

Season influence on serum kisspeptin level and its association with hormonal levels and semen kinematics of buffalo bulls (*Bubalus bubalis*)

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Abstract

Kisspeptin has an important role in the stimulation of hypothalamic-pituitary-gonadal axis in term of pubertal development, release of reproductive and metabolic hormones and ultimately affecting the fertility. The aim of the present study was to evaluate the serum kisspeptin level and its correlation with semen quality and selected hormones in buffalo bulls during the summer and spring seasons. Semen and blood samples from eight Nili-Ravi buffalo bulls (age: 9.21 ± 1.02 years) were collected. Semen was analysed using computer-assisted semen analysis. Serum concentrations of kisspeptin, gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, cortisol, triiodothyronine (T3), thyroxin (T4) and insulin like growth factor (IGF-1) were estimated using enzyme-linked immunosorbent assay kits. Kisspeptin was neither affected by seasons and nor correlated with semen parameters and hormones. Higher levels of GnRH, LH, cortisol, IGF-1, total motility (TM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL) and linearity (LIN) were recorded in summer compared to spring. Correlations of GnRH versus IGF-1 and LH, LH versus IGF-1 and cortisol, FSH versus T4 and testosterone, testosterone versus T3 and T4 and T3 versus T4 were observed. The GnRH and IGF-1 were positively associated with TM, VAP, VSL, VCL and LIN. The LH was correlated with VSL, straightness and LIN. In conclusion, GnRH, LH, and IGF-1 correlations with semen parameters can be used to indicate semen quality. The buffalo bulls are well-adapted and can give quality semen in the summer season.

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Introduction

Kisspeptin also called as metastatin,¹ is the peptide product of the Kiss1 gene stimulating gonadotropin releasing hormone (GnRH) secretion having vital reproductive function.² Cell bodies of kisspeptin neurons are mainly reported in two areas of the hypothalamus in mammals. One population is in the arcuate nucleus (ARC) of buffalo³ and cattle.⁴ The other population is in the pre-optic area (POA) of buffalo³ and cattle.⁵ Kisspeptin and its receptors Kiss1r have also been characterized in the buffalo brain.⁶ Higher expressions of Kiss1 gene and CYP gene (a rate-limiting enzyme for steroidogenesis in testes) have been previously described in post-pubertal animals compared to pre-pubertal bulls.⁷ Interestingly, findings of higher expression of mRNA in the POA than the ARC^{3,7} are

contrary to cattle, in which Kiss1 mRNA expression is higher in ARC than POA of the hypothalamus. The role of kisspeptin in steroidogenesis and functional development of spermatozoa is evident from the co-increase of Kiss1 gene expression with steroidogenic enzymes genes including CYP11A1, HSD3B1, CYP17 and CYP19.⁸ The Kiss1 and Kiss1r are also expressed in testicular tissue and the kisspeptin secreted by testes could contribute to circular concentration of kisspeptin.^{9,10} This suggests that there may be involvement of kisspeptin in the fertility of bulls by altering the steroidogenic activity of testes.

Different factors can influence the reproductive efficiency in buffalo bulls including season as well as congenital, nutritional, and hormonal variations.¹¹ Seasons can affect the fertility of buffalo bull semen used in artificial insemination.¹² The fertility rate following

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artificial insemination with cryopreserved semen is lower in buffalo than cattle ascribing to the low-grade cryopreserved semen.¹³ Administration of exogenous kisspeptin has been found to stimulate the secretion of luteinizing hormone (LH) from the anterior pituitary in buffalo¹⁴ and cattle.¹⁵ So, it can be suggested that kisspeptin may affect the buffalo fertility. There is a body of evidence describing the effects of seasons on semen quality in cattle^{16,17} and buffaloes.^{12,18,19} However, seasonal fluctuation of kisspeptin and its association with semen quality and other hormonal levels in buffalo bull are yet to be elucidated. Therefore, the present study aimed at investigating the serum kisspeptin level and its association with semen attributes and selected hormones like kisspeptin, GnRH, follicle-stimulating hormone (FSH), LH, testosterone, insulin like growth factor (IGF-1), triiodothyronine (T3), thyroxin (T4) and cortisol during the summer and spring seasons.

