Indian Journal of Animal Sciences **92** (5): 580–584, May 2022/Short communication
https://doi.org/10.56093/ijans.v92i5.106785

Relative expression profile of Kisspeptin (Kiss1-Kiss1r) and gonadotrophin receptor in the ovarian follicular tissue and their association in the buffalo

G K MISHRA[1], M K PATRA[2*], L KIPJEN SINGH[3], V UPMANYU[2], S CHAKRAVARTI[2], M KARIKALAN[2], M PANIGRAHI[2], S K SINGH[2], G K DAS[2], K NARAYANAN[4] AND H KUMAR[2]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 31 October 2020 ; Accepted: 17 February 2022

Keywords: Buffalo, Kisspeptin, Ovarian tissue, Receptors

Kisspeptin was identified as ligand for the orphan G-protein coupled receptor 54 (GPR54), also termed as Kiss1r (Lee *et al.* 1996) that regulates reproductive processes in human and rats (Terao *et al.* 2004, Castellano *et al*. 2006, Hu *et al*. 2018). In the ovary, expression of kisspeptin (Kiss1-Kiss1r) has been observed in the granulosa cells of pre-ovulatory follicles of mice (Gaytan *et al*. 2014), Siberian hamsters (Shahed and Young 2009) and bitch (Cielesh *et al*. 2017). In the rat ovary, the expression of Kiss1r was consistent throughout the stages of estrous cycle; however, the level of Kiss1 expression fluctuated in a cyclic-dependent manner, with a robust increase preceding ovulation during pro-estrus. In addition, immature rat ovaries showed low levels of Kiss1 mRNA, which were significantly enhanced by gonadotropin priming (Castellano *et al*. 2006). Ovarian kisspeptin expression seems to be controlled by preovulatory LH surge and putatively associated in ovulation, tissue remodelling and steroidogenesis (Laoharatchatathanin *et al*. 2015). Kisspeptin suppressed the recruitment of follicles by decreasing their sensitivity to FSH and regulate the developing follicle population during aging in rats (Fernandois *et al*. 2016). These concepts indicate that kisspeptin could act as an essential gatekeeper in many reproductive functions at ovarian level (Roa and Tena-Sempere 2007). Till date, the intra-ovarian role of kisspeptinergic system is not well established in farm animals including buffalo.

The water buffalo (*Bubalus bubalis*), is a seasonally polyestrus animal and its reproductive efficiency is often compromised by biological and management problems. Delayed puberty, prolonged postpartum anestrus, summer anestrus, silent heat and extended inter-calving intervals impede the realization of optimum reproductive efficiency in the buffalo (Nanda *et al*. 2003). Our recent studies documented the presence of kisspeptinergic system in the hypothalamus and corpus luteum of buffalo and highlighted its expression patterns in hypothalamus of cyclic and acyclic buffalo in the breeding season (Mishra *et al.* 2019 a, b) and association with luteal steroidogenesis (Mishra *et al.* 2019c). The present study was undertaken to explore the expression of kisspeptinergic system and its association with gonadotrophin receptors in the ovarian follicular tissue at different stages of estrous cycle in buffalo.

Present address: [1]College of Veterinary Sciences and Animal Husbandry, Anjora, Dau Shri Vasudev Chandrakar Kamdhenu Viswavidhyalaya, Durg, Chhattisgarh. [2]ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. [3]ICAR-National Dairy Research Institute, Karnal, Haryana. [4]ICAR-Indian Veterinary Research Institute, Bengaluru, Karnataka. ✉Corresponding author email: drmanas01@gmail.com

