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RESEARCH ARTICLE

Immunocytochemical Detection of Kisspeptin Receptor and Its Association with Motility of Buffalo Bull (*Bubalus bubalis*) Spermatozoa

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ABSTRACT

Kisspeptin is a powerful regulator of the hypothalamic-pituitary-gonadal axis. It acts through its receptor GPR54 to regulate sexual maturation, oogenesis, spermatogenesis and fertilization. The present research was implemented to evaluate the presence of kisspeptin receptor on various regions of buffalo bull spermatozoa and to decipher its relationship with different motility parameters of the fresh spermatozoa. Standard swim-up protocol was performed on fresh ejaculates from Nili-Ravi buffalo bulls to obtain three hypothetical layers, having spermatozoa with enhanced motility and normal morphology. The progressive sperm motility was measured in each layer by using phase contrast microscopy. Methanol fixed sperm smears from each layer were processed for standard immunocytochemistry procedure for the detection of kisspeptin receptor using specific antibodies. Maximum GPR54 immunoreactivity was observed in the upper regions (head, neck/midpiece) and moderate immunoreactivity was seen in the lower region (tail) of the spermatozoa in all the three layers. No significant difference in total GPR54 expression was observed in the spermatozoa from three layers and no relationship was observed between percentage motility and GPR54 like ir. Present findings suggest that the buffalo sperm motility is not influenced by GPR54 expression on different regions of the sperm. However, robust GPR54 expression in dorsal areas of buffalo sperm, would raise possibility of a covert function of kisspeptin in the bubaline sperm biology.

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INTRODUCTION

Kisspeptin (Kp), a neurohormone, is essential for the activity of reproductive axis (Franssen and Tena-Sempere, 2018). Kisspeptin peptide is a member of RF-amide superfamily of neuropeptides that has Arg-Phe-NH_2 C- terminal sequence. After Kp discovery, different groups independently identified in the rat's brain, an orphan G protein-coupled membrane receptor i.e GPR54 now known as Kiss1r with high affinity for Kp (Uenoyama *et al*., 2019).

Both Kiss1 the gene encoding Kp, and GPR54 mRNA is present in the hypothalamus especially in the mediobasal hypothalamus, preoptic area, arcuate nucleus and anteroventral paraventricular area, where it has a central action in controlling gonadotropins secretion in different species, suggesting the reproduction regulating role of kisspeptin-GPR54 pathway (Chaikhun *et al*., 2016; Chaikhun-Marcou *et al*., 2018; Mishra *et al*., 2019). Kiss1-GPR54 signaling is fundamental to GnRH-driven fertility (Leon *et al*., 2016). Mutation in Kp or in GPR54 are the main reasons for various types of reproductive axis disabilities such as, iodiopathic hypogonadotropic hypogonadism (Kotani *et al*., 2014), central precocious puberty (Oh *et al*., 2017) and Normosmic idiopathic hypogonadotrophic hypogonadism (Chelaghma *et al*., 2018).

Besides its prominent expression at the hypothalamic levels mentioned earlier, GPR54 mRNA or protein is also reported in several reproductive tissues such as in mice Leydig cells, spermatid cells, seminiferous tubules and testes (Hua *et al*., 2013; Hsu *et al*., 2014), in the ovaries of cats (Tanyapanyachon *et al*., 2018), dogs (Cielesh *et al*., 2017) and pigs (Basini *et al*., 2018) and in the testes of human (Feng *et al*., 2019) and frogs (Chianese *et al*., 2017). Moreover, in rhesus monkeys, positive immunostaining of GPR54 was noted in round spermatocytes, seminiferous tubules, Sertoli cells and spermatids (Tariq *et al*., 2013) and in the perimeter of the seminiferous epithelium (Irfan *et al*., 2016).

KISS1and KISS1R immunoreactivity has also been reported in human sperm cells (Pinto *et al*., 2012). Intense immunostaining for KISS1 and KISS1R was detected in the equatorial region, which plays a pivotal role in the fusion of male and female gametes. In majority of the cells, positive immune labelling for KISS1 and KISS1R was detected around the neck part, which suggests its role in the sperm motility. Also, the immunoreactivity of both KISS1 and KISS1R were detected in the midpiece region in a lower number of the sperm (Trevisan *et al*., 2018).

However, the presence of GPR54 and its feasible role in the regulation of freshly ejaculated buffalo sperm motility is still unknown. Objective of this research was to investigate the expression of GPR54 on different regions of buffalo bull spermatozoa and its relationship with motility of the fresh sperm cells. Our hypothesis was this, that GPR54 expression will be the greatest on the sperms with highest motility.

