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SHORT COMMUNICATION

Possible Role of Kisspeptin in Regulation of Motility Spectrum of Buffalo Bull Spermatozoa: A Preliminary Study

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ABSTRACT

This study was designed to determine possible effects of human kisspeptin-10 (KP-10) on motility spectrum of buffalo bull spermatozoa. Semen was collected from three adult Nili-Ravi buffalo bulls twice a week for three-weeks, split into five aliquots and diluted with different doses (0, 0.2, 2.0, 20.0 and 40.0 µM) of KP-10 in 0.1% BSA-PBS. The aliquots were incubated for 2, 6, 12 and 24 min at 37°C and evaluated through Computer-Assisted Sperm Analysis (CASA) system for sperm motility parameters. Ethanol fixed smears were processed for immuno-cytochemical detection of KP. Results showed that KP-10 exposure (mostly at 20.0 and 40.0 µM) for different incubation time-points significantly ($P<0.05$) improved CASA sperm motilities, velocities, and rapid subpopulation than control. Furthermore, buffalo bull spermatozoa showed KP-10 expression in their tail region. This study identified the stimulatory effect of human KP-10 on buffalo bull sperm motility parameters during *in vitro* incubation. Thus, human KP-10 can be potentially used to enhance quality of buffalo bull sperm during incubation at 37°C *in vitro*.

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INTRODUCTION

Kisspeptin is a neurohormone encoded by Kiss1 gene (Gottsch *et al.,* 2009) and shows its functional activity through its functional receptor, G-protein-coupled receptor-54 (GPR54). Kisspeptin plays a key role in the activation of reproductive axis following the release of GnRH (Tena-Sempere, 2010). GPR54 mRNA receptors are present in the medio-basal hypothalamus, preoptic area, and arcuate nucleus, where they show a central action in controlling gonadotropins secretion in different species, suggesting the role of kisspeptin-GPR54 pathway in reproduction (Hsu *et al*., 2014; Mishra *et al*., 2019). A direct effect of kisspeptin at the testicular level has been observed in non-human primates (Tariq and Shabab, 2017). In another study, Pinto *et al.* (2012) have demonstrated the presence of kisspeptin receptors on mature human spermatozoa with a definite pattern of cellular distribution and functional role of kisspeptin. Additionally, kisspeptin and its receptors were found in the sperm midpiece, a region related to the control of energetic requirements and Ca^{2+} buffering and sperm motility (Pinto *et al.,* 2012). Moreover, stimulatory effect of human kisspeptin on gonadotrophins secretion has also been reported in bovines (Ahmed *et al.,* 2009).

However, there is relatively little information regarding the role of kisspeptin in influencing motility pattern of buffalo bull spermatozoa. Therefore, the present study was undertaken to investigate the possible effects of human kisspeptin-10 exposure on sperm kinematics and to access site of expression of kisspeptin on buffalo bull spermatozoa during *in vitro* incubation at 37°C.

MATERIALS AND METHODS

Experimental animals: In this study, three adult Nili-Ravi buffalo bulls, aged 6-7 years and maintained under standard conditions at the National Agriculture Research Centre, Islamabad, Pakistan (longitude 73.8° East, latitude 33.4° North) were used for semen collection. The animals were fed with green fodder (10% body weight), cotton seed cakes and wheat bran (3 kg per animal daily)

collectively at 10:00-10:30 AM, while clean drinking water was available 24 hours a day.

Semen collection and initial evaluation: Semen collection from each bull was carried out once a week for a period of three-weeks, using an artificial vagina (42°C). After initial evaluation for ejaculatory volume, sperm motility and concentration, each ejaculate was divided into 5 aliquots and diluted at (37°C) in 0.1% BSA-PBS supplemented with 0.0 µM (control, C), 0.2 µM (D1), 2.0 µM (D2), 20.0 µM (D3) and 40.0 µM (D4) of human kisspeptin-10 (Metastin, Calbiochem, Darmstadt, Germany) to achieve final sperm concentration of 25 million per ml. Each dose of KP-10 was added to the 50 ml of extender.

