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Kiss 1 and *GPR54* mRNA expression, endocrine profile, follicular development and onset of estrus following kisspeptin administration in pre-pubertal mithun heifers

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ABSTRACT

Present study was undertaken to find out the effect of exogenous kisspeptin administration on *Kiss1* and *GPR54* mRNA expression, plasma endocrine profile and follicular development in pre-pubertal mithun heifers. Mithun heifers (20), age between 22–26 months, were selected and divided randomly into two groups (n=10). Before starting the experiment, all animals were examined by rectal palpation as well as through ultrasonography to ascertain the pre-pubertal status and blood samples were collected. All the animals in group A (treatment) were injected with kisspeptin (Metastin) @ 1.3 µg/kg body wt. at every 3 days interval up to day 20 while in group B (control), normal saline was injected as placebo. Blood samples were collected on the day of injection and on the day of estrus in control as well as treatment group. Trans-rectal ultrasonography was also conducted at every 3 days interval till day 20 and at estrus to study the follicular development. Results revealed significant increase in *Kiss1* and *GPR54* mRNA expression following kisspeptin administration as compared to control. Level of FSH and Estradiol 17β was higher in treatment group while no difference was reported in plasma progesterone in control and treatment group. Increased numbers of medium and large follicle were recorded in treatment group while control group showed only small and medium follicles. Early onset of estrus was reported in treatment group than in control. It may be concluded that exogenous administration of kisspeptin increased expression of *Kiss1* and *GPR54* mRNA; peripheral FSH and estradiol concentration, increased follicular growth and early onset of estrus in treated heifers than in control.

Key words: Endocrine profile, Metastin, Mithun, *GPR54, Kiss1*

Mithun (*Bos frontalis*) is a massive domesticated rare ruminant species mainly reared for meat purpose and geographically located only in four states in India viz. Nagaland, Arunachal Pradesh, Manipur and Mizoram. Indian livestock species including mithun suffer different reproductive failure viz. anestrus, late maturity, poor estrus cycle, delayed ovulation, long post partum calving intervals etc. (Chaudhari *et al*. 2012). The age at puberty and age at first calving is significantly higher in mithun i.e. 27 to 36 months and 40 to 48 months, respectively (Mondal *et al*. 2014). Physiologically, puberty is triggered by the activation of neurons in the forebrain which produce a neuroendocrine substrate to stimulate GnRH (Saito *et al*. 2012). Basal release of GnRH from the hypothalamus into the hypophyseal portal circulation maintains tonic gonadotropin secretion from the pituitary and results in follicular development and steroidogenesis in the ovaries (Knobil *et al*. 1980). In the last decade, kisspeptin, a peptide encoded by the *Kiss1* gene, has attracted attention as a key molecule in the regulation of GnRH/luteinizing hormone (LH/FSH) release in many mammalian species including rodents, ruminants and primates (Tena Sempore 2006, Naniwa *et al*. 2013). G-protein coupled receptor 54 (*GPR54*), a member of the rhodopsin family, act as the endogenous receptor of kisspeptin.

Exogenous repetitive administration of kisspeptin induces pulsatile GnRH/LH release in various species (Catary *et al*. 2007, Ezzat *et al*. 2009, Joseph *et al.* 2015). Kisspeptin have been shown to increase circulating concentrations of LH and FSH secretion in pre-pubertal cattle and estrogen secretion, followed by the preovulatory LH surge in acyclic ewes and cattle (Ezzat *et al.* 2009). Keeping in view the above mentioned facts, it may be hypothesized that exogenous administration of kisspeptin may induce estrus in prepubertal heifers and help in advancing the age of puberty in mithun. No such studies investigating whether exogenous Kisspeptin can induce puberty in pre-pubertal Mithun heifers have been conducted. Therefore, the present study was undertaken to find out the effect of exogenous administration of Kisspeptin (kp–10)

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on induction of estrus, expression of *Kiss1* and *GPR54*, endocrine profile in pre-pubertal mithun heifers.