Collection of buffalo genitalia and classification of phase of estrous cycle: In the present study, buffalo genitalia (n=8/stage) were collected at a local slaughterhouse within 30 min of exsanguinations and transported to the laboratory on ice. The stage of estrous cycle was classified as early luteal (EL, day 1-5), mid luteal (ML, day 6-14), follicular (FL, day 15-21) and acyclic (Ali *et al*. 2003, Mishra *et al.* 2018). Presence of sub-clinical endometritis was ruled out through endometrial cytology (Boby *et al*. 2017). Retrospective estimation of serum progesterone and estradiol was done by radioimmunoassay (Mishra *et al.* 2018). Ovarian tissue having medium to large follicle (size 8-12 mm) was harvested and a portion was collected in RNAlater (Qiagen GmbH, Germany) and stored at –80ºC until analysis. Total RNA was extracted using TRIzol® Reagent (Ambion Life Technologies, USA) and the quality of RNA samples were assessed in NanoDrop spectrophotometer. The good quality RNA samples having $A_{260/280}$ at 1.8 to 2.0 were used in the study. DNase I was added to eliminate gDNA in the extracted total RNA and reverse transcribed into cDNA using RevertAid M-MuLV Reverse Transcriptase enzyme using oligod$T_{(18)}$ primers. Primers for buffalo Kiss1, Kiss1r, FSHR and LHR were designed using Primer Quest tool of Integrated DNA Technologies (Table 1).

The PCR cyclic conditions were optimized in 25 μL reaction mixture using Taq PCR Master Mix Kit (Qiagen, Germany) in a gradient thermal cycler (SureCycler 8800, Agilent Technologies, Great Britain) and the amplicon was confirmed in 1.5% (w/v) agarose gel electrophoresis. Each target gene was amplified using QuantiTect™ SYBR® Green PCR Master Mix (Qiagen GmbH, Germany) in

Table 1. Primers used in qPCR for amplification of different target genes in the ovarian tissue of buffalo (*Bubalus bubalis*)

Target gene	Oligonucleotide sequences (5’>>>>>3’)	Length (bp)	Efficiency (%)	Product size (bp)	Annealing temperature (°C)	Accession No.
Kiss1	F’-GCT TTC CTG GCA GCT GAT	18	99.7	99	55.0	MF168937
	R’-TGC GAG CCT GTG GTT CTA	18				
Kiss1r	F’-GAA GTC AGG CAG CGG TAG TC	20	97.3	229	55.0	XM_006050477
	R’-AGC ATC AAC AGT GGC AAA CA	20				
FSHR	F’-GCA GAG GCA GAA GAA AGC AG	20	100.0	122	54.1	NM_174061/
	R’-TCC GTT AGA GCA GTG ACA GAG	21				Pandey et al. (2010)
LHR	F’-CAG TGT GCT CCT GAA CCA GA	20	98.2	192	55.0	DQ858168
	R’-GTC TGC AAA GGA GAG GTT GC	20				
β-actin	F’-GAC ATC AAG GAG AAG CTC TG	20	98.4	214	55.0	DQ661647
	R’-TGG AAT TGA AGG TAG TTT CG	20				

Kiss1, Kisspeptin; Kiss1r, Kisspeptin receptor; FSHR, Follicle stimulating hormone receptor; LHR, Luteinizing hormone receptor; F’, Forward primer. R’, Reverse primer.

Table 2. Relative fold changes ($2^{-\Delta\Delta Ct}$) of Kiss1, Kiss1r and gonadotropin receptors in the ovarian tissue at different stages of estrous cycle and acyclicity in the buffalo (*Bubalus bubalis*)

Target gene	Early Luteal		Mid Luteal		Follicular		Acyclic		P value*
	Mean	SED	Mean	SED	Mean	SED	Mean	SED	
Kiss1	28.64[a]	0.01	66.79[a]	1.32	14.09[a]	0.42	1.00[b]	1.32	0.001
Kiss1r	12.27[ab]	0.02	77.26[a]	3.87	22.37[ab]	2.72	1.00[b]	0.72	0.021
FSHR	0.09	1.21[a]	0.27[a]	1.17	15.08[b]	1.32	1.00[ab]	1.61	0.003
LHR	1.79	2.25	19.27	2.11	5.03	3.64	1.00	2.92	0.334