Materials and Methods

The study was conducted after the approval from the Institutional Review Committee for Biomedical Research, University of Veterinary and Animal Sciences, Lahore, Pakistan (No. DR/1978).

The study was carried out during two seasons including the hot humid summer season (August-September 2017) and the spring season (February-March 2018) at Semen Production Unit, Qadirabad, Punjab, Pakistan (30.7189° N, 73.2514° E). Seasons were classified according to the previous study at the same station.²⁰ Blood and semen samples were collected from eight Nili-Ravi buffalo bulls of an average age of 9.21 ± 1.02 years. Blood was collected from the jugular vein in a plain vacutainer twice a season with one-week interval. Serum was separated by centrifuging the blood sample at 3,000 rpm for 15 min and the supernatant was collected using micropipette by tilting the vacutainer at 45° and stored at -40.00 °C for further analyses.

Hormonal assays. Serum concentration of kisspeptin was estimated using bovine kisspeptin enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Pars Biochem Co, Ltd. Nanjing, China). The serum concentration of GnRH was determined using the bovine ELISA kits (Elabscience Biotechnology Inc., Houston, USA). Testosterone, FSH, LH and IGF-1 were estimated with the help of commercial ELISA kits (Fine Biotech Co., Ltd, Wuhan, China). The T3, T4 and cortisol were measured using ELISA kits (Monobind Inc., Lake Forest, USA). All ELISA plates were read on Epoch™ micro-plate spectrophotometer (Biotek Instruments Inc., Winooski, USA).

Semen collection and evaluation. Semen samples were collected using a standard technique as described earlier.²¹ Briefly, the semen samples were collected in graduated tubes using artificial vagina. The bulls were

allowed normally a false mount before collection of semen. After the collection of semen, each ejaculate was held in a water bath at 37.00 °C until evaluated for volume, individual motility percentage and sperm concentration per ejaculate for inclusion and exclusion in the study. Spermatozoa showing $\geq 70.00\%$ motility along with concentration of $> 1.00 \times 10^9$ spermatozoa mL⁻¹ were further processed for cryopreservation.

Semen processing. The final concentration of 30.00×10^6 spermatozoa per 0.50 mL French semen straw was ensured by diluting the sample with regular a semen extender. These straws were kept in a cooling cabinet for 90 min at 4.00 °C and equilibrated for 2 hr at the same temperature.¹² Thereafter, straws were placed at 4.00 cm above liquid nitrogen surface vapours for 10 min. Semen straws were directly immersed into the liquid nitrogen and stored until analysed.²²

Computer-assisted semen analysis (CASA). Sperm motility and other kinematics were assessed by equilibrating the frozen-thawed semen in 37.00 °C water bath for 10 min. Then, the post-thawed semen parameters like total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; $\mu\text{m sec}^{-1}$), curvilinear velocity (VCL; $\mu\text{m sec}^{-1}$), straight-line velocity (VSL; $\mu\text{m sec}^{-1}$), straightness (STR; %; ratio of VSL/VAP) and linearity (LIN; %; ratio of VSL/VCL) were analysed with the CASA (CEROS; Hamilton Thorne Biosciences, Beverly, USA). Standard settings of CASA were used to analyse bubaline semen sample.¹²

