**ARTICLE HISTORY (17-241)**

**ABSTRACT**

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 female 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 1.25 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 hypothalamic 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, 1.5 - 2 years, 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 (Sothibhandhu, 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 was performed following the previous 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 immuno-
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.

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
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.

REFERENCES


