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RESEARCH ARTICLE

Kiss1 mRNA and Its Protein Distribution in Preoptic and Arcuate Hypothalamic Nuclei in Pre-Pubertal Female Swamp Buffaloes

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The relationship between kisspeptin and GnRH releasing in many species has been studied including post-pubertal buffalo, but not in pre-pubertal buffalo. The aims of this study were to detect the localization of *Kiss1*mRNA and the distribution of kisspeptin protein in the preoptic (POA) and arcuate (ARC) hypothalamic nuclei of pre-pubertal swamp buffaloes. Brains were collected from ten pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5-2 years, 2-2.5 years and 2.5-3 years; two animals each age) and processed for paraffin blocks. Four-micron paraffin sections of the POA and ARC hypothalamic nuclei were prepared. The present research found evidence of *Kiss1* mRNA in the cytoplasm of the neuronal soma and some small neuronal cells using the in-situ hybridization technique in all ten heifers. Using the immunohistochemistry technique, kisspeptin proteins expressed a weak intensity in the cellular process of neurons. The distribution of kisspeptin immunoreactions were found mainly in the POA hypothalamic nucleus in the juvenile group (<1 years) and only in the ARC area in the 1-2.5 years old pre-pubertal group. However, there was no kisspeptin reaction in both hypothalamic nuclei in the 2.5-3 years (peri-pubertal) group ($P<0.01$). This study provides evidence of *Kiss1* mRNA and kisspeptin protein in the hypothalamus of pre-pubertal buffaloes. This suggests that kisspeptin may be involved in reproductive development and may influence puberty onset in swamp buffalo heifers.

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INTRODUCTION

Puberty is the sexual transition from immaturity to maturity involving body growth and development (related with leptin in adipose tissue and growth hormone) (Kadokawa *et al*., 2008; Smith *et al*., 2010). The onset of puberty is triggered by the activation of neurons in the forebrain which produce a neuroendocrine substrate to stimulate GnRH (Saito *et al*., 2012). In 2003, researchers found that mutations of GPR54 were associated with hypogonadotropic hypogonadism in humans (de Roux *et al*., 2003; Seminara *et al*., 2003). These studies demonstrate that kisspeptin-GPR54 signaling is necessary for pubertal activation of GnRH neurons and reproductive function both of which play a pivotal role in the control of the hypothalamic- pituitary-gonadal (HPG) axis (Roseweir and Millar, 2009; Tsukamura and Maeda, 2011). The late puberty onset of swamp buffalo is the main problem affecting their reproductive efficiency (Chaikhun *et al*., 2012). Our previous studies suggest that kisspeptin is a key controller of reproductive function in post-pubertal swamp buffalo in relation to the HPG axis (Chaikhun *et al*., 2013, 2016; Chaikhun-Marcou*et al*., 2014a, 2014b, 2016). However, no research has been done in pre-pubertal buffalo in relation to puberty development and onset. In order to further this basic research, the objectives of this study were to determine the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of pre-pubertal swamp buffalo of various ages.

MATERIALS AND METHODS

The experimental procedures involving animals were approved by Chulalongkorn University Animal Care and Use Committee in accordance with the university regulations and policies governing the care and use of laboratory animals (No.13310007).

Sample: Brains were collected from ten pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5-3 years; two animals each age) from slaughter houses. The samples were processed using the same protocol of our previous study (Chaikhun *et al.*, 2016). Briefly, the animals were perfused by a 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) into a common carotid artery within 15 min of the animal's death. All buffaloes were non-cycling. The hypothalami were collected and fixed in 4% paraformaldehyde for 24 hr. The samples were embedded in paraffin blocks and stored at room temperature. The paraffin block of the POA and ARC hypothalamic nuclei of buffaloes were identified by the anatomical structure identification used in a previous study of buffalo cows (Chaikhun *et al.*, 2016).

In situ hybridization of *Kiss1* mRNA: The ISH protocol was modified following our previous studies (Sotthibandhu, 2009; Chaikhun *et al*., 2016).