Semen assays. Eosin-nigrosine staining technique was used to evaluate the viability of spermatozoa.²³ Briefly, 0.67 g of eosin B (Merck, Darmstadt, Germany) was mixed with 10.00 g nigrosine (Merck) and 0.90 g of NaCl in 100 mL of distilled water to prepare working solution of stain. Smear was stained instantly using the working solution and observed for liveability under a phase-contrast microscope (LX400; Labomed, Los Angeles, USA) at 400×. Sperm membrane integrity was assessed by the hypo-osmotic swelling test.²⁴ The hypo-osmotic solution was prepared by dissolving 1.35 g fructose and 0.73 g of sodium citrate in 100 mL distilled water. Final osmolarity was kept at osmotic pressure of 150 mOsmol kg⁻¹ (Osmomat 030; Gonotec, Berlin, Germany). The sample was mixed with the working solution and evaluated for membrane integrity based on tail coiling. The percentage of the normal apical ridge was assessed by a method already explained.²⁵ Briefly, the semen sample was mixed in 10:1 ratio with 1.00% solution of formal citrate and analysed for intactness of apical ridge. The DNA damage was detected by the acridine orange (AO) staining technique.²⁶ Briefly, the air-dried smear was immersed in Carnoy's solution (glacial acetic acid and methanol: 1:3). Slides were incubated for 7 min at 60.00 °C in tampon solution (0.30 M Na₂HPO₄ + 0.10 M citric acid: 1:16 at pH: 2.50) after complete fixation. Thereafter, the smear was

stained (in dark) with AO stain (1,000 µg mL⁻¹ in distilled water; Sigma-Aldrich, St. Louis, USA) for 5 min after rinsing with water. The slides were analysed immediately under fluorescent microscope (Labomed).

Statistical analysis. Data were presented as mean ± SEM and analysed using SPSS (version 20.0; IBM Corp., Armonk, USA). Data were evaluated for normal distribution by Shapiro-Wilk test. Based on distribution, seasonal data were compared using paired sample *t*-test and Wilcoxon test. The associations between semen and hormonal attributes were assessed using Pearson's and Spearman's correlations according to the data normality. Level of significance was set at *p* < 0.05.

Results

Seasonal effect on hormonal and semen profiles.

Seasonal differences of hormonal levels are presented in Table 1. Kisspeptin concentration was similar in the summer and spring seasons. The concentration of GnRH, LH and IGF-1 was higher (*p* < 0.05) in the summer compared to the spring season. Cortisol was tended to be higher (*p* = 0.06) in the summer compared to the spring season. The concentration of T4 was higher (*p* < 0.05) in the spring season compared to the summer season. Post-thawed semen analysis revealed that the values of TM, VAP, VSL, VCL and LIN were higher (*p* < 0.05) in the summer than spring. Other kinetic and morphological features of sperm remained unaffected by season (Table 2).

Table 2. Semen parameters during summer and spring seasons.

Parameters	Summer	Spring	<i>p</i> -value
Total motility (%)	85.13 ± 1.62	75.70 ± 2.63	0.035
Progressive motility (%)	30.75 ± 4.68	29.21 ± 2.03	0.529
Average path velocity (µm sec ⁻¹)	75.19 ± 3.81	48.41 ± 4.75	0.006
Straight line velocity (µm sec ⁻¹)	65.84 ± 3.94	40.19 ± 4.38	0.008
Curvilinear velocity (µm sec ⁻¹)	109.60 ± 4.48	84.19 ± 7.10	0.023
Straightness (%)	85.56 ± 1.30	82.06 ± 1.07	0.092
Linearity (%)	60.44 ± 2.07	46.81 ± 1.21	0.002
Viability (%)	74.75 ± 2.74	70.55 ± 2.27	0.670
Hypo-osmotic swelling test (%)	72.82 ± 1.01	69.23 ± 1.92	0.085
Normal apical ridge (%)	73.50 ± 1.36	70.57 ± 1.72	0.137
DNA integrity (%)	97.75 ± 0.36	97.17 ± 0.32	0.088

Table 3. Correlation of serum kisspeptin with endocrine profiles of buffalo bulls.

