



Exogenous kisspeptin (kp-10) resumes cyclicity in postpartum anestrus mithun cows

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Received: 20 September 2018; Accepted: 16 January 2019

ABSTRACT

Present study was conducted to assess the effect of exogenous kisspeptin administration on estrus induction, Kiss1 gene expression, endocrinological profiles and follicular development in post partum (PP) anestrus mithun cows. Animals who failed to resume cyclicity after day 90–100 of parturition were selected. Experimental cows were examined thoroughly through per-rectal as well as ultrasonographic examination to confirm the anestrus status. PP anestrus healthy mithun cows (12), aged 5–6 years (body condition score: 5–6 of 10) were selected with uniform anestrus status and randomly divided into 2 groups namely treatment (Gr I) and control (Gr II), each group consist 6 cows. Anestrus cows in Gr I were injected with kisspeptin @ 1.30 µg per kg body weight at 3 day interval for 21 days after 90–100 days of post parturition whereas in control group, normal saline was injected as placebo. Blood samples were collected on the days of injection and at estrus in both the groups. Trans-rectal ultrasonography was done at 3 days interval till onset of estrus and ovulation to study the follicular development. Exogenous kisspeptin administration caused significantly early resumption of cyclicity in treatment as compared to control (24.64±10.43 vs 66.56±14.66 days) and significantly increased kiss1 and GPR54 mRNA expression in treatment as compared to control on the day of estrus (1.943±0.29 vs 0.424±0.062 and 1.84±0.31 vs 0.416±0.082, respectively). Similarly, circulating level of estradiol and follicle stimulating hormone (FSH) increased gradually after exogenous administration of kisspeptin, which reached peak on the day of estrus in treatment group (25.36±1.27 pg/ml and 15.65±1.22 ng/ml, respectively) whereas no significant difference was observed between days of treatment in control group except on the day of estrus (11.29±1.76 pg/ml and 9.86±1.06 ng/ml, respectively). Level of estradiol and FSH on the day of estrus was significantly higher in treatment as compared to control whereas non-significant difference was observed in plasma progesterone concentration. Number of medium and large follicles increased in treatment whereas only small follicles were observed in control group. Improved endocrinological profiles, follicle development and kiss gene profiles in post partum anestrus mithun cows following exogenous kisspeptin indicates that kisspeptin induced or resumed the cyclicity in early.

Key words: Anestrus, Endocrinological profiles, Estrus induction, Follicular development, *Kiss 1*, Mithun, Post-partum

Mithun (*Bos frontalis*), pride of North-Eastern hilly (NEH) region of India, is heavily built, semi domesticated bovine species. Chaudhari *et al.* (2012) reported that Indian livestock species including mithun suffer different reproductive failure including anestrus, late maturity, poor estrus expression, delayed ovulation, long post partum calving intervals. Shisode *et al.* (2009) reported that mithun breeds throughout the year and no definite breeding season is observed. He further reported that mithun is polyestrous animal and inter-calving period of 400 days; however, Giasuddin *et al.* (2003) reported day's open of 172 days and inter-calving interval of 465 and 838 days for natural service and artificial insemination, respectively in mithun.

Duration of postpartum acyclicity/anestrus is influenced

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by suckling status, nutritional status, calving season and age as in beef cows (Yavas and Walton 2000). Although uterine involution begins and ovarian follicular waves resume soon after parturition, dominant follicles of these waves fail to ovulate due to failure to undergo terminal maturation. As a result, postpartum anovulatory dominant follicles are smaller than the ovulatory follicles in cyclic cows. Failure of postpartum dominant follicles to undergo terminal maturation is due to absence of appropriate LH pulses, a prerequisite for follicular terminal maturation prior to ovulation.

Kisspeptin (kiss-1), a peptide encoded by the *Kiss1* gene, has attracted attention as a key molecule in the regulation of gonadotrophin releasing hormone (GnRH)/Gonadotropic hormones (LH/FSH) release in many mammalian species including rodents, ruminants and primates (Tena 2006, Naniwa *et al.* 2013). Kisspeptin and its receptor (GPR54) have emerged as key players in regulation of reproduction in animals. Pratheesh *et al.* (2013) reported that kisspeptin

stimulate the secretion of gonadotropin from pituitary by stimulating the release of GnRH from the forebrain after the activation of GPR54, which is expressed by GnRH neurons. Kisspeptin is expressed abundantly in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) of forebrain. Kisspeptin neurons express the estrogen receptor and androgen receptor and these cells are direct targets for the action of gonadal steroid in both male and female animal.

