รายงานวิจัยฉบับสมบูรณ์

โครงการบทบาทของคิสเป็บทีนในการควบคุมการเจริญพันธุ์ของกระบือปลักสาวในประเทศไทย

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สัญญาเลขที่ RSA5680048

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและจุฬาลงกรณ์มหาวิทยาลัย

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับการสนับสนุนทุนวิจัยจากสำนักงานกองทุนสนับสนุนการวิจัยร่วมกับ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย และจุฬาลงกรณ์มหาวิทยาลัย และการศึกษาวิจัยนี้ได้รับความอนุเคราะห์เป็นอย่างดีในความร่วมมือในการทำงานทางห้องปฏิบัติการพยาธิวิทยา ศูนย์ชัณสูตรโรคสัตว์ คณะสัตวแพทยศาสตร์ มหาวิทยาลัยเทคโนโลยีมหานคร ขอขอบคุณ อ.สพ.ญ. สุวริน ภาวสุทธิไพศิฐ และ อ.น.สพ. ชณัตญาณประภาศิริ ที่มีส่วนช่วยเหลืองานทางด้านห้องปฏิบัติการ และ Mr. Philippe Marcou ที่มีส่วนช่วยเหลือและให้คำแนะนำด้านภาษา

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คิสเป็บทีนเป็นโปรตีนที่สร้างจากเซลล์ประสาทโดยผลิตจากยีน *KISS1* ในกลุ่มเซลล์ประสาทไฮโปทาลามัส บริเวณพรีออพติค (พีโอเอ) และอาร์คูเอท นิวเคลียส (เออาร์ซี) ซึ่งพบว่า มีส่วนในการควบคุมของแกนการทำงานส่วนไฮโปทาลามัส ต่อมใต้สมอง และอวัยวะสืบพันธุ์ในสัตว์หลายชนิด รวมถึงสัตว์เคี้ยวเอื้อง ส่งผลต่อการเข้าสู่ภาวะเจริญพันธุ์ และการทำงานของระบบสืบพันธุ์ ซึ่งการศึกษาของคณะผู้วิจัยก่อนหน้านี้ได้พบหลักฐานเบื้องต้นว่า คิสเป็บทีนมีบทบาทในการควบคุมการทำงานของระบบสืบพันธุ์ในแม่กระบือปลัก แต่ยังไม่มีการศึกษาในกระบือสาว การศึกษานี้เพื่อสำรวจบทบาทของคิสเป็บทีนต่อการเจริญพันธุ์ในกระบือปลักสาวในประเทศไทย พบหลักฐานของ *KISS1* mRNA โดยการใช้เทคนิคอินซิตู ไฮบริไดเซชัน ในกระบือทั้งหมด10 ตัว ตั้งแต่อายุ <1- 3 ปี (ช่วงก่อนวัยเจริญพันธุ์) และพบโปรตีนคิสเป็บทีนด้วยการใช้เทคนิคอิมมูโนฮิสโตเคมิสทรีในกลุ่มเซลล์ประสาทไฮโปทาลามัสบริเวณพีโอเอของลูกกระบือ และพบในตำแหน่งบริเวณเออาร์ซีเฉพาะกระบือรุ่นอายุ 1-2.5 ปี แต่ไม่พบในกระบือใกล้เข้าสู่วัยเจริญพันธุ์ 2.5-3 ปี นอกจากนี้ยังตรวจพบลักษณะการพบร่วมกันระหว่างตัวรับคิสเปบทีนและเซลล์ประสาทโกนาโดทรอปิน รีลิซซิง ฮอร์โมน (จีเอ็นอาร์เอช) ด้วยเทคนิคดับเบิล อิมมูโนฟลูออเรสเซนต์ ในบริเวณเดียวกันในทุกตัว (ประมาณ 50%ของจำนวนเซลล์ประสาททั้งหมดที่ศึกษา) และยังพบเซลล์ประสาทจีเอ็นอาร์เอชเดี่ยวกระจายอยู่ทั่วไป ซึ่งผลการศึกษาที่ได้รับนี้แสดงให้เห็นได้ว่าคิสเป็บทีนมีความสัมพันธ์กับการเจริญพันธุ์กระบือปลักไทยโดยมีการควบคุมการทำงานของเซลล์ประสาทจีเอ็นอาร์เอชในระดับหนึ่ง และคิสเป็บทีนน่าจะมีบทบาทที่เกี่ยวข้องกับการพัฒนาการของระบบสืบพันธุ์เพื่อเข้าสู่ช่วงการเจริญพันธุ์ในกระบือปลักสาวในประเทศไทย

คำหลัก : กระบือปลักสาว, คิสเป็บทีน, การเจริญพันธุ์

Abstract

Project Code : RSA5680048

Project Title : Role of kisspeptin in control of puberty of swamp buffalo heifer in Thailand

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Kisspeptin is a neuropeptide and is produced from the *KISS1* gene in hypothalamic nuclei found mainly in the preoptic area (POA) and the arcuate nucleus (ARC). It has been found to control the hypothalamic-pituitary-gonadal axis in relation to puberty onset and reproductive functions in many animals including ruminants. Although our previous study found that kisspeptin plays role in cycling buffalo cow reproduction, no studies have been done on the relationship of kisspeptin and puberty in buffalo heifers. This study investigated the role of kisspeptin in the puberty stage of buffalo heifers. This research found evidence of *KISS1* mRNA using the in situ hybridization technique in all 10 heifers (age between <1-3 years old pre-pubertal buffaloes). Kisspeptin protein presented 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 by using the immunohistochemistry technique. However, there was no kisspeptin reaction in both hypothalamic nuclei in the 2.5-3 years (peri-pubertal) group. Moreover, structural interactions were found between kisspeptin receptors and gonadotropin releasing hormone (GnRH) neurons as revealed by double immunofluorescent in the same areas (50% of the total neurons) in both POA and ARC areas. Also, single GnRH neurons were distributed in the POA and ARC hypothalamic nuclei. These results suggest that kisspeptin may control GnRH neurons on some level and may therefore be related to buffalo heifer puberty. This study indicates that kisspeptin may be involved in reproductive development and may influence puberty onset in swamp buffalo heifers in Thailand.

Keywords : Swamp buffalo heifer, Kisspeptin, Puberty

Introduction

Factors that influenced in the decreasing population of swamp buffalo in Thailand included high meat consumption and improper farm management. In addition, low fertility problem is a major source of productive loss of buffalo in Thailand. The limitations of reproductive performance in buffalo include many unique features such as; inherent late maturity, a prolonged intercalving interval, decreased ovarian function, poor estrus and poor fertility.

The delay in puberty and the sequential delay in the age of first conception cause infertility and represented a major source of economic loss in buffalo heifers, and that lead to the low reproductive performance of the buffalo species, especially in swamp buffaloes, thus lengthening the non-productive life. Many factors effect age at puberty, such as breed, season, climate, nutrition, growth rate, management, health status and biostimulatory effect of bulls. In addition, the long intercalving period of buffalo cow is another one of the main problems in buffalo's reproductive efficiency. However, the present research project will focus on study research in pre-puberty and post-puberty of swamp buffalo heifer in Thailand.

In present decade, kisspeptin has been a fascinating topic in the reproductive spotlight. Kisspeptin is a neuropeptide, produced mainly in the hypothalamus from the Kiss1 gene. This gene was initially discovered as a tumor- suppressor gene in human malignant melanoma. Then in the year 2003, researchers found that mutations of the G protein- coupled receptor (GPR54), which is the strongly cognate receptor of kisspeptin, were associated with hypogonadotropic hypogonadism in mice and humans. There are many kisspeptin distribution and function studies (both in vitro and in vivo) in mammals. In ruminants kisspeptin has been studied mainly in sheep and to some extent in goats and cattle, but not in buffalo.

Previous research indicates, however, that kisspeptin-GPR54 signaling is a key regulator of puberty, reproductive functioning and fertility via the hypothalamic pituitary gonadal axis, which produces a neuroendocrine substrate to stimulate gonadotropin releasing hormone and have the potential to replay feedback effects from the sex hormones to GnRH neurons. In ewes, Kiss1 is expressed in the arcuate nucleus to forward signals relevant to negative feedback regulation of gonadotropin releasing hormone (GnRH), and is also responsible for positive feedback regulation of GnRH at the time of the preovulatory GnRH/luteinizing hormone (LH) surge via the hypophyseal portal circulation. Although there are Kiss1 cells in the preoptic area, whether they play a role in the sex steroid feedback regulations of GnRH secretion has not been discovered yet, especially in Thai swamp buffalo.

Kisspeptin research has been ongoing in many animals both in vitro and in vivo. The main point of most of these studies is to gain fundamental knowledge of, and information about, the mechanism of kisspeptin in the reproductive system. In normal condition animals it has been discovered that kisspeptin has a role involving cooperation with other neurons and hormones. Some

studies have tried to focus on different status animals such as seasonal breeders (sheep and horses) and anestrous animals. Innovative uses of kisspeptin might possibly be applied in farm management in the future, for example, using kisspeptin to control the estrous cycle with or without other exogenous hormonal treatments, solving infertility problems which have causes related to kisspeptin, and controlling reproductive management in the non-breeding season as well as in the normal season for optimal production. However, more research on a variety of animals should provide more information, which could help increase the potential applications of kisspeptin hereafter.

Kisspeptin is a family of structurally related peptides, encoded by the Kiss1 gene, which act via the G-protein-coupled receptor, GPR54 (or Kiss1R). Mammalian has demonstrated that kisspeptin play an essential stimulatory role in the neuroendocrine control of reproductive maturation and function. These stimulatory actions are primarily conducted by activation of GnRH neurons via direct synaptic contacts or indirect trans-synaptic inputs. Initial observation that the lack of functional GPR54 in humans and mice leads to absence of puberty and sexual immaturity indicated the important physiological role of kisspeptin in mammalian puberty.

Literature review

Kisspeptin (syn. metastin) was discovered in 1996 by Lee et al. (1996) who identified the Kiss1 gene for suppressing metastasis in human malignant melanoma. Kiss1 was named for the home of the famous Hershey chocolate Kiss (Hershey, Pennsylvania, USA) which is manufactured in the area where the gene was discovered (Gottsch et al., 2009). Kisspeptin are peptide hormones which encode a 145- amino acid peptide that produces various lengths (10-54) of biologically active peptide (Gottsch et al., 2009; Hashizume, et al., 2010). The larger peptides contain some variability between species, whereas the 10 amino acid C-terminus peptide is stable and has the potency to activate its receptors (Lee et al., 1996; Muir et al., 2001; Ohtaki et al., 2001; Richard et al., 2008). G protein- coupled receptor (GPR54 or Kiss1r) is the strongly cognate receptor of kisspeptin. Kisspeptin and GPR54 have been found within the hypothalamus, brainstem, spinal cord, pituitary, ovary, prostate, liver, pancreas, intestine, aorta, coronary artery, umbilical vein and placenta (Lee et al., 1996; Muir et al., 2001; Ohtaki et al., 2001; Mead et al., 2007; Richard et al., 2008; Roseweir and Millar, 2009). 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 gonadotropin releasing hormone (GnRH) neurons and reproductive functioning , both of which play a pivotal role in the control of the hypothalamic pituitary gonadal (HPG) axis involving follicular development, ovulation, spermatogenesis and steroidogenesis (Roseweir and Millar, 2009; Tsukamura and Maeda, 2011). In ruminants, kisspeptin has been studied mainly in sheep and to some extent in goat and cattle, but not in buffalo.

Kisspeptine and GnRH neurons expression and distribution in the hypothalamus

The expression and distribution of kisspeptin, GPR54 and GnRH neurons are different in each species due to differences in anatomy (Colledge, 2008). In situ hybridization (ISH) and immumohistochemistry (IHC) are the main techniques for researching kisspeptine, GPR54 and GnRH gene or protein expression, and localization. Many kisspeptin, GPR54 and GnRH distribution studies have been done in mammals such as mice (Gottsch et al., 2004), hamsters (Revel et al., 2006), rats (Irwig et al., 2004; Smith et al., 2006), horses (Decourt et al., 2008), pigs (Tomikawa et al., 2010) monkeys (Rometo et al., 2007), humans (Rometo et al., 2007) and sheep (Estrada et al., 2006; Smith et al., 2007).

In ewes, Kiss1 neurons were found in arcuate neucleus (ARC) and preoptic area (POA) regions with a greater cell density in ARC by ISH (Estrada et al., 2006; Smith et al., 2007). Several anti-kisspeptin antiboies have been created for localization of kisspeptin neurons by IHC. Some reports noticed that cross-reaction with other RF-amide peptides and the non-specific reactivity of anti-kisspeptin antisera in IHC might be the reason that Kiss1 expression has not been detected by ISH in some locations, thus anti-protein antibodies should be validated for specificity (Colledge, 2008). From the IHC technique, kisspeptin-immunoreactive (Kp-ir) cells have been mainly found in the ARC, POA and dorsomedial nucleus (DMN) (Rometo et al., 2007; Franceschini et al., 2006). GnRH cell bodies are presented both in the POA and ARC (Colledge, 2008).

Connections and actions of kisspeptin on GnRH neurons

The hypothesis that kisspeptin neurons stimulate GnRH neurons directly is proven by many immunofluorescence data reports. In ewes, Kp-ir fibres were detected in the POA where the GnRH neurons reside, in spite of the fact that co- localization studies were not reported (Franceschini et al., 2006). Additionally, Kp-ir fibres were found to extend from the ARC into the external neurosecretory zone of the median eminence (ME) (Franceschini et al., 2006; Pompolo et al, 2006). These terminals might be causative for the kisspeptin that has been identified in the ewe hypophyseal portal blood (Smith et al, 2008) Despite this data, the function of these connections is still unknown, but kisspeptins might have a non-synaptic action at the ME level to activate GnRH release (Ramaswamy et al., 2008). Moreover, in sheep the kisspeptin cell bodies are located closer to the GnRH cell bodies than in rodents (Colledge, 2008).

GnRH neurons can be directly acted on by kisspeptin to begin a sustained depolarization event. The ability of GnRH neurons to respond to kisspeptin signals is progressively regulated, with the percentage of responsive GnRH neurons raising from 25% in the pre-pubertal period to more than 90% in adults. This raise in GnRH responsiveness during the prepubetal period reflects an increasing in Kiss1 gene expression rather than GPR54 expression (Han et al., 2005). The study in the POA region of mice found that kisspeptin causes membrane depolarization ranging from 5 mV to 22 mV in about 90% of GnRH neurons (Han et al., 2005; Liu et al., 2008) and depolarization was sustained,

lasting for up to 30 mins after kisspeptin removal. GPR54 expression by GnRH neurons appears to be not sexually dimorphic since to no sex differences were detected in the number of GnRH neurons (Liu et al., 2008).

Kisspeptin and puberty

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., 2008a; Kadokawa et al., 2008b; 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., 2011).

Many studies in mammals indicate that kisspeptin and GPR54 are key regulators of puberty due to the programmed increase in Kiss1 mRNA, GPR54 mRNA (only in females), which have been observed in the anteroventral periventicular neucleus (AVPV), the POA, and the ARC areas (through the immunoreactive method) which can in turn cause an increase in GPR54 sensitivity to kisspeptin (possibly due to increase in receptors at the cell surface). Activation of the kisspeptin system facilitates increased pulsatile and surge modes of GnRH from GnRH neurons (in the POA and the ME), then GnRH awakens the reproductive axis bringing about pubertal maturation via hypophyseal portal circulation to stimulate the production and release of gonadotropins such as luteinizing hormones (LH) and follicle-stimulating hormones (FSH) (Kadokawa et al., 2008a; Roseweir and Millar, 2009). GnRH releasing is regulated by gonadal steroid feedback action (Tsukamura and Maeda, 2011).

Kisspeptin and Hypothalamic Pituitary Gonadal axis

It is well known that steroid hormones fluctuate across the female estrous cycle and feedback from gonads regulate the HPG axis. Kisspeptin neurons express estrogen receptor α (ERα), progesterone receptor (PR) and androgen receptor (AR), and hence have the potential to relay feedback effects on the GnRH neuron (Hashizume et al., 2010). Many studies found that ovariectomy (OVX) and estrogen replacement in animals effects kisspeptin expression in different region of hypothalamus as we call “differential estrogen regulation” (Smith et al., 2005; Clarkson and Herbison, 2006; Adachi et al., 2007). The two modes of GnRH secretion are the estrogen- induced ovulatory surge of GnRH/LH, and pulsatile, basal GnRH/LH releasing modes.

The model is most well developed in rodents. On one hand, the Kiss1 neurons in the APVP are directly stimulated by estrogen effects via ERα (predominant in females). These neurons in turn directly stimulate GnRH neurons through GPR54 expressed on the cell bodies. This positive feedback of estrogen effect on APVP Kiss1 neurons climaxes in the GnRH/LH surge, which generates the preovulatory LH surge which, in turn, triggers ovulation. On the other hand, the pulsatile GnRH/LH release from ARC kisspeptin neurons (present in both female and male) drives tonic secretion of gonadotropin which mainly controls folliculogenesis and steroidogenesis and is negatively regulated

by estrogen. Additionally, it appears that positive feedback occurs at the level of GnRH cell bodies, with estrogen responsive cells in the AVPV projecting directly to GnRH neurons, whereas negative feedback occurs primarily at the GnRH terminal level by an indirect (inter-neuronal) pathway (from estrogen-sensitive neurons in the ARC) (Wintermantel et al., 2006; Smith et al., 2010; Tsukamura and Maeda, 2011).

In ewes, kisspeptin cells in the ARC are poised to play a role in the negative feedback control of GnRH/LH secretion by sex steriods which has been proven by OVX stimulation, estrogen and progesterone replacement (Smith et al., 2007). Practically, all kisspeptin cells in the ARC of the ewe brain express ERα and PR (Franceschini et al., 2006; Smith et al., 2007). The major site of sex steroid negative feedback action is the mediobasal hypothalamus (Caraty et al., 1998). Furthermore, the positive feedback effects of estrogen is also seen in the ARC (specifically, in caudal region) which up regulation of Kiss1 mRNA immediately before the preovulatory GnRH/LH surge (Estrada et al., 2006).

Therefore, kisspeptin cells in the ovine ARC appear to regulate in both negative and positive feedback control of GnRH by sex steroids and show a significant difference from the Kiss1 regulatory model presented in rodents. It is possible that subpopulations of kisspeptin cells respond differently to these stimuli (Smith, 2009). Moreover, the nature of the estrogen stimulus might induce different responses. There are three types of estrogen feedback important for GnRH releasing in ewes: short-term negative feedback, long-term negative feedback, and transient positive feedback (Smith, 2009). It is thought that kisspeptin cells may be able to discriminate between the more chronic effects of constant estrogen levels, inducing negative feedback, from the more acute increase in estrogen during the late follicular phase of the estrous cycle, inducing the switch to transient positive feedback (Smith, 2009).

Early research suggests no regulatory effect of sex steroids on Kiss1 mRNA expression in the POA. However, one study using twice the number of POA tissue sections reported chronic estrogen replacement after OVX increased the Kiss1 mRNA expression and kisspeptin protein in the POA, and half of the kisspeptin expressing cells co-expressed ERα. Although estrogen acts in the ARC at the mediobasal hypothalamus, not the POA, to induce the GNRH/LH surge, it is possible that POA kisspeptin cells in sheep participate in the E-induced preovulatory LH surge, similar to AVPV neurons in rodents. Thus, the sheep POA kisspeptin neurons would most likely be stimulated indirectly by the positive feedback control of estrogen (Smith, 2009).

In summary, Kiss1 genes mainly appear in the hypothalamus of ruminants and other mammals to provide the negative and positive feedback regulation of GnRH secretion by gonadal steroids. In ewes, which are used as representatives of ruminants, the kisspeptin cells in the ARC are balanced to play a role in the steroid negative feedback control of GnRH. Also, the kisspeptin cells in the mediodorsal hypothalamus are involved in the surge mode, and kisspeptin expression is

increased immediately before the preovulatory GnRH/LH surge. The process involved in the regulation and function of the kisspeptin cells in the POA area, however, is still not understood and further studies need to be done.

Since variations have been found between species due to differences in their anatomy and physiology, and since no studies have been done in buffalo, the exact nature of how these processes function in buffalo is unknown. Therefore, the objective of this study to understand the role of kisspeptin in control of reproductive function and the potential applications of kisspeptin to manage reproduction in buffalo heifer.