Parameters	T3	T4	GnRH	Kisspeptin	IGF-1	FSH	LH	Testosterone	Cortisol
T3	1.00	0.54*	0.16	-0.10	0.19	0.40	0.04	0.61*	-0.14
T4		1.00	-0.44	0.29	-0.02	0.65**	-0.15	0.58*	-0.28
GnRH			1.00	-0.08	0.74**	-0.47	0.55*	0.07	0.49
Kisspeptin				1.00	0.37	0.02	-0.11	0.12	0.01
IGF-1					1.00	0.02	0.66**	0.30	0.42
FSH						1.00	-0.02	0.61*	0.14
LH							1.00	0.16	0.58*
Testosterone								1.00	0.17
Cortisol									1.00

GnRH: Gonadotropin releasing hormone; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; T3: Triiodothyronine; T4: Thyroxine; IGF-1: Insulin like growth factor.

Correlation coefficient with * is significant at *p* < 0.05; while, ** is significant at *p* < 0.01.

Correlation of endocrine status with semen parameters. As shown in Table 3, kisspeptin did not show any association with under-studied hormones. However, GnRH was positively correlated (*p* < 0.05) with IGF-1 and LH. Testosterone was positively correlated (*p* < 0.05) with T3, T4 and FSH. The FSH has a positive correlation (*p* < 0.05) with T4. Cortisol was positively correlated (*p* < 0.01) with LH. The T3 was positively correlated (*p* < 0.05) with T4. The serum concentration of kisspeptin did not exhibit any correlation with the semen attributes. However, GnRH was positively correlated (*p* < 0.05) with TM, VAP, VSL, VCL and LIN (Table 4). The LH also showed a positive correlation (*p* < 0.05) with VSL, STR and LIN. The IGF-1 had a positive correlation (*p* < 0.05) with TM, VAP, VSL and LIN (Table 4).

Table 1. Serum hormonal concentration during summer and spring seasons.

Hormones	Summer	Spring	<i>p</i> -value
T3 (ng mL ⁻¹)	0.90 ± 0.02	0.93 ± 0.04	0.341
T4 (µg dL ⁻¹)	4.06 ± 0.16	4.63 ± 0.26	0.009
GnRH (pg mL ⁻¹)	79.54 ± 2.32	56.61 ± 0.32	0.012
Kisspeptin (ng L ⁻¹)	27.91 ± 3.34	27.27 ± 2.64	1.000
IGF-1 (ng mL ⁻¹)	283.16 ± 20.31	198.29 ± 14.35	0.002
FSH (mIU mL ⁻¹)	8.51 ± 0.69	10.10 ± 0.69	0.091
LH (mIU mL ⁻¹)	5.14 ± 0.40	3.75 ± 0.21	0.024
Testosterone (ng mL ⁻¹)	2.36 ± 0.11	2.38 ± 0.25	0.902
Cortisol (nmol L ⁻¹)	10.23 ± 1.36	5.42 ± 1.50	0.056

GnRH: Gonadotropin releasing hormone; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; T3: Triiodothyronine; T4: Thyroxine; IGF-1: Insulin like growth factor.

Table 4. Correlation of reproductive hormones with semen attributes of buffalo bulls.

Parameters	GnRH	Kisspeptin	FSH	LH	Testosterone	T3	T4	IGF-1	Cortisol
Total motility (%)	0.75**	0.20	-0.24	0.34	0.19	0.03	-0.15	0.54*	0.16
Progressive motility (%)	-0.09	-0.07	-0.01	-0.15	0.19	0.06	0.13	-0.19	-0.45
Average path velocity ($\mu\text{m sec}^{-1}$)	0.75**	-0.04	-0.47	0.49	0.04	0.06	-0.38	0.52*	0.09
Straight line velocity ($\mu\text{m sec}^{-1}$)	0.76**	-0.06	-0.46	0.53*	0.06	0.07	-0.38	0.51*	0.09
Curvilinear velocity ($\mu\text{m sec}^{-1}$)	0.70**	-0.02	-0.46	0.34	0.01	0.15	-0.28	0.48	-0.00
Straightness (%)	0.49	-0.08	-0.30	0.59*	0.12	0.09	-0.23	0.35	-0.08
Linearity (%)	0.71**	-0.05	-0.41	0.68**	0.04	-0.09	-0.46	0.53*	0.23
Viability (%)	0.04	0.10	-0.35	0.05	-0.19	-0.28	-0.27	-0.05	0.04
Hypo-osmotic swelling test (%)	0.32	0.03	-0.36	0.23	-0.23	-0.34	-0.20	0.21	-0.11
Normal apical ridge (%)	0.12	0.06	-0.39	0.10	-0.34	-0.72**	-0.49	-0.09	0.27
DNA integrity (%)	0.34	-0.07	-0.20	0.52*	0.05	-0.14	-0.18	0.26	0.19