Preparation of cRNA probe: Plasmid DNA, inserted with a section of ovine *Kiss1* gene (GenBank accession no. DQ059506) at the length of 375 base pairs, was generated by GenScript, NJ, USA. In confirmation from our previous study's assumptions, 94% of the tested sequences showed significant alignments between the predicted buffalo and ovine *Kiss1* sequences when analyzed by the BLASTN 2.2.30+ program (Chaikhun *et al*., 2016). The plasmid DNA was digested with SpeI (Promega, WI, USA) for preparation of a sense probe (negative result indicator) and NotI (Promega, WI, USA) for preparation of an anti-sense (positive result indicator) probe using a DIG-labeling *in vitro* transcription kit (Roche, Mannheim, Germany). In situ hybridization on paraffin sections were performed following the previous study protocol (Chaikhun *et al*., 2016). The images of the *Kiss1* mRNA signals were taken under a light microscope (Axiolab, Zeiss, Oberkochen, Germany). The results were reported as "positive" (no percentage calculations are possible with this technique) if *Kiss1* mRNA could be detected by a purple stain reaction in the cytoplasm of neurons in the anti-sense probe applied samples or "negative" if *Kiss1* mRNA could not be detected by a purple stain reaction in the sense probe applied samples. The images were captured by Axivision software (Axiolab, Zeiss, Oberkochen, Germany). As a positive control for tissue and for the specificity of the probe, the POA and ARC hypothalamic nuclei of ewe were treated using the same protocol for both of the anti-sense and sense probes.

Immunohistochemistry of kisspeptin: The sections from the POA and ARC hypothalamic nuclei blocks of samples were prepared at 4 microns for kisspeptin immunohistochemical study. The protocol of immunohistochemistry was the same protocol used in our previous study (Chaikhun *et al*., 2016). Briefly, antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 70°C. The non-specific binding was blocked using 10% normal horse serum (Gibco, NY, USA) for 20 min at RT. Then the sections were incubated with a 1:500 dilution of a rabbit anti-mouse kisspeptin-10 antibody (Millipore catalog number AB9754, MA, USA) at 4°C overnight (16 hr). In the final step, 3, 3'-diaminobenzidine (DAB, Dako, Glostrup, Denmark), a chromogen, was added to visualize bound enzyme (brown color) on the observed samples for 5 min. Positive controls for antibody and tissue specificity were prepared using ewe and buffalo cow POA and ARC hypothalamic nuclei paraffin sections. Negative controls for antibody specificity were conducted using PBS. Negative controls for tissue specificity were the white matter area of the central nervous system, which is an area known to have no kisspeptin expression. Two observers checked and counted the reaction results together from a single DAB staining session under a light microscope (Axiolab, Zeiss, Oberkochen, Germany). Neurons in the cytoplasm which appeared to be stained brown were counted as kisspeptin "positive" or kisspeptin-immunoreactive (ir) neurons and non- brown stained neurons were identified as kisspeptin "negative" neurons. Then counterstain by hematoxylin, was applied on the sample slides. After that the number of kisspeptin-ir cells randomly found in the 100 mm^2 area per slide taken from each of the buffalo cow's POA and ARC hypothalamic nuclei (one randomly selected slide from the POA and ARC of each buffalo) were counted. The images were captured by Axivision software (Axiolab, Zeiss, Oberkochen, Germany).

Statistical analysis: The expression of *Kiss1* mRNA in the POA and ARC hypothalamic nuclei were detected through in situ hybridization. The *Kiss1* mRNA expression in the POA and ARC hypothalamic nuclei were explained by descriptive statistics.

For the analysis of the immunohistochemical reactions to kisspeptin, the kisspeptin-ir cells in the POA and ARC from each buffalo were calculated as a percentage by dividing the number of positive neurons by the total counted neurons and then multiplying by 100. This figure was then averaged across animals to calculate a mean (±SE). The comparison of the average number of kisspeptin-ir cells between the POA and ARC were analyzed by paired t-test ($P<0.05$). The intensity of immunohistochemical reaction of kisspeptin between the POA and ARC were graded into 3 levels; 1=weak, 2=moderate, and 3=intense, and analyzed by paired t-test ($P<0.05$). The distribution of kisspeptin-ir neurons was described.