*P value was estimated using the ΔCt value of target genes and acyclic stage served as calibrator.

a Real-time qPCR (Agilent Aria.01Mx, Great Britain). Briefly, templates 10 ng (1 µL) were added to 0.2 µL forward and reverse primers (10 pmol each), and 5 µL 2× SYBR Green Master Mix to make a final volume of 10.0 µL. The thermal cyclic conditions were: hot start denaturation for 15 min at 95°C, 40 cycles of a three segmented amplification and quantification program (denaturation 15 sec at 95°C, annealing for 30 sec at the primer specific temperature (Table 1), extension for 30 sec at 72°C and a melting step by gradual heating from 55 to 95°C at the rate 1°C/s with acquisition of fluorescent data. Further, reaction with no template controls containing all components except the template cDNA was included in each qPCR that gave consistently no background signals. β-actin served as endogenous control to generate ΔCt of the target genes, since its expression was consistent across the stages of estrous cycle in the ovarian tissue as compared to GAPDH. The threshold cycle (Ct) value of β-actin varied between 19.15±0.55 to 20.58±0.83 in the ovarian follicular tissue and there was no significant variation in different cyclic stages. The relative expression of target genes was expressed as $\log_2$fold change (Livak and Schmittgen 2001). Standard error of deviation (SED) was calculated by using formula:

$$SED = \text{square root } \{(\text{Standard deviation of gene of interest}^2/n_1) + (\text{Standard deviation of gene of reference}^2/n_2)\}$$

where n_1 and n_2 are number of observations.

The Ct value of the reference gene was tested for normality by Shapiro-Wilk test. The level of significance of the mean ΔCt value for target genes was compared by one-way ANOVA with Tukey’s *post hoc* test, where equality of variance was assumed and Levene’s test was non-significant. Whereas, Dunnet C was used if there was inequality of variance. The association of target genes in each group was determined based on the correlation coefficient using Spearman’s rho. The difference of mean values for all data analyzed with $P<0.05$ was considered as significant, whereas $0.05<P<0.10$ was considered as tendency. All the figures were prepared in GraphPad Prism version 6.0.

In the past decade, kisspeptin research was primarily focussed on the regulation of GnRH release. Extra-hypothalamic localization of kisspeptin was previously reported in the pituitary gland, kidney, liver, cecum, colon, small intestine, stomach, uterus and placenta (Terao *et al*. 2004, Murphy 2005, Vikman and Ahren 2009, Pinilla *et al*. 2012). In the present study, expression of ovarian Kiss1 transcripts was abundant in the cyclic than acyclic stage. The fold change of Kiss1 transcript was significantly ($P<0.01$) up-regulated in ML (66.79 folds) followed by EL (28.64 folds) and FL (14.09 folds) stages against the acyclic buffalo (Table 2). Similarly, the Kiss1r expression was highest at ML (77.26 folds). Our study indicated clearly demonstrated that Kiss1 and Kiss1r transcripts were not only present in the follicular compartment of buffalo ovary, but their expression varies depending on the phase of the

Table 3. Correlation matrix[*] for the differentially expressed genes (Kiss1, Kiss1r) and gonadotropin receptors in the ovarian tissue at different stages of estrous cycle and acyclicity in the buffalo (*Bubalus bubalis*)

Stages of cyclicity	Gene	Kiss1r	FSHR	LHR
Early Luteal	Kiss1	-0.012	0.262	-0.500
	Kiss1r		-0.495*	-0.999
	FSHR			0.771
Mid Luteal	Kiss1	0.124	0.415**	-0.600
	Kiss1r		-0.272	-0.600
	FSHR			-0.257
Follicular	Kiss1	0.045	0.407**	0.371
	Kiss1r		-0.363*	0.771
	FSHR			-0.714
Acyclic	Kiss1	-0.245	0.405*	-0.714
	Kiss1r		-0.625**	-0.429
	FSHR			-0.214

[*]Spearman's rank correlation coefficient's (rho) was calculated on ΔCt value. *, $P<0.05$; **, $P<0.01$.

estrous cycle (Fig. 1). Besides, the expression of FSHR was also found to be up-regulated at FL (15.08 folds) stage in response to follicular activity and subsequently observed to be down-regulated at EL (0.09 times) and ML (0.27 times) stage (Table 2). However, the fold change of LHR did not show any significant difference among the groups, although it was moderately up-regulated in ML (19.27 folds) and FL (5 folds).