Diluted samples were incubated at 37°C and evaluated for sperm motility patterns at different time points i.e. 2 min (T1), 6 min (T2), 12 minutes (T3) and 24 min (T4) of incubation, using CASA system.

Computer-assisted sperm analysis (CASA) system: At each time-point sperm motility parameters were assessed (37°C) by CASA (Hamilton Throne Biosciences CEROS, Japan). A 7µl drop of sample from each aliquot was placed on glass-slide and examined for total motile sperm (TM, %), progressive motile sperm (PM, %), sperm showing rapid velocity (RV, %), average-path velocity (VAP, µm/sec), straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), beat cross frequency (BCF, Hz), and straightness (STR, %). Approximately 200 spermatozoa were evaluated for each aliquot. Based on the CASA sperm motility pattern, four subpopulations of spermatozoa (rapid, hyperactivated, low and poor motile) were identified.

Immuno-staining: Immuno-cytochemical procedure was adopted to determine KP-10 expression in spermatozoa. For this purpose, 5 µl of diluted semen was smeared on frosted-slides, air-dried and fixed in chilled methanol (-20°C, 20 min). For blocking non-specific binding sites, after adding rabbit serum (10%), slides were kept in a humidified chamber for 120 min and incubated with PBS as KP-10 omitted control (Hussain *et al*., 2020). Then slides were incubated with primary antibody (1:120,000 in PBS/0.05%Triton-X/0.1%) and kept at 4°C for 48 hours in humidified chamber. Slides were then incubated (90 min) with secondary antibodies for Kisspeptin AF 488 (Abcam, Cambridge, UK), rabbit antisheep; 1:400, diluted in PBS/0.05% Triton-X/0.1%, followed by washing with PBS (thrice). Smears were then mounted, and cover slipped, dried overnight at 4°C and examined under fluorescent microscope (AMEP-4615, Washington, USA) at different magnifications and screen shots were taken as per general rule for the use of microscope. Smear treated without primary antibody lack fluorescence whereas, smears treated with both primary and secondary antibodies displayed fluorescence.

Statistical analysis: Statistical analyses were performed using Minitab. No interaction was observed between bull (n=3) and treatments with GLM procedure, so bull data were pooled and analyzed by ANOVA followed by Tukey's method for pairwise comparisons between the treatment means. The differences were considered significant at $P<0.05$.

RESULTS

Effect of KP-10 on CASA sperm motility parameters: As shown in Table 1, sperm TM was higher ($P<0.05$) in D4 than control and D1 at 2 and 6 min exposure to KP-10. However, at 12 and 24 min exposure to KP-10, sperm TM was higher ($P<0.05$) in all KP-10 treated groups than control. Sperm PM was significantly higher ($P<0.05$) in all treatment groups than control at 2 min exposure to KP-10. At 6 and 12 min exposure to KP-10, sperm PM was significantly higher in D2, D3 and D4 groups than control and D1 group. Sperm RV was higher ($P<0.05$) in D4 than control group at 2 and 6 min exposure to KP-10. At 12 and 24 min exposure, sperm showed higher RV values in D3 and D4 groups compared to control ($P<0.05$). Sperm VAP, VSL, VCL, BCF, and STR were higher ($P<0.05$) in D4 group than control at all-time points, except that there was no difference in STR between KP-10 treated and control groups.

Effects of KP-10 on CASA sperm sub-populations: In the present study, the prevalence of rapid CASA sperm subpopulation was significantly higher ($P<0.05$) in D4 as compared to control at all incubation time-points (Table 2). However, the effects of different concentrations of KP-10 on other three CASA sperm populations (hyper-activated, low and poor motile) at all four incubation time-points were non-significant.