Objectives

To understand the role of kisspeptin in control of reproductive function and the potential applications of kisspeptin to manage reproduction in swamp buffalo heifer. The objectives of this project are:

- To detect the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of pre-pubertal swamp buffaloes

- To investigated the characteristic of kisspeptin receptor co-localized in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in pre-pubertal swamp buffaloes for determination the interaction of KISS1R and GnRH neurons in hypothalamus of swamp buffalo heifer.

Materials and methods

Experiment 1

Title: Evidence of *Kiss1* mRNA and Its Protein Distribution in Preoptic and Arcuate Hypothalamic Nuclei in Pre-pubertal Female Swamp Buffaloes (*Bubalus bubalis*)

Objective: To detect the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of heifer buffaloes.

Experiment 2

Title: Characteristic of Kisspeptin Receptor Co-localized in GnRH Neurons in the Preoptic Area and Arcuate Hypothalamic Nuclei in Pre-pubertal Female Swamp Buffaloes (*Bubalus bubalis*)

Objective: To investigated the characteristic of kisspeptin receptor co-localized in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in pre-pubertal buffaloes

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 10pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5 -3 years; 2 animals each age) from slaughterhouses. 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 (Fig.1). All buffaloes were non-cycling. Estrous cycle identification of the animals was determined based on observation of the postmortem ovarian morphology (Ali et al., 2003) and plasma progesterone analysis by radioimmunoassay. After removing the brains, the hypothalami were collected and fixed in 4% paraformaldehyde for 24 hr (Fig.2-5). The samples were embedded in paraffin blocks and stored at room temperature (RT) (Fig.6-7). The paraffin block of the POA and ARC hypothalamic nuclei of buffaloes were identified by the anatomical structure identification in previous study in the buffalo cows (Chaikhun et al., 2016).

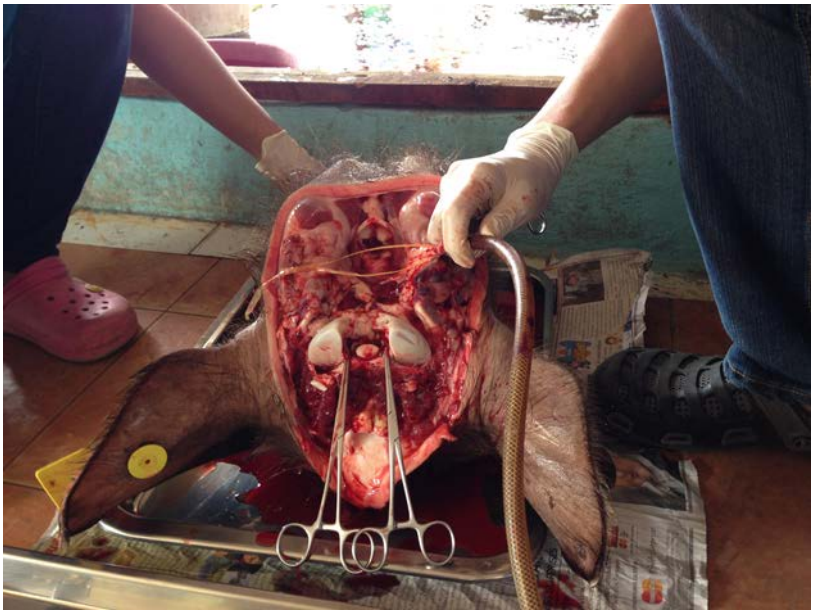

Figure 1 The infusion of the4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) into a common carotid artery

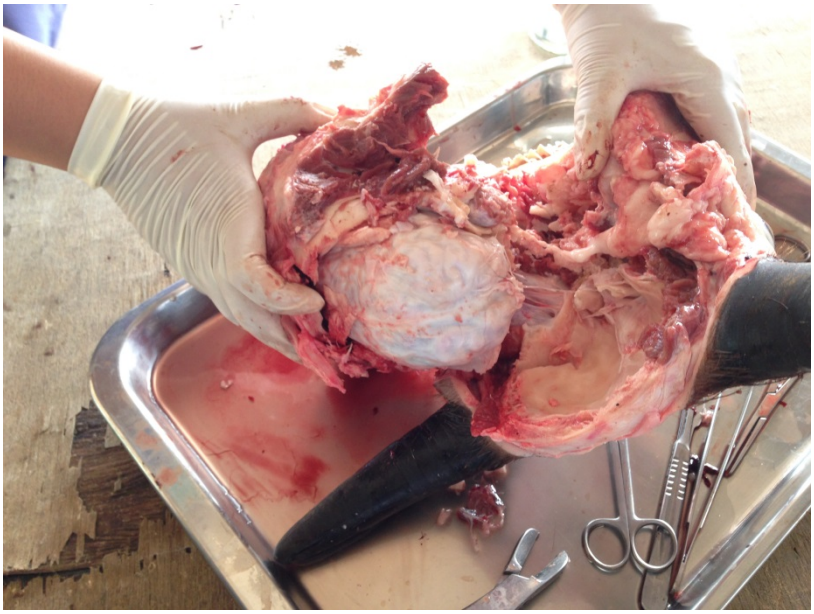

Figure 2 The brain are removing from the skull gently

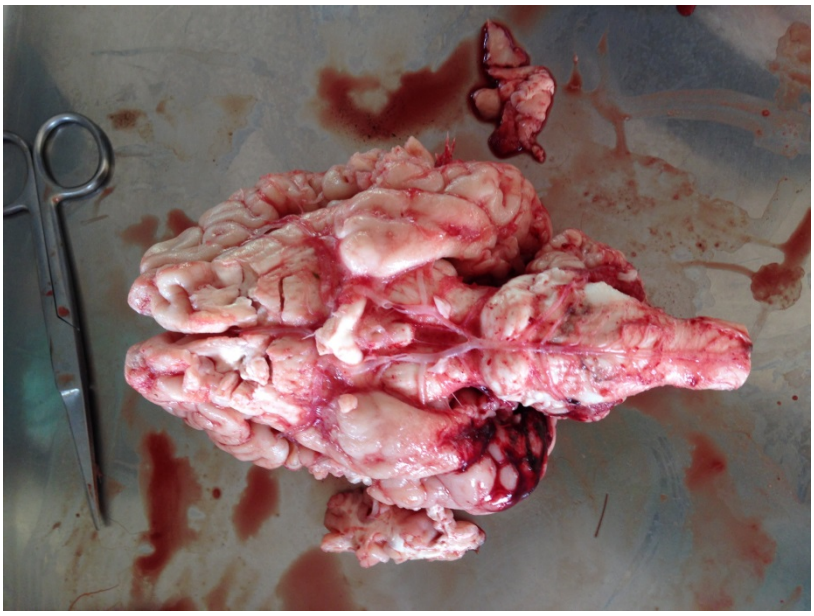

Figure 3 The brain is completely removed from the skull. It is firmed by the fixative.

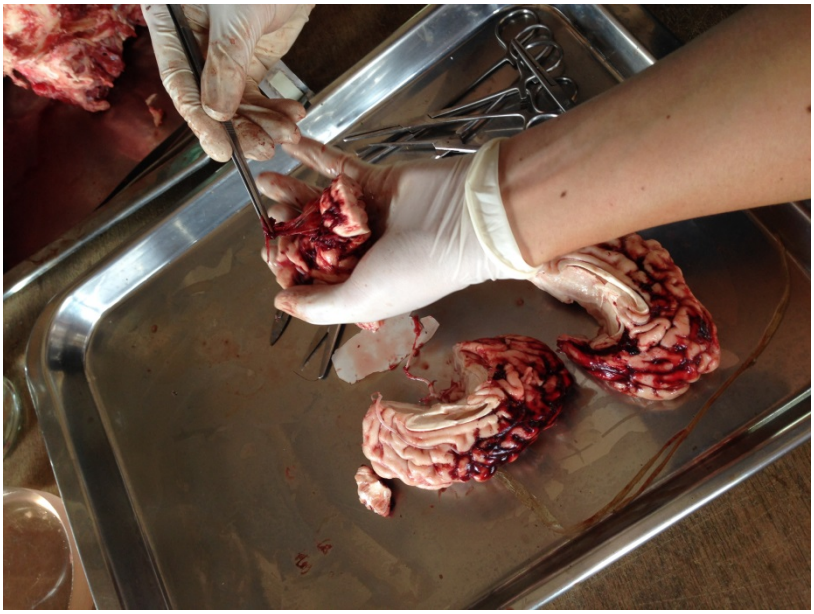

Figure 4 The hypothalamus is dissected.

Figure 5 The hypothalamus appearance after fixing in the 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hr.

Figure 6 The hypothalamus is trimmed and set in the cassettes for tissue processing with RNAse free condition (manual processing).

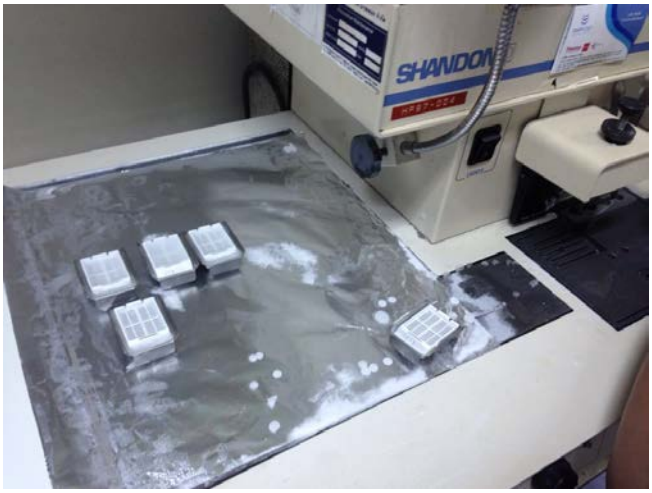

Figure 7 The preparation of the paraffin block samples under the RNAse free condition.

Experiment 1

Title: Evidence of *Kiss1* mRNA and Its Protein Distribution in Preoptic and Arcuate Hypothalamic Nuclei in Pre-pubertal Female Buffaloes (*Bubalus bubalis*)

Objective: To detect the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of heifer buffaloes.

In situ hybridization of Kiss1 mRNA

The ISH protocol was modified following our previous studies(Chaikhun et al., 2016; Sotthibandhu, 2009).

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. Themain reason for using the ovine *Kiss1*cRNA probe for the ISH in this study is that there is no reported research on the *Kiss1* sequence in buffalo and it is thus not commercially available. Also buffalo areruminants and seasonal (short-day) breeders similar to sheep (Borghese and Mazzi, 2005; Montiel and Ahuja, 2005). The ISH technique has been used to detect specific mRNA in specific cells (Yang et al., 1999). In addition, the proven cross-reactivity of gene probes to the same mRNA in different animals (but still of the same ruminant type) by Butler et al. (1994) indicated to us that this may be a sensitive and useful tool for our preliminary observations. In confirmation of our previous study's assumptions, 94% of the tested sequences showed significant alignments between the predicted buffalo and ovine *Kiss-1* 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

Four-micron paraffin sections of the POA and ARC hypothalamic nuclei were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol prepared with a 0.1% dimethyl pyrocarbonate (DMPC; Sigma, MO, USA) treated distilled water. The rehydratedsections were immersed in a citrate buffer (Bio-Optica, Milano, Italy) (pH 6.0) and autoclaved for 10 min at 121 °C. Then the endogenous alkaline phosphatase was blocked by treatment of the section with 0.2 N HCl for 10 min at RT. After that the sections were post-fixed in 4% paraformaldehyde in a 10 mM phosphate buffer saline (PBS; Bio-Optica, Milano, Italy) (pH 7.2) for 10 min at RT. After autoclaving, and between each step up until post-fixation, the sections were washed in PBS.

Prehybridization was conducted by treating the sections withahybridization cocktail (Hybridization cocktail 50% formamide, Amresco, Ohio, USA) for 1 hr at RT. A complimentary RNA probe was heated at 95 °C for 5 min and then diluted with a hybridization cocktail. The hybridization was done at 45 °C for 20 hr in hybridizer (S2451-30, Dako, Glostrup, Denmark) saturated with 50% of

2x sodium chloride- sodium citrate buffer (SSC; AppliChem, Darmstadt, Germany) and 50% formamide (Sigma, MO, USA). The sections were stringently washed in 50% of 2x SSC and 50% formamide for 1 hr, followed with 2x SSC containing 0.03% Brij-35 (30% Brij-35, Sigma Aldrich, USA) for 30 min, twice. Then the sections were washed in 0.5x SSC containing 0.03% Brij-35 for 30 min, and twice in 0.2x SSC containing 0.03% Brij-35 for 30 min. Each step of stringentwashing was conducted at 45 °C. Next, the sections were equilibrated in a 0.1 M Tris-HCl buffer in 150 mMNaCl (pH 7.5). Then unspecific bindings were blocked by incubating the sections with a blocking reagent (DIG nucleic acid detection kit, Roche, Mannheim, Germany) for 30 min at RT, followed by a sheep anti-digoxigenin antibody conjugated with alkaline phosphatase (DIG wash and block buffer set, Roche, Mannheim, Germany). The sections were applied using 1x NBT/BCIP (DIG nucleic acid detection kit, Roche, Mannheim, Germany) to detect mRNA signals. 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 kisspeptinimmunohistochemical study. Paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol. Antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 70 °C. Following this the endogenous peroxidase activity was blocked by incubating in 1% hydrogen peroxide (QRëC, New Zealand) in methanol (Merck, Darmstadt, Germany) for 30 min at RT. 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 - this antibody is the same #566 antibody used in the study by Franceschini et al. (2006)) at 4 °C overnight (16 hr). This antibody has showna species reactivity in ovine, rat and mouse but a very low level of reactivity with human kisspeptin10. In addition, its’ specificityhas been provennot to be inhibited by other hypothalamic peptides which are in the RFamide family of peptides (such as the PrRPused in radioimmunoassay, also the GnIH, NFF, Chemerin, QRFP and PrRPused inimmunostaining) (Franceschini et al., 2006; Goodman et al., 2007). After that, a biotinylated universal antibody and streptavidine horseradish peroxidase (LSAB+System-HRP™, catalog number K0679, Dako, Glostrup, Denmark) were incubated for 1 hr and 50 min respectively at RT. 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. In each step, the sections were washed in a 10 mM phosphate buffer saline (PBS; Bio-Optica, Milano, Italy) (pH 7.2). This PBS also was used for diluents of the antibody and other reagents. Positive controls for antibody and tissue specificity were prepared using ewe POA and ARC hypothalamic nuclei paraffin sections.Negative controls for antibody specificitywere conducted using PBS(instead of a primary antibody application)in combination with 10% normal horse serum, which was applied for non-specific binding blocking of primary antibody. 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 counter- stained by hematoxylin, was applied on the sample slides. After that the number of kisspeptin-ir cells randomly found in the100 mm^2area per slidetaken 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 fromeach cow were calculated as a percentage by dividing the number of positive neurons by the total counted neurons and then multiplying by 100. This figure wasthen 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.

Experiment 2

Title: Characteristic of Kisspeptin Receptor Co-localized in GnRH Neurons in the Preoptic Area and Arcuate Hypothalamic Nuclei in Pre-pubertal Female Swamp Buffaloes (*Bubalus bubalis*)

Objective: To investigated the characteristic of kisspeptin receptor co-localized in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in pre-pubertal buffaloes.

Double label immunohistochemistry for KISS1R and GnRH.

Paraffin sections of the POA and ARC hypothalamic nuclei were prepared at 4 microns on superfrost-slides (Fisher Brand, Thermo Fisher Scientific, MA, USA) and were processed by the standard double label- immunohistochemical method. Paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol. Antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 121 °c. Non-specific binding was blocked using 1% normal goat serum (Gibco, NY, USA) for 20 min at RT. The sections were incubated with a primary rabbit anti-human KiSS1R/GPR54 polyclonal antibody (1:100 dilution) (Bioss, catalog number bs-2501R, MA, USA) overnight (16 hr) at 4 °C. A secondary goat anti-rabbit IgG (H+L) antibody (1:500 dilution) (Alexa fluor 488, catalog number A-11008, Life Technologies, CA, USA) was applied to the sections at RT for 2 hr. A mouse anti-mammalian GnRH monoclonal antibody (1:100 dilution) (Chemicon, catalog number MAB5456, CA, USA) was applied to the sections overnight (16 hr) at 4°C. A 1:100 dilutionof secondary goat anti-mouse IgG (H+L) (Alexa fluor 568, catalog number A-11004, Life Technologies , CA, USA) was applied to the slides and kept at RT for 2 hr then washed with PBS. All antibodies were diluted with 10 mM phosphate buffer saline (PBS; Bio-Optica, Milano, Italy) (pH 7.2). In each of the aforementioned washing steps, the sections were washed in 10 mM PBS 3 times for 5 minutes each. The washing step following the 1st secondary antibody application consisted of the sample slides being washed in 10 mM PBS 3 times for 10 minutes each, by a slow shaking in aluminum foil covered Coplin jars. The sections were then mounted and covered by an anti-fade reagent (Molecular probes, catalog number P36930, CA, USA). The double labeled immunoreactivity was observed under a fluorescent microscope (Axiolab, Zeiss, Oberkochen, Germany). A single observer counted the number of KISS1R-ir cells, GnRH-ir cells and co-localized-ir cells found in 100 mm^2 area slides from each of the buffalo cow's POA and ARC hypothalamic nuclei. The images were captured by Axivision software (Axiolab, Zeiss, Oberkochen, Germany). The layers of images were combined in Adobe Photoshop.

Controls and specificity

Antibody validation and cross-reactivity studies of the rabbit anti-human KiSS1R/GPR54 polyclonal antibody used in this study (Bioss, catalog number bs-2501R, MA, USA), have been carried out in humans, rats and mice by the source company (Bioss, MA, USA). Positive controls for antibody and tissue specificity were prepared using brain sections from wild-type mice. Negative controls for antibody specificity were performed using brain sections from *Gpr54* gene knockout (KO/*Gpr54*) mice

and also by omitting the primary antibody. Negative controls for tissue specificity were performed using the white matter area of the central nervous system of buffalo and mice - an area known to have no KISS1R expression.

The mouse anti- mammalian GnRH monoclonal antibody (Chemicon, catalog number MAB5456, CA, USA), has been used in buffaloes previously (Chaikhun et al., 2016; Zerani et al., 2012). Positive controls for antibody and tissue specificity were prepared using ewe POA and ARC hypothalamic nuclei paraffin sections. Negative controls for antibody specificity were conducted using PBS (instead of a primary antibody application) in combination with 1% normal goat serum, which was applied to reduce the non-specific binding of the primary antibody. Negative controls for tissue specificity were the white matter area of the central nervous system of eve and buffalo- which are known to have no GnRH expression.

Statistical analysis

The number of each type of immunoreactive cells with co-localization or non co-localization were calculated as a percentage of the total number and then were averaged across animals to calculate a mean (± SE). The comparison of the average number of each type of immunoreactive cells (with co-localization or non-co-localization) between the POA and ARC were analyzed by a t-test ($P<0.05$). Characterizations of co-expression and none co-expression were described.

Results

All 10 animals were the pre-pubertal female swamp buffaloes by plasma progesterone analysis (<1 ng/ml) and ovarian morphological inspection.

Experiment 1

In situ hybridization of Kiss1 mRNA

The expression of *Kiss1* mRNA using an antisense *Kiss1*cRNA probe was detected in the cytoplasm of neuronal soma in the majority of neurons with great intensity reaction in both the POA (Fig. 9, 11Aand 12) and ARC hypothalamic nuclei (Fig.10, 11B and 13) of all age buffalo samples. *Kiss1*mRNA was also found in some small neuronal cells (Fig. 9E, 10E) which were distinguished from glia cells by their vesicular nuclei. Interestingly, there is a synapsis evidence between 2 kisspeptin neurons (Fig.11A3) There was no signal of *Kiss1* mRNA in the buffalo POA and ARC sections in which the sense *Kiss-1*cRNA probe was applied (Fig. 9B, 9D, 10B and 10D) and these were considered as negative control reactions. Positive control reactions were prepared using the buffalo cow POA (Fig. 10E and 10F) and ARC (Fig. 11E and 11F) hypothalamic nuclei paraffin sections.

Table1 The expression of *Kiss1* mRNA using an antisense (AS) and a sense (S) *Kiss1*cRNA probe in both the POA and the ARC hypothalamic nuclei of each buffalo. Remarks: Reaction intensity in the green highlight means the result from the 1st trial but the red highlight means the results from a 2nd trail (repetition).