MATERIALS AND METHODS

Animals: In the present study, four sexually mature and trained Nili Ravi buffalo bulls (*Bubalus bubalis*) aged 5-7 years with body weight 500-600 kg were selected from Animal Reproduction and Genetics Programme, National Agriculture Research Centre (NARC), Islamabad, Pakistan. These bulls were primarily considered and used for semen collection under standard conditions at the NARC. Bulls were individually housed and were properly nourished daily at 10:00-10:30AM and 4:00PM.

Semen collection: Semen was taken from each bull in a collection yard with artificial vagina (AV) at 42℃, during the month of March (low breeding season). From each animal two ejaculates were taken, the first one was in the morning 9-10 AM and the second ejaculate was collected half an hour later. Both samples were collected before feeding. After collection semen was transported quickly into the laboratory in an incubator within no time to avoid any external shocks and kept at 37°C in the heating block.

Preliminary analysis: After semen collection within 10-15 minutes of holding period, preliminary analysis was carried out to find the semen volume (ml), colour, sperm progressive motility (%) and its concentration (ml). Ejaculates having greater than 60 percent motility and more than 0.5×10^9 sperm/ml were used for swim up.

Swim up: After initial analysis, semen was diluted using standard protocol. About 0.5ml of extended semen samples were made with the help of a proper dilution medium (PBS+1mg BSA/ml). Then separation of different qualities of the spermatozoa was carried out by the technique of "Direct Swim Up" as described earlier by Parrish and Foote (1987) for both ejaculates of a bull. In this motility enhancement technique, initially, 2.5ml modified Tyrode's medium i.e the sperm TALP (Bavister and Yanagimachi, 1977) was taken in special culture tubes, pre-warmed in the water bath at 37˚C. Then 0.5ml diluted semen was suspended at the bottom of tube through a plastic pasture pipette. After an incubation period of 30-45 minutes at 37˚C, three layers of semen were formed i.e. on the top, middle and bottom of the culture tube. The whole of the experimental setup for the desired method was carried out in the water bath at 37˚C. From each layer (of approximately 1 ml) 20µl semen was taken in the eppendorf tubes to check viability of the sperm and also progressive motility under phase contrast microscope. Then 200 µl semen from each layer was taken on the frosted glass slide (Santa Cruz; Biotechnology, Taxas, USA) for smear formation. In this way three smears were made from each semen sample. The smears were dried under room temperature and fixed in chilled methanol at -20˚C for 20 minutes. Later, these slides were processed for standard immunocytochemistry (ICC) procedure.

Viability test: Standard hypo-osmotic swelling test (HOST) was used to check sperm's viability, originally illustrated by (Jeyendran *et al*., 1984). Two different solutions were prepared by dissolving 1.35 g fructose and 0.73 g sodium citrate in 0.1l of refined water. The osmolality of final solution was approximately 150 mOsm/ kg. The procedure was carried out by mixing 50 µL of the specimen containing spermatozoa with 500 µL of pre-warmed hypo-osmotic solution (37˚C) for 30-45 minutes. Incubated sample was observed under a phase-contrast microscope at 40X magnification. Approximately 200 sperm cells were counted in at least 10 different screen shots, with more than 10 spermatozoa in each field. Sperm cells having normal cell membrane were represented by coiled sperm tail.

Immunocytochemistry: To observe the GPR54 like immunoreactivity (ir) on fixed buffalo bull spermatozoa, the smears were processed for standard immunocyto-chemistry protocol. From each animal, three semen smears were obtained for GPR54 immunostaining. While three smears obtained randomly from one animal were used as control slides.

For immunostaining smears were incubated in anti-GPR54 antibody (Catalogue no. H-048-61; Phoenix Pharmaceuticals, Inc., Burlingame, California, USA, used at 1:500, diluted in PBS containing 0.03% TritonX-100, 0.05% BSA and 10% normal goat serum) for 48 hours in humidified chamber at 4ºC. Next smears were incubated in Alexa Flour 488 goat anti rabbit (Catalogue no. ab150077; Abcam, Cambridge, UK secondary antibody, used at 1:400, diluted in PBS containing 0.03% TritonX-100 and 0.05% BSA) for 2 hours in dark at room temperature. At the end, coverslip was placed over the slide and kept at 4ºC for drying, overnight.

Microscopy: Fluorescent microscope (AMEP-4615, Evos, Bothel, Washington, USA) was used to observe GPR54 like ir at different magnifications and screen shots were taken.