Kisspeptin induces pulsatile GnRH/LH release in various species (Caraty *et al.* 2007, Ezzat *et al.* 2009, Joseph *et al.* 2015) suggesting that kisspeptin–GPR54 signaling plays a pivotal role in regulation of reproductive functions. Moenter *et al.* (1992) reported that in the ovaries of cows, antral follicle growth is stimulated by the action of gonadotropin and basal release of GnRH from the hypothalamus into hypophyseal portal circulation maintain tonic gonadotropin secretion from pituitary, resulting in follicular development and steroidogenesis in the ovaries (Knobil 1980). It has been shown that exogenous repetitive administration of kisspeptin have been shown to increase circulating concentrations of LH and FSH secretion in pre-pubertal cattle and estrogen secretion, followed by the pre-ovulatory LH surge in acyclic ewes and cattle (Ezzat *et al.* 2009). Since inter-calving period and days of calving are quite long in mithun and studies to reduce post partum interval has not been reported earlier. The objective of the present study was to reduce the inter-calving interval by reducing post partum interval in anestrus mithun cows following exogenous administration of metastin (kisspeptin analogue).

MATERIALS AND METHODS

Selection of animals: Twelve mithun cows (age 5–6 years, body condition score: 5–6 of 10), who failed to resume cyclicity after 90–100 days of parturition were considered as anestrus and were selected from mithun farm of the institute. All the animals were maintained under uniform management and nutritional condition throughout the experiment (farm schedule). Experimental cows were examined thoroughly through per-rectal palpation as well as ultrasonographic examination to confirm the anestrus status and were selected with uniform anestrus status. Ovaries were scanned with a linear array trans-rectal probe (7.5 MHz transducer). Post-partum anestrus status was confirmed by absence of >4 mm follicle or functional corpus luteum in a weekly scanning of ovaries over a period of 21 days following 90–100 days of post parturition. To standardize counting of follicles, each ovary was scanned from end to end to identify the positions of the corpus luteum and antral follicles. Animals were randomly divided into two groups, treatment (Gr I) and control (Gr II), each group consisted 6 cows.

Kisspeptin administration and hormone estimation: Anestrus cows in Gr I were injected intramuscularly with kisspeptin @ 1.30 µg per kg body weight at 3 day interval for 21 days after 90–100 days of post parturition whereas in control group, normal saline was injected with equal

quantity as placebo. The blood samples were collected by venipuncture of jugular vein in heparin tubes (20 IU of heparin/mL of blood) from cows from both control and treatment groups. The blood samples were centrifuged at $1200 \times g$ for 15 min at 4°C. The plasma samples were separated rapidly, labelled properly and preserved at –80°C in deep freezer for hormone estimation. Serum oestrogen and progesterone were estimated by using radioimmunoassay technique (Immunotech, France). Analytical sensitivity for estimation of oestradiol and progesterone was 6.2 pg/ml and 0.32 ng/ml, respectively. The intra- and inter assay coefficients of variation of oestradiol and progesterone were 12.1 and 11.2% and 8 and 12%, respectively. Follicle stimulating hormone (analytical sensitivity: 0.1 mIU/mL; intra- and inter-assay coefficients of variation: 6.47 and 9.54%, respectively) was estimated with commercially available bovine ELISA kit (MyBiosource, San Diego, CA, USA) by optical density (λ 450 nm) in 96-well clear polypropylene microplate using a MRC Microplate Reader (UT-2100C, Israel).