No	Age (year)	Reaction intensity	POA		Reaction intensity	ARC	
			AS	S		AS	s
H1	<1	Great	✓	✓	Great	✓	✓
H2	<1	Great	✓	✓	Great	✓	✓
H3	1-1 ½	Great	✓	✓	Great	✓	✓
H4	1-1 ½	Great	✓	✓	Great	✓	✓
H5	1 ½ -2	Great	✓	-	Great	✓	✓
H6	1 ½ -2	Great	✓	✓	Great	✓	✓
H7	2-2 ½ years	Great	✓	✓	Great	✓	✓
H8	2- 2 ½	Great	✓	✓	Great	✓	✓
H9	2 ½ -3	Great	✓	✓	Great	✓	✓
H10	2 ½ -3	Great	✓	✓	Great	✓	✓
C5 Control	7	Great	✓	✓	Great	✓	✓

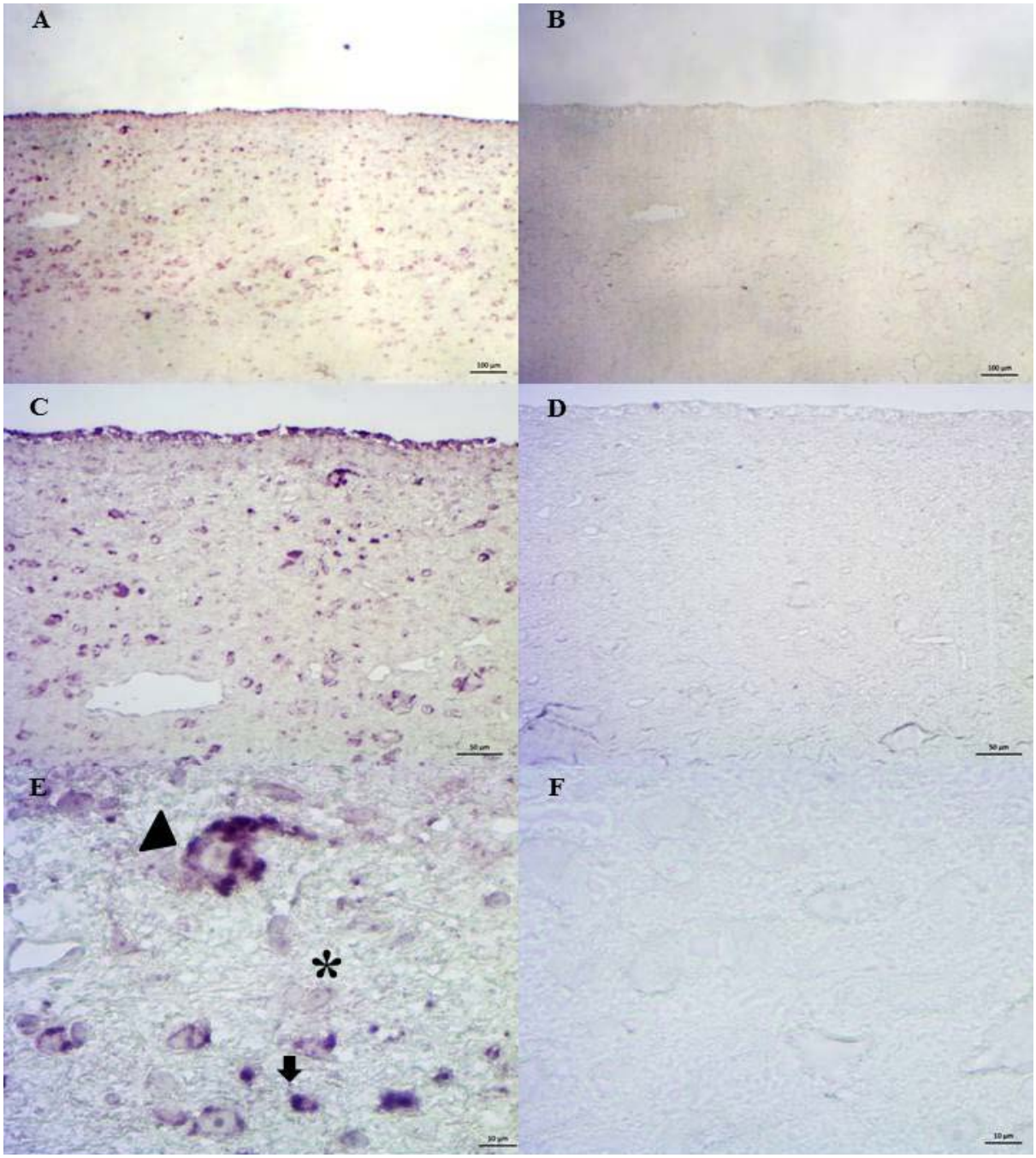

Figure 9*Kiss1* mRNA in the POA hypothalamic nucleus of buffalo samples, is visible in the anti-sense (positive result) of *Kiss1* mRNA samples (A, C and E) and is not expressed in the sense (negative result) of *Kiss1* mRNA samples (B, D and F). *Kiss1* mRNA expressions are localized in the cytoplasm of a neuron (arrow head) and a small neuronal cell (full arrow) but not in a 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.

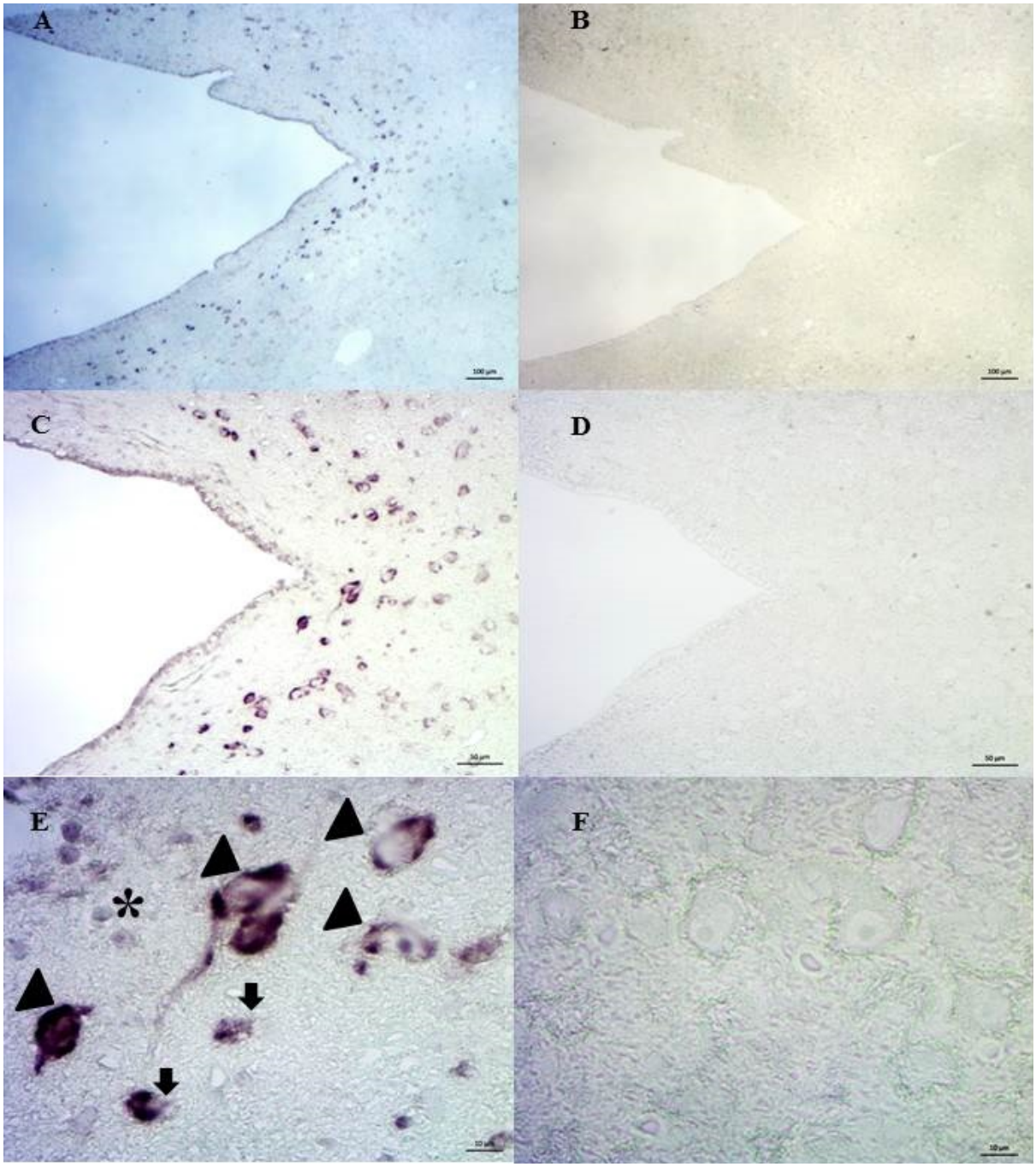

Figure 10 In the ARC hypothalamic nucleus of buffalo samples, *Kiss1*mRNA is visible in the anti-sense (positive result) of *Kiss1* mRNA sample (A, C and E) but not expressed in the sense (negative result) of *Kiss1* mRNA sample (B, D and F). The *Kiss1* mRNA is localized in the cytoplasm of a neuron with some strong signal (arrow heads) but no signal in other small neurons (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.

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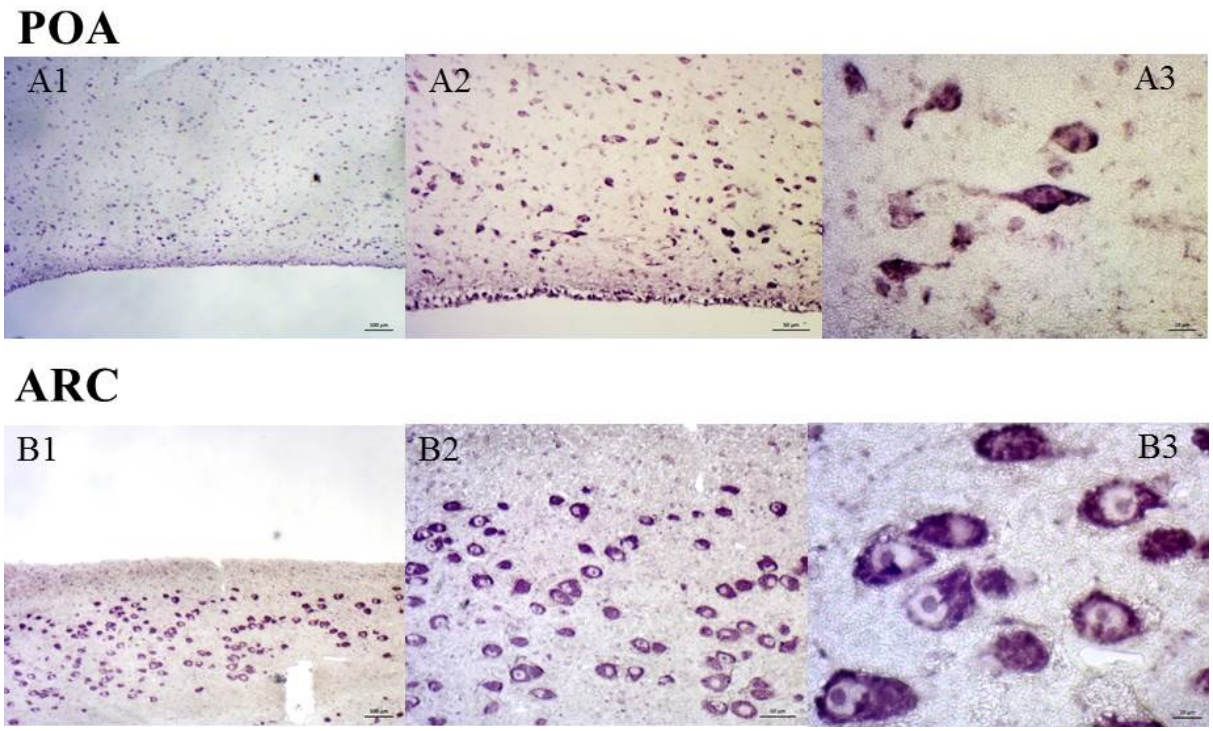

Figure 11 The *Kiss1* mRNA neuron shows scattered distribution in POA area (A1 and A2), controversially to the ARC area which is accumulated distribution pattern (B1 and B2). There is a synapsis evidence between 2 kisspeptin neurons in A3. Scale bar is 100 μm in A1 and B1, 50 μm in A2 and B2 but it is 10 μm in A3 and B3.

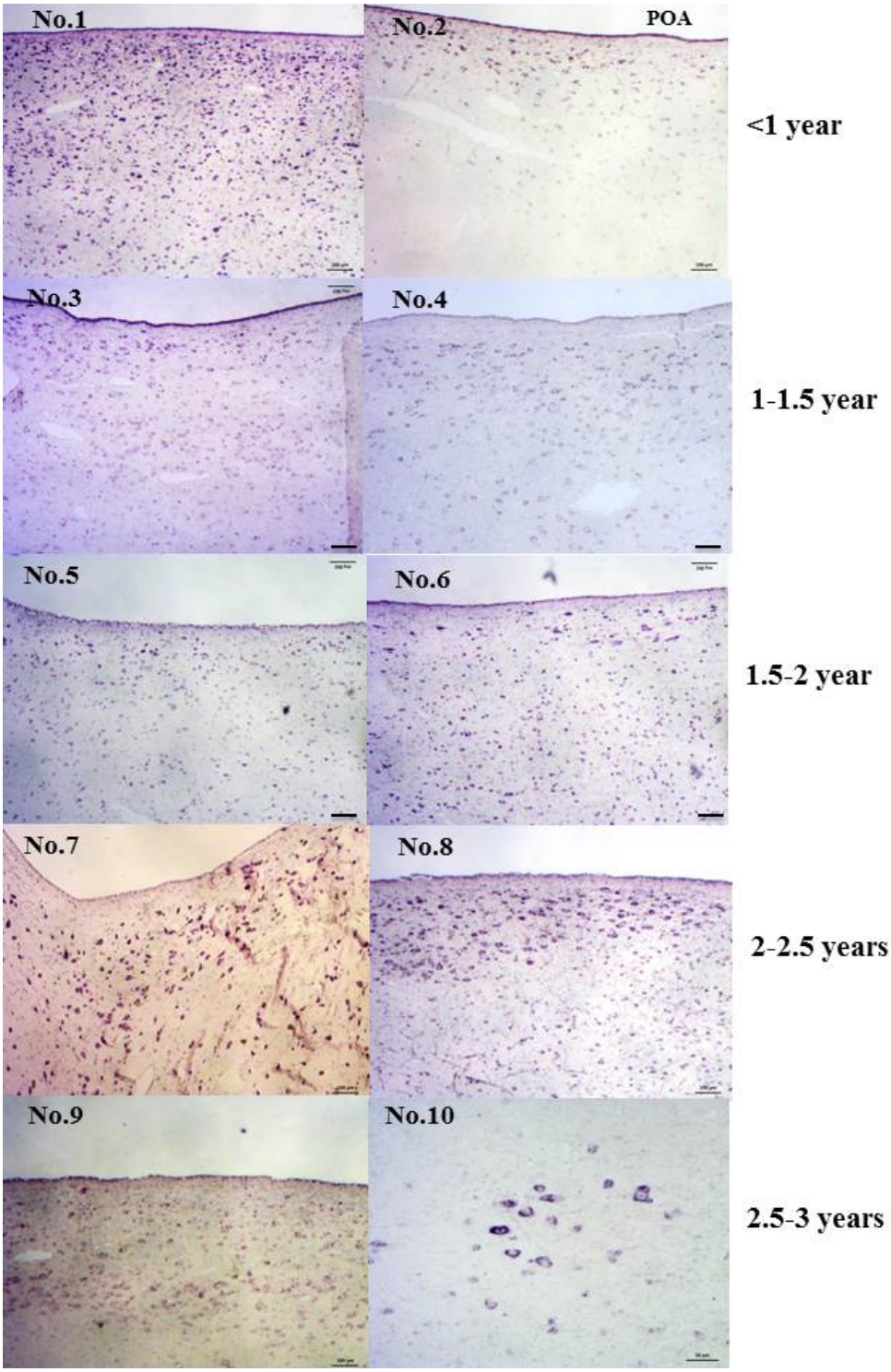

Figure 12 In POA hypothalamic nuclei,*Kiss1* mRNA expressions are presented in every age of pre-pubertal buffaloes. There are great intensities in ependymal cells and neuronal cells.Scale bar is 100 μm (No.1-9) but it is 50 μm in No.10.

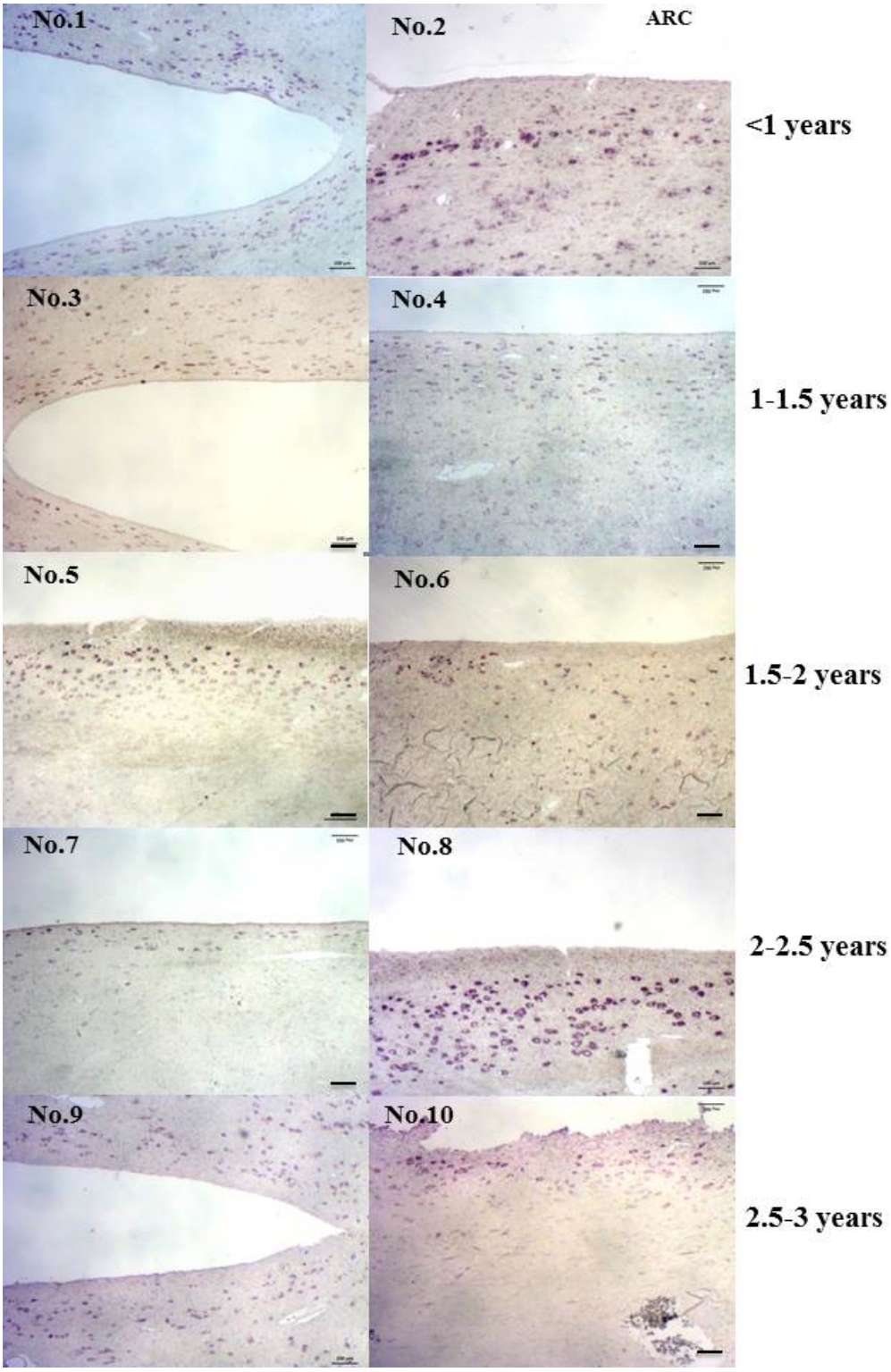

Figure13 In ARC hypothalamic nuclei, *Kiss1* mRNA expressions are presented in every age of pre-pubertal buffaloes. There are great intensities in neuronal cells that nearby the 3rd ventricle. Scale bar is 100 µm.