MATERIALS AND METHODS

Selection of animals: Mithun heifers (20), maintained at ICAR-National Research Centre on Mithun, Nagaland (India), aged between 22–26 months were selected for the study. All the animals were maintained under same management and nutritional regimen throughout the experiment. Pre-pubertal animals were selected after rectal palpation and ultrasound scanning of reproductive organs, follicular activity and hormonal profiles. Ovaries in each heifer were scanned with a linear array trans-rectal probe (7.5 MHz transducer). Pre-pubertal stage was confirmed by absence of >4 mm follicle or corpus luteum in a weekly scanning of ovaries over a period of 22 days. Animals were randomly divided into two groups; control (n=10) and treatment (n=10). To standardize counting of follicles, each ovary was scanned from end to end to identify the positions of the corpus luteum and antral follicles.

Kisspeptin administration and blood collection: Kp-10 (kisspeptin) @ 1.3 µg/kg body wt. was injected intramuscularly in treatment group at every three days interval till day 20. Control group was administered with equal quantity of normal saline as placebo. Blood samples (10 ml) were collected in 15 ml sterile tube containing 6% EDTA, just after injection, by jugular vein puncture from both control and treatment group. Plasma was separated by centrifugation @ 4,000 rpm for 10 min. Plasma samples were stored in –20°C for hormones estimation (FSH, Estradiol-17β and Progesterone).

Leukocyte separation and RNA isolation: Leukocytes were separated from the blood samples using erythrocyte lysis buffer (10×) containing 0.2 g disodium EDTA salt, 41.2 g Ammonium chloride (NH_4Cl), 5 g $KHCO_3$ dissolved in 500 ml of distilled water. Lysis buffer (1×) was prepared by dissolving 100 ml of 10× buffer in 900 ml of distilled water. Samples were centrifuged at 4,000 rpm for 10 min. Lysis and centrifugation were repeated 3–4 times to obtain a white pellet of cells. Finally, it was dissolved in 500 µl of PBS and kept at –20°C for storage. Total RNA was extracted from blood cells as per the standard protocol using standard kit (QIAGEN RNase mini kit; USA).The quality of RNA was taken into account when the ratio of optical density at 260 nm/280 nm was >1.9. The RNA thus isolated was stored at –20°C for future use.

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

Primer name	Primer sequence	Product size (bp)	Annealing temperature
GAPDH	F-5'-CCTGGAGAAACC TGCCAAGT-3' R-5'-GCCAAATTCATTGT CGTACCA-3'	218	58°C
Kiss1	F-5'-GGGCCCGGAGA AAGGCTTTG-3' R-5' TGTGGGAGCACAGT GGTCTTTGC-3'	526	70°C
GPR54	F-5'-CAGTTCATTG CCCATTAGGG-3' R-5' GAAGGGAGTGTG TGGAGCAGAG-3'	408	62°C

cDNA isolation and amplification: RNAs were reverse transcribed to produce cDNA using standard kit (Thermo Scientific Revert Aid H Minus First cDNA synthesis kit; USA). The PCR cyclic conditions of selected target genes (*Kiss1* and *GPR54*) and house-keeping gene (*GAPDH*) were standardized by using PCR master mix and different annealing temperatures in a thermal cycler (Table 1). The initial denaturation was done at 95°C for 5 min followed by 32 cycles of cyclic denaturation at 95°C for 30 sec, different annealing temperature (Table 1) for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 8 min. The amplified PCR product was verified by 1.5% (w/v) agarose gel electrophoresis using 100 bp DNA ladder.