RESULTS

In situ hybridization of *Kiss1* mRNA: The expression of *Kiss1 mRNA* using an anti-sense *Kiss1 cRNA* probe was detected in the cytoplasm of neuronal soma in the majority of neurons with an intense reaction in both the POA (Fig. 1, 3A and 4) and ARC hypothalamic nuclei (Fig. 2, 3B and 5) of all buffalo samples, regardless of the animals age. *Kiss1* mRNA was also found in some small

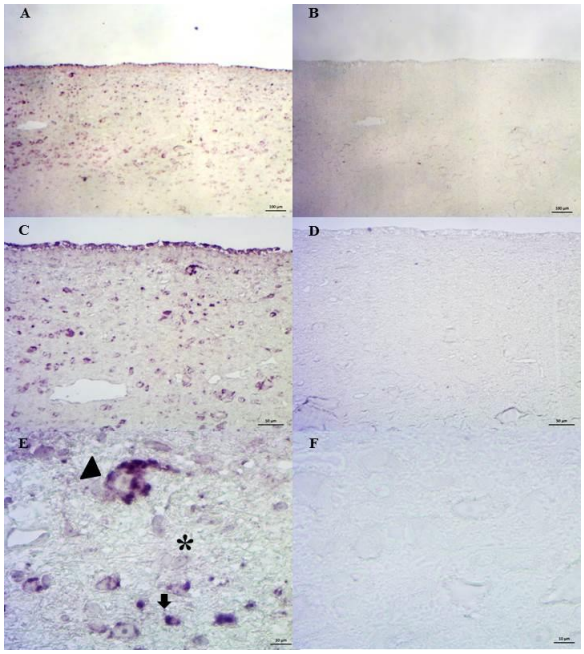

Fig. 1: *Kiss1* mRNA in the POA hypothalamic nucleus of buffalo, is visible in the anti-sense (positive result) of *Kiss1* mRNA in samples A, C and E, and is not expressed in the sense (negative result) of *Kiss1* mRNA in samples B, D and F. *Kiss1* mRNA expressions are localized in the cytoplasm of a neuron (arrow head) and a small neuronal cell (full arrow). A non-expressed neuron is shown (asterisk) in E. Scale bar is 100 µm in A and B, 50 µm in C and D but it is 10 µm in E and F.

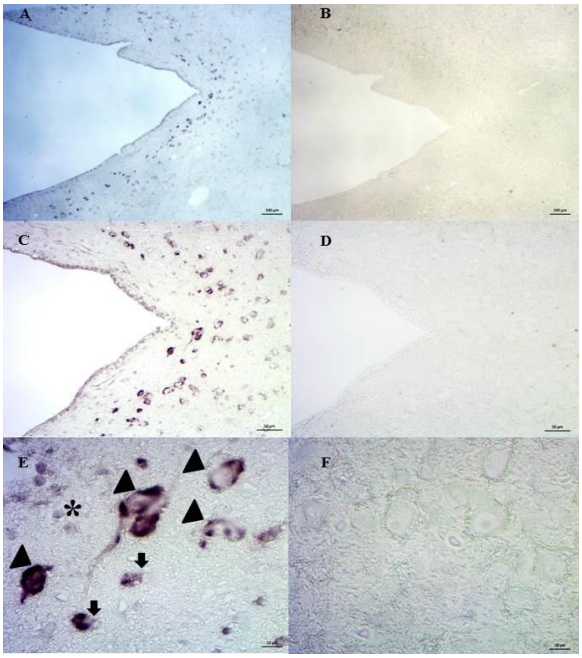

Fig. 2: In the ARC hypothalamic nucleus of buffalo, *Kiss1* mRNA is visible in the anti-sense (positive result) of *Kiss1* mRNA in samples A, C and E. It is not expressed in the sense (negative result) of *Kiss1* mRNA in samples B, D and F. *Kiss1* mRNA are localized in the cytoplasm of neurons with strong signals (arrow heads) and small neurons (full arrows) but no signal in a small neuron (asterisk) in E. Scale bar is 100 µm in A and B, 50 µm in C and D but it is 10 µm in E and F.