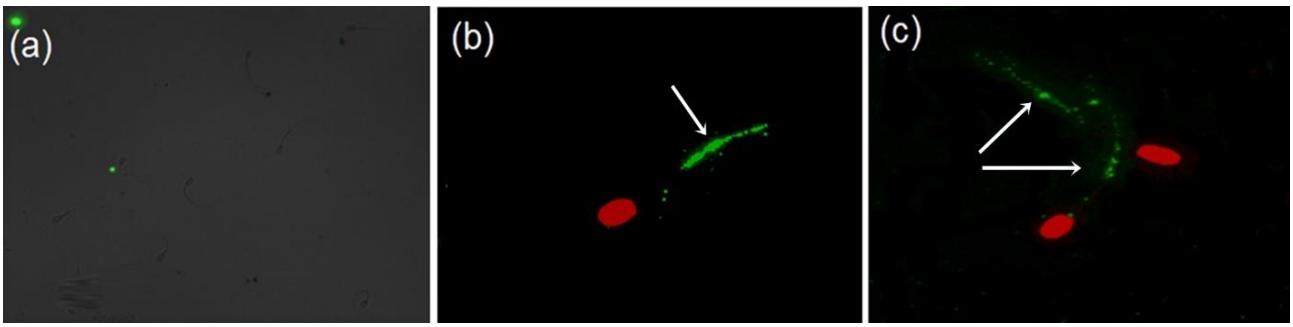

Fig. 1: Fluorescent microscopic images showing kisspeptin immunoreactivity of the buffalo bull spermatozoa. Control smears (a) were processed without primary antibody and displayed no immunoreactivity. Primary and secondary antibodies treated smears (b and c) displayed green fluorescence in the tail region (arrows) of buffalo bull sperm (100X).

Table 1: Effect of different doses of KP-10 on CASA sperm motility parameters at different incubation times

Incubation time	Treatment groups	CASA sperm motility parameters							
		TM (%)	PM (%)	RV (%)	VAP (µm/sec)	VSL (µm/sec)	VCL (µm/sec)	BCF (Hz)	STR (%)
Time 1	C (0 µM)	89.24±1.27b	53.78±1.21b	64.8±1.89b	120.15±2.70b	90.33±1.32b	138.78±2.03b	37.51±1.27b	81.59±1.48b
(2 minutes)	D1 (0.2 µM)	89.56±1.02b	59.33±1.21a	69.73±1.54ab	122.13±1.57ab	93.67±1.09ab	142.78±1.59ab	39.75±1.32ab	83.9±1.53ab
	D2 (2 µM)	90.89±1.12ab	62.17±1.62a	70.51±1.35ab	120.44±1.63ab	96.46±2.96ab	143.11±1.36ab	40.91±1.11ab	85.04±1.46ab
	D3 (20 µM)	91.89±9.73ab	63.45±1.37a	70.27±1.27ab	124.67±1.73ab	95.89±1.75ab	154.32±7.15ab	41.23±1.23ab	87.34±1.75ab
	D4 (40 µM)	94.32±1.32a	64.58±1.08a	74.14±1.80a	127.67±1.36a	99.56±1.07a	157.03±5.67a	42.24±0.72a	88.33±1.70a
Time 2	C (0 µM)	82.78±1.23b	44.11±1.09b	51.8±1.09b	99.33±1.36b	86.78±2.33b	119.11±2.46b	32.03±0.78b	75.56±0.99b
(6 minutes)	D1 (0.2 µM)	83.67±1.34b	47.67±1.14b	56.37±1.45ab	103.89±1.59ab	89.00±1.62ab	124.89±1.60ab	33.35±0.71ab	77.53±1.26ab
	D2 (2 µM)	85.56±1.36ab	55.22±1.35a	57.28±1.41ab	105.44±1.93ab	91.22±1.89ab	125.33±2.42ab	34.22±0.94ab	79.11±0.89ab
	D3 (20 µM)	87.56±1.06ab	56.78±1.66a	57.44±1.69ab	103.56±1.43ab	93.11±1.71ab	128.56±1.53ab	34.87±1.56ab	80.15±1.48ab
	D4 (40 µM)	89.33±0.91a	59.56±1.09a	62.69±2.12a	109.33±3.09a	95.67±1.63a	129.33±3.71a	36.98±0.99a	81.78±1.32a
Time 3	C (0 µM)	71.11±1.21b	37.11±0.77b	35.33±1.34b	80.56±1.38b	72.89±1.67b	96.78±3.28b	26.74±0.43b	60.40±0.86b
(12 minutes)	D1 (0.2 µM)	77.78±1.87a	40.22±0.94b	39.67±1.13ab	84.67±1.05ab	74.78±1.43ab	100.44±3.04ab	27.87±0.87ab	61.46±0.64ab
	D2 (2 µM)	80.67±1.35a	45.89±0.87a	39.44±1.07ab	86.33±1.12ab	75.11±1.52ab	103.22±1.89ab	28.08±0.88ab	61.96±1.10ab
	D3 (20 µM)	81.11±1.06a	45.00±1.19a	40.22±1.34a	87.11±2.32ab	77.56±1.07ab	105.56±1.56ab	29.08±1.12ab	64.22±1.02ab
	D4 (40 µM)	82.78±1.36a	48.78±0.64a	42.44±1.08a	88.44±2.44a	78.67±0.91a	107.89±2.10a	30.63±0.30a	65.33±1.09a
Time 4	C (0 µM)	53.78±1.27b	12.89±0.95	12.11±1.22b	59.89±1.31b	57.11±2.23a	69.44±2.22b	20.11±0.89b	51.2±1.35
(24 minutes)	D1 (0.2 µM)	64.11±1.59a	20.78±1.14	14.44±1.36ab	60.56±1.06ab	60.11±1.49ab	71.11±1.93ab	21.22±1.01ab	51.88±1.45
	D2 (2 µM)	64.22±1.30a	21.11±0.77	14.89±1.18ab	62.44±1.42ab	60.22±0.95ab	73.89±2.10ab	22.67±0.93ab	53.83±1.43
	D3 (20 µM)	65.22±1.04a	22.22±0.94	17.78±1.30a	63.89±1.25ab	62.89±1.11ab	76.67±2.15ab	24.11±1.29ab	54.17±1.20
	D4 (40 µM)	69.22±1.31a	22.56±1.20	19.56±1.33a	65.67±1.63a	63.22±0.95a	78.67±1.80a	24.65±1.12a	55.95±1.28