The correlation matrix of differentially expressed genes in the ovarian tissue (Table 3) revealed that the expression of Kiss1 was moderately correlated with FSHR only at ML (ρ=0.415) and FL (ρ=0.407). However, the Kiss1r expression had a negative correlation with FSHR at all stage of cyclic as well as acyclic conditions. In concurrence with our study, a prominent expression of kisspeptin and Kiss1r was documented in the ovary of different species like rat (Terao *et al*. 2004, Castellano *et al*. 2006, Ricu *et al*. 2012), hamster (Shahed and Young 2009), marmoset and woman (Gaytan *et al*. 2009), doe and sow (Inoue *et al*. 2009, Saadeldin *et al*. 2012) and dog (Cielesh *et al*. 2017). The degree of kisspeptin expression depends on

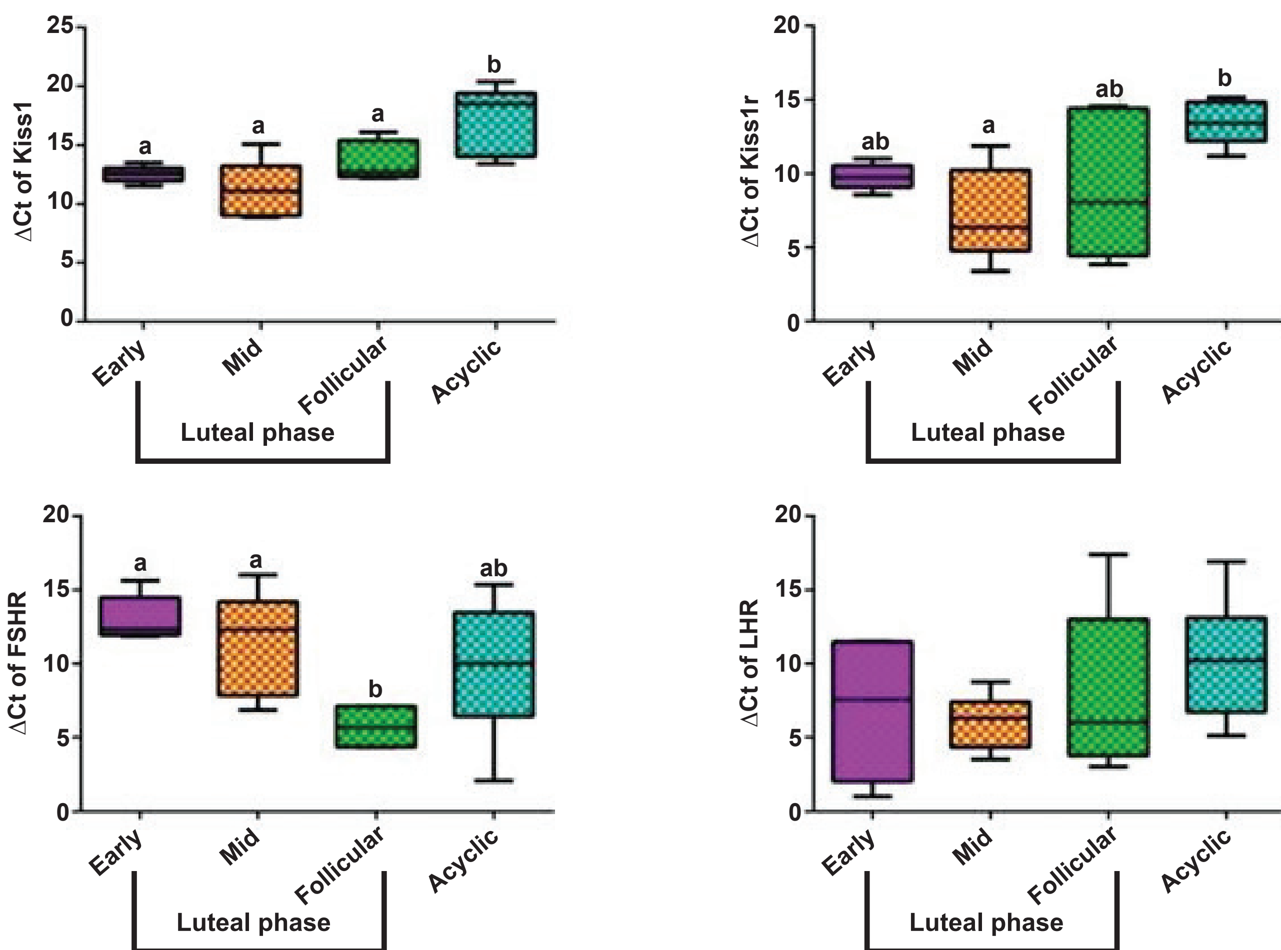

Fig. 1. Differential expression of Kiss1, Kiss1r and gonadotropin receptors in the ovarian tissue of the buffalo. Each Box plot with the whiskers indicates the median, maximum and minimum ΔCt values of Kiss1; Kiss1r; FSHR and LHR at different stages of cyclic and acyclic condition.

the stage of estrous cycle in the rat (Castellano *et al*. 2006, Gaytan *et al*. 2009). Emerging evidence indicated that ovarian kisspeptin is involved mainly in folliculogenesis (Fernandois *et al*. 2016), oocyte maturation (Saadeldin *et al*. 2012) and ovulation (Laoharatchatathanin *et al*. 2015, Fernandois *et al*. 2016). In another study, it was shown that intra-ovarian expression of Kiss1 is up-regulated by the sympathetic innervation of the ovary (Fernandois *et al*. 2017) and is associated with the follicular development in the aging rat (Ricu *et al*. 2012). Preovulatory LH surge induces kisspeptin production in the granulosa cells of the rat (Laoharatchatathanin *et al*. 2015). The presence of Kiss1 and Kiss1r in the large and small luteal cells of early and mid CL that are associated in luteal steroidogenesis have also been documented (Mishra *et al.