Leukocyte separation and RNA isolation: Blood leukocytes were separated using erythrocyte lysis buffer (10×) consisting 41.2 g ammonium chloride (NH_4Cl), 0.2 g disodium EDTA salt and 5 g KHCO_3 thoroughly dissolved in 500 mL of double distilled water from the blood samples. The required lysis (1×) buffer was arranged by diluting the 100 mL of 10× buffer into 900 mL of double distilled water properly. These samples were centrifuged thoroughly at $1200 \times g$ for 10 min. The process of lysis as well as centrifugation was 3–4 times repeated to obtain a clear white pellet of cells. The white pellet was dissolved properly in 500 µl of phosphate buffer saline (PBS) and preserved at –80°C for storage for further use. Total RNA was extracted as per the standard protocol by using commercially available kit (RNase mini kit, Qiagen; USA) from blood cells. Quality of RNA was tested and taken into account when the ratio of optical density at 260 nm/280 nm was >1.9. The RNA thus isolated was stored at –80°C for future use.

cDNA isolation and amplification: By using commercially available standard kit (Thermo Scientific Revert Aid H Minus First cDNA synthesis kit; USA), the total RNAs were reverse transcribed into cDNA. By using commercial PCR master mix as well as different annealing temperatures in a PCR thermal cycler, the PCR cyclic conditions of selected target genes such as *Kiss1* and *GPR54* were standardized (Table 1). The final amplified PCR product was run and verified by using 1.5% (w/v) agarose gel electrophoresis using 100 bp DNA ladder.

Quantitative real-time PCR: The selected target genes such as *Kiss1* and *GPR54* were thoroughly amplified with real-time PCR system using commercially available standard kit (Quantifast SYBR Green, Qiagen, USA). The reaction mixture was prepared for qRT-PCR and run qRT-PCR as per the instruction of manufacturer in real-time PCR (Thermo Scientific Piko-Real real time PCR, Finland). Threshold cycle (Ct) value for target as well as house-keeping genes was measured for final evaluation of fold of

Table 1. Primers used for amplification of *GAPDH*, *Kiss1* and *GPR54* gene

Primer name	Primer sequence	Product size (bp)	Annealing temperature
<i>GAPDH</i>	F-5'-CCTGGAGAAACCTGCCAAGT-3' R-5'-GCCAAATTCATTGTCGTACCA-3'	218	58°C
<i>Kiss1</i>	F-5'-GGGCCCCGAGAAAGGCTTTG-3' R-5'-TGTGGGAGCACAGTGGTCTTTGC-3'	526	70°C
<i>GPR54</i>	F-5'-CAGTTCATTGCCCATAGGG-3' R-5'-GAAGGGAGTGTGTGGAGCAGAG-3'	408	62°C

gene expression. Dissociation curve were analyzed for each amplified product of the target genes to verify the specificity and sensitivity of the product and corrected out any false amplification due to the primer-dimer complications. The mean fold change (n-fold) for each selected standard gene was assessed by using the relative quantification method ($2^{-\Delta\Delta C_t}$) as explained by Livak and Schmittgen (2001).

Statistical analysis: The statistical analysis of the data was performed as per standard procedure (Snedecor and Cochran 1994). Analysis of variance (ANOVA) was performed using a generalized liner model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC) and treatment means were separated using Student–Newman–Keuls (SNK) multiple range test. Tables present the non-transformed data. The data used in the study were tested for normality before analysis using Shapiro–Wilk statistics. Means were analyzed by one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences between the different days of the experiment with treatment or without treatment, and student t test between the treatment and control groups on different experimental days on endocrinological and gene expression profiles using the SAS software/PC computer program. Differences with $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Kiss1 and GPR54 expression: Data analysis revealed a significant ($P < 0.05$) increase in expression of *Kiss1* and *GPR54* mRNA following exogenous kisspeptin administration in post-partum anestrus mithun cows (Table 2). Relative expression of target genes (*Kiss1* and *GPR54*) were significantly higher ($P < 0.05$) at day of estrus in treatment as compared to control group (1.943 ± 0.29 and 1.84 ± 0.31 fold, respectively). Kisspeptin neurons are involved in regulation of pulsatile release of GnRH, which is important in maintenance of the sensitivity of the pituitary gonadotrophin such as FSH and LH to GnRH stimulation, thus follicular development and maturation of follicle occurred (Navarro *et al.* 2004). Repetitive exogenous administration of metastin causes increased pulsatile GnRH/FSH and LH release in rats (Tovar *et al.* 2006), ovine species (Caraty *et al.* 2007) and monkeys (Plant *et al.* 2006). Similarly, the results of the present study indicated the kisspeptin neurons in the brain play an important role in regulation of pulsatile GnRH/LH release. Caraty *et al.*