Immunohistochemistry of kisspeptin

The percentage of kisspeptin reactive cells (Fig. 14) and intensity in each age of 10 heifers and each hypothalamic area are showed in Table 2. The results showed weak reactions of kisspeptin located in the cytoplasm of the neuronal soma in a less than 1 year old of age in both the POA and ARC hypothalamic nuclei which the number of kisspeptin neurons in the POA (45.24%) was higher than the ARC area (22.22%). Another same age calf found few kisspeptin neurons in the POA (2.44%) but none in the ARC area. In 1-2.5 years old of age group expressed kisspeptin reactions only in the ARC area (Fig.15) but none in the POA (Fig.16). All of the heifers 2.5-3 years old of age showed none of kisspeptin expression in both areas. There was no difference in kisspeptin reaction intensity between the POA and ARC hypothalamic nuclei and each 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. 17a, c. The negative control presented no non-specific reactions (Fig. 17b, d).

Table 2 The percentage of kisspeptin protein reactions populations and intensity of reactions in the POA and ARC hypothalamic areas in all age of 10 heifers.

Heifer ID	Age (year)	POA		ARC	
		Kisspeptin-ir cells (%)	Intensity	Kisspeptin-ir cells (%)	Intensity
H1	<1	45.24	weak	22.22	weak
H2	<1	2.44	weak	0	weak
H3	1-1.5	0	No	21.74	weak
H4	1-1.5	0	No	65	weak
H5	1.5-2	0	No	94.12	weak
H6	1.5-2	0	No	53.12	weak
H7	2-2.5	0	No	54.84	weak
H8	2-2.5	0	No	12	weak
H9	2.5-3	0	No	0	no
H10	2.5-3	0	No	0	no

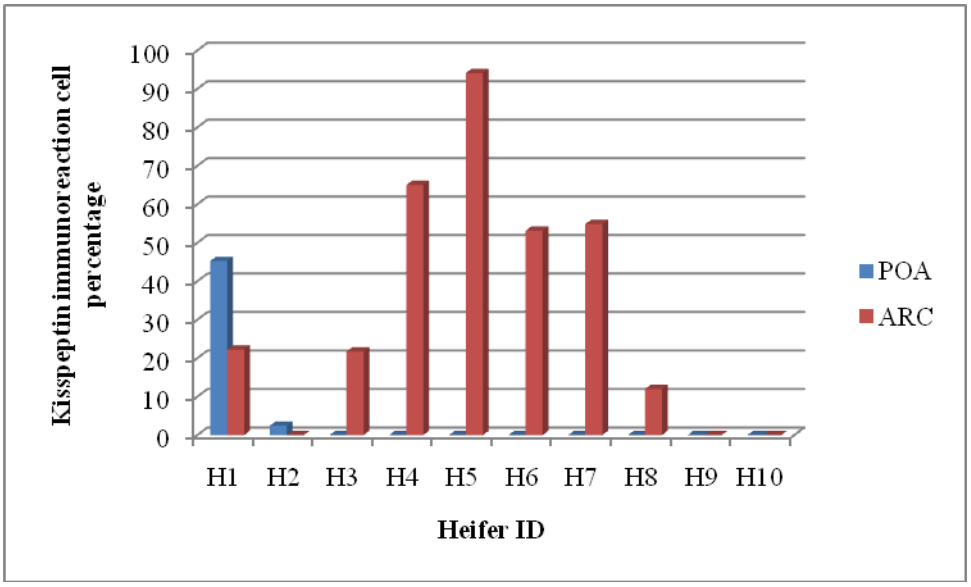

Figure14 The percentage of kisspeptin reactive neurons in each age of 10 pre-pubertal female swamp buffalo in the POA (blue) and the ARC (red) hypothalamic areas.

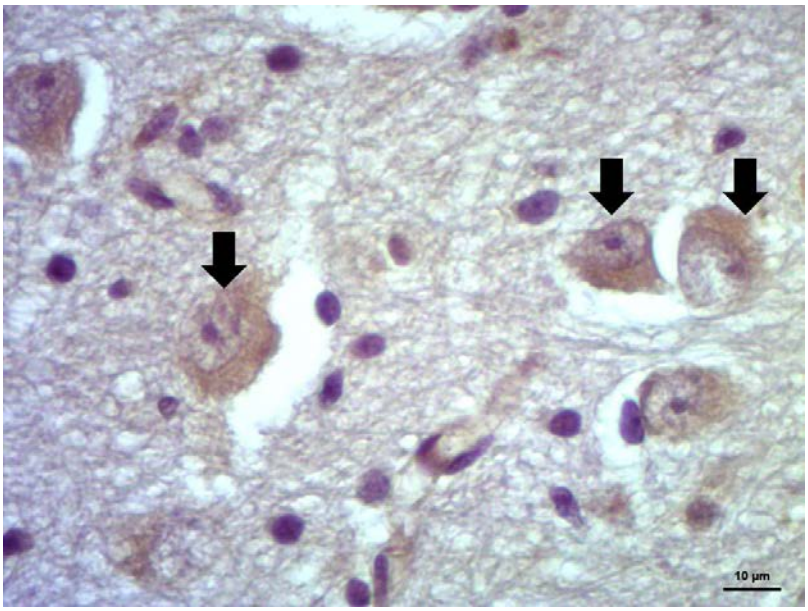

Figure15 The kisspeptin reactive proteins show in cytoplasm of neurons with weak intensity (arrow) in the ARC are in 1-2.5 years old of age groups. Scale bar 10 µm.

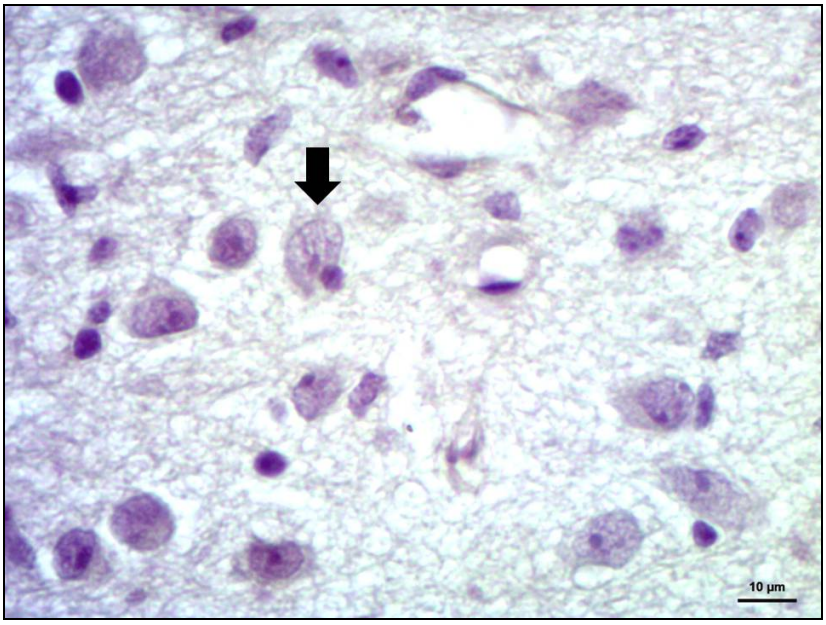

Figure16 None of the kisspeptin reactive protein present in any cytoplasm of neuron (arrow) in the POA in 1-2.5 years old of age groups. Scale bar 10 µm.

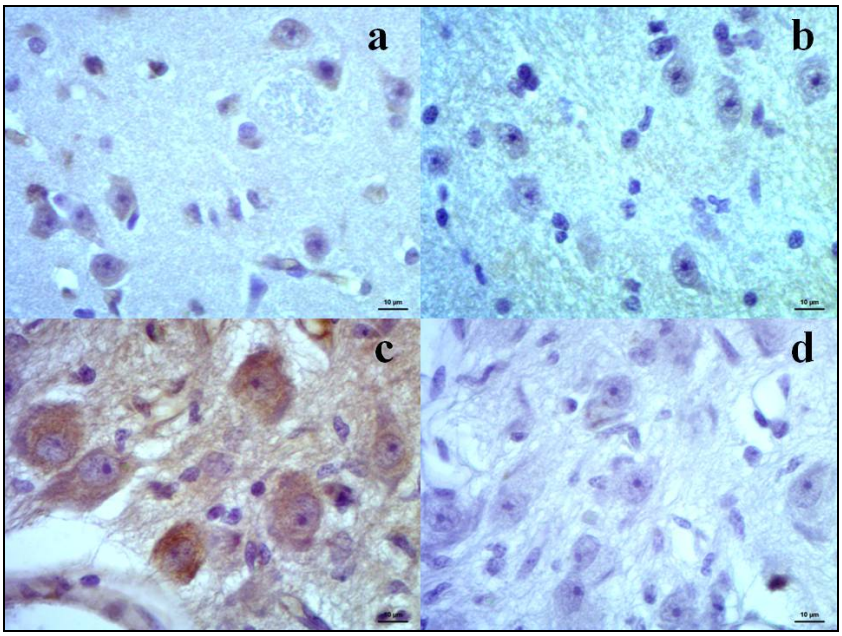

Figure17 The positive (left) and negative controls (right) for immunohistochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in the ewe (a, b) and the buffalo cow (c, d). Scale bar 10 µm.

Experiment 2

The pattern of immunoreactivity for GnRH-ir appeared in the cytoplasm of neuronal soma and formed granulation in some neurons. KISS1R-ir located mainly in cytoplasm and some in nucleus of neurons. Double label results showed that all observed KISS1R-ir were co-localized with GnRH-ir neurons (Fig.18). The population of KISS1R-ir co-localized in GnRH neurons in each hypothalamic nuclei in all 10 pre-pubertal buffaloes is showed in Table 3 and Fig. 19. The co-localized neuron population in the POA (52.58±17.71%) was no statistical different with the ARC (58.56±13.79%) in all age groups (P>0.05). There were the single GnRH-ir neuron populations in the POA (37.77±19.69%) was similar as in the ARC (36.32±11.88%) (P>0.05). There were none of both KISS1R and GnRH reactions in neurons (non-ir neurons) in the POA (8.65±5.34%) and the ARC (5.12±2.58%) areas (P>0.05).

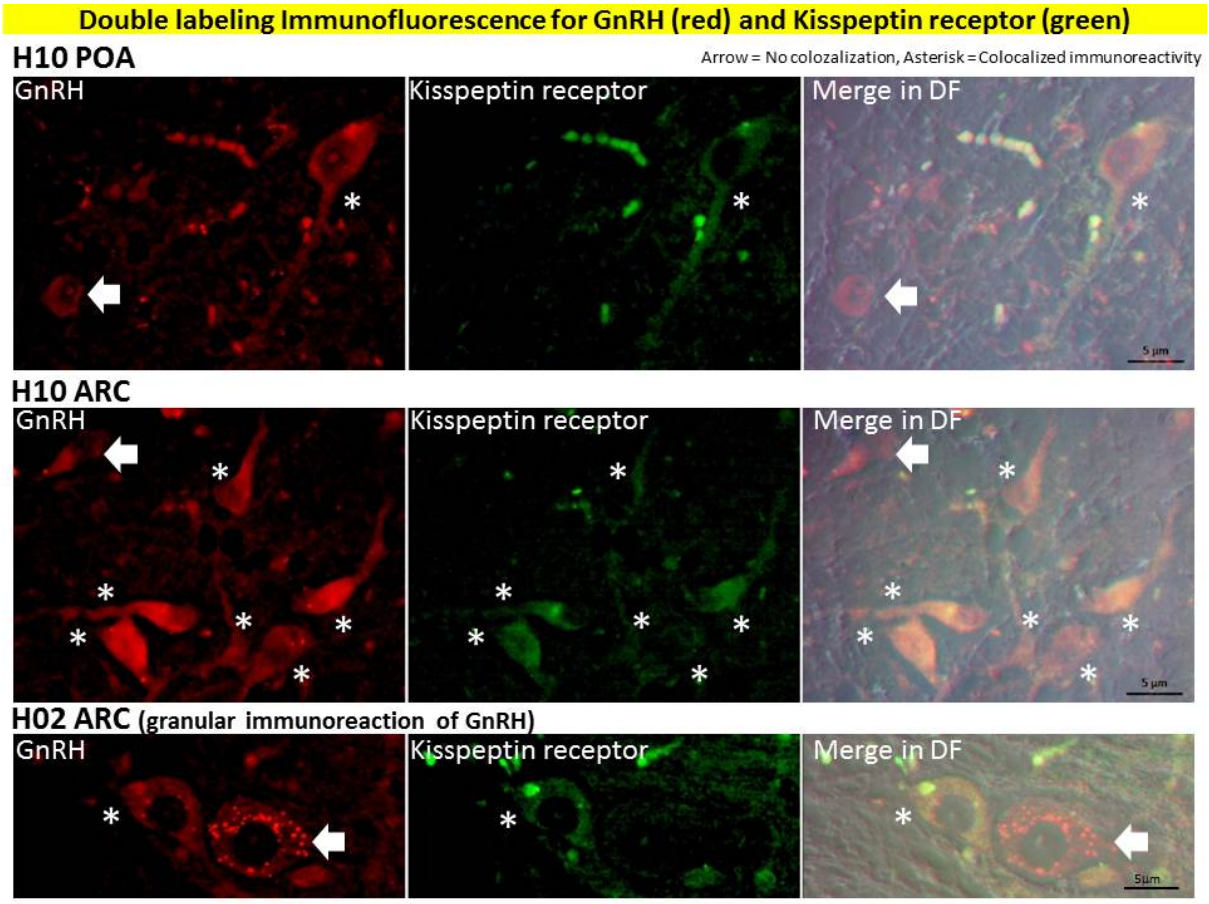

Figure 18 The KISS1R immunoreations are co-localized with GnRH neurons in the POA and ARC hypothalamic nuclei (asterisks). However, there are some neurons express only GnRH reactions in both hypothalamic nuclei which granular immunoreaction appear in some neurons (arrows), scale bar 5 µm

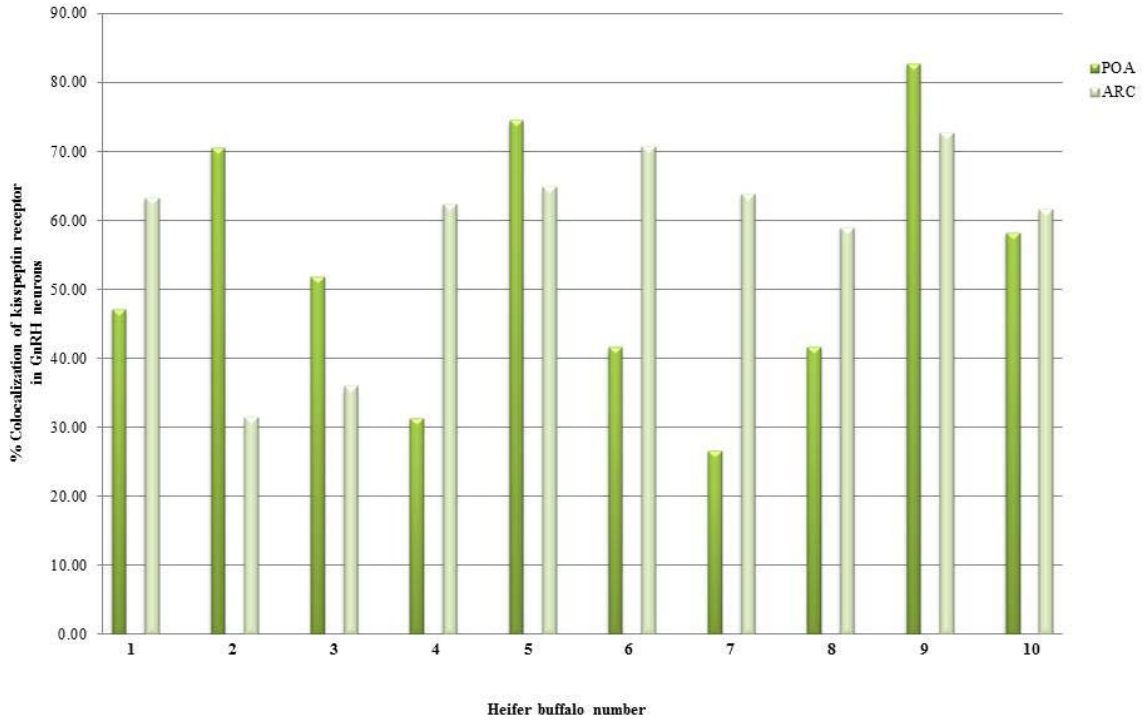

Figure 19 The KISS1R-ir co-localized in GnRH-ir neurons population (%) in the POA and ARC areas in each pre-pubertal buffaloes.

Table3 The co-localized population of KISS1R immunoreations with GnRH neurons in the POA and ARC hypothalamic nuclei in pre-pubertal buffaloes

Heifer ID	Age (year)	Area (Total neuron in 100 mm^2)	%Immunoreactive neurons (n)			% Non-reactive neurons (n)
			Co-localized (n)	GnRH (n)	KISS1R (n)	
H1	<1	POA (157)	47.13 (74)	43.31 (68)	0 (0)	9.55 (15)
		ARC (226)	63.24 (143)	27.88 (63)	0 (0)	8.85 (20)
H2	<1	POA (230)	70.43 (162)	13.91 (32)	0 (0)	15.65 (36)
		ARC (277)	31.41 (87)	61.37 (170)	0 (0)	7.22 (20)
H3	1-1.5	POA (552)	51.81 (286)	28.08 (155)	0 (0)	20.11 (111)
		ARC (359)	35.93 (129)	55.71 (200)	0 (0)	8.36 (30)
H4	1 -1.5	POA (458)	31.22 (143)	63.10 (289)	0 (0)	5.68 (26)
		ARC (436)	62.39 (272)	29.82 (130)	0 (0)	7.80 (34)
H5	1.5-2	POA (470)	74.47 (350)	18.72 (88)	0 (0)	6.81 (32)
		ARC (481)	64.86 (312)	31.81 (153)	0 (0)	3.33 (16)
H6	1.5-2	POA (373)	41.55 (155)	54.69 (204)	0 (0)	3.75 (14)
		ARC (400)	70.75 (283)	25.50 (102)	0 (0)	3.75 (15)
H7	2-2.5	POA (409)	26.65 (109)	65.53 (268)	0 (0)	7.82 (32)
		ARC (384)	63.80 (245)	34.64 (133)	0 (0)	1.56 (6)
H8	2-2.5	POA (377)	41.64 (157)	51.99 (196)	0 (0)	6.37 (24)
		ARC (472)	58.90 (278)	36.23 (171)	0 (0)	4.87 (23)
H9	2.5-3	POA (404)	82.67 (334)	7.43 (30)	0 (0)	9.90 (40)
		ARC (377)	72.68 (274)	23.87 (90)	0 (0)	3.45 (13)
H10	2.5-3	POA (450)	58.22 (262)	40.89 (184)	0 (0)	0.89 (4)
		ARC (401)	61.60 (247)	36.41 (146)	0 (0)	2.00 (8)

Discussions

Experiment 1

The present study's detection and localization of *Kiss1* mRNA in all age of pre-pubertal swamp buffaloes and both POA and ARC hypothalamic nuclei which similar to the post-pubertal swamp buffaloes(Chaikhun et al., 2016). Itpossibly suggests that the buffaloes could be able to produce *Kiss1* mRNA in these hypothalamic nuclei in all ages.Also, these evidences could be determine that kisspeptin is a master pubertal and reproductive function activator in swamp buffalo which similar to other mammals (Estrada et al., 2006; Goodman et al., 2007; Rometo et al., 2007).