* 2019c). Therefore, the present study suggests that the expression of Kiss1 and Kiss1r are putatively associated with folliculogenesis and intrafollicular steroidogenic regulation. However, functional studies are warranted to unravel the key extra-hypothalamic role of kisspeptinergic system in regulation of ovarian function.

From this study, it could be concluded that Kiss1 and Kiss1r are expressed in the buffalo ovarian follicle and their expression is associated with stage of estrous cycle.

SUMMARY

In the past decade, kisspeptin research was primarily focussed on the regulation of GnRH release in hypothalamus. Present study was designed to explore the expression of extra-hypothalamic kisspeptinergic (Kiss1-Kiss1r) system in the follicular compartment of buffalo ovary. Buffalo genitalia (n=32) were collected immediately after exsanguinations and categorized into early luteal (EL), mid luteal (ML), follicular (FL) and acyclic (n=8 per group), based on the gross ovarian morphology. Ovarian follicular tissue samples were subjected to total RNA extraction, cDNA synthesis and qPCR amplification of Kiss1, Kiss1r, follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) along with an endogenous control (β-actin) gene. The expression of ovarian Kiss1 transcripts was abundant in the cyclic than acyclic stage. The fold change was significantly upregulated in ML (66.79 fold) followed by EL (28.64 fold) and FL (14.09 fold) stages against the acyclic stage (calibrator). Similarly, the Kiss1r expression was highest at ML (77.26 fold). The expression of FSHR was upregulated at FL (15.08 fold) stage in response to follicular activity and subsequently observed to be down regulated at EL (0.09 times) and ML (0.27 times). Further, the expression of Kiss1 was positively correlated with FSHR only at ML and FL. From this study, it could be concluded that Kiss1 and Kiss1r are expressed in the buffalo ovarian follicle and their expression is associated with the stage of estrous cycle.