GnRH: Gonadotropin releasing hormone; FSH: Follicle-stimulating hormone; LH: Luteinizing hormone; T3: Triiodothyronine; T4: Thyroxine; IGF-1: Insulin like growth factor.

Correlation coefficient with * is significant at $p < 0.05$; while, ** is significant at $p < 0.01$.

Discussions

Several studies regarding seasonal effects on hormonal profiles^{11,20} as well as semen quality parameters^{18,27} have already been reported in buffalo bulls. In the present study, the serum concentration of kisspeptin was not affected by seasons in buffalo bulls. Besides, there was no association of kisspeptin with under-studied hormones as well as semen parameters. It has been observed that the kisspeptin neurons are higher in POA than ARC of the hypothalamus in buffalo^{3,7} and there is also an evidence of no change in kisspeptin expression at POA in different seasons.²⁸ So, it may be possible that testicular kisspeptin may also interact with circulating kisspeptin¹⁰ or some other factors involved in consistent concentration of kisspeptin in both seasons. Recent findings of no association between Kiss1r on spermatozoa and sperm motility²⁹ also support this correlation. It is well documented that kisspeptin acts on the hypothalamus to release GnRH^{2,30} and a higher level of GnRH in summer without any alteration in kisspeptin was observed in this study. So, it can be inferred that there may be some other pathways which are also controlling the release of GnRH. In spite of the inhibitory role of cortisol on GnRH,³¹ it did not affect the GnRH in our study. To find out the reason, ewe model was used and elaborated that cortisol does not affect the hypothalamus in the absence of gonadal steroids still needing further studies to be fully elucidated.³² Higher GnRH in the summer season may also be due to longer day length. The GnRH is released from the hypothalamus and acts on gonadotrophs in the anterior pituitary to release FSH and LH which have receptors on Sertoli cells and Leydig cells, respectively.³³ These gonadotropins control male fertility by controlling the steroidogenesis and spermatogenesis. In our study, hormonal correlations showed that the GnRH concentration was positively correlated with LH and IGF-1. This may be due to the fact that the acute or prolonged shock may cause GnRH neuron stimulation as well as an increase in LH pulse³⁴ and GnRH neurons are co-expressed with IGF-1 neurons.³⁵

However, the GnRH did not show any correlation with FSH which may be due to the difference in frequency of GnRH pulse. Accordingly, higher GnRH pulse frequency may suppress the release of FSH.³⁶ The GnRH and LH levels were positively correlated with semen parameters being comparable with the previous studies on buffaloes.²⁷ Exogenous administrations of GnRH has also shown a positive effect on semen parameters in cattle bulls.³⁷ In our study, a higher level of LH was observed in the summer season compared to the spring season. We had also observed a positive correlation between LH and cortisol, which is consistent with the study in which the higher cortisol resulted in increased concentration of LH.³⁸ Although the exact reason for this is not fully known, there may be some other factors altering the sensitivity of anterior pituitary.^{39,40} A constant concentration of testosterone at different ambient temperatures may be due to its metabolism.⁴¹ A positive correlation of testosterone with ejaculatory volume without any effect on sperm kinematics of Nili Ravi buffalo bulls,⁴² agrees with our study. These results were also consistent with previous studies,^{43,44} showing that testosterone does not have any significant correlation with semen parameters. Among metabolic hormones, IGF-1 has an important role in male fertility due to its receptors on Sertoli cells, Leydig cells and spermatozoa.⁴⁵ The higher serum concentration of IGF-1 was found in males with normal sperm motility than those with abnormal sperm motility.^{46,47}

We observed better semen quality during summer which may be due to the reason that our studied time (August-September) was closer to breeding season peak.¹⁹ Improved semen parameters were also documented during the summer in buffalo.^{18,48} However, our results are in contradiction with the study,⁴⁹ observing no significant difference of motility in different seasons; while, other parameters were better in the spring and autumn seasons. It can be inferred that in buffalo, the testosterone and thyroid hormones might not be affected by season; however, the higher levels of GnRH, LH and IGF-1 may increase most of the spermatogenic parameters.