The results of our study's immunohistochemistry testing found the kisspeptin protein reactions located in the cytoplasm of neurons in only some samples of the pre-pubertal buffaloes. We also detected the evidence of 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, the kisspeptin reactions showed mostly in the POA in the <1 year of age group, which suggests that the POA might be a main area related to kisspeptin expression in the early age or juvenile. 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 pre-pubertal age. Surprisingly, all of the heifers in 2 .5-3 years of age no showed any expression of kisspeptin in both areas, which this age close to puberty or peri-puberty of swamp buffalo(Chaikhun et al., 2012). Controversially, the post-pubertal buffalo's kisspeptin protein reactions were detected in both follicular and luteal phases and both POA and ARC areas. But the population of kisspeptin neurons in the POA was greater than the ARC area. The variation in kisspeptin protein distribution might not be only depend on *Kiss1* mRNA distribution but also on a possible difference in 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(Caraty et al., 2007; Colledge, 2008; Cui et al., 2015; Han et al., 2005; Liu et al., 2014; Overgaard et al., 2013; Poling and Kauffman, 2013). 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 our variation of kisspeptin distribution in pre-pubertal ages; in 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), kisspeptin and the release of growth hormone and kisspeptin and the release of prolactin (Kadokawa et al., 2008; Whitlock et al., 2008).These studies support our variation of kisspeptin distribution results that kisspeptin protein from hypothalamus possibly not just only involved in reproductive puberty but also in other metabolic purposes in the <1 to 2.5 years old pre-pubertal buffaloes. Remarkably, there was no kisspeptin protein reaction at 2.5-3years old group. This may

involve a temporary stop of kisspeptin production before the onset of puberty, or kisspeptin is produced but goes to other hypothalamic nuclei. Generally, kisspeptin expression is regulated by estrogen via estrogen receptor alpha (ERα) in kisspeptin neurons, especially peri-pubertal period. Estradiol 17β is reduced in the late juvenile stage of development. Low or no presentation of ERα in kisspeptin neuron may activate onset of puberty(Han et al., 2005; Mayer et al., 2010; Mayer and Boehm, 2011). Therefore, colocalization of ERα in kisspeptin neurons in pre-pubertal group of buffalo should be done in further study for more information.

Conclusion

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, kisspeptin proteins were localized in subpopulations of neurons in the POA greater than the ARC area in the 6 months old group. There were only the ARC area presented the kisspeptin reactions in 1-2 years old group. Interestingly, there was no of any kisspeptin reactions in either the POA and ARC areas in the 2 ½ years old group. These results suggest possibility of the kisspeptin might involve in pre-pubertal buffalo in different function and mode of reaction depend on age and hypothalamic nuclei. The kisspeptin-kisspeptin receptor-GnRH- ERα relationship should be researched in next step.

Experiment 2

Our present study found more than 50% of the co- localized KISS1R immunoreations with GnRH neuron population in both POA and ARC hypothalamic nuclei in the pre-pubertal female buffaloes. This indicates the kisspeptin possibly regulates GnRH function in the juvenile and the pre-pubertal buffaloes in some aspects. In post-pubertal buffaloes, the co- localized KISS1R immunoreations with GnRH neuron population was more than 80% in both hypothalamic areas in the previous study (Chaikhun-Marcou et al., 2016). These results are suggested that kisspeptin-kisspeptin receptor signaling could involves in female buffalo reproductive development and function via GnRH controlling through all ages of pre- and post- pubertal periods. Similarly, a study in mice have found the percentage of GnRH neurons responding to kisspeptin (*in vivo*) increases by age from juvenile (25%), pre-pubertal (50%) and adult (>90%) (Han et al., 2005). According to 35% approximately of single GnRH immunoreactive neurons were detected in both hypothalamic areas. Thus, most of GnRH neurons might not be regulated by kisspeptin. By contrast, our previous study found the co-localized KISS1R immunoreations in every GnRH neurons and the KISS1R immunoreations were detected in many other non-GnRH immunoreactive neurons(Chaikhun-Marcou et al., 2016). The results point to the possible on difference active mode, function and mechanism of the kisspeptin on GnRH releasing between pre- and post- pubertal reproductive function.

In pre-pubertal period, the GnRH is generally released in basal level (tonic mode) just enough to controls reproductive development. Also, some neuroendocrine or proteins might be a major factor that involve in reproductive development and body growth development such as leptin rather than kisspeptin(Manfredi-Lozano et al., 2016; Whitlock et al., 2008). The kisspeptin and it's receptor expression (*Kiss1* mRNA, KISS1R) were detected in low level which also have been reported in other pre-pubertal animal(Colledge et al., 2010; Han et al., 2005). But kisspeptin is a major factor that control the GnRH releasing for reproductive cycle regulation in post-pubertal period(Clarkson et al., 2009; Colledge, 2008). The population of the co- localized KISS1R immunoreations in GnRH neurons was no different between the POA and ARC hypothalamic nuclei**.** There is a variation of the number of co-localized neurons on the age of pre-pubertal buffalo similar to post-pubertal buffaloes.

Conclusion

The co- localized KISS1R immunoreations with GnRH neuron population in both POA and ARC hypothalamic nuclei was detected in the pre-pubertal female buffaloes in this study. However, there is around 50% of single GnRH immunoreactive neurons (without KISS1R immunoreations). It might indicate that kisspeptin could regulates GnRH releasing in some level for reproductive development and puberty in pre-pubertal female buffaloes which might not be the same of post-pubertal period.

Summary

Our previous and present researches have studied the role of kisspeptin on hypothalamic-pituitary-ovarian axis in female swamp buffalo reproduction. In pre-pubertal buffalo, we have done in hypothalamic level for kisspeptin and GnRH relationship for puberty development and puberty onset. Remarkably, we found the significantly difference of those results compare to the post-pubertal buffalo. The summary of our research from the Fig.20, the *Kiss1* mRNA has been expressed in juvenile to adult in both POA and ARC hypothalamic nuclei. However, kisspeptin protein has identified only in the cytoplasm of neurons in the POA during the juvenile with weak reaction. Then in the 1-2.5 years old as pre-pubertal age, the kisspeptin protein has presented only in the ARC area. But there is no any kisspeptin protein reaction in both areas in peri-pubertal age (2.5-3 years). When the buffalo passed thru puberty, the kisspeptin protein can be detected in both areas with intensive reaction. Interestingly, although the population of co-localized Kiss1r-ir in GnRH neurons in the pre-pubertal buffalo (50%) seems no different to the post-pubertal buffalo (80%) but the single or non-co-localization neuron in the pre- and post-pubertal buffalo is an interesting difference. The single GnRH neuron is a half population of all the GnRH-ir neurons in the pre-pubertal swamp buffaloes. This is opposite to the post-pubertal swamp buffaloes which the single Kiss1r-ir neuron is an only non-co-localized neuron population.

We provide evidence that kisspeptin- kisspeptin receptor signaling develops across puberty at the level of the GnRH neurons in the female buffaloes significantly, which is similar to other animals (Colledge et al., 2010; d'Anglemont de Tassigny and Colledge, 2010; Decourt et al., 2008; Dhillo et al., 2007; Han et al., 2005; Mayer et al., 2010; Mayer and Boehm, 2011). The previous reported have proved that the administration of exogeneous kisspeptin can induce the puberty onset in female mice, rats(d'Anglemont de Tassigny et al., 2008; Maguire et al., 2011), goats(Hashizume et al., 2010; Saito et al., 2012) and cattle (Whitlock et al., 2008). These results have suggested the knowledge for the kisspeptin-kisspeptin receptor signaling within the GnRH neuronal network may be a “gatekeeper” for onset of puberty (Maguire et al., 2011; Seminara et al., 2003). The mode of action, mechanism and other elements that effect on puberty onset and reproductive function in swamp buffalo still need to be explored.

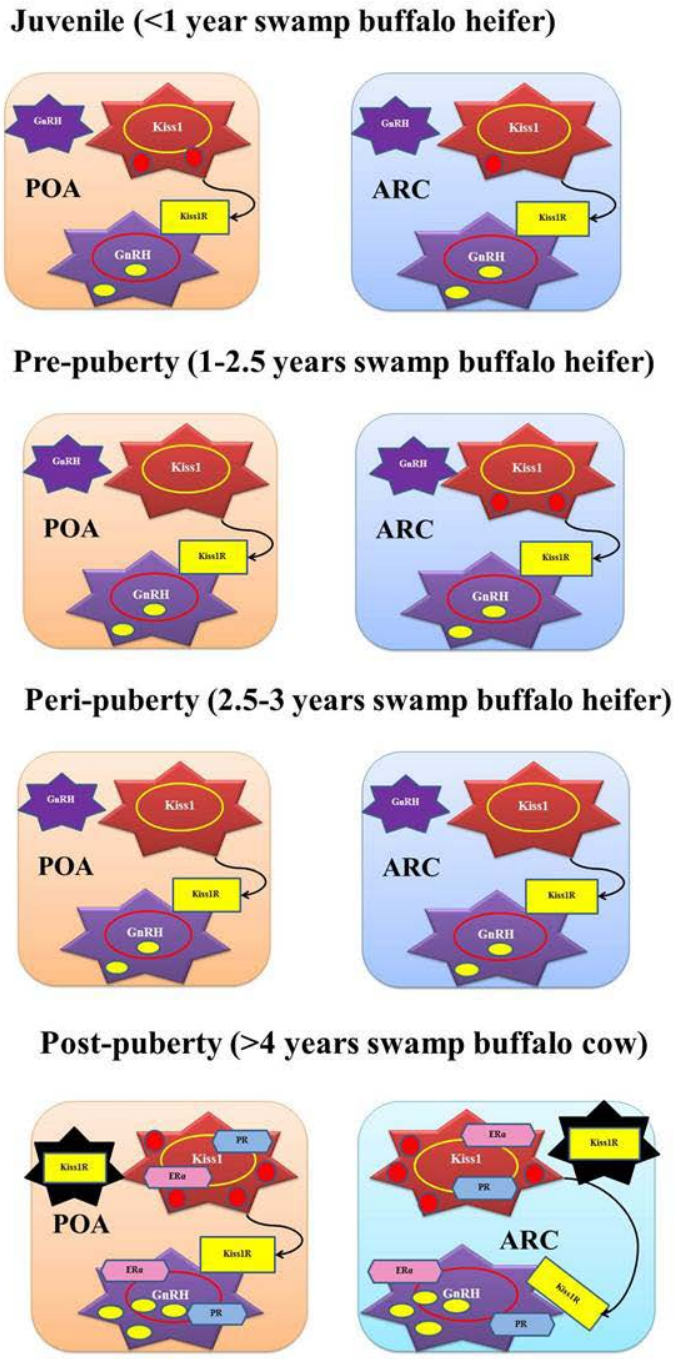

Figure 20 Comparison of localization and distribution of *Kiss1* m-RNA, kisspeptin protein, kisspeptin receptor (KISS1R) and GnRH expressions in the POA and ARC hypothalamic nuclei in difference age of pre- and post- pubertal female swamp buffaloes.

References

Adachi, S., Yamada, S., Takatsu, Y., Matsui, H., Kinoshita, M., Takase, K., Sugiura, H., Ohtaki, T., Matsumoto, H., Uenoyama, Y., Tsukamura, H., Inoue, K., Maeda, K.I. 2007. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rates. J Reprod dev. 53: 367-378.

Ali, A., A. K. Abdel-Razek, S. Abdel-Ghaffar and P. S. Glatzel. 2003. Ovarian follicular dynamics in buffalo cows (Bubalus bubalis). Reproduction in domestic animals = Zuchthygiene 38:214-218.

Butler, W. R., S. P. O'Connor, M. A. Yakrus and W. M. Gross. 1994. Cross-reactivity of genetic probe for detection of Mycobacterium tuberculosis with newly described species Mycobacterium celatum. Journal of clinical microbiology 32:536-538.

Caraty, A. Fabre-Nys, C., Delaleu, B., Locatelli, A., Bruneau, G., Karsch, F.J. 1998. Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe. Endocrinology. 139: 1752-1760.

Caraty, A., J. T. Smith, D. Lomet, S. Ben Said, A. Morrissey, J. Cognie, B. Doughton, G. Baril, C. Briant and I. J. Clarke. 2007. Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 148:5258-5267.

Chaikhun-Marcou, T., P. Sotthibandhu, V. Kyle, S. Hwa Yeo, W. Henry Colledge and S. Suadsong. 2016. Evidence of kisspeptin receptor expression in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes. The Thai Journal of Veterinary Medicine 46:10.

Chaikhun, T., R. Hengtrakunsin, F. de Rensis, M. Techakumphu and S. Suadsong. 2012. Reproductive and dairy performances of Thai swamp buffaloes under intensive farm management. Thai J Vet Med 42:81-85.

Chaikhun, T., C. Yanprapasiri, P. Sotthibandhu and S. Suadsong. 2016. Kiss-1 mRNA/kisspeptin distribution in preoptic and arcuate nuclei of cycling buffalo (Bubalus bubalis) hypothalamus. Pak Vet J 36:93-97.

Clarkson, J., Herbison, A.E. 2006. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology. 147: 5817-5825.

Clarkson, J., X. d'Anglemont de Tassigny, W. H. Colledge, A. Caraty and A. E. Herbison. 2009. Distribution of kisspeptin neurones in the adult female mouse brain. Journal of neuroendocrinology 21:673-682.

Colledge, W. H. 2008. GPR54 and kisspeptins. Results and problems in cell differentiation 46:117-143.

Colledge, W.H. 2008. Kisspeptins and GnRH neuronal signaling. Trends Endocrinol Metab. 20: 115-121.Decourt, C., Tillet, Y., Caraty, A., Franceschini, I., Briant, C. 2008. Kisspeptin immunoreactive neurons in the equine hypothalamus Interactions with GnRH neuronal system. J Chem Neuroanat. 36: 131-137.

Colledge, W. H., H. Mei and X. d'Anglemont de Tassigny. 2010. Mouse models to study the central regulation of puberty. Molecular and Cellular Endocrinology 324:12-20.

Cui, P., C. Yang, K. Zhang, X. Gao, L. Luo, Y. Tian, M. Song, Y. Liu, Y. Zhang, Y. Li, X. Zhang, S. Su, F. Fang and J. Ding. 2015. Effect of estrogen on the expression of GnRH and kisspeptin in the hypothalamus of rats during puberty. Theriogenology 84:1556-1564.

d'Anglemont de Tassigny, X. and W. H. Colledge. 2010. The role of kisspeptin signaling in reproduction. Physiology (Bethesda, Md.) 25:207-217.

d'Anglemont de Tassigny, X., L. A. Fagg, M. B. Carlton and W. H. Colledge. 2008. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 149:3926-3932.

Decourt, C., Y. Tillet, A. Caraty, I. Franceschini and C. Briant. 2008. Kisspeptin immunoreactive neurons in the equine hypothalamus: Interactions with GnRH neuronal system. Journal of Chemical Neuroanatomy 36:131-137.

De Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E. 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. U.S.A. 100: 10972-10976.

De Tassigny, X.A and Colledge, W.H. 2010. The role of kisspeptin signaling in reproduction. Physiology. 25: 207-217.

Dhillo, W. S., K. G. Murphy and S. R. Bloom. 2007. The neuroendocrine physiology of kisspeptin in the human. Reviews in endocrine & metabolic disorders 8:41-46.

Estrada, K. M., C. M. Clay, S. Pompolo, J. T. Smith and I. J. Clarke. 2006. Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. Journal of neuroendocrinology 18:806-809.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet and A. Caraty. 2006. Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. Neuroscience Letters 401:225-230.

Goodman, R. L., M. N. Lehman, J. T. Smith, L. M. Coolen, C. V. de Oliveira, M. R. Jafarzadehshirazi, A. Pereira, J. Iqbal, A. Caraty, P. Ciofi and I. J. Clarke. 2007. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752-5760.

Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F., Seminara, S., Clifton, D.K., Steiner, R.A. A role for kisspeptin in the regulation of gonadotropin secretion in the mouse. Endocrinology. 145: 4073-4077.

Han, S.K., Gottsch, M.L., Lee, K.J., Popa, S.M., Smith, J.T., Jakawich, S.K., Clifton, D.K., Steiner, R.A., Herbison, A.E. 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci. 25: 11349–11356.

Hashizume, T., H. Saito, T. Sawada, T. Yaegashi, A. A. Ezzat, K. Sawai and T. Yamashita. 2010. Characteristics of stimulation of gonadotropin secretion by kisspeptin-10 in female goats. Animal reproduction science 118:37-41.

Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M., Cunningham, M.J., Gottsch, M.L., Clifton, D.K., Steiner, R.A. 2004. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KISS-1 mRNA in male rat. Neuroendocrinology. 80: 264-272.

Kadokawa, H., S. Suzuki and T. Hashizume. 2008. Kisspeptin-10 stimulates the secretion of growth hormone and prolactin directly from cultured bovine anterior pituitary cells. Animal reproduction science 105:404-408.

Keen, K.L., Wegner, F.H., Bloom, S.R., Ghatei, M.A., Terasawa, E. 2008. An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. Endocrinology. 149: 4151-4157.

Lee, J.H., Miele, M.E., Hicks, D.J.,Phillips, K.K., Trent, J.M., Weissman, B.E., Welch, D.R. 1996. KiSS-1, a novel human malignant melanoma metastasis suppressor gene. Journal of the National Cancer Institute. 88: 1731-1737.

Liu, R.M., Lee, K., Herbison, A.E. 2008. Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/ calcium-dependent pathway regulating multiple ion channels. Endocrinology. 149: 4605-4614.

Liu, R.M., H. Li, D.M. Zhang, Y.F. Lv, X.Y. Lin and H.Q. Zhang. 2014. Expression of Kisspeptin/kiss1r System is Down-regulated in the Hypothalamic Arcuate Nucleus of Pubertal Male Rats with High-fat-diet. Journal of Reproduction and Contraception 25:1-11.

Manfredi-Lozano, M., J. Roa, F. Ruiz-Pino, R. Piet, D. Garcia-Galiano, R. Pineda, A. Zamora, S. Leon, M. A. Sanchez-Garrido, A. Romero-Ruiz, C. Dieguez, M. J. Vazquez, A. E. Herbison, L. Pinilla and M. Tena-Sempere. 2016. Defining a novel leptin–melanocortin–kisspeptin pathway involved in the metabolic control of puberty. Molecular Metabolism 5:844-857.

Maguire, J. J., H. R. Kirby, E. J. Mead, R. E. Kuc, X. d'Anglemont de Tassigny, W. H. Colledge and A. P. Davenport. 2011. Inotropic Action of the Puberty Hormone Kisspeptin in Rat, Mouse and

Human: Cardiovascular Distribution and Characteristics of the Kisspeptin Receptor. PloS one 6:e27601.

Mayer, C., M. Acosta-Martinez, S. L. Dubois, A. Wolfe, S. Radovick, U. Boehm and J. E. Levine. 2010. Timing and completion of puberty in female mice depend on estrogen receptor α-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences 107:22693-22698.

Mayer, C. and U. Boehm. 2011. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci 14:704-710.

Mead, E.J., Maguire, J.J., Kuc, R.E., Davenport, A.P. 2007. Kisspeptins are novel potent vasoconstrictors in humans, with a discrete localization of their receptor, G protein-coupled receptor 54, to atherosclerosis-prone vessels. Endocrinology. 148: 140–147.

Montiel, F. and C. Ahuja. 2005. Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. Animal reproduction science 85:1-26.

Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekeres, P.G., Sarau, H.M., Chambers, J.K., Murdock, P., Steplewski, K., Shabon, U., Millert, J.E., Middleton, S.E., Darker, J.G., Larminie, C.G.C., Wilson, S., Bergsma, D.J., Emson, P., Faull, R., Philpott, K., Harrison, D.C. 2001. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem. 276: 28969–28975.

Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., Fujino, M. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature. 411: 613–617.

Overgaard, A., M. Tena-Sempere, I. Franceschini, E. Desroziers, V. Simonneaux and J. D. Mikkelsen. 2013. Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. Peptides 45:85-90.

Poling, M. C. and A. S. Kauffman. 2013. Organizational and activational effects of sex steroids on kisspeptin neuron development. Frontiers in neuroendocrinology 34:3-17.

Pompolo, S., Pereira, A., Estrada, K.M., Clarke, I.J. 2006. Colocalization of kisspeptin and gonadotropin-releasing hormone in the ovine brain. Endocrinology. 147: 804-810.

Ramaswamy, S., Guerriero, K.A., Gibbs, R.B., Plant, T.M. 2008. Structural interactions between kisspeptin and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey (*Macaca mulatta*) as revealed by double immunofluorescence and confocal microscopy. Endocrinology. 149: 4387-4395.