TM=Total motile sperm, PM=Progressive motile sperm, RV=Sperm with Rapid velocity, VAP=Average-path velocity, VSL=Straight-line velocity, VCL=Curvilinear velocity, BCF=Beat cross frequency, STR=Straightness. Values in the same column with different letters for each incubation time differ significantly (P<0.05).

Table 2: Effects of different doses of KP-10 on CASA sperm subpopulation at different incubation times

Incubations time	Treatment groups	CASA sub-populations			
		Rapid subpop (%)	Hyperactivated subpop (%)	Low subpop (%)	Poor subpop (%)
Time 1	C (0 µM)	49.85±2.14b	18.96±1.66	24.67±1.52	8.01±0.75
(2 minutes)	D1 (0.2 µM)	54.22±2.16ab	14.28±1.11	21.81±2	5.76±0.72
	D2 (2 µM)	55.77±2.28ab	12.93±1.11	24.23±0.92	5.55±0.69
	D3 (20 µM)	56.02±2.79ab	14.86±0.96	26.44±0.57	5.51±0.7
	D4 (40 µM)	58.97±1.22a	13.58±0.37	23.52±1.45	6.23±0.73
Time 2	C (0 µM)	44.14±1.47b	16.34±0.6	28.03±0.88	8.15±1.57
(6 minutes)	D1 (0.2 µM)	48.32±1.76ab	14.73±0.38	27.09±1.55	5.73±1.08
	D2 (2 µM)	50.34±2.13ab	16.57±1.58	25.04±2.09	4.62±0.77
	D3 (20 µM)	51.1±2.08ab	14.28±0.53	24.93±1.04	6.06±0.62
	D4 (40 µM)	52.23±1.15a	15.05±1.19	26.56±1.31	9.76±1.36
Time 3	C (0 µM)	40.95±0.69b	16.5±1.61	26.88±1.52	12.75±2.22
(12 minutes)	D1 (0.2 µM)	41.85±1.22ab	13.71±0.91	29.54±1.89	11.72±1.36
	D2 (2 µM)	42.32±1.33ab	14.37±0.6	29.88±1.46	12.11±1.38
	D3 (20 µM)	43.48±1.87ab	15.13±1.21	26.58±1.57	11.68±1.02
	D4 (40 µM)	47.14±1.79a	18.44±1.26	28.34±0.42	6.73±0.78
Time 4	C (0 µM)	37.79±1.01b	23.07±0.76	28.03±0.39	7.38±0.63
(24 minutes)	D1 (0.2 µM)	43.35±2.26ab	17.94±1.33	28.04±0.52	9.1±1.16
	D2 (2 µM)	44.22±1.76ab	19.43±1.8	28.34±0.61	11.02±1.59
	D3 (20 µM)	44.61±2.6ab	21.17±2.1	28.63±0.87	9.93±1.21
	D4 (40 µM)	47.17±2.09a	23.72±0.88	27.85±0.46	8.2±0.96