Collectively, kisspeptin was not influenced by the season in our experimental conditions. Further studies using molecular techniques regarding the kisspeptin neuron stimulation in different seasons are required. However, positive correlations of GnRH, LH and IGF-1 with semen traits may be used as potential indicators of semen quality. Better semen quality in hot weather shows that buffalo bulls are adapted to this season and semen collected in this season can also be cryopreserved for artificial insemination without any insecurity.

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Conflict of interest

There is no conflict of interest in any part of the research and manuscript.

References

- Ohtaki T, Shintani Y, Honda S, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 2001; 411(6837): 613-617.
- de Roux N, Genin E, Carel JC, et al. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* 2003; 100(19):10972-10976.
- Chaikhun T, Yanprapasiri C, Sothibandhu P, et al. Kiss-1 mRNA/kisspeptin distribution in preoptic and arcuate nuclei of cycling buffalo (*Bubalus bubalis*) hypothalamus. *Pak Vet J* 2016; 36(1):93-97.
- Tanco VM, Whitlock BK, Jones MA, et al. Distribution and regulation of gonadotropin-releasing hormone, kisspeptin, RF-amide related peptide-3, and dynorphin in the bovine hypothalamus. *Peer J* 2016; 4: e1833. doi: 10.7717/peerj.1833.
- Husna M, Flatscher-Bader T, Lehnert S, et al. Gene expression of GnRH, kisspeptin, neuropeptide Y and receptors for estrogen and leptin in the hypothalamus of suckled and weaned beef cows. *J Tropical Agric Food Sci* 2012; 40:245-255.
- Mishra GK, Patra MK, Singh LK, et al. Kiss1 and its receptor: molecular characterization and immunolocalization in the hypothalamus and corpus luteum of the buffalo. *Anim Biotechnol* 2019; 30(4):342-351.
- Rehman H, Shahzad MK, Rabbani I, et al. Potential role of Kisspeptin and Cytochrome P mRNA genes in pubertal development and spermatogenesis in buffalo bulls (*Bubalus bubalis*). In proceedings: British Society of Animal Sciences Annual Conference. Edinburgh, UK 2019; 176.
- Samir H, Nagaoka K, Karen A, et al. Investigation the mRNA expression of KISS1 and localization of kisspeptin in the testes of Shiba goats and its relationship with the puberty and steroidogenic enzymes. *Small Ruminant Res* 2015; 133:1-6.
- Tariq AR, Shahab M, Clarke IJ, et al. Kiss1 and Kiss1 receptor expression in the rhesus monkey testis: a possible local regulator of testicular function. *Cent Eur J Biol* 2013; 8(10):968-974.
- Salehi S, Adeshina I, Chen H, et al. Developmental and endocrine regulation of kisspeptin expression in mouse Leydig cells. *Endocrinology* 2015; 156(4): 1514-1522.
- Koonjaenak, S, Chanatinart V, Aiumlamai S, et al. Seasonal variation in semen quality of swamp buffalo bulls (*Bubalus bubalis*) in Thailand. *Asian J Androl* 2007; 9(1):92-101.
- Ahmed H, Andrabi SMH, Jahan S. Semen quality parameters as fertility predictors of water buffalo bull spermatozoa during low-breeding season. *Theriogenology* 2016; 86(6):1516-1522.
- Andrabi SMH, Ansari MS, Ullah N, et al. Duck egg yolk in extender improves the freezability of buffalo bull spermatozoa. *Anim Reprod Sci* 2008; 104(2-4): 427-433.
- Pottapenjara V, Rajanala SR, Reddy C, et al. Kisspeptin modulates luteinizing hormone release and ovarian follicular dynamics in pre-pubertal and adult Murrah buffaloes. *Front Vet Sci* 2018; 5:149. doi: 10.3389/fvets.2018.00149.
- Naniwa Y, Nakatsukasa K, Setsuda S, et al. Effects of full-length kisspeptin administration on follicular development in Japanese Black beef cows. *J Reprod Dev* 2013; 59(6):588-594.
- Koivisto MB, Costa MTA, Perri SHV, et al. The effect of season on semen characteristics and freezability in *Bos indicus* and *Bos taurus* bulls in the southeastern region of Brazil. *Reprod Domest Anim* 2009; 44(4):587-592.
- Fiaz M, Usmani RH, Abdullah M, et al. Evaluation of semen quality of Holstein Friesian and Jersey bulls maintained under subtropical environment. *Pak Vet J* 2010; 30(2):75-78.
- Mallick S, Aggarwal A, Prakash BS. Seasonal changes in semen quality and correlation with plasma hormone profiles in Karan Fries bulls. *Biol Rhythm Res* 2016; 47(6):967-974.
- Ahmed H, Andrabi SMH, Anwar M, et al. Use of post-thaw semen quality parameters to predict fertility of water buffalo (*Bubalus bubalis*) bull during peak breeding season. *Andrologia* 2017; 49(4):e12639. doi: 10.1111/and.12639.
- Javed MT, Khan A, Ali M. Influence of season on seminal plasma testosterone and oestrogen in healthy and