Revel, F.G., Saboureau, M., Masson-Pevet, M., Pevet, P., Mikkelsen, J.D., Simonneaux, V. 2006. KISS-1: a likely candidate for the photoperiodic control of reproductive in seasonal breeders. Chronobiol Int. 23: 277-287.

Richard, N., Galmiche, G., Corvaisier, S., Caraty, A., Kottler, M.L. 2008. KiSS-1 and GPR54 genes are co-expressed in rat gonadotrophs and differentially regulated *in vivo* by oestradiol and gonadotrophin-releasing hormone. J neuroendocrinology. 20: 381-393.Rometo, A. M., S. J. Krajewski, M. L. Voytko and N. E. Rance. 2007. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. The Journal of clinical endocrinology and metabolism 92:2744-2750.

Rometo, A.M., Krajewski, S.J., Voytko, M.L., Rance, N.E. 2007. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibulur nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab. 92: 2744-2750.

Roseweir, A.K., Millar, R.P. 2009. The role of kisspeptin in the control of gonadotrophin secretion. Human Reproduction Update. 15:2, 203-212.

Saito, H., T. Sawada, T. Yaegashi, Y. Goto, J. Jin, K. Sawai and T. Hashizume. 2012. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. Animal science journal = Nihon chikusan Gakkaiho 83:487-492.

Seminara, S. B., S. Messager, E. E. Chatzidaki, R. R. Thresher, J. S. Acierno, Jr., J. K. Shagoury, Y. Bo-Abbas, W. Kuohung, K. M. Schwinof, A. G. Hendrick, D. Zahn, J. Dixon, U. B. Kaiser, S. A. Slaugenhaupt, J. F. Gusella, S. O'Rahilly, M. B. Carlton, W. F. Crowley, Jr., S. A. Aparicio and W. H. Colledge. 2003. The GPR54 gene as a regulator of puberty. The New England journal of medicine 349:1614-1627.

Smith, J.T., Acohido, Clifton, D.K., Steiner, R.A. 2006. KISS-1 neurons are direct targets for leptin in the ob/ob mouse. J Neuroendocrinol. 18: 298-303.

Smith, J.T., Dungan, H.M, Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., Steiner, R.A. 2005. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology. 146: 2976-2984.

Smith, J.T., Popa, S.M., Clifton, D.K., Hoffman, G.E., Steiner, R.A. 2006. Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. 26: 6687-6694.

Tomikawa, J., Homma, T., Tajima, S., Shibata, T., Inamoto, Y., Takase, K., Inoue, N., Ohkura, S., Uenoyama, Y., Maeda, K., Tsukamura, H. 2010. Molecular characterization and estrogen regulation of hypothalamic KISS gene in the pig. Biol Reprod. 82: 313-319.

Tsukamura, H. and Maeda, K. 2011. GnRH pulse generation and its adaptation to the manipulation of follicular development and ovulation. Thai J. Vet. Med. Suppl. 41: 69-72.

Whitlock, B. K., J. A. Daniel, R. R. Wilborn, S. P. Rodning, H. S. Maxwell, B. P. Steele and J. L. Sartin. 2008. Interaction of estrogen and progesterone on kisspeptin-10-stimulated luteinizing hormone and growth hormone in ovariectomized cows. Neuroendocrinology 88:212-215.

Wintermantel, T.M., Campbell, R.E., Porteous, R., Bock, D., Grone, H.J., Todman, M.G., Korach, K.S., Greiner, E., Perez, C.A., Schutz, G., Herbison, A.E. 2006. Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. Neuron. 52: 271-280.

Yang, H., I. B. Wanner, S. D. Roper and N. Chaudhari. 1999. An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 47:431-446.

Zerani, M., G. Catone, M. Maranesi, A. Gobbetti, C. Boiti and F. Parillo. 2012. Gonadotropin-releasing hormone 1 directly affects corpora lutea lifespan in Mediterranean buffalo (Bubalus bubalis) during diestrus: presence and in vitro effects on enzymatic and hormonal activities. Biology of reproduction 87:45.

การเผยแพร่ผลงานวิจัยที่เกี่ยวข้อง

1. Chaikhun-Marcou, T., P. Sotthibandhu, and S. Suadsong. 2014. *Kisspeptin and the hypothalamic pituitary gonadal axis in buffalo*. In: Proceeding of the 11th Annual Conference of the Asian Reproductive Biotechnology Society, Sukosol Hotel, Bangkok, Thailand; November 2-8, 2014. Page 19. (Oral presentation)

2. Polnok, S., Wannapake, K., Boonnual, S., Kawijai, P., Chaimongkol, K., Hanrin, W., Srinak, W., Hattapanit, P., Sotthibandhu, P., Suadsong, S., and Chaikhun-Marcou, T. 2014. *Study of the optimal conditions for estrogen receptors alpha and progesterone receptors detection in buffalo uterus (Bubalusbubalis) by immunohistochemistry technique.* In: Proceeding of the 8thMahanakorn Veterinary Conference on November 14, 2014. Bangkok, Thailand. Page 8-10. (Oral presentation).

3. T. Chaikhun-Marcou, P. Sotthibandhu, C. Yanprapasiri, S.Suadsong, 2015. Localization of Kisspeptin Receptors in Buffalo Heifer Pituitary Glands**.** In proceeding ofThe 14th Chulalongkorn University Veterinary Conference (CUVC2015): Responsible for Lives. Royal Paragon Hall, Siam Paragon, Bangkok, Thailand. 20-22 April, 2015. 213. (Poster presentation).

4. Hanrin, W., Hattapanit, P., Srinak, W., Yanprapasiri, C., Sotthibandhu, P., Suadsong, S. and Chaikhun-Marcou, T. 2015. Immunofluorescence Colocalization Study of Estrogen Receptors Alpha and Progesterone Receptors in GnRH Neurons in Preoptic Area and Arcuate Hypothalamic Nuclei in Buffalo Cows. In: Proceeding of ICVS 2015 on November 11-13, 2015. Impact Forum, Nonthaburi, Thailand. On CD proceeding. (Oral presentation).

5. Chaikhun-Marcou, T.,Yanprapasiri, C., Samran, W.,Sotthibandhu, P. and Suadsong, S. 2015. *Character of Kisspeptin Receptors Immunoreactions in Pituitary Glands of Cycling Buffaloes (Bubalusbubalis).* In: Proceeding of 5th SAADC 2015 on October 27-30, 2015. Dusit Thani Pattaya Hotel, Thailand. Page 132. (Oral presentation).

6. T. Chaikhun-Marcou, P. Sotthibandhu, C. Yanprapasiri and S. Suadsong. 2016. Understanding of Puberty in Buffalo: The Relation of Age and Kisspeptin Signaling Expression in Pre- and Postpubertal Hypothalamic Nuclei in Swamp Buffalo. In: Proceeding of TOAC 2016 on January 4-6, 2016. Chaum Regent Beach Resort, Petchaburi, Thailand.

7. T. Chaikhun-Marcou, P. Sotthibandhu, C. Yanprapasiri, S. Pavasutthipaisit and S.Suadsong. *Differences in Kisspeptin Localization in the Pre- and Post- Pubertal Buffalo's Preoptic Area and Arcuate* (CUVC2016): Research in Practice. Chamchuree 10 Building, Bangkok, Thailand. 20-22 April, 2016. Thai J Vet Med Suppl. 2016, 46 : 421-422. (Poster presentation).

8. T. Chaikhun-Marcou, P. Sotthibandhu, C. Yanprapasiri, S. Pavasutthipaisit and S.Suadsong**.** Colocalization of Estrogen Receptor AlphainKisspeptin Neuronsin the Pre- Pubertal Buffalo's Preoptic Area and ArcuateHypothalamic Nuclei. In proceeding of The 16th Chulalongkorn University Veterinary Conference (CUVC2017): Research in Practice. Queen Sirikit National Convention Center, Bangkok, Thailand. 22-24 March, 2017. Thai J Vet Med Suppl. 2017, 47 : 233-234. (Poster presentation).

งานวิจัยตีพิมพ์ที่เกี่ยวข้อง

1. Chaikhun, T.,Sotthibandhu, P. and Suadsong, S. 2013. The Role of Kisspeptin Signaling in Reproduction of Ruminants.Thai J Vet Med. 42(1): 81-85.

2. Chaikhun, T., Sotthibandhu, P. and Suadsong, S. 2013. Localization of GnRH Receptors in Buffalo Cow Pituitary Gland in Follicular and Luteal Phases. Buffalo Bulettin. 32 (Special issue2): 468-472.

3. Chaikhun- Marcou, T.,Sotthibandhu, P. and Suadsong, S. 2014. Evidence of Sex Steroid Hormone Receptors in the Preoptic Area and Arcuate Hypothalamic Nuclei in Cycling Buffaloes. Thai J Vet Med. 44 (suppl 1): 199-200.

4. Chaikhun, T., Yanprapasiri, C., Sotthibandhu, P. and Suadsong, S. 2016. Kiss-1 mRNA/Kisspeptin Distribution in Preoptic and Arcuate Nuclei of Cycling Buffalo *(Bubalus bubalis)* Hypothalamus. Pakistan Vet J. 36(1): 93-97.

5. Chaikhun-Marcou, T., Sotthibandhu, P., Kyle, V., Yeo, S.H., Colledge, W.H., Suadsong, S. 2016. Evidence of Kisspeptin Receptor Expression in GnRH Neurons in the Preoptic Area and Arcuate Hypothalamic Nuclei in Cycling Buffaloes.Thai J Vet Med. 46(3): 381-390.

งานวิจัยที่คาดว่าจะตีพิมพ์ในวารสารนานาชาติต่อไป

1. *Kiss1* mRNA and Its Protein Distribution in Preoptic and Arcuate Hypothalamic Nuclei in Pre-pubertal Female Swamp. (Manuscript1)

2. Characteristics of Kisspeptin Receptor Co-localized in GnRH Neurons in the Preoptic Area and Arcuate Hypothalamic Nuclei in Buffalo Heifers (*Bubalus bubalis*). (Manuscript2)

ภาคผนวก

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

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STATEMENT OF NOVELTY

Previous studies have been done on kisspeptin and reproductive function in post-pubertal swamp buffalo in relation to the hypothalamic- pituitary- gonadal axis. The present study in the pre-pubertal swamp buffaloes found evidence of kisspeptin localization and distribution in the preoptic area (POA) and arcuate nucleus (ARC) hypothalamic nuclei similar to post-pubertal buffaloes in general. *Kiss1* mRNA signals and kisspeptin proteins were also detected in the cytoplasm of neuronal soma and some small neuronal cells in every age range. Interestingly, the kisspeptin immunoreactive neuron population in buffalo of different ages varied and the intensity of reactions was lower (weak) compared to the post-pubertal buffalo sample. A high percentage of kisspeptin immunoreactive neurons were found in the POA area in the juvenile group (<1 year), but the ARC area was the only area that expressed kisspeptin immunoreactions in the pre-pubertal group (1-2.5 years). Surprisingly, there was no kisspeptin immunoreaction in any hypothalamic nuclei in the peri-pubertal group (2.5-3 years). This distribution pattern is atypical and different from the post-pubertal buffalo previously studied.

ABSTRACT

The relationship between kisspeptin and GnRH releasing in many species has been studied including post-pubertal buffalo, but not pre-pubertal buffalo. The aims of this study were to detect the localization of *Kiss-1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of pre-pubertal swamp buffaloes. Brains were collected from 10 pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5 -3 years; 2 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 10 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
Keywords: swamp buffalo, pre-puberty, distribution, *Kiss1*, kisspeptin, hypothalamus

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' findings suggest that kisspeptin is a key controller of reproductive function in post-pubertal swamp buffalo in relation to the hypothalamic- pituitary- gonadal axis (Chaikhun et al., 2013; Chaikhun-Marcou et al., 2014a; Chaikhun-Marcou et al., 2014b; Chaikhun-Marcou et al., 2016; Chaikhun et al., 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 MEDTHODS

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 10 pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5 -3 years; 2 animals each age) from slaughterhouses (Fig.1). 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 (RT). 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 *Kiss-1* 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 counter- stain 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 cow 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 *Kiss-1* mRNA using an antisense *Kiss-1*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. *Kiss-1*mRNA was also found in some small neuronal cells (Fig. 1E, 2E) which were distinguished from glia cells by their vesicular nuclei. Interestingly, there is evidence of a synapsis between 2 kisspeptin neurons (Fig.3A3) There was no signal of *Kiss-1* mRNA in the buffalo POA and ARC sections in which the sense *Kiss-1*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 10 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 a 6 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 year old age 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 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 to 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.

CONCLUSION

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 .

Authors' contributions

Authors declare the contribution that all authors prepared the document for the study funding and designed the experiment. PS, CY, SP and TC performed the experiment and PS and TC analyzed the results. TC prepared the manuscript. All authors reviewed, revised the manuscript for intellectual contents and approved the final version critically.

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References

Caraty A, Smith JT, Lomet D, et al., 2007. Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. Endocrinology 148(11): 5258-5267.

Chaikhun-Marcou T, Sotthibandhu P, Kyle V, et al., 2016. Evidence of kisspeptin receptor expression in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes. The Thai Journal of Veterinary Medicine 46(3): 10.

Chaikhun-Marcou T, Sotthibandhu P andSuadsong S, 2014a. Evidence of sex steroid hormone receptors in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes. Proc "The 2nd Symposium of the Thai Society for Animal Reproduction", Demonstration Room, 60th year Building, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, pp:199-200.

Chaikhun-Marcou T, Sotthibandhu P, Suthikrai W, et al., 2014b. Comparative effects of administration of kisspeptin-10 and GnRH on LH secretion in buffalo cows. Proc "World Congress on Reproductive Biology 2014", Edinburgh, Scotland, pp:(online) https://www.google.co.th/?gws_rd=cr,ssl&ei=4ofEVtruI9CZuQSyw4L4Bw#q=kisspeptin+in+buffalo.

Chaikhun T, Hengtrakunsin R, de Rensis F, et al., 2012. Reproductive and dairy performances of Thai swamp buffaloes under intensive farm management. Thai J Vet Med 42: 81-85.

Chaikhun T, Sotthibandhu P andSuadsong S, 2013. Localization of GnRH receptors in buffalo cow pituitary gland in follicular and luteal phases. Buffalo Bulletin 32 (Special issue): 468-472.

Chaikhun T, Yanprapasiri C, Sotthibandhu P, et al., 2016. Kiss-1 mRNA/kisspeptin distribution in preoptic and arcuate nuclei of cycling buffalo (Bubalus bubalis) hypothalamus. Pak Vet J 36(1): 93-97.

Colledge WH, 2008. GPR54 and kisspeptins. Results Probl Cell Differ 46: 117-143.

Cui P, Yang C, Zhang K, et al., 2015. Effect of estrogen on the expression of GnRH and kisspeptin in the hypothalamus of rats during puberty. Theriogenology 84(9): 1556-1564.

de Roux N, Genin E, Carel JC, et al., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci U S A 100(19): 10972-10976.

Estrada KM, Clay CM, Pompolo S, et al., 2006. Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback. J Neuroendocrinol 18(10): 806-809.

Goodman RL, Lehman MN, Smith JT, et al., 2007. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148(12): 5752-5760.

Han SK, Gottsch ML, Lee KJ, et al., 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25(49): 11349-11356.

Kadokawa H, Matsui M, Hayashi K, et al., 2008. Peripheral administration of kisspeptin-10 increases plasma concentrations of GH as well as LH in prepubertal Holstein heifers. J Endocrinol 196(2): 331-334.

Liu R-r, Li H, Zhang D-m, et al., 2014. Expression of Kisspeptin/kiss1r System is Down-regulated in the Hypothalamic Arcuate Nucleus of Pubertal Male Rats with High-fat-diet. Journal of Reproduction and Contraception 25(1): 1-11.

Manfredi-Lozano M, Roa J, Ruiz-Pino F, et al., 2016. Defining a novel leptin–melanocortin–kisspeptin pathway involved in the metabolic control of puberty. Molecular Metabolism 5(10): 844-857.

Mayer C, Acosta-Martinez M, Dubois SL, et al., 2010. Timing and completion of puberty in female mice depend on estrogen receptor α-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences 107(52): 22693-22698.

Mayer C and Boehm U, 2011. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci 14(6): 704-710.

Overgaard A, Tena-Sempere M, Franceschini I, et al., 2013. Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. Peptides 45: 85-90.

Poling MC and Kauffman AS, 2013. Organizational and activational effects of sex steroids on kisspeptin neuron development. Front Neuroendocrinol 34(1): 3-17.

Rometo AM, Krajewski SJ, Voytko ML, et al., 2007. Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. J Clin Endocrinol Metab 92(7): 2744-2750.

Roseweir AK and Millar RP, 2009. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 15(2): 203-212.

Saito H, Sawada T, Yaegashi T, et al., 2012. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. Anim Sci J 83(6): 487-492.

Seminara SB, Messager S, Chatzidaki EE, et al., 2003. The GPR54 gene as a regulator of puberty. N Engl J Med 349(17): 1614-1627.

Smith MS, True C andGrove KL, 2010. The neuroendocrine basis of lactation-induced suppression of GnRH: role of kisspeptin and leptin. Brain Res 1364: 139-152.

Sotthibandhu P. 2009. Morphology studies of mammalian taste chemosensory cells. Thesis for PhD degree, Laboratory of Veterinary Anatomy, Kitasato University, Japan.

Tsukamura H and Maeda K, 2011. GnRH pulse generation and its adaptation to the manipulation of follicular development and ovulation. Thai J Vet Med Suppl 41: 69-72.

Whitlock BK, Daniel JA, Wilborn RR, et al., 2008. Interaction of estrogen and progesterone on kisspeptin-10-stimulated luteinizing hormone and growth hormone in ovariectomized cows. Neuroendocrinology 88(3): 212-215.

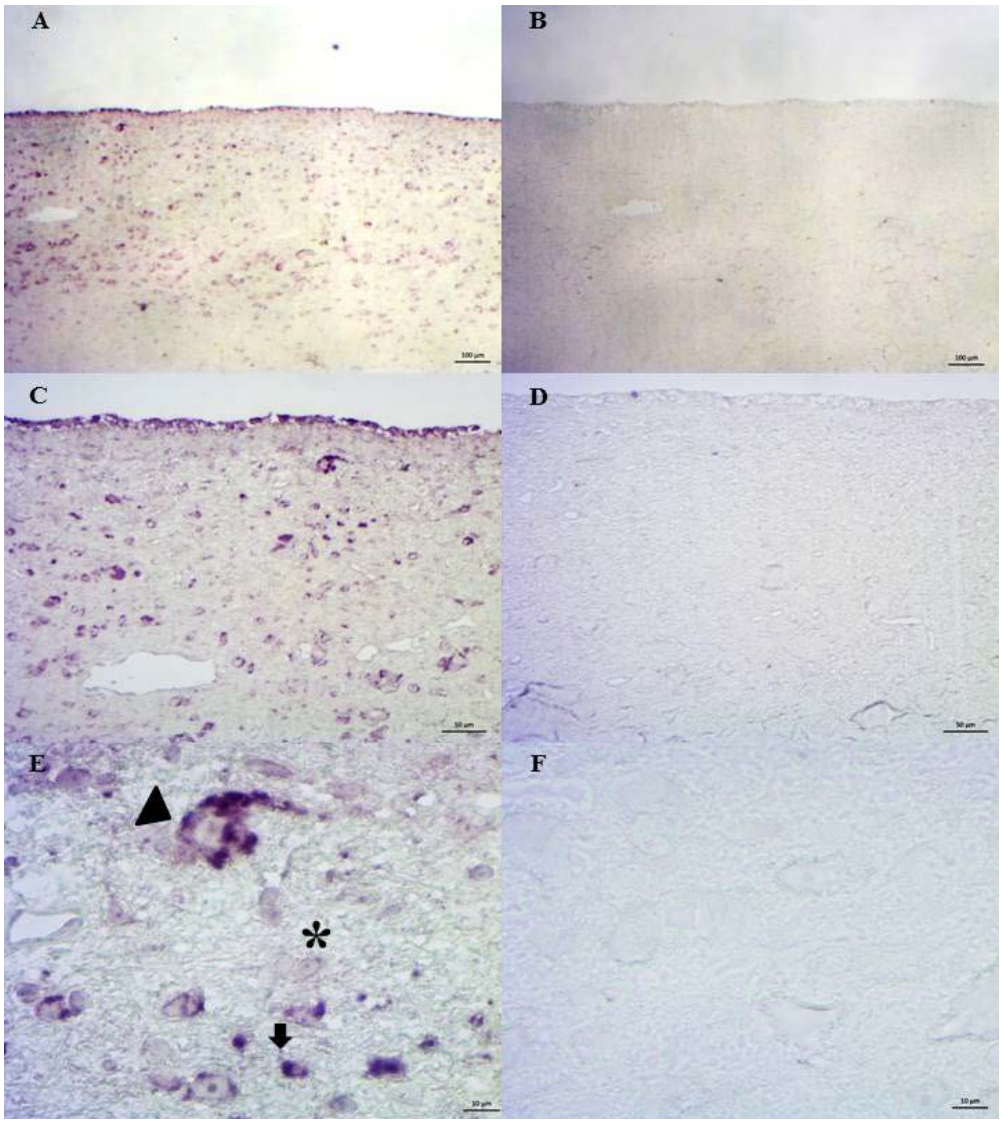

Fig. 1: *Kiss1* mRNA in the POA hypothalamic nucleus of buffalo, is visible in the anti-sense (positive result) of *Kiss1* mRNA in samples A, C and E, and is not expressed in the sense (negative result) of *Kiss1* mRNA in samples B, D and F. *Kiss1* mRNA expressions are localized in the cytoplasm of 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.