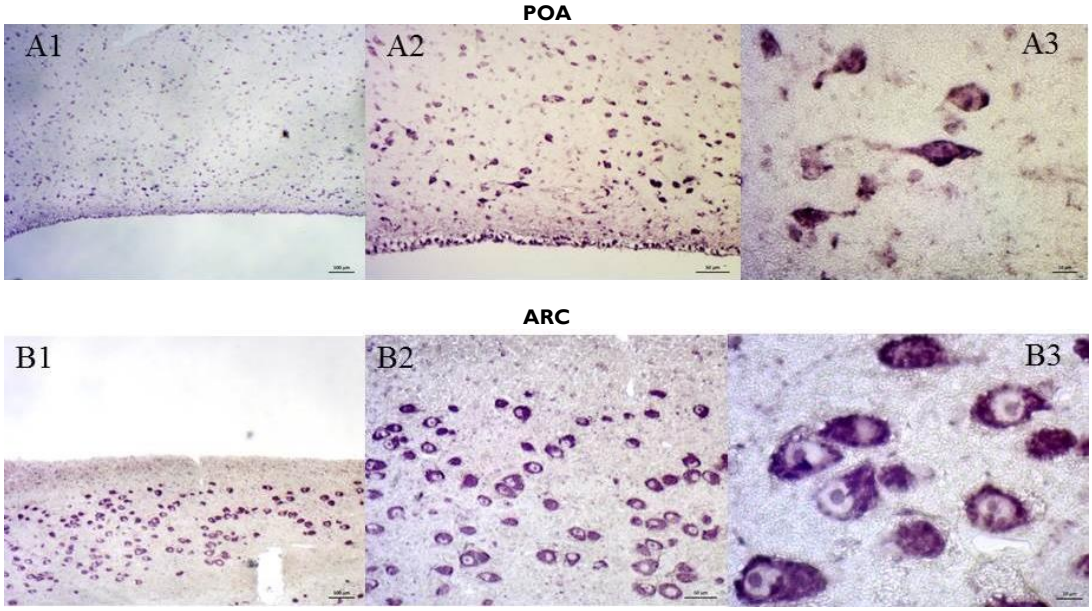

Fig. 3: The *Kiss1* mRNA neuron shows scattered distribution in the POA area (A1 and A2), compared to the ARC area which shows a more tightly clustered distribution pattern (B1 and B2). There is evidence of a synapsis between two kisspeptin neurons in A3. Scale bar is 100 µm in A1 and B1, 50 µm in A2 and B2 but it is 10 µm in A3 and B3.

neuronal cells (Fig. 1E, 2E) which were distinguished from glia cells by their vesicular nuclei. Interestingly, there is evidence of a synapsis between two kisspeptin neurons (Fig. 3-A3). There was no signal of *Kiss1* mRNA in the buffalo POA and ARC sections in which the sense *Kiss1* cRNA probe was applied (Fig. 1B, 1D, 2B and 2D) and these were considered as negative control reactions. Positive control reactions were prepared using the buffalo cow POA (Fig. 2E and 2F) and ARC (Fig. 3E and 3F) hypothalamic nuclei paraffin sections.

Immunohistochemistry of kisspeptin: The percentage of kisspeptin reactive cells in each age of ten heifers and each hypothalamic area are shown in Fig. 6. The results showed weak reactions of kisspeptin located in the cytoplasm of the neuronal soma in six months old heifer in both the POA and ARC hypothalamic nuclei and the number of kisspeptin neurons in the POA (45.24%) was higher than the ARC area (22.22%). Another calf of the same age had a few kisspeptin neurons in the POA (2.44%) but none in the ARC area. The 1-2 years old age