Statistical analysis: Total number of GPR54 like (ir) spermatozoa of each smear was calculated followed by the calculation of mean±SEM of GPR54 like ir sperms per smear for each animal. Data were analyzed using one-way and two-way repeated measures ANOVA followed by the post hoc Tukey's test. An association between sperm motility and GPR54 expression was examined by correlation procedure (GraphPad Prism *version5.01*, GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Swim up motility: TALP (sperm motility-stimulating media) based Swim Up procedure arranged the sperm in three layers according to motility (Fig. 1). One-way ANOVA along with Tukey's Multiple Comparison Test revealed a significant difference (P<0.0001) between the three layers containing spermatozoa with respect to the progressive motility. The spermatozoa in the top most layers showed the highest motility (P<0.005) as compared to that in middle and lower layers. The hypo-osmotic swelling (HOS) test showed that the viability of the spermatozoa also increased from lower to upper layer, supporting the measurements obtained from sperms Swim Up.

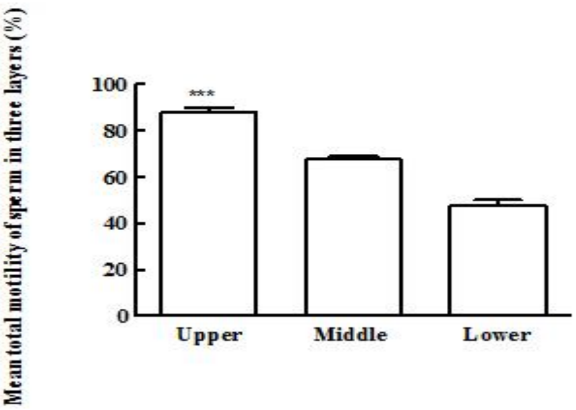

Fig. 1: Comparison of mean ± SEM swim up motility of buffalo bull spermatozoa (n=8) in the upper, middle and lower layers. Swim up motility was significantly higher (***P<0.0001) in the uppermost layer as compared to that in the middle and lower layers.

GPR54 expression: GPR54 like ir was observed in 100% of sperm cells in smears (n=24) from all the three layers for all samples ejaculates (n=8) through fluorescent microscopy. GPR54 like ir was detected in head, neck/midpiece and tail regions of spermatozoa in different layers (Fig. 2a, 2b, 2c). Through visual observation a dense GPR54 expression was observed throughout the neck/midpiece regions of the spermatozoa locating in all the three layers. While, a 50-60% of the spermatozoa of all the three layers showed positive immunoreactivity in the tail region. However, no staining was observed with primary antibody omitted control spermatozoa prepared randomly from any buffalo bull negative control (Fig. 2d). Comparison of GPR54 like ir in different regions within each layer showed that GPR54 like ir was significantly lower in tail areas as compared to other regions (Fig. 3).

Interlayer comparison of mean number of GPR54 like ir in particular region observed in 200 studied sperm is presented in Fig. 4a. Two-way repeated measure ANOVA showed no effect of layer on GPR54 like ir. Similarly, mean total GPR54 like ir found at various regions of sperm observed in 200 studied sperm is compared across three layers. One-way ANOVA demonstrated no significant variation in number of GPR54 like ir sperm coming from the three layers (Fig. 4b). Moreover, no significant correlation was observed between total GPR54 like ir with respect to percentage motility of each layer of each animal (Fig. 4c).

DISCUSSION

This is the first study that evaluated the regional expression of GPR54 on buffalo spermatozoa having different motility qualities in order to understand the effect of kisspeptin signaling in monitoring sperm motility. Buffalo semen is widely used for artificial insemination (AI) and revealing the role of kisspeptin and its receptor in physiology of buffalo sperm could therefore, have translational implications. Present study clearly demonstrated the expression of GPR54 like ir in all regions of the sperm cells having different motility. Visually, very dense immunoreactivity was observed in neck/midpiece region. Our findings are consistent in this regard with the findings of Pinto *et al*. (2012) in the human sperms. It is also important to mention here that the expression of kisspeptin in the buffalo spermatozoa was also observed to be region specific (unpublished data).