Quantitative real time PCR: The target genes (*Kiss1* and *GPR54*) and housekeeping gene (*GAPDH*) were amplified in real-time PCR system using standard kit (Quantifast SYBR Green, QIAGEN; USA). The reaction mixture for qRT-PCR was prepared and run as per the manufacturer instruction in real-time PCR (Thermo Scientific Piko Real real time PCR, Finland). The threshold cycle (C_t) value for each target and house-keeping genes was recorded for evaluation of fold of expression. The dissociation curve for each amplified product of the target genes was analyzed to verify the specificity of the product and rule out any false amplification due to primer-dimer. The mean fold change (n-fold) for each gene was determined by using the relative quantification method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen (2001). The difference in threshold cycle value (C_t) of target genes and house-keeping gene (*GAPDH*) for each sample were considered for calculation of fold expression and expressed as ΔC_t. The $\Delta\Delta C_t$ of target gene in treatment group was calculated by deducting the average ΔC_t of target gene of control animals (normalized calibrator) from the ΔC_t of target gene of treatment group. The fold of expression of target gene in pre-pubertal mithun was finally estimated as $2^{-\Delta\Delta Ct}$.

RESULTS AND DISCUSSION

Kiss1 and GPR54 expression: The results revealed that there was a significant increase in *Kiss1* and *GPR54* mRNA expression following Metastin administration at estrus as evident by agarose gel electrophoresis (Fig. 1). The quantitative expression of *Kiss*1 and *GPR54* mRNA in treatment and control group were also compared at day 0, day 20 and at estrus. The results revealed that the relative expression of *Kiss1* and *GPR54* mRNA were significantly higher at day 20 in treated group as compared to control. However, no significant change in expression level was recorded at day 0 as well as on the day of estrus.The significant variation ($P<0.05$) in fold expression of *Kiss1*

Table 2. Relative expression of *Kiss1* and *GPR54* in control and treatment group

Days	Group of animal	*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	1.962 ± 0.23[b]	1.861 ±0.33[b]

*Values having different superscripts (a, b) in a column differ significantly (P<0.05) for a particular day of treatment.

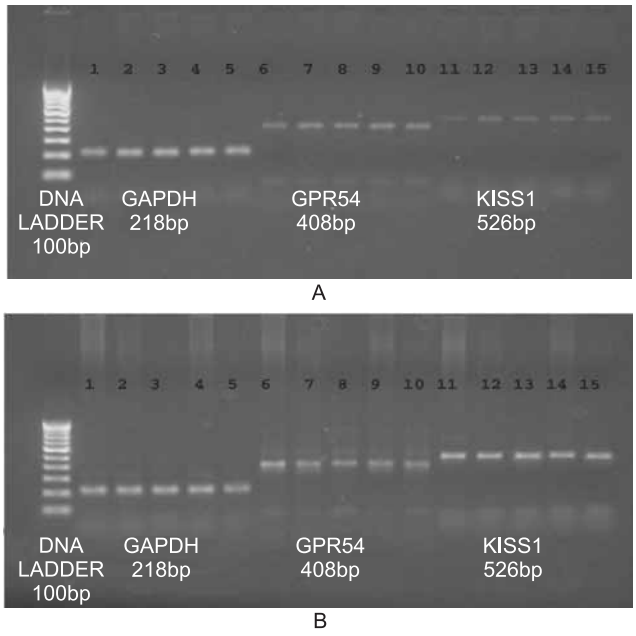

Fig. 1. (A) Agarose electrophoresis gel depicting the expression of *GAPDH* (lane 1–5), *GPR54* (lane 6–10), and *Kiss1* (lane 11–15) in control. (B) Agarose electrophoresis gel depicting the expression of *GAPDH* (lane 1–5), *GPR54* (lane 6–10), and *Kiss1* (lane 11–15) in treatment group.

and *GPR54* mRNA (1.962±0.23 and 1.86±0.33) at day 0, 20 and at estrus were also observed within the group (Table 2). It was reported that kisspeptin and *GPR54* are involved in the developmental activation of the GnRH neuronal network on induction of puberty. An increase in the pulsatile