- abnormal buffalo bulls and their relationship with other semen parameters. *Vet Arh* 2000; 70(3): 141-150.
21. Jainudeen MR, Bongso TA, Dass S. Semen characteristics of the swamp buffalo (*Bubalus bubalis*). *Anim Reprod Sci* 1982; 4(3):213-217.
 22. Rasul Z, Anzar M, Jalali S, et al. Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. *Anim Reprod Sci* 2000; 59 (1-2):31-41.
 23. Björndahl L, Söderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 2003; 18(4):813-816.
 24. Jayendran RS, Van der Ven HH, Perez-Pelaez M, et al. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984; 70(1):219-228.
 25. Khan MIUR, Ijaz A. Assessing undiluted, diluted and frozen-thawed Nili-Ravi buffalo bull sperm by using standard semen assays. *Ital J Anim Sci* 2007; 6(Suppl. 2):784-787.
 26. Fayyaz MH, Ahmad M, Ahmad N. Survival of buffalo bull spermatozoa: effect on structure and function due to alpha-lipoic acid and cholesterol-loaded cyclodextrin. *Andrologia* 2017; 49(4):e12652. doi: 10.1111/and.12652.
 27. Javed MT, Khan A, Kausar R. Effect of age and season on some semen parameters of Nili-Ravi buffalo (*Bubalus bubalis*) bulls. *Vet Arh* 2000; 70(2):83-94.
 28. Smith JT, Clay CM, Caraty A, et al. KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology* 2007; 148(3):1150-1157.
 29. Hussain A, Nabi W, Zubair H, et al. Immunocytochemical detection of kisspeptin receptor and its association with motility of buffalo bull (*Bubalus bubalis*) spermatozoa. *Pak Vet J* 2020; doi: 10.29261/pakvetj/2020.007.
 30. Gutiérrez-Pascual E, Martínez-Fuentes AJ, Pinilla L, et al. Direct pituitary effects of kisspeptin: activation of gonadotrophs and somatotrophs and stimulation of luteinising hormone and growth hormone secretion. *J Neuroendocrinol* 2007; 19(7):521-530.
 31. Padmanabhan V, Keech C, Convey EM. Cortisol inhibits and adrenocorticotropin has no effect on luteinizing hormone-releasing hormone-induced release of luteinizing hormone from bovine pituitary cells *in vitro*. *Endocrinology* 1983; 112(5):1782-1787.
 32. Ralph CR, Lehman MN, Goodman RL, et al. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? *Reproduction* 2016; 152(1):R1-R14.
 33. Walker WH, Cheng J. FSH and testosterone signaling in Sertoli cells. *Reproduction* 2005; 130(1):15-28.
 34. Łapot M, Ciechanowska M, Malewski T, et al. The effect of stress on the expression of GnRH and GnRH receptor genes in the discrete regions of the hypothalamus and pituitary of anestrous ewes. *Reprod Biol* 2007; 7(1):55-71.
 35. Daftary SS, Gore AC. IGF-1 in the brain as a regulator of reproductive neuroendocrine function. *Exp Biol Med (Maywood)* 2005; 230(5):292-306.