Values in the same column with different letters for each incubation time differ significantly (P<0.05).

Expression of KP in buffalo spermatozoa: The immuno-cytochemical study of KP-10 treated spermatozoa revealed the presence of green fluorescence in the tail region (Fig. 1). This shows that Kisspeptin receptors are located in tail region of buffalo bull spermatozoa.

DISCUSSION

Kisspeptin a neurohormone, which acts through its receptors and regulates reproductive activity of an animal at the levels of hypothalamus and pituitary (Tena-Sempere, 2010). Receptors of this hormone are localized in mature human sperm (Pinto *et al.,* 2012), and are involved in regulation and control of flagella movement (Publicover *et al.,* 2008). This study investigated KP-10 effects on motion kinetics of buffalo bull spermatozoa during *in vitro* storage at 37°C. Moreover, site for localization of KP receptors in buffalo bull spermatozoa was also investigated.

Results of the present study revealed that KP-10 exposure (mostly 20.0 or 40.0µM) significantly improved sperm motility of buffalo bull spermatozoa at different incubation time-points at 37°C compared with the control group. Moreover, KP-10 exposure improved VAP, VSL, VCL and BCF of buffalo bull spermatozoa than control. Similarly, rapid subpopulation of buffalo bull spermatozoa also showed a significant increase over control group when exposed to 40.0µM KP-10. It has been reported that kisspeptin exposure results in important fluctuations in human sperm motility (Pinto *et al.,* 2012). Initially, sperm build up a progressive motility in comparatively normal and linear patterns that are compulsory for spinning and transportation through the female reproductive tract (Ahmed *et al.,* 2016). These findings suggest that KP-10 acts as a mediator of motility by increasing forward movement and progressiveness of buffalo bull spermatozoa during *in vitro* storage.

To the best of our knowledge, this is the first study in which the presence of KP-10 receptors on buffalo bull

spermatozoa was investigated. The occurrence of kisspeptin in the tail region of buffalo spermatozoa identified a novel function of kisspeptin in spermatozoa activity. It is postulated that kisspeptin stimulates different signal transduction pathways, suggesting a modulatory role of kisspeptin in progressive sperm motility.

Conclusions: From this study, it may be suggested that human KP-10 has stimulatory effects on semen quality of buffalo bull evaluated in terms of CASA sperm motility parameters. Thus, human KP-10 can be potentially used to enhance quality of buffalo bull sperm during incubation at 37°C *in vitro*.

Authors contribution: LK carried out execution of study and manuscript preparation; SS assisted in manuscript preparation; HA assisted in initial evaluation of semen and manuscript write up; SMHA was involved in overall supervision of semen collection and its analysis; HZ, RB and BTK helped in manuscript preparation and revision; MS proposed study design and helped in manuscript preparation and review.

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