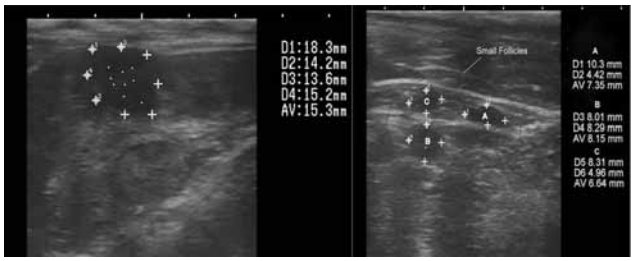

Fig. 2. Ultrasonography picture of ovary showing (A) Graffian follicles (B) Small follicles.

secretion of GnRH/LH appears to be an important event leading to puberty and release of GnRH/LH is pulsatile in all prepubertal rats, human, heifers. Kisspeptin and its receptors (*Kiss1R/GPR54*) have emerged as key players in the regulation of reproduction in animals. Shahab *et al.* (2005) reported that *Kiss1* mRNA levels detected by real time PCR increased with puberty in both male and female monkeys. In intact females, but not in agonadal males, *GPR54* mRNA levels in the hypothalamus increased by 3 fold from the juvenile to mid-pubertal stages. Our results revealed that *Kiss*1 and *GPR54* genes are expressed more abundantly at day 20 (approximately 2 folds higher) in treatment than in control group.

Endocrine profile: Results of hormonal assay revealed that the level of FSH and estradiol 17β was higher in treatment group while no difference was reported in plasma progesterone in control and treatment group (Table 3). The peak FSH concentration found at the day of estrus (7.29±0.39) in treatment group was significantly (P<0.05) higher than control animals. Ezzat *et al.* (2009) reported that peripheral injection of human Kp-10 induced FSH secretion in prepubertal cattle but Naniwa *et al.* (2013) reported that full-length bovine kisspeptin (Kp-53) (0.2 or 2 nmol/kg) did not stimulate FSH secretion. Adams *et al.* (1994) reported that a biphasic FSH peak was found during estrous cycle in cattle heifer and FSH concentration closely followed the LH pattern observed during the cycle and FSH concentrations attained peak levels on the day of estrus. Dhali *et al*. (2005) reported peak FSH concentration (6.52±0.22) on day 2 or 3 before estrus followed by the second peak concentration on estrus day.

In the present study, it was recorded that there was gradual increase in estradiol 17β concentration form day 0

Table 3. Endocrine profile of FSH, estradiol 17β and progesterone following metastin administration

Hormonal assay	Group	Day 0	Day 4	Day 8	Day 12	Day 16	Day 20	Day of estrus
FSH	Control	4.86±0.35	4.91±0.45	5.11±0.98	5.08±0.39	5.16±0.42	5.21±0.44	7.29±0.39
	Treatment	4.92±0.65	4.96±0.62	5.29±0.57	5.61±0.48	5.65±0.36	6.13±0.52	7.43±0.41
Estradiol 17β	Control	9.19±0.88	9.58±0.96	10.11±1.02	9.24±1.13[A]	10.5±1.03[A]	10.22±0.86[A]	25.65±1.56
	Treatment	9.32±0.89	9.77±1.03	12.39±0.88	16.77±1.35[B]	20.36±1.11[B]	23.01±0.96[B]	26.96±1.77
Progesterone	Control	0.234±0.065	0.285±0.093	0.316±0.092	0.285±0.08	0.206±0.096	0.305±0.088	0.186±0.035
	Treatment	0.285±0.071	0.226±0.096	0.302±0.012	0.266±0.085	0.255±0.095	0.311±0.072	0.211±0.014

*Values having different superscripts (A, B) in a column differ significantly (P<0.05).