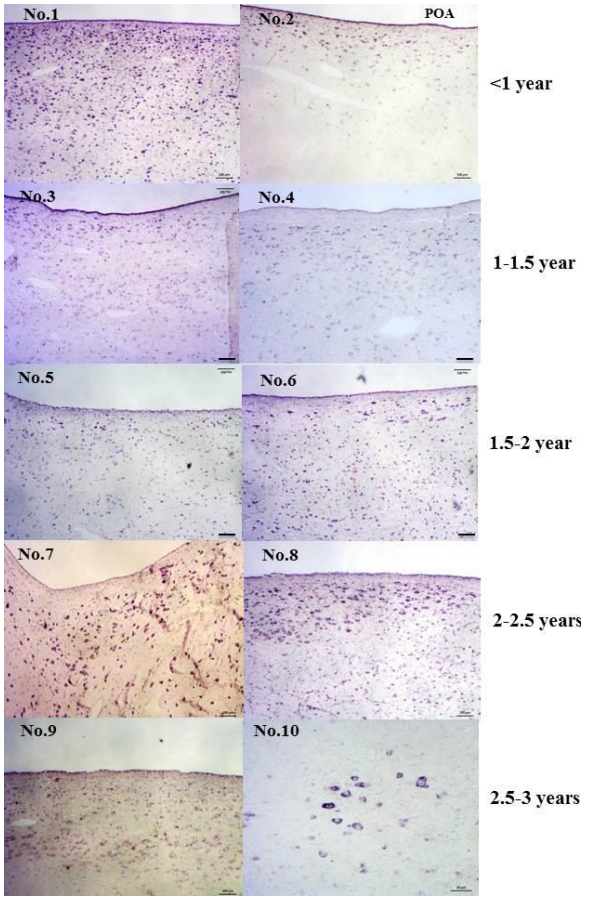

Fig. 4: In POA hypothalamic nuclei, *Kiss1* mRNA expressions are presented in samples from pre-pubertal buffaloes of different ages. There are intense reactions visible in ependymal cells and neuronal cells. Scale bar is 100 µm (No.1-9) but it is 50 µm in No.10.

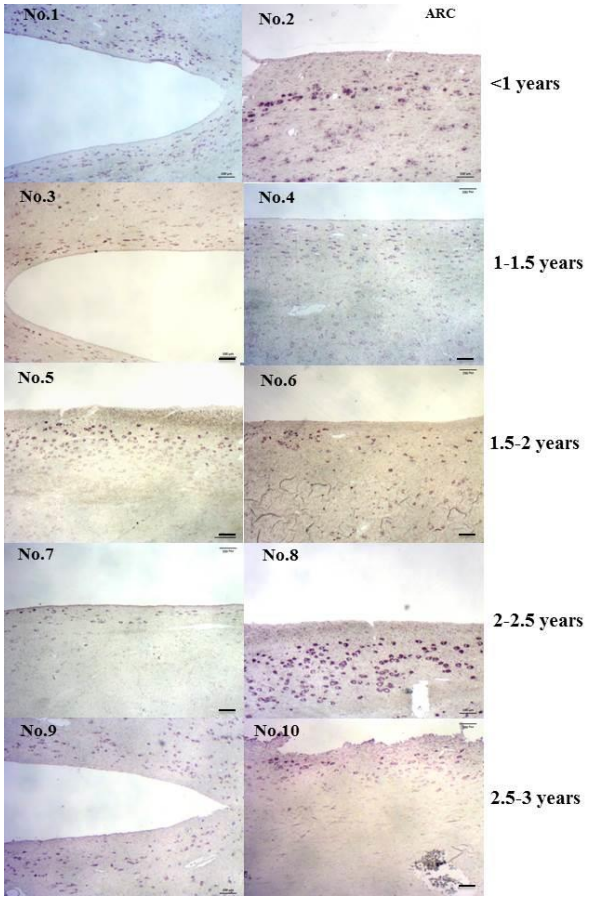

Fig. 5: In ARC hypothalamic nuclei, *Kiss1* mRNA expressions are presented in samples from pre-pubertal buffaloes of different ages. There are intense reactions visible in neuronal cells near the 3rd ventricle. Scale bar is 100 µm.

