Lakhimi cows treated with exogenous kisspeptin ($P < 0.05$). Observations were in support of Leonardi *et al.* (2020) in cows. Significant increase in maximum size of the DFs during both waves in treated Lakhimi cows were in agreement with the Khan *et al.* (2019), Rajin *et al.* (2022), De Lima *et al.* (2020) and Kaya *et al.* (2023) (Tables 1 to 4, Fig. 2). In the present study, DF reached maximum size during the luteal phase, persisted for 3-6 days and then underwent atresia while second wave DF reached its maximum size during luteal regression and ovulated. This finding

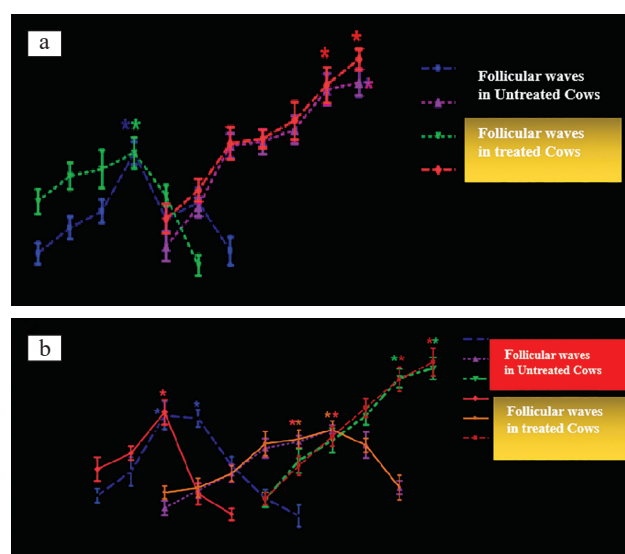


Fig. 2. Graphical representation showing the effect of Kisspeptin on follicular dynamics of Lakhimi cows with two (a) and three (b) follicular waves during the estrous cycles.

matches the reports by Miura *et al.* (2019). Administration of intravenous kisspeptin has induced accelerated growth of ovarian follicles, early onset of atresia, and shorter duration of persistence of wave during both 2 and 3 waves in Lakhimi cows compared to the control group. Findings were consistent with the findings of Pottapenjara *et al.* (2018), Khan *et al.* (2019), Dogan *et al.* (2020), Rajin *et al.* (2022) and De Lima *et al.* (2020).

Hormonal profile in Lakhimi cows concerning follicular dynamics: The trend of serum progesterone (ng/ml) did not show any difference in untreated and treated cows, however significantly higher concentrations were recorded on the 8th, 10th, 16th, and 18th day ($P < 0.05$) which is inconsistent with Leonardi *et al.* (2020), Guo *et al.* (2022), Matta *et al.* (2023). A wide range of serum progesterone levels were observed during the follicular phase and luteal phase of the estrous cycle in Lakhimi cows treated with kisspeptin. The serum concentration of estradiol was significantly higher in treated group on the day before estrus (day 20) and on the day of heat (day 0) in the treatment group compare to the control group, similar observations were also recorded

Table 1. Different ovarian follicular characteristics (Mean±SE) during two-wave cycles in treated and untreated Lakhimi cows

Characteristics	Follicular wave			
	1 st wave (Anovulatory)		2 nd wave (Ovulatory)	
	Control	Treatment	Control	Treatment
Day of wave emergence	1.30±0.30	0.90±0.27	11.11±0.47	10.11±0.27*
No. of follicles (>3mm)	3.6±0.42	4.8±0.42*	4.5±0.49	5.5±0.45*
Day of emergence of DF	5.8±0.42	4.9±0.42	15.04±0.60	13.85±0.35*
Maximum diameter of DF	8.85±0.49	9.85±0.49	13.34 ±0.54*	14.8 ±0.54*
Day of maximum diameter	6.96±0.40	6.06±0.40	20.65±0.30	19.65±0.30
Day of onset of atresia	8.97±0.30	8.12±0.70	-	-
Termination of wave	13.88±0.42	12.88±0.42	-	-
Duration of persistence of wave	14.65	14.32	10.84±0.65	10.25±0.65

*, Level of significance ($P > 0.05$).

Table 2. Different ovarian follicular diameter (Mean±SE) during two-wave cycles in treated and untreated Lakhimi cows

Day	1 st wave (Anovulatory)		2 nd wave (Ovulatory)	
	Control	Treatment	Control	Treatment
Day 0	4.4000±0.64721	6.71±0.62	0	0
Day 2	5.5000±0.59193	7.61±0.77	0	0
Day 4	6.117±0.44666	7.91±0.806	0	0
Day 6	8.8967±0.59165	8.91±0.85	0	0
Day 8	5.7950±0.57762	6.88±0.64	0	5.89±0.72
Day 10	6.5983±0.59200	3.97±0.64	4.5083±0.65518	7.10±0.58
Day 12	4.5117±0.61159	0	6.4950±0.41261	9.20±0.69
Day 14	0	0	8.9100±0.54472	9.35±0.59
Day 16	0	0	9.1950±0.53611	9.58±0.60
Day 18	0	0	9.5950±0.59092	11.55±0.84
Day 20	0	0	11.4200±0.68172	12.65±0.54
Day 21	0	0	11.7117±0.55980	12.75±0.40

*, Level of significance (P > 0.05).