to day 20. The mean estradiol 17β in treatment group was significantly higher (P<0.05) as compared to control. The higher concentration of estradiol 17β was recorded at day 20 (23.01±1.4) in treatment group of animals; but in control at day 20, it was significantly lower. A previous study had shown that a continuous Kp-10 infusion elicited a prolonged LH release and then estrogen secretion, followed by the pre-ovulatory LH surge in acyclic ewes (Sebert *et al*. 2010). Intermittent kisspeptin administration is more effective at maintaining GnRH secretion in the treated animals than continuous kisspeptin infusion, which produces desensitization of *GPR54* receptors (Plant *et al*. 2006). The mean progesterone level showed that there was no significant difference in both treatment group and control animals. Dhali *et al*. (2005) reported that in mithun the progesterone concentration was increased gradually till day 6 or 7 following estrus and attained the peak level between day 11 and 15 of the cycle. On the other hand, estradiol 17β concentration varied significantly throughout the estrus cycle and attained the peak between day 14 and 17, and came down to the basal level thereafter before the onset of the next estrus. It was reported that estradiol 17β and progesterone act together to cause physiological and behavioural estrus in bovine species (Hafez and Hafez 1993). The contribution of progesterone to the inhibition or facilitation of estrus behaviour depends on the concurrent progesterone action in number of different neural sites in the hypothalamus and subsequent changes in progesterone receptors in any of these areas. The high progesterone concentration during luteal phase increase the number of estradiol 17β receptors in the mediobasal hypothalamus and therefore increases the sensitivity to estradiol 17β (Catary *et al*. 2007).

Follicular development and induction of puberty: Repeated administration of kisspeptin-10 has been found to enhance the pubertal process in mithun as observed by ultrasonography. The ultrasonographic pictures of ovaries changed following kisspeptin (Metastin) injection in all the animals (Fig. 2). It was observed that there was increase in numbers of medium and large follicles (>4 mm diameter) in treatment group; while in control group, only small follicles were seen. Naniwa *et al*. (2013) reported that follicular development is facilitated in cows administered peripherally with full-length kisspeptin. Administration of 2 nmol/kg of Kp-53 induced elevation of LH concentrations from the basal level for up to 4 h, and the LH response to kisspeptin administration reflects the response of follicular growth after the kisspeptin injection. In addition, developmental studies examining hypothalamic kisspeptin and *GPR54* mRNA levels have found correlations between transcript expression levels and the onset of puberty in the rat (Navarro *et al*. 2004) and monkey (Shahab *et al.* 2005). Average diameter of graffian follicle was recorded as 14.60 mm (largest 15.30 mm) while medium follicles average was 7.96 mm.

Estrus was exhibited in treatment group after 8–24 days (avg. 12.5±6.81 days) in kisspeptin injected group; while in control group, estrus was shown after 120–155 days (avg. 135±5.15 days) after cessation of treatment (Table 4). The treated animals exhibit slightly swollen vulva and hyperemic vaginal mucosa which were absent in control group. Studies reported that kisspeptin stimulate the secretion of gonadotropins from the pituitary by stimulating the release of GnRH from forebrain after activation of *GPR54*. Kisspeptin neurons express the estrogen receptor and the androgen receptor, and these cells are direct targets for the action of gonadal steroids in both male and female animals, suggesting that kisspeptin signalling could mediate the neuroendocrine events that trigger the onset of puberty. Hence, as a whole, these investigations have laid the foundation for the concept that kisspeptin-GPR54 signaling within the GnRH neuronal network may be a "gatekeeper" for puberty onset (Seminara and Kaiser 2005).

Table 4. Estrus response in pre-pubertal mithun heifers following Metastin (kp-10) administration

Group	Age (months) at the start of exp.	Interval from cessation of treatment to estrus		Duration of estrus (h)	
		Mean±S.E. (days)	Range (days)	Mean ±S.E. (days)	Range (days)
Treatment	22.40± 0.83	12.5± 6.81[a]	8-24	17.31± 2.26	16-20
Control	23.50± 0.82	135± 5.15[b]	120–155	18.10± 2.32	15–21

*Values having different superscripts (a,b) in a column differ significantly (P<0.05).

In conclusion, results of the present experiment clearly showed the differential expression patterns of *Kiss1* and *GPR54* genes and their importance during pubertal process in mithun and exogenous administration of kp-10 @1.3 µg/kg b.wt. induces early onset of puberty.

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