Kisspeptin acts through KISS1R and activates a series of processes important for enhancing the sperm motility parameters (Pinto *et al*., 2012). In the present study, major GPR54 ir was observed around the neck / midpiece and head portions. As these areas have been established to be important for motility (Hsu *et al*., 2014; Trevisan *et al*., 2018), our finding will suggest kisspeptin signalling may participate in control of motility. Sperm motility is essential for normal fertilization (Chang and Suarez, 2010). Indeed, exposure of kisspeptin to human spermatozoa resulted in enhanced motility (Pinto *et al*., 2012) and buffalo spermatozoa (unpublished data). Another finding of the present research showed that the GPR54 like ir was significantly lower in the tail region as compared to the other parts in sperm with different motility, which coincides with the study of Pinto *et al*. (2012), where a significant decrease in the expression of kisspeptin receptor was noted in the tail region when contrasted with the head part of human sperm. The presence of GPR54 especially at the head region of the spermatozoa highly suggests a local function of kisspeptin signalling, which may cause motility or alterations in the plasma membrane [Ca^{2+}] ion levels, resulting in the enhancement of buffalo bull spermatozoa. Although, principle result of the current research showed that regional GPR54 expression was similar in buffalo sperm showing different motility. GPR54 expression was similar in spermatozoa with clearly different motility characteristics. Our result appears to be in contrast to observation of Pinto *et al*. (2012), where kisspeptin exposure enhanced human sperm motility, but was decreased after the application of KISS1R antagonist, p234. Also our unpublished data supports the findings of Pinto *et al*. (2012), where the exposure of human kisspeptin-10 indicated a significant enhancement in rapid motile buffalo sperm.

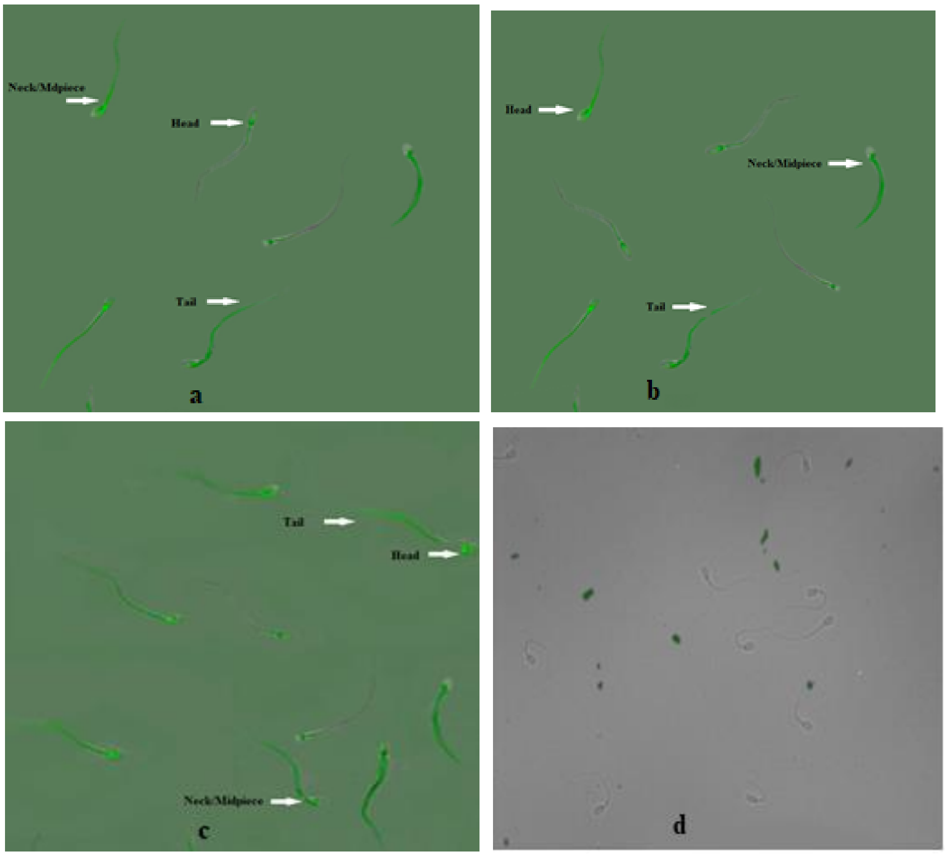

Fig. 2: Representative photomicrographs showing GPR54 like immunoreactivity of buffalo bulls spermatozoa of all the bulls in all three layers i-e upper, middle and lower (a, b, c respectively). Fluorescent immunocytochemistry was carried out using goat anti rabbit Alexa Fluor 488 secondary antibody. White arrows indicate GPR54 like-ir. Control smears (d) were processed without primary antibody and showed no immunoreactivity. All images were taken at 40x magnification.