Table 3. Different ovarian follicular characteristics (Mean±Se) of three-wave cycles in treated and untreated Lakhimi cows

Characteristics	Follicular wave					
	1 st (Anovulatory)		2 nd Anovulatory)		3 rd (Ovulatory)	
	Control	Treatment	Control	Treatment	Control	Treatment
Day of Wave Emergence	0.88±0.36	0.70±0.36	7.55±0.69	7.02±0.49	12.56±0.51	10.8±0.54*
No. of Follicles (>3mm)	3.93±0.57	4.7±0.67	2.94±0.30	3.94±0.50	3.86±0.33	5.25±0.33*
Day of emergence of DF	4.88±0.30	4.35±0.30	9.46±0.31	8.46±0.38	15.77±0.62	14.77±0.62*
Maximum diameter of DF	9.71±0.87	10.4±0.54	8.41±0.26	8.66±0.26	12.81±0.89	13.94±0.45
Day of maximum diameter	6.56±0.47	5.94±0.47	15.71±0.76	14.71±0.76	20.78±0.33	20.46±0.33
Day of onset of atresia	8.02±0.54	7.60±0.54	15.46±0.60	15.02±0.60		
Day of termination of wave	14.56±0.42	14.20±0.42	19.55±0.47	18.55±0.49		
Duration of persistence of wave	14.36±0.40	13.36±0.40	11.9±0.60	10.9±0.60	7.33±0.47	7.24±0.47

*, Level of significance (P > 0.05).

Table 4. Mean±SE of the follicle diameter (mm) at different days in three-wave cycles in treated and untreated Lakhimi cows

Days	1 st Follicular wave		2 nd Follicular wave		3 rd Follicular wave	
Day 0	-	-	-	-	-	-
Day 2	4.51±0.43	6.10±0.75				
Day 4	5.94±0.91	7.01±0.43				
Day 6	9.71±0.87	9.81±0.69	3.66±0.47	4.51±0.40	-	
Day 8	9.72±0.54	4.51±0.66	4.82±0.49	5.11±0.59	-	
Day 10	6.63±0.54	3.31±0.37	5.911±0.56	6.21±0.47	-	4.63±0.40
Day 12	4.54±0.55		7.58±0.60	7.81±0.78	4.28±0.46	6.81±0.61
Day 14	3.20±0.70		7.90±0.38	8.21±0.56	6.78±0.77	8.07±0.62
Day 16	-		8.71±0.26	8.80±0.55	7.81±0.78	9.91±0.59
Day 18	-		7.72±0.822	7.91±0.44	9.69±0.58	11.72±0.76
Day 20	-		4.91±0.44	4.92±0.78	12.20±0.59	12.91±0.87
Day 21	-		-		12.81±0.69	13.25±0.57

*, Level of significance (P > 0.05).

by Khan *et al.* (2019) in Mithun and Rajin *et al.* (2022). Kisspeptin greatly increases GnRH/LH release and GnRH neuron firing activity which may be involved in promoting serum concentration of estradiol, LH (Rizzo *et al.* 2018). Kaya *et al.* (2023) reported that estrogen concentrations increased with follicular development, increase KP-10 concentrations, with a positive feedback mechanism.

Significant LH profile were recorded on day 1 of the

estrous cycle in treated group as compared to untreated group and it maintained at very low concentration on other days of estrous cycle. This is in agreement with Rajin *et al.* (2022), Macedo *et al.* (2021), and Pottapenjera *et al.* (2018). It is also relevant that the follicular development is mainly stirred by the action of LH but not FSH after DF selection, as previously reported (Bruke *et al.* 2022, Macedo *et al.* 2021 and Vikram *et al.* 2023). (Supplementary Table 1,

Supplementary Fig.1).

Serum kisspeptin: The significant enhancement in serum Kisspeptin level on day 1, 6, 20 of estrous cycle of Lakhimi cows were observed in treated cows ($P < 0.05$) compared to untreated ones, which is in support with observations made by D'Occhio *et al.* (2020). The emergence of the first peak of kisspeptin a day before preovulatory LH surge may be required for ovulation, as kisspeptin is a potent gonadotropin secretagogue in animals. The other peak of concentrations of kisspeptin during early (day 6) and day 20 may be required to increase blood FSH for follicular wave emergence in bovines (D'Occhio *et al.* 2020, Macedo *et al.* 2021 and Vikram *et al.* 2023). The relative abundance of the transcripts encoding *Kiss1* and *Kiss1R* genes was found to be higher on the day of estrus and decreased till the 11th day of the estrous cycle, becoming negligible on day 13th of the estrous cycle. Kisspeptin concentration was recorded to be the highest two days before the beginning of the next estrous cycle and correspondingly the two genes *KiSS1* and its receptor gene (*KiSS1R*) have also been expressed more abundantly than any other days of the cycle. Similar patterns of *KiSS1* and its receptor gene (*KiSS1R*) expression were reported by Mondal *et al.* (2015), Khan *et al.* (2019), D'Occhio *et al.* (2020) and Bruke *et al.* (2022) (Supplementary Table 1 and Supplementary Fig. 2).

It can be concluded that administration of intravenous exogenous Kisspeptin-10 @ 1.3 µg/kg body weight augmented the growth of ovarian follicles, mean diameter of dominant follicles and numbers of follicles during each wave. The mean concentration of ovarian steroids (LH, Estrogen, and progesterone) was significantly enhanced, maintaining the normal dynamics with more abundant expression patterns of transcripts encoding *KiSS1* and *KiSS1R* genes during different days of the estrous cycle. Successful reproductive performance can be assessed through success of conception and offspring performance, which may be evaluated in future line of work for Lakhimi breed of cow.

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