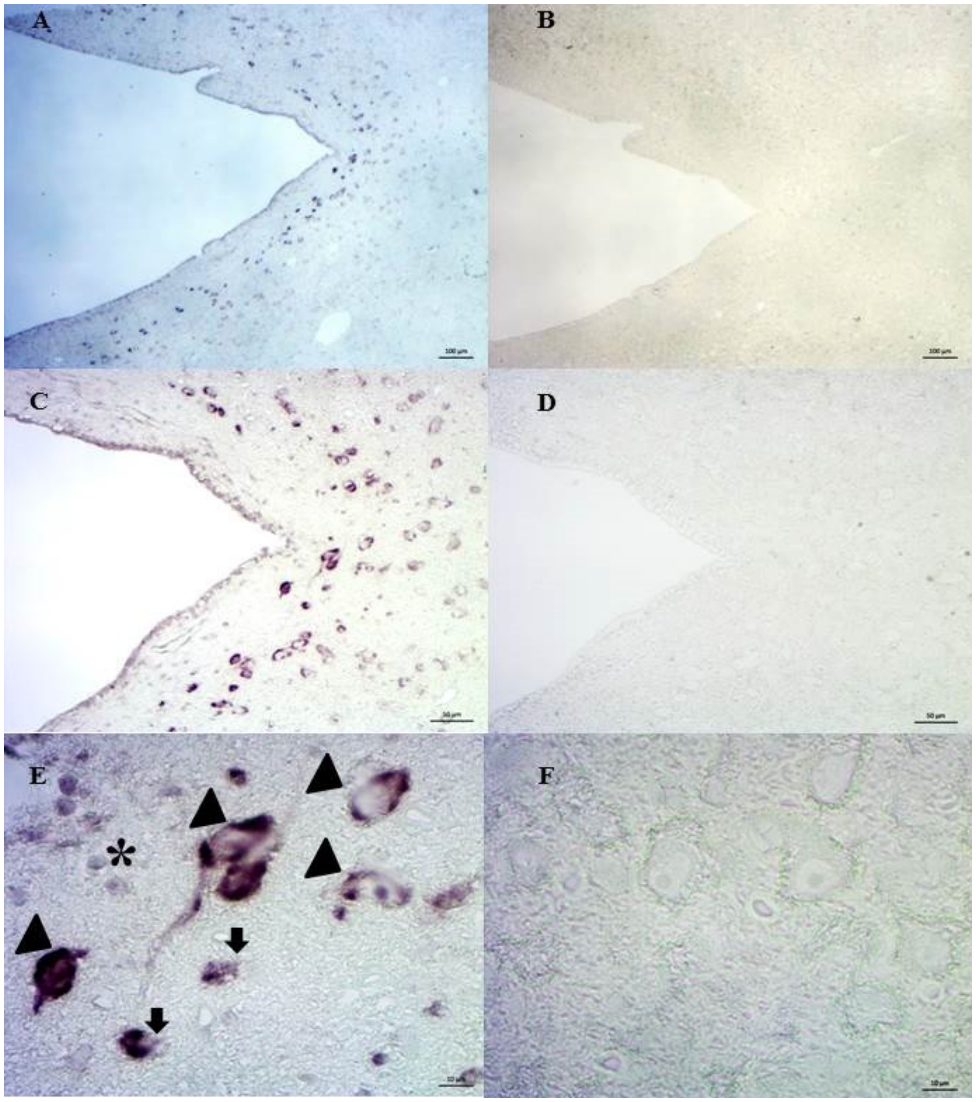

Fig. 2: In the ARC hypothalamic nucleus of buffalo, *Kiss1* mRNA is visible in the anti-sense (positive result) of *Kiss1* mRNA in samples A, C and E. It is not expressed in the sense (negative result) of *Kiss1* mRNA in samples B, D and F. *Kiss1* mRNA is localized in the cytoplasm of a neuron with a strong signal (arrow heads) but no signal in other small neurons (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.

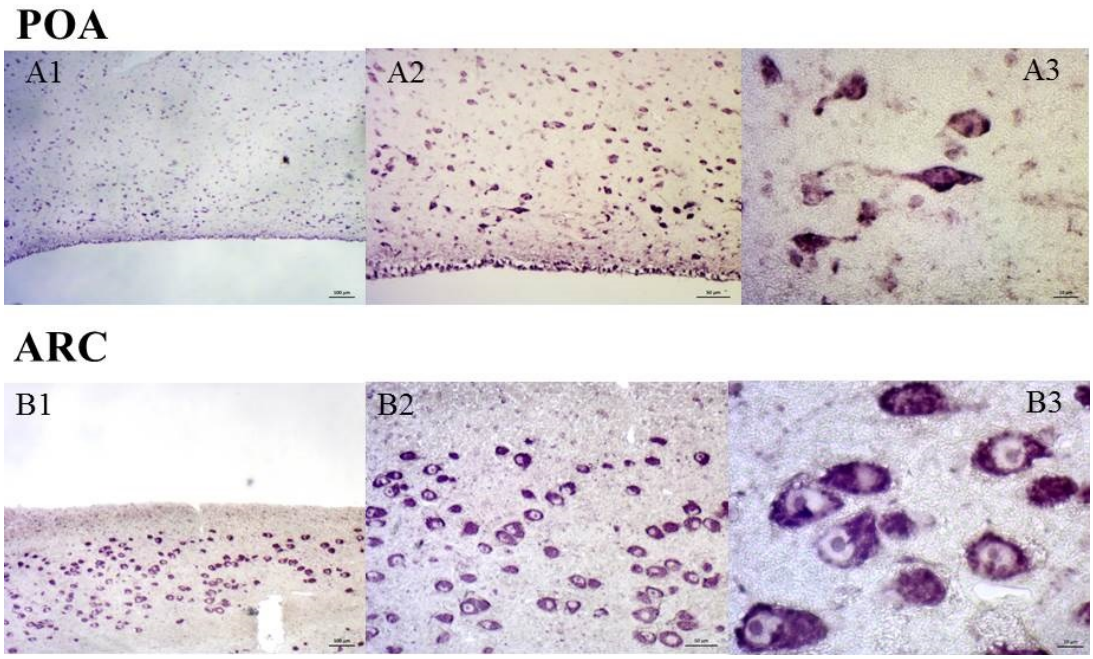

Fig. 3: The *Kiss1* mRNA neuron shows scattered distribution in POA area (A1 and A2), compared to the ARC area which shows a more tightly clustered distribution pattern (B1 and B2). There is evidence of a synapsis between 2 kisspeptin neurons in A3. Scale bar is 100 µm in A1 and B1, 50 µm in A2 and B2 but it is 10 µm in A3 and B3.

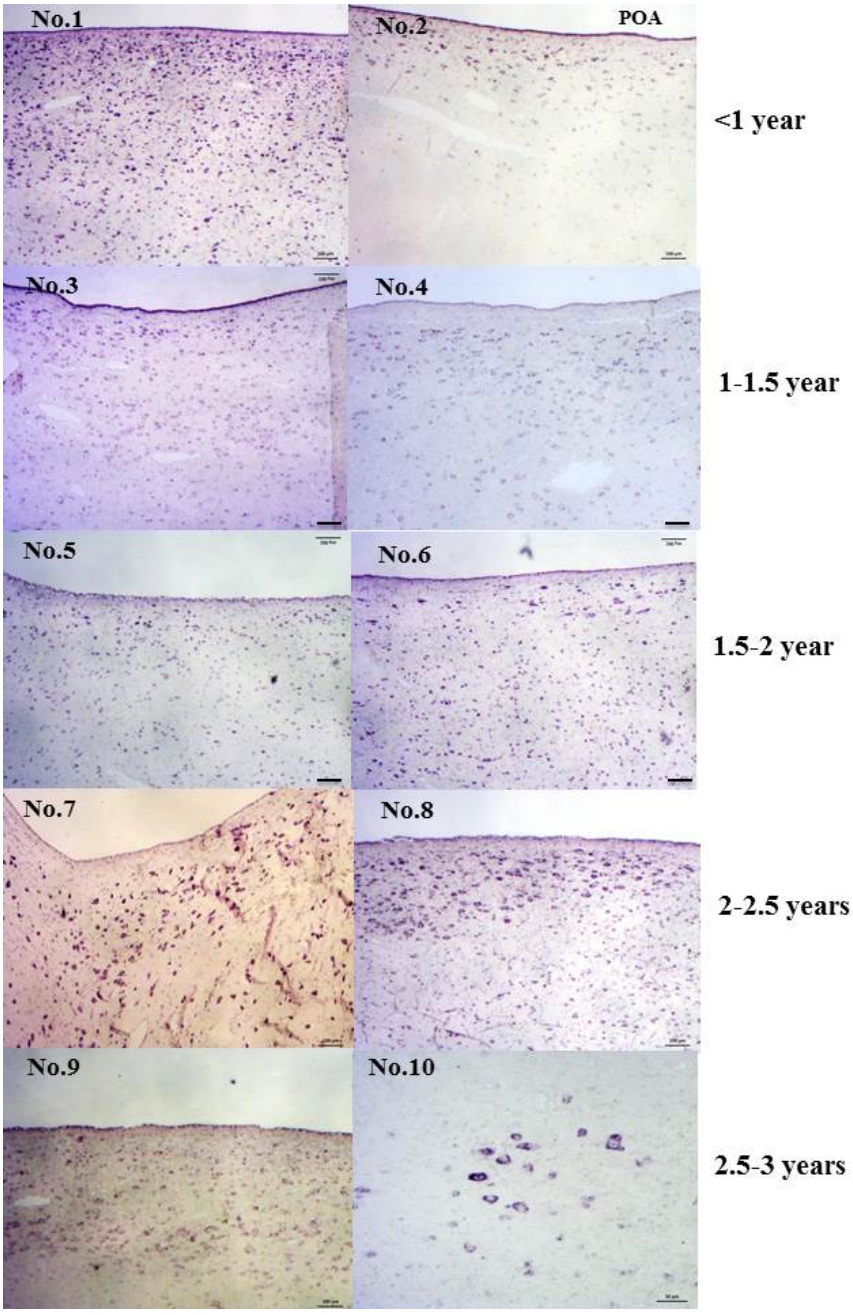

Fig. 4: In POA hypothalamic nuclei, *Kiss1* mRNA expressions are presented in samples from pre-pubertal buffaloes of different ages. There are intense reactions visible in ependymal cells and neuronal cells. Scale bar is 100 µm (No.1-9) but it is 50 µm in No.10.

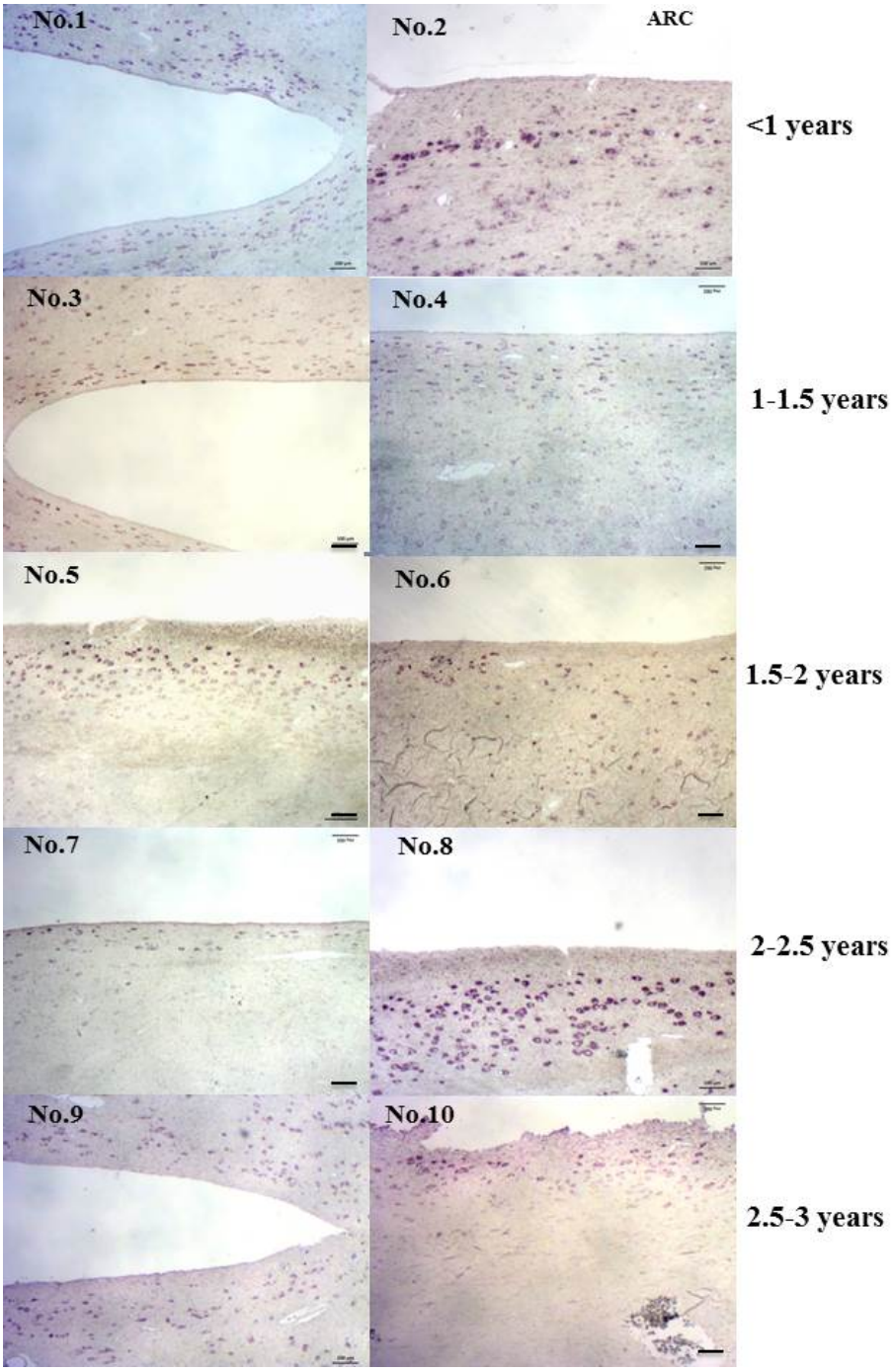

Fig. 5: In ARC hypothalamic nuclei, *Kiss1* mRNA expressions are presented in samples from pre-pubertal buffaloes of different ages. There are intense reactions visible in neuronal cells near the 3rd ventricle. Scale bar is 100 µm.

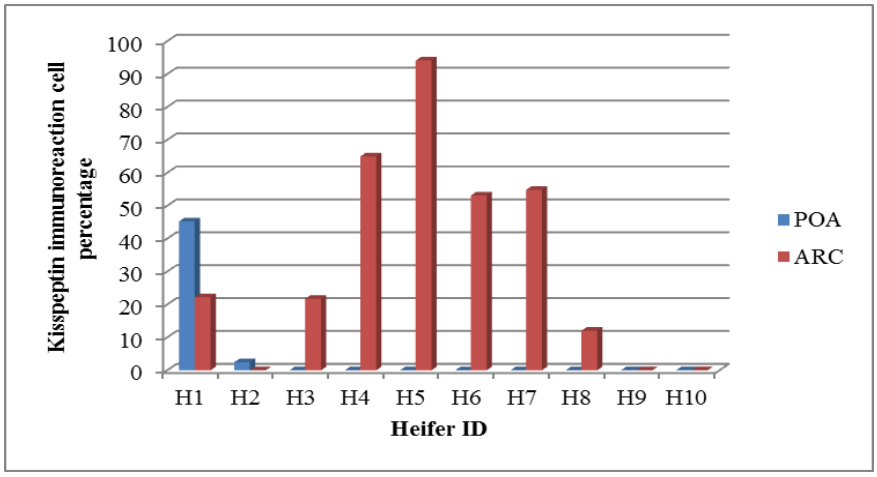

Fig. 6: The percentage of kisspeptin reactive neurons in the POA (blue) and the ARC (red) hypothalamic areas from 10 pre-pubertal female swamp buffalo of different ages.

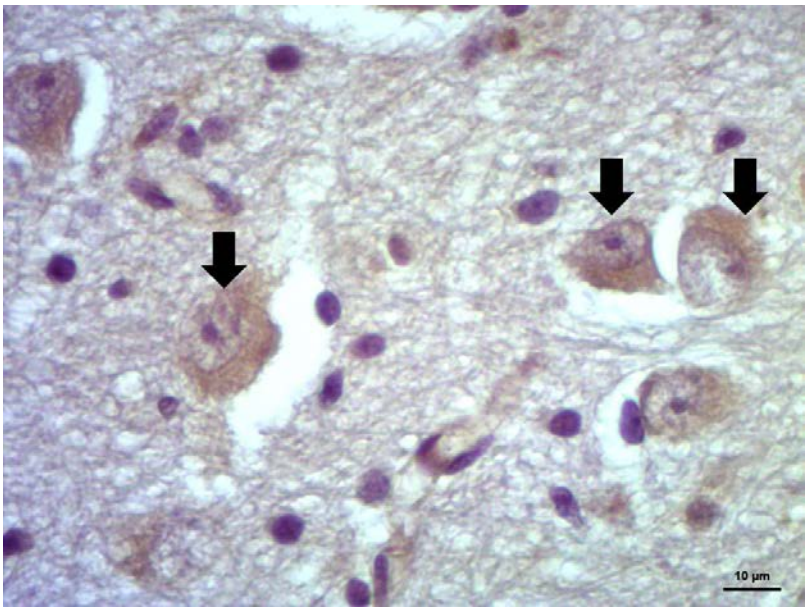

Fig. 7: Kisspeptin reactive proteins are visible in the cytoplasm of neurons with weak intensity (arrow) in the ARC of a 1-2.5 years old pre-pubertal female swamp buffalo. Scale bar 10 µm.

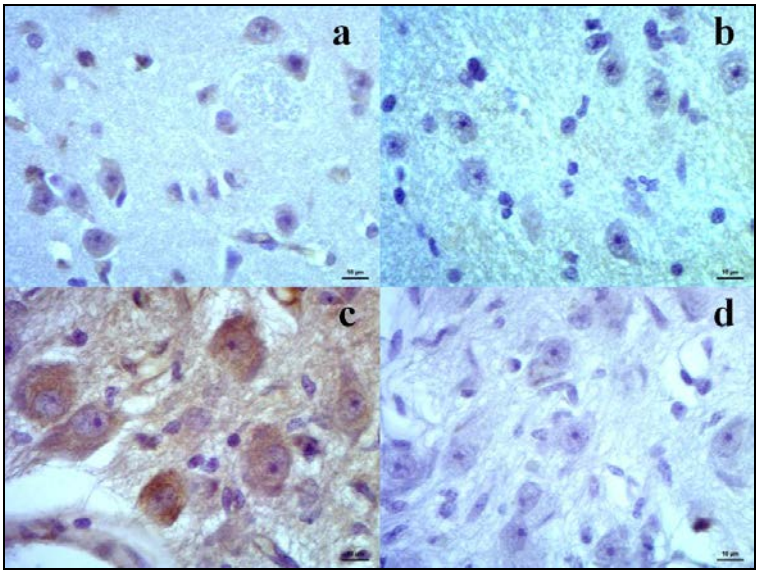

Fig. 8: The positive (left) and negative controls (right) for immunohistochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in a ewe (a, b) and a buffalo cow (c, d). Scale bar 10 µm.