ACKNOWLEDGEMENTS

The study was partly supported by an intramural grant (Project code: IXX13018) and Early Career Research Award (Project no. ECR/2017/002998), Science and Engineering Research Board, Department of Science and Technology, Government of India to Dr Manas Kumar Patra. The authors thank the Director, ICAR-Indian Veterinary Research Institute, Izatnagar for facilitating the work.

REFERENCES

Ali A, Abdel-Razek A K, Abdel-Ghaffar S and Glatzel P S. 2003. Ovarian follicular dynamics in buffalo cows (*Bubalus bubalis). Reproduction in Domestic Animals* **38**: 214–18.

Boby J, Kumar H, Gupta H P, Jan M H, Singh S K, Patra M K, Nandi S, Abraham A and Krishnaswamy N. 2017. Endometritis increases pro-inflammatory cytokines in follicular fluid and cervico-vaginal mucus in the buffalo cow. *Animal Biotechnology* **28**(3): 163–67.

Castellano J M, Gaytan M, Roa J, Vigo E, Navarro V M, Bellido C, Dieguez C, Aguilar E, Sánchez-Criado J E, Pellicer A, Pinilla L, Gaytan F and Tena-Sempere M. 2006. Expression of KiSS-1 in rat ovary: Putative local regulator of ovulation? *Endocrinology* **147**: 4852–62.

Cielesh M E, McGrath B M, Scott C J, Norman S T and Stephen C P. 2017. The localization of kisspeptin and kisspeptin receptor in the canine ovary during different stages of the reproductive cycle. *Reproduction in Domestic Animals* **52**(S2): 24–28.

Fernandois D, Na E, Cuevas F, Cruz G, Lara H E and Paredes A H. 2016. Kisspeptin is involved in ovarian follicular development during aging in rats. *Journal of Endocrinology* **228**: 161–70.

Fernandois D, Cruz G, Na E K, Lara H E and Paredes A H. 2017. Kisspeptin level in the aging ovary is regulated by the sympathetic nervous system. *Journal of Endocrinology* **232**(1): 97–105.

Gaytan F, Gaytan M, Castellano J M, Romero M, Roa J, Aparicio B, Garrido N, Sánchez-Criado J E, Millar R P, Pellicer A, Fraser H M and Tena-Sempere M. 2009. KiSS-1 in the mammalian ovary: Distribution of kisspeptin in human and marmoset and alterations in KiSS-1 mRNA levels in a rat model of ovulatory dysfunction. *American Journal of Physiology-Endocrinology Metabolism* **296**(3): E520–31.

Gaytan F, Garcia-Galiano D, Dorfman M D, Manfredi-Lozano M, Castellano J M, Dissen G A, Ojeda S R and Tena-Sempere M. 2014. Kisspeptin receptor haplo-insufficiency causes premature ovarian failure despite preserved gonadotropin secretion. *Endocrinology* **155**: 3088–97.

Hu K L, Zhao H, Chang H M, Yu Y and Qiao J. 2018. Kisspeptin/Kisspeptin receptor system in the ovary. *Frontier in Endocrinology* (*Lausanne*) **8**: 365.

Inoue N, Hirano T, Uenoyama Y, Tsukamura X, Okamura H and Maeda K. 2009. Localization of kisspeptin and gonadotrophin releasing hormone (GnRH) in developing ovarian follicles of pigs and goats. *Biology of Reproduction* **81**: 551.