Table 2. Effect of exogenous kisspeptin administration on relative expression of *Kiss1* and *GPR54* in anestrus mithun cows

Days	Group of animals	Kiss1	GPR54
Day 0	Control	0.424±0.062	0.416±0.082
	Treatment	0.429±0.091	0.421±0.09
Day 20	Control	0.495±0.021 ^a	0.426±0.09 ^a
	Treatment	1.46±0.233 ^b	1.338±0.43 ^b
Day of estrus	Control	0.512±0.09 ^a	0.446±0.076 ^a
	Treatment	1.943±0.29 ^b	1.841±0.31 ^b

Within columns means with different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment groups for different days.

(2007) observed that administration of kp-10 through intravenously stimulated ovulation during the anestrus (non-breeding) season and strongly suggested that metastin is a key factor in the neuroendocrine control of seasonal effect on onset of estrus cycle and the annual reproductive cycle in sheep and goat. Similarly, Li *et al.* (2012) also observed that Kisspeptin receptor expression and Kiss1R mRNA in GnRH neurons is higher during the breeding than during non-breeding season in ovine and Kiss1R mRNA expression in GnRH neurons is considerably reduced by kisspeptin (Kp-10) administration of ewes during non-breeding season but not by steroid treatment of ovariectomized ewes. Kisspeptin mediated release of GnRH has been demonstrated in ewe indirectly (Messenger *et al.* 2005) and in female rhesus monkeys (Keen *et al.* 2008) and also by inhibition of kisspeptin response in rats by exogenous administration of GnRH antagonist.

Endocrine profile: Estradiol 17β and FSH concentration were significantly ($P < 0.05$) higher in treatment than control group whereas non-significant difference was reported in plasma progesterone concentration between experimental groups (Table 3). Highest concentration of FSH was reported on the day of estrus (9.86 ± 1.06 ng/ml) in treatment group which was significantly ($P < 0.05$) higher than in control group. In mithun, Dhali *et al.* (2005) reported highest FSH concentration of 6.52 ± 0.22 ng/ml on day 2 or 3 before estrus followed by second peak observed on day of estrus. Navarro *et al.* (2004) observed that kisspeptin signaling play a role in modulating the GnRH pulsatile release.

Follicular development and induction of cyclicity in postpartum anestrus animals: Repeated and frequent supplementation of kisspeptin has been observed to induce estrus in postpartum anestrus cows as observed by

Table 3. Effect of exogenous kisspeptin administration on endocrine profile of follicle stimulating hormone (FSH), estradiol-17 α and progesterone in anestrus mithun cows

Hormonal profiles	Experimental groups	Experimental days						
		Day 0	Day 4	Day 8	Day 12	Day 16	Day 20	Day of estrus
FSH (ng/ml)	Control	4.81 $\pm$ 0.33	4.95 $\pm$ 0.44	5.01 $\pm$ 0.97	5.03 $\pm$ 0.59	5.11 $\pm$ 0.49	5.26 $\pm$ 0.44 ^a	9.86 $\pm$ 1.06 ^a
	Treatment	4.92 $\pm$ 0.65	4.96 $\pm$ 0.62	5.29 $\pm$ 0.51	5.63 $\pm$ 0.42	5.69 $\pm$ 0.39	6.19 $\pm$ 0.53 ^b	15.65 $\pm$ 1.22 ^b
Estradiol- 17 β (pg/ml)	Control	9.21 $\pm$ 0.88	9.59 $\pm$ 0.96	10.19 $\pm$ 1.02	9.24 $\pm$ 1.13 ^a	10.56 $\pm$ 1.03 ^a	10.73 $\pm$ 0.86 ^a	11.29 $\pm$ 1.76 ^a
	Treatment	9.32 $\pm$ 0.89	9.78 $\pm$ 1.03	12.49 $\pm$ 0.88	16.77 $\pm$ 1.35 ^b	21.36 $\pm$ 1.11 ^b	23.01 $\pm$ 0.96 ^b	25.36 $\pm$ 1.27 ^b
Progesterone (ng/ml)	Control	0.23 $\pm$ 0.07	0.28 $\pm$ 0.09	0.32 $\pm$ 0.09	0.29 $\pm$ 0.08	0.21 $\pm$ 0.09	0.31 $\pm$ 0.09	0.19 $\pm$ 0.03
	Treatment	0.28 $\pm$ 0.07	0.22 $\pm$ 0.09	0.32 $\pm$ 0.05	0.26 $\pm$ 0.08	0.26 $\pm$ 0.08	0.31 $\pm$ 0.07	0.22 $\pm$ 0.04

Within columns means with different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment groups for different days.