 36. Jayes FC, Britt JH, Esbenshade KL. Role of gonadotropin-releasing hormone pulse frequency in differential regulation of gonadotropins in the gilt. *Biol Reprod* 1997; 56(4):1012-1019.
 37. Sajjad M, Ali S, Akhter S, et al. Effect of gonadotropin releasing hormone on semen characteristics in Nili-Ravi buffalo bulls. *Pak Vet J* 2007; 27(3):153-154.
 38. Liptrap RM, Raeside JL. Effect of cortisol on the response to gonadotrophin releasing hormone in the boar. *J Endocrinol* 1983; 97(1):75-81.
 39. Kawate N, Inaba T, Mori J. Effects of progesterone and cortisol on the release of gonadotropin-releasing hormone from the perfused pituitary stalk-median eminence and on luteinizing hormone release from the pituitary of cows. *Anim Reprod Sci* 1993; 34(2): 93-100.
 40. Wagenmaker ER, Breen KM, Oakley AE et al. Cortisol interferes with the estradiol-induced surge of luteinizing hormone in the ewe. *Biol Reprod* 2009; 80(3):458-463.
 41. Minton JE, Wettemann RP, Meyerhoeffler DC, et al. Serum luteinizing hormone and testosterone in bulls during exposure to elevated ambient temperature. *J Anim Sci* 1981; 53(6):1551-1558.
 42. Sajjad M, Ali S, Ullah N, et al. Blood serum testosterone level and its relationship with scrotal circumference and semen characteristics in Nili-Ravi buffalo bulls. *Pak Vet J* 2007; 27(2):63-66.
 43. de Oliveira Souza LW, Andrade AFC, Celeghini ECC, et al. Correlation between sperm characteristics and testosterone in bovine seminal plasma by direct radioimmunoassay. *Rev Bras Zootec* 2011; 40(12):2721-2724.
 44. Rajak SK, Tripathi UK, Attapuram NM, et al. Relationship of blood and seminal plasma testosterone concentrations with semen quality in crossbred bulls. *Indian J Dairy Sci* 2014; 67(67):162-167.
 45. Handelsman DJ, Spaliviero JA, Scott CD, et al. Identification of insulin-like growth factor-I and its receptors in the rat testis. *Acta Endocrinol (Copenh)* 1985; 109(4):543-549.
 46. Kumar P, Suman, Pawaria S, et al. Serum and seminal plasma IGF-1 associations with semen variables and effect of IGF-1 supplementation on semen freezing capacity in buffalo bulls. *Anim Reprod Sci*

- 2019;204:101-110.
47. Lee HS, Park Y-S, Lee JS, et al. Serum and seminal plasma insulin-like growth factor-1 in male infertility. *Clin Exp Reprod Med* 2016; 43(2):97-101.
48. Ramadan TA, Taha TA, Samak MA, et al. Seasonal and monthly variations in semen characteristics of Egyptian buffalo bulls. *Alex J Agric Res* 2009; 54(2):13-23.
49. Hameed S, Masood S, Zaneb H, et al. Semen characteristics as influenced by seasonal and climatic variations in Nili-Ravi buffalo breeding bulls. *J Anim Plant Sci* 2017; 27(6):1750-1757.