Laoharatchatathanin T, Terashima R, Yonezawa T, Kurusu S and Kawaminami M. 2015. Augmentation of metastin/kisspeptin mRNA expression by the proestrous luteinizing hormone surge in granulosa cells of rats: implications for luteinization. *Biology of Reproduction* **93**(1): 1–9.

Lee J H, Miele M E, Hicks D J, Phillips K K, Trent J M, Weissman B E and Welch D R. 1996. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *Journal of the National Cancer Institute* **88:** 1731–37.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene

expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**(4): 402–08.

Mishra G K, Patra M K, Sheikh P A, Teeli A S, Kharayat N S, Karikalan M, Bag S, Singh S K, Das G K, Narayanan K and Kumar H. 2018. Functional characterization of corpus luteum and its association with peripheral progesterone profile at different stages of estrous cycle in the buffalo. *Journal of Animal Research* **8**(3): 507–12.

Mishra G K, Patra M K, Singh L K, Upmanyu V, Chakravarti S, Karikalan M, Singh S K, Das G K, Kumar H and Narayanan K. 2019a. Kiss1 and its receptor: Molecular characterization and immunolocalization in the hypothalamus and corpus luteum of the buffalo. *Animal Biotechnology* **30**(4): 342–51.

Mishra G K, Patra M K, Singh L K, Sheikh P A, Upmanyu V, Chakravarti S, Karikalan M, Sonwane A, Singh S K, Das G K, Kumar H and Narayanan K. 2019b. Expression of kisspeptin and its receptor in the hypothalamus of cyclic and acyclic buffalo (*Bubalus bubalis*). *Theriogenology* **139**: 167–77.

Mishra G K, Patra M K, Singh L K, Upmanyu V, Chakravarti S, Karikalan M, Bag S, Singh S K, Das G K, Kumar H and Narayanan K. 2019c. Expression and functional role of kisspeptin and its receptor in the cyclic corpus luteum of buffalo (*Bubalus bubalis*). *Theriogenology* **130**: 71–8.

Murphy K G. 2005. Kisspeptins: Regulators of metastasis and the hypothalamic pituitary gonadal axis. *Journal of Neuroendocrinology* **7**(8): 519–25.

Nanda A S, Brar P S and Prabhakar S. 2003. Enhancing reproductive performance in dairy buffalo: major constraints and achievements. *Reproduction Supplement* 27-36.

Pinilla L, Aguilar E, Dieguez C, Millar R P and Tena-Sempere M. 2012. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiological Reviews* **92**: 1235–1316.

Ricu M A, Ramirez V D, Paredes A H and Lara H E. 2012. Evidence for a celiac ganglion-ovarian kisspeptin neural network in the rat: Intraovarian anti-kisspeptin delays vaginal opening and alters estrous cyclicity. *Endocrinolology* **153**: 4966–77.

Roa J and Tena-Sempere M. 2007. KiSS-1 system and reproduction: Comparative aspects and roles in the control of female gonadotropic axis in mammals. *General and Comparative Endocrinology* **153**: 132–40.

Saadeldin I M, Koo O J, Kang J T, Kwon D K, Park S J, Kim S J, Moon J H, Oh H J, Jang G and Lee B C. 2012. Paradoxical effects of kisspeptin: It enhances oocyte *in vitro* maturation but has an adverse impact on hatched blastocysts during *in vitro* culture. *Reproduction and Fertility Development* **24**(5): 656–68.

Shahed A and Young K A. 2009. Differential ovarian expression of KiSS-1 and GPR54 during the estrous cycle and photoperiod induced recrudescence in Siberian hamsters (*Phodopus sungorus*). *Molecular Reproduction and Development: Incorporating Gamete Research* **76**(5): 444–52.

Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T and Shintani Y. 2004. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* **1678**: 102–10.

Vikman J and Ahrén B. 2009. Inhibitory effect of kisspeptins on insulin secretion from isolated mouse islets. *Diabetes, Obesity and Metabolism* **11**(s4): 197–201.