Table 4. Estrus response in post Partum mithun cows following Metastin (kp-10) administration

Experimental groups	Age at the start of experiment (months)	Interval from initiation of treatment to estrus (days)		Duration of estrus (hrs)	
		Mean $\pm$ S.E.	Range	Mean $\pm$ S.E.	Range
Treatment (Gr I; n=6)	61.56 $\pm$ 2.33	24.64 $\pm$ 10.43 ^a	13–36	17.37 $\pm$ 2.29	16–21
Control (Gr II; n=6)	62.23 $\pm$ 2.82	86.56 $\pm$ 14.66 ^b	51–102	18.15 $\pm$ 2.38	15–22

Within columns means with different superscripts (a, b) differ significantly ($P < 0.05$) between control and treatment groups

ultrasonography method. Follicular development was observed following exogenous kisspeptin administration in treatment group. Medium and large follicles (> 4 mm diameter) increased significantly in treatment group whereas only small follicles were observed in control group. Naniwa *et al.* (2013) also reported enhanced follicular development in postpartum anestrus mithun cows injected peripherally with full-length metastin (kisspeptin). Exogenous injection of 2 nmol/kg of kisspeptin (Kp-53) enhanced elevation of LH level from the basal level for up to 4 h and LH response to kisspeptin injection suggested the response of follicular growth or development after the kisspeptin administration. Kisspeptin signal is involved in regulation of the reproductive cycle of species especially that are showing seasonal breeding. Several other species have also shown seasonal breeding pattern that coordinate the time of birth with threshold environmental conditions. Reduction of kisspeptin expression has been reported in sheep during the anestrus (non-breeding) season (Smith *et al.* 2006) and in also hamster (Greives *et al.* 2007). Supplementation of kisspeptin during the anestrus or non-breeding season can induce the reproductive axis and stimulate the testicular growth in hamster (Revel *et al.* 2006) and ovulation in ewe (Caraty *et al.* 2007).

Induction of estrus: Estrus was exhibited on day 13–36 (average 24.64 $\pm$ 10.43 days) of kisspeptin injection in treatment group whereas in control group, estrus expression was observed on day 51–102 (86.56 $\pm$ 14.66) after initiation of treatment (Table 4). The treatment group of cows expressed swollen vulva and hyperemic vaginal mucosa. Earlier studies reported that kisspeptin induces the gonadotropins secretion from the anterior pituitary by stimulating the release of GnRH from hypothalamus or

forebrain after *GPR54* activation. Moreover, Smith *et al.* (2005) reported kisspeptin stimulated the release of sex steroids from gonads have positive feedback effect on the Kiss I neuron expression in AVPV nucleus whereas negative feedback effect on arcuate nucleus neurons. The suppression of kisspeptin activity by sex steroids in the ARC appears to be mediated by estrogen receptors (ER) in the female. These observations suggest that kisspeptin neurons in the ARC and AVPV of female provide tonic drive to GnRH-neuronal activity, which is modulated by the negative/positive feedback effects of gonadal steroids (estradiol in the female). Similar results were observed in the present study. Improved endocrinological profiles, follicle development and kiss gene profiles in post partum anestrus mithun cows following exogenous kisspeptin administration indicates that kisspeptin induced or resumed the cyclicity in early. This will facilitate to minimize the inter-calving interval and improve the production and reproduction performance of post partum anestrus mithun cows.

ACKNOWLEDGEMENTS

Authors are thankful to the Department of Biotechnology, Government of India, New Delhi for providing financial support and Director, ICAR-NRC on Mithun, Medziphema, Nagaland for providing the research facilities.

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