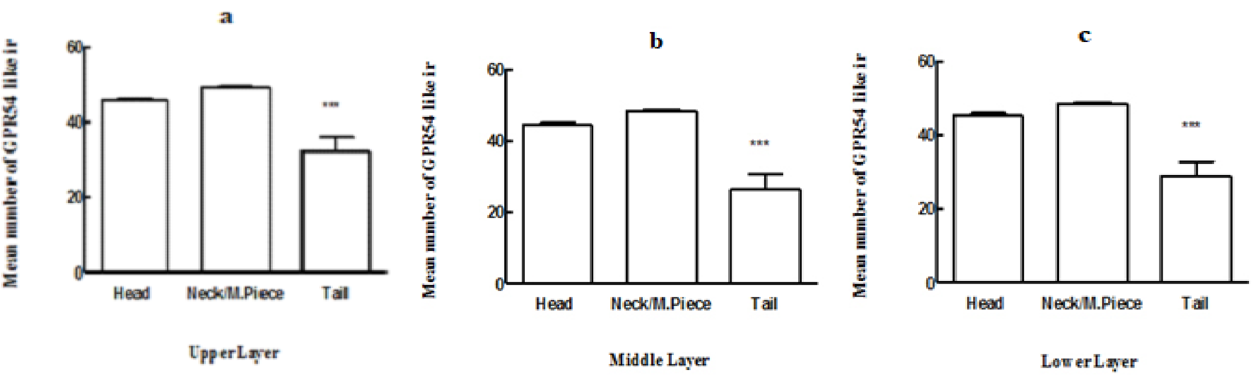

Fig. 3: Comparison of mean ± SEM GPR54 like-ir on different sections of buffalo bull spermatozoa (n=8, Cell n=200), residing in the uppermost layer (a), middle layer (b) and lower layer (c). GPR54 like ir was significantly (***P<0.0001) lower in the tail region as compared to the other parts in all the three layers.

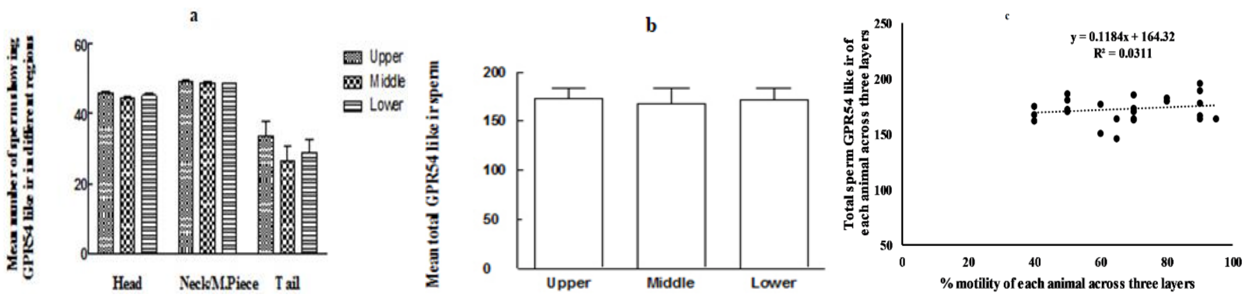

Fig. 4: Comparison of mean number of GPR54 like ir sperm showing in different regions, observed out of 200 studied sperm across three layers (a), comparison of mean total GPR54 like ir sperm/ 200 sperm observed from three layers (b) and comparison of total sperm GPR54 like ir in all animals across three layers (upper, middle and lower) with respect to %age motility of each layer for each animal (c).

Conclusions: The present study demonstrates that GPR54 is expressed in all parts of the freshly ejaculated buffalo bull spermatozoa, in which 100 % of the immune staining was observed in the upper regions (head, neck/midpiece) and the lower region (tail) showed 50-60% positive immune labelling. Moreover, GPR54 expression was similar in spermatozoa with clearly different motility parameters, which suggests that the buffalo sperm motility is not influenced by GPR54 expression on different regions of the sperm.

There may be a number of reasons for the non-significant association of GPR54 expression with the enhancement of motility of buffalo bull spermatozoa. It may be due to species difference; alternatively, it may be possible that endogenous kisspeptin signalling does not participate in mechanism underlying motility of buffalo bulls spermatozoa unlike human's sperm where a significant enhancement in sperm progressive motility was noted. In this regard our previous observation in enhancement of buffalo bull sperm motility by exogenous kisspeptin may have been pharmacological and not physiological. Therefore, it is important that exposure of sperm cells to exogenous kisspeptin in physiological doses be studied to decipher physiological role of kisspeptin signalling regulating sperm motility. Alternatively, the notion that kisspeptin is involved in influencing other sperm functions through a covert pathway, cannot be excluded.

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Authors contribution: AH execution of study and manuscript preparation; WN, assisted in experimental set up; HZ, helped in carrying out ICC and article reviewing; RB, Statistics and article reviewing; HA, helped in semen collection and its initial analysis; SMHA, overall supervision of semen collection and its analysis; MS, study design and manuscript preparation.

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