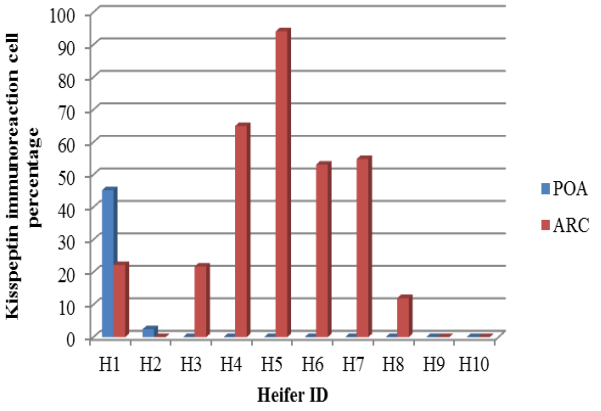

Fig. 6: The percentage of kisspeptin reactive neurons in the POA (blue) and the ARC (red) hypothalamic areas from ten pre-pubertal female swamp buffaloes of different ages.

group expressed kisspeptin reactions in the ARC area (Fig.7) but none in the POA. All of the heifers >2 ½ years of age showed no kisspeptin expression in both areas. In all buffalo samples that expressed a kisspeptin reaction there was no difference in intensity between the POA and ARC hypothalamic nuclei regardless of age group, which were both graded as weak (level 1) (P>0.5). The positive control for immunohistochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in the ewe and the buffalo cow are shown in Fig. 8a, c. The negative control presented no non-specific reactions (Fig. 8b, d).

DISCUSSION

The present study's detection and localization of *Kiss1* mRNA in all age groups of pre-pubertal swamp buffaloes in both POA and ARC hypothalamic nuclei was similar to our previous studies' findings in post-pubertal swamp buffaloes (Chaikhun *et al*., 2016). This suggests that buffaloes of all ages may be able to produce *Kiss1* mRNA in these hypothalamic nuclei. Also, this evidence may indicate that kisspeptin is a master pubertal and reproductive function activator in swamp buffalo which is similar to its function in other mammals (Estrada *et al*., 2006; Goodman *et al*., 2007; Rometo *et al*., 2007).

The results of our study's immunohistochemistry testing found kisspeptin protein reactions located in the cytoplasm of neurons in only some samples of the pre-pubertal buffaloes. We also detected evidence that kisspeptin was synthesized by *Kiss1* mRNA in the less than 1 year to 2.5 years old pre-pubertal swamp buffaloes but not in the 2.5-3 years old group. Interestingly, kisspeptin reactions showed mostly in the POA of the <1 year of age group, which suggests that the POA might be the main area related to kisspeptin expression in early age or juvenile buffalo. However, the 1-2.5 years old heifers presented kisspeptin reactions only in the ARC area, which suggests that kisspeptin is also involved in this area between 1 and 2.5 years old, which is the pre-pubertal age. Surprisingly, all of the heifers in the 2.5-3 years of age range showed no expression of kisspeptin in both

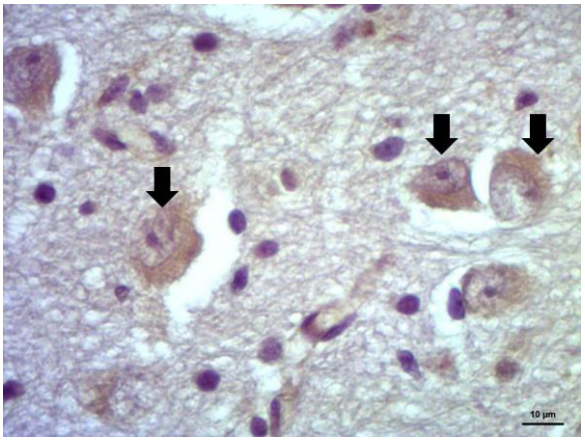

Fig. 7: Kisspeptin reactive proteins are visible in the cytoplasm of neurons with weak intensity (arrow) in the ARC of a 1-2.5 years old pre-pubertal female swamp buffalo. Scale bar 10 µm.

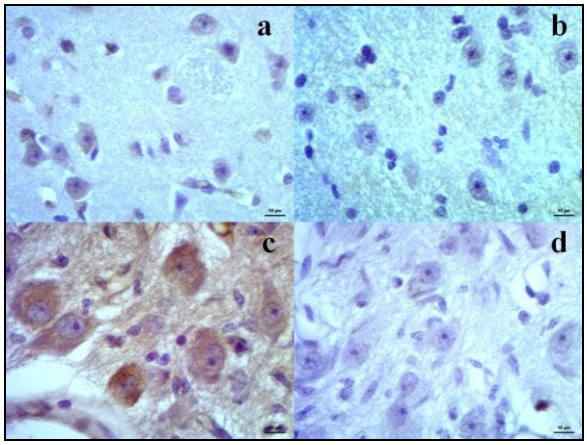

Fig. 8: The positive (left) and negative controls (right) for immuno-histochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in a ewe (a, b) and a buffalo cow (c, d). Scale bar 10 µm.

areas, which is the age close to puberty or the peri-puberty of swamp buffalo (Chaikhun *et al*., 2012). Conversely, kisspeptin protein reactions in post-pubertal buffalo were detected in both the follicular and luteal phases in both the POA and ARC areas. But the population of kisspeptin neurons in the POA was greater than in the ARC area. This variation in kisspeptin protein distribution might not be only dependent on *Kiss1* mRNA distribution but may also reflect a possible difference in the role of kisspeptin, its active mode in each species, age ranges, sex steroid hormone effects, nutritional status, anatomical and physiological variations, and differences in the volume of kisspeptin synthesized from different hypothalamic nuclei (Han *et al*., 2005; Caraty*et al*., 2007; Colledge, 2008; Overgaard *et al*., 2013; Poling and Kauffman, 2013; Liu *et al*., 2014; Cui *et al*., 2015). It is possible that kisspeptin in the ARC of post-pubertal buffalo may have a different role in its active mode, which may account for this difference in kisspeptin neuron distribution (Chaikhun *et al*., 2016).

There are a few possible reasons for the variation of kisspeptin distribution in pre-pubertal ages noted in our study. Pre-pubertal animal research has found the relationship between kisspeptin coordinates with other elements such as; the leptin-melanocortin-kisspeptin pathway and puberty (Manfredi-Lozano *et al*., 2016), the release of growth hormones, and the release of prolactin (Kadokawa *et al*., 2008; Whitlock *et al*., 2008). These studies support our finding of a variation of kisspeptin distribution results and suggest that kisspeptin protein from the hypothalamus may not just be involved in reproductive puberty but also in other metabolic functions in <1-2.5 years old pre-pubertal buffaloes. Remarkably, there was no kisspeptin protein reaction in 2.5-3 years old group. This may be the result of a temporary stop of kisspeptin production before the onset of puberty, or kisspeptin may be produced but goes to other hypothalamic nuclei. Generally, kisspeptin expression is regulated by estrogen via estrogen receptor alpha (ERα) in kisspeptin neurons, especially during the peri-pubertal period. Estradiol 17β is reduced in the late juvenile stage of development. Low or no presentation of ERα in kisspeptin neurons may activate the onset of puberty (Han *et al*., 2005; Mayer *et al*., 2010; Mayer and Boehm, 2011). Therefore, further studies involving the co-localization of ERα in kisspeptin neurons in pre-pubertal buffalo should be done for more information.

Conclusions: Our present study found *Kiss1* mRNA and kisspeptin protein in the hypothalamus of pre-pubertal buffalo which provides fundamental data on kisspeptin and its relation to buffalo puberty development. *Kiss1* mRNA was expressed in some neurons of both the POA and the ARC hypothalamic nuclei in all age groups. However, more kisspeptin proteins were localized in subpopulations of neurons in the POA than in the ARC area in the 6 months old group. Only the ARC area presented kisspeptin reactions in the 1-2 years old group. Interestingly, there were no kisspeptin reactions in either the POA or the ARC areas in the 2.5-3 years old group. These results suggest the possibility that kisspeptin in pre-pubertal buffalo may have different functions and modes of action that vary with age and with specific hypothalamic nuclei (POA versus ARC). Kisspeptin and the kisspeptin receptor-GnRH- ERα relationship should be researched next.

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Authors contribution: All authors collaborated in the design of this experiment and in the preparation of the documents. PS, CY, SP and TC performed the experiment. PS and TC analyzed the results. TC prepared the manuscript. All authors reviewed and revised the manuscript for intellectual contents and approved the final version

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