Original full length article

Characteristics of Kisspeptin Receptor Co-localized in GnRH Neurons in the Preoptic Area and Arcuate Hypothalamic Nuclei in Buffalo Heifers (*Bubalus bubalis*)

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STATEMENT OF NOVELTY

Few studies have been done on kisspeptin and reproductive function in relation to the hypothalamic- pituitary- gonadal axis in post-pubertal buffalo. The present study found structural interactions between kisspeptin receptors and gonadotropin releasing hormone (GnRH) neurons as revealed by double immunofluorescent (□ 50% of the total neurons) in both preoptic area (POA) and arcuate nucleus (ARC) hypothalamic nuclei. Also, single GnRH neurons appeared as granular formations in the cytoplasm of neuronal soma and were distributed in both the POA and ARC hypothalamic nuclei. This suggests that kisspeptin might be involved in GnRH activation during the pre-pubertal period in swamp buffalo heifers.

ABSTRACT

Many studies in mammals indicate that kisspeptin and GPR54 (kisspeptin receptor or KISSR) are key regulators of puberty. Our previous study suggested that kisspeptin plays a significant role in GnRH releasing in post-pubertal buffalo reproductive functions but no studies on this in the puberty phase of their development have been done . The present study was designed to investigate the characteristics of KISS1R immunoreactions (ir) in the GnRH neurons in the POA and ARC hypothalamic nuclei in 5 different ages (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5 -3 years; 2 animals each age) of 10 buffalo heifers by double-labeling immunohistochemistry. In both the POA and ARC areas, KISS1R immunoreactivity (ir) was detected mainly in the cytoplasm, although some was also found in the nucleus of neuronal soma. The GnRH-ir appeared as granular formations in the cytoplasm of neuronal soma. The co-localized KISS1R-ir and GnRH-ir neuron population in the POA (52.58±17.71%) was similar to that found in the ARC (58.56±13.79%) (P>0.05). The single GnRH-ir neuron population in the POA (37.77±19.69%) was also the same as in the ARC (36.32±11.88%). Double labeling showed that all observed GnRH-ir neurons were co-localized with KISS1R-ir. These findings present evidence of kisspeptin receptors in the GnRH neurons in buffalo POA and ARC areas and suggests that kisspeptin has a functional role in GnRH release in pre-pubertal swamp buffaloes.

Keywords: swamp buffalo, heifer, gonadotropin releasing hormone, hypothalamus, kisspeptin receptor

INTRODUCTION

Buffaloes (*Bubalus bubalis*), are common domestic animals used for meat and milk supply. Nevertheless, this species has many unique reproductive limitations. Delays in puberty (and the subsequent delay in the age of first conception) can cause infertility and represent a major source of economic loss in buffalo, leading to low reproductive performance and a lengthening of their non-productive life (Chaikhun et al., 2010; Chaikhun et al., 2012). Many studies in mammals indicate that kisspeptin and GPR54 are key regulators of puberty due to the programmed increase of *Kiss1* mRNA and *GPR54* mRNA, which have been observed in the the POA, and the ARC areas and which can, in turn, cause an increase in GPR54 sensitivity to kisspeptin. Activation of the kisspeptin system facilitates increased pulsatile and surge modes of GnRH from GnRH neurons. GnRH then awakens the reproductive axis and brings about pubertal maturation (via hypophyseal portal circulation) to stimulate the production and release of gonadotropins such as LH and FSH (Kadokawa et al., 2008; Roseweir and Millar, 2009). GnRH releasing is regulated by gonadal steroid feedback action (Tsukamura and Maeda, 2011). Our previous study indicates that kisspeptin might interact with the buffalo HPO axis by activating GnRH neurons via its receptors (KISS1R and GPR54) in combination with sex steroid hormone interactions in the POA and ARC hypothalamic nuclei of cycling buffalo cows (Chaikhun et al., 2013; Chaikhun-Marcou et al., 2014a; Chaikhun-Marcou et al., 2014b; Chaikhun-Marcou et al., 2016; Chaikhun et al., 2016). Kisspeptin's role in buffalo puberty development, however, and the mechanism behind this, is not completely understood. The objective of this study was to investigate the characteristics of kisspeptin receptors co-localized in GnRH neurons in the POA and in the ARC hypothalamic nuclei of pre-pubertal swamp buffaloes.

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).

Samples

Brains were collected from 10 pre-pubertal female buffaloes (<1 year, 1-1.5 years, 1.5 -2 years, 2-2.5 years and 2.5 -3 years; 2 animals each age). 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 which was detected by postmortem ovarian morphology and plasma progesterone analysis (<1 ng/ml). After removing the brains, the hypothalami were collected and fixed in 4% paraformaldehyde for 24 hr. The samples were embedded in paraffin blocks and stored at room temperature (RT).

Double label immunohistochemistry for KISS1R and GnRH.

Paraffin sections of the POA and ARC hypothalamic nuclei were prepared at 4 microns and were processed by the standard double label- immunohistochemical method as in the previous study (Chaikhun-Marcou et al., 2016). Briefly, antigen retrieval in a citrate buffer (pH 6.0) was done for 10 min at 121 °c. Non-specific binding was blocked using 1% normal goat serum (Gibco, NY, USA) for 20 min at RT. The sections were incubated with a primary rabbit anti-human KiSS1R/GPR54 polyclonal antibody (1:100 dilution) (Bioss, catalog number bs-2501R, MA, USA) overnight (16 hr) at 4 °C. A secondary goat anti-rabbit IgG (H+L) antibody (1:500 dilution) (Alexa fluor 488, catalog number A-11008, Life Technologies, CA, USA) was applied to the sections at RT for 2 hr. A mouse anti-mammalian GnRH monoclonal antibody (1:100 dilution) (Chemicon, catalog number MAB5456, CA, USA) was applied to the sections overnight (16 hr) at 4°C. A 1:100 dilution of secondary goat

anti-mouse IgG (H+L) (Alexa fluor 568, catalog number A-11004, Life Technologies , CA, USA) was applied to the slides and kept at RT for 2 hr then washed with PBS. The double labeled immunoreactions were observed under a fluorescent microscope (Axiolab, Zeiss, Oberkochen, Germany). A single observer counted the number of KISS1R-ir cells, GnRH-ir cells and co-localized-ir cells found in 100 mm^2 area slides from each of the buffalo cow's POA and ARC hypothalamic nuclei. The images were captured by Axivision software (Axiolab, Zeiss, Oberkochen, Germany). The layers of images were combined in Adobe Photoshop.

Controls and specificity

Antibody validation and cross-reactivity studies of the rabbit anti-human KiSS1R/GPR54 polyclonal antibody used in this study have been carried out in humans, rats and mice by the source company (Bioss, MA, USA). Positive controls for antibody and tissue specificity were prepared using brain sections from wild-type mice and buffalo cow samples. Negative controls for antibody specificity were performed using brain sections from *Gpr54* gene knockout (KO/*Gpr54*) mice and also by omitting the primary antibody. Negative controls for tissue specificity were performed using the white matter area of the central nervous system of buffalo and mice - an area known to have no KISS1R expression.

The mouse anti- mammalian GnRH monoclonal antibody has been used in buffaloes previously (Zerani et al., 2012; Chaikhun et al., 2016). Positive controls for antibody and tissue specificity were prepared using ewe POA and ARC hypothalamic nuclei paraffin sections. Negative controls for antibody specificity were conducted using PBS in combination with 1% normal goat serum. Negative controls for tissue specificity were the white matter area of the central nervous system of eve and buffalo - which are known to have no GnRH expression.

Statistical analysis

The number of each type of immunoreactive cells with co-localization or non co-localization were calculated as a percentage of the total number and then were averaged across animals to calculate a mean (± SE). The comparison of the average number of each type of immunoreactive cells (with co-localization or non-co-localization) between the POA and ARC were analyzed by a t-test ($P<0.05$). Characterizations of co-expression and non co-expression were described.

RESULTS

Immunoreactions for GnRH-ir were observed in the cytoplasm of neuronal soma and appeared as granular formations in some neurons. The KISS1R-ir were located mainly in cytoplasm although some were also found in the nucleus of neurons. Double label results showed that all observed KISS1R-ir were co-localized with GnRH-ir neurons (Fig.1). The population of KISS1R-ir co-localized in GnRH neurons in each hypothalamic nuclei of all 10 pre-pubertal buffaloes is shown in Fig. 2. The co-localized neuron population in the POA (52.58±17.71%) was not significantly different from the ARC (58.56±13.79%) in all age groups tested ($P>0.05$). The single GnRH-ir neuron populations in the POA (37.77±19.69%) were also similar to those in the ARC (36.32±11.88%) ($P>0.05$). Some neurons (non-ir neurons) in both the POA (8.65±5.34%) and the ARC (5.12±2.58%) areas ($P>0.05$) had, however, no KISS1R or GnRH reactions at all.

DISCUSSION

Our present study found more than 50% of KISS1R immunoreations were co-localized with GnRH neuron populations in both the POA and ARC hypothalamic nuclei of the pre-pubertal female buffaloes tested. This suggests that kisspeptin may be involved in regulating GnRH functions in juvenile and pre-pubertal buffaloes. In a previous study of post

– pubertal buffaloes (Chaikhun-Marcou et al., 2016) KISS1R immunoreactions were co-localized with the GnRH neuron population in more than 80% of the samples in both hypothalamic areas. These results imply that kisspeptin and kisspeptin receptor signaling could be involved in female buffalo reproduction and functions via GnRH controlling in both their pre- and post- pubertal phases of development. Similarly, a study in mice has found the percentage of GnRH neurons responding to kisspeptin (*in vivo*) increases by age from juvenile (25%), pre-pubertal (50%) and adult (>90%) (Han et al., 2005).

In our study approximately 35% of the GnRH immunoreactive neurons detected in both hypothalamic areas were non co- localized. Thus, many of these GnRH neurons may not be regulated by kisspeptin. By contrast, our previous study found all KISS1R immunoreactions co- localized with GnRH neurons and KISS1R immunoreations were even detected in many other non-GnRH immunoreactive neurons (Chaikhun-Marcou et al., 2016). These results point to the possibility of different active modes, functions and mechanisms of kisspeptin and its relation to GnRH releasing in the pre- and post- pubertal phases of buffalo reproductive development.

In the pre-pubertal period, GnRH is generally released on a basal level (tonic mode) just enough to control reproductive development. Also, some neuroendocrine hormones or proteins might be a major factor in reproductive development and body growth - such as leptin ,rather than kisspeptin (Whitlock et al., 2008; Manfredi-Lozano et al., 2016). Kisspeptin and its' receptor expression (*Kiss1* mRNA, KISS1R) were detected in low levels - which has also been reported in other pre-pubertal animals (Han et al., 2005; Colledge et al., 2010). But kisspeptin is a major factor that controls GnRH releasing for reproductive cycle regulation in the post-pubertal period (Colledge, 2008; Clarkson et al., 2009). The percentage of KISS1R immunoreations co- localized with GnRH neurons was not significantly different between the POA and ARC hypothalamic nuclei**.** There was a variation in the number of co-localized neurons found in samples from pre-pubertal buffalo of different ages that was similar to the differences found in post-pubertal buffaloes of different ages.

Our previous and present researches have studied the role of kisspeptin in the hypothalamic-pituitary-ovarian axis in female swamp buffalo reproduction. In pre-pubertal buffalo, we studied the relationship on the hypothalamic level of kisspeptin and GnRH in relation to puberty development and puberty onset. Remarkably, we found a significant difference in those results compared to post-pubertal buffalo. The summary of our research is found in figure 3, which shows *Kiss1* mRNA as expressed in juvenile to adult buffalo samples in both POA and ARC hypothalamic nuclei. However, kisspeptin protein was identified only in the cytoplasm of neurons in the POA of the samples from juvenile buffalo - with a weak reaction. Then in the 1-2.5 years old pre-pubertal samples, the kisspeptin protein has presented only in the ARC area. There is no kisspeptin protein reaction in both areas in the samples from the peri-pubertal age (2.5-3 years). When the buffalo have passed thru puberty, kisspeptin proteins can be detected in both areas with an intense reaction. Interestingly, although the population of Kiss1r-ir co-localized with GnRH neurons in the pre-pubertal buffalo (50%) seems no different to the post-pubertal buffalo (80%) but the single or non-co-localization neuron in the pre- and post-pubertal buffalo is an interesting difference. Non co- localized GnRH neurons represent half the population of all GnRH-ir neurons in the pre-pubertal swamp buffalo samples. This is the opposite of our studies' findings for post-pubertal swamp buffaloes in which there was no non co-localized neuron population.

This study provides evidence that both kisspeptin's function and its receptor signaling in female buffalo changes during puberty in relation to GnRH neurons - which is similar to research findings in other animals (Han et al., 2005; Dhillo et al., 2007; Decourt et al., 2008; Colledge et al., 2010; d'Anglemont de Tassigny and Colledge, 2010; Mayer et al., 2010; Mayer and Boehm, 2011). Previous reports have proved that the administration of exogeneous

kisspeptin can induce the onset of puberty in female mice, rats (d'Anglemont de Tassigny et al., 2008; Maguire et al., 2011), goats (Hashizume et al., 2010; Saito et al., 2012), cattle (Whitlock et al., 2008) and humans (Jayasena et al., 2014). These results suggest that kisspeptin and kisspeptin receptor signaling within the GnRH neuronal network may be a "gatekeeper" for the onset of puberty (Seminara et al., 2003; Maguire et al., 2011). The mode of action, mechanism and other elements that effect puberty onset and reproductive functions in swamp buffalo still need to be explored.

CONCLUSION

KISS1R immunoreations co- localized with the GnRH neuron population in both POA and ARC hypothalamic nuclei were detected in pre-pubertal female buffaloes in this study. However, around 50% of GnRH immunoreactive neurons were not co-localized with KISS1R in this population. This may indicate that kisspeptin regulates GnRH releasing for reproductive development and puberty in pre-pubertal female buffaloes in a manner which is different from that of post-pubertal buffaloes.

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Authors' contributions

Authors declare the contribution that all authors prepared the document for the study funding and designed the experiment. PS, SP and CY performed the experiment and TC analyzed the results. TC prepared the manuscript. All authors reviewed, revised the manuscript for intellectual contents and approved the final version critically.

REFERENCES

Chaikhun-Marcou T, Sotthibandhu P, Kyle V, et al., 2016. Evidence of kisspeptin receptor expression in GnRH neurons in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes. The Thai Journal of Veterinary Medicine 46(3): 10.

Chaikhun-Marcou T, Sotthibandhu P andSuadsong S, 2014a. Evidence of sex steroid hormone receptors in the preoptic area and arcuate hypothalamic nuclei in cycling buffaloes. Proc "The 2nd Symposium of the Thai Society for Animal Reproduction", Demonstration Room, 60th year Building, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, pp:199-200.

Chaikhun-Marcou T, Sotthibandhu P, Suthikrai W, et al., 2014b. Comparative effects of administration of kisspeptin-10 and GnRH on LH secretion in buffalo cows. Proc "World Congress on Reproductive Biology 2014", Edinburgh, Scotland, pp:(online) https://www.google.co.th/?gws_rd=cr,ssl&ei=4ofEVtruI9CZuQSyw4L4Bw#q=kisspeptin+in+buffalo.

Chaikhun T, Hengtrakunsin R, de Rensis F, et al., 2012. Reproductive and dairy performances of Thai swamp buffaloes under intensive farm management. Thai J Vet Med 42: 81-85.

Chaikhun T, Sotthibandhu P andSuadsong S, 2013. Localization of GnRH receptors in buffalo cow pituitary gland in follicular and luteal phases. Buffalo Bulletin 32 (Special issue): 468-472.

Chaikhun T, Tharasanit T, Rattanatep J, et al., 2010. Fertility of swamp buffalo following the synchronization of ovulation by the sequential administration of GnRH and PGF(2)alpha combined with fixed-timed artificial insemination. Theriogenology 74(8): 1371-1376.

Chaikhun T, Yanprapasiri C, Sotthibandhu P, et al., 2016. Kiss-1 mRNA/kisspeptin distribution in preoptic and arcuate nuclei of cycling buffalo (Bubalus bubalis) hypothalamus. Pak Vet J 36(1): 93-97.

Clarkson J, d'Anglemont de Tassigny X, Colledge WH, et al., 2009. Distribution of kisspeptin neurones in the adult female mouse brain. J Neuroendocrinol 21(8): 673-682.

Colledge WH, 2008. GPR54 and kisspeptins. Results Probl Cell Differ 46: 117-143.

Colledge WH, Mei H andd'Anglemont de Tassigny X, 2010. Mouse models to study the central regulation of puberty. Molecular and Cellular Endocrinology 324(1–2): 12-20.

d'Anglemont de Tassigny X and Colledge WH, 2010. The role of kisspeptin signaling in reproduction. Physiology (Bethesda) 25(4): 207-217.

d'Anglemont de Tassigny X, Fagg LA, Carlton MB, et al., 2008. Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals. Endocrinology 149(8): 3926-3932.

Decourt C, Tillet Y, Caraty A, et al., 2008. Kisspeptin immunoreactive neurons in the equine hypothalamus: Interactions with GnRH neuronal system. Journal of Chemical Neuroanatomy 36(3–4): 131-137.

Dhillo WS, Murphy KG andBloom SR, 2007. The neuroendocrine physiology of kisspeptin in the human. Rev Endocr Metab Disord 8(1): 41-46.

Han SK, Gottsch ML, Lee KJ, et al., 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25(49): 11349-11356.

Hashizume T, Saito H, Sawada T, et al., 2010. Characteristics of stimulation of gonadotropin secretion by kisspeptin-10 in female goats. Anim Reprod Sci 118(1): 37-41.

Jayasena CN, Abbara A, Veldhuis JD, et al., 2014. Increasing LH pulsatility in women with hypothalamic amenorrhoea using intravenous infusion of Kisspeptin-54. The Journal of Clinical Endocrinology & Metabolism 99(6): E953-E961.

Kadokawa H, Matsui M, Hayashi K, et al., 2008. Peripheral administration of kisspeptin-10 increases plasma concentrations of GH as well as LH in prepubertal Holstein heifers. J Endocrinol 196(2): 331-334.

Maguire JJ, Kirby HR, Mead EJ, et al., 2011. Inotropic Action of the Puberty Hormone Kisspeptin in Rat, Mouse and Human: Cardiovascular Distribution and Characteristics of the Kisspeptin Receptor. PLOS ONE 6(11): e27601.

Manfredi-Lozano M, Roa J, Ruiz-Pino F, et al., 2016. Defining a novel leptin–melanocortin–kisspeptin pathway involved in the metabolic control of puberty. Molecular Metabolism 5(10): 844-857.

Mayer C, Acosta-Martinez M, Dubois SL, et al., 2010. Timing and completion of puberty in female mice depend on estrogen receptor α-signaling in kisspeptin neurons. Proceedings of the National Academy of Sciences 107(52): 22693-22698.

Mayer C and Boehm U, 2011. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. Nat Neurosci 14(6): 704-710.

Roseweir AK and Millar RP, 2009. The role of kisspeptin in the control of gonadotrophin secretion. Hum Reprod Update 15(2): 203-212.

Saito H, Sawada T, Yaegashi T, et al., 2012. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. Anim Sci J 83(6): 487-492.

Seminara SB, Messager S, Chatzidaki EE, et al., 2003. The GPR54 gene as a regulator of puberty. N Engl J Med 349(17): 1614-1627.

Tsukamura H and Maeda K, 2011. GnRH pulse generation and its adaptation to the manipulation of follicular development and ovulation. Thai J Vet Med Suppl 41: 69-72.

Whitlock BK, Daniel JA, Wilborn RR, et al., 2008. Interaction of estrogen and progesterone on kisspeptin-10-stimulated luteinizing hormone and growth hormone in ovariectomized cows. Neuroendocrinology 88(3): 212-215.

Zerani M, Catone G, Maranesi M, et al., 2012. Gonadotropin-releasing hormone 1 directly affects corpora lutea lifespan in Mediterranean buffalo (Bubalus bubalis) during diestrus: presence and in vitro effects on enzymatic and hormonal activities. Biol Reprod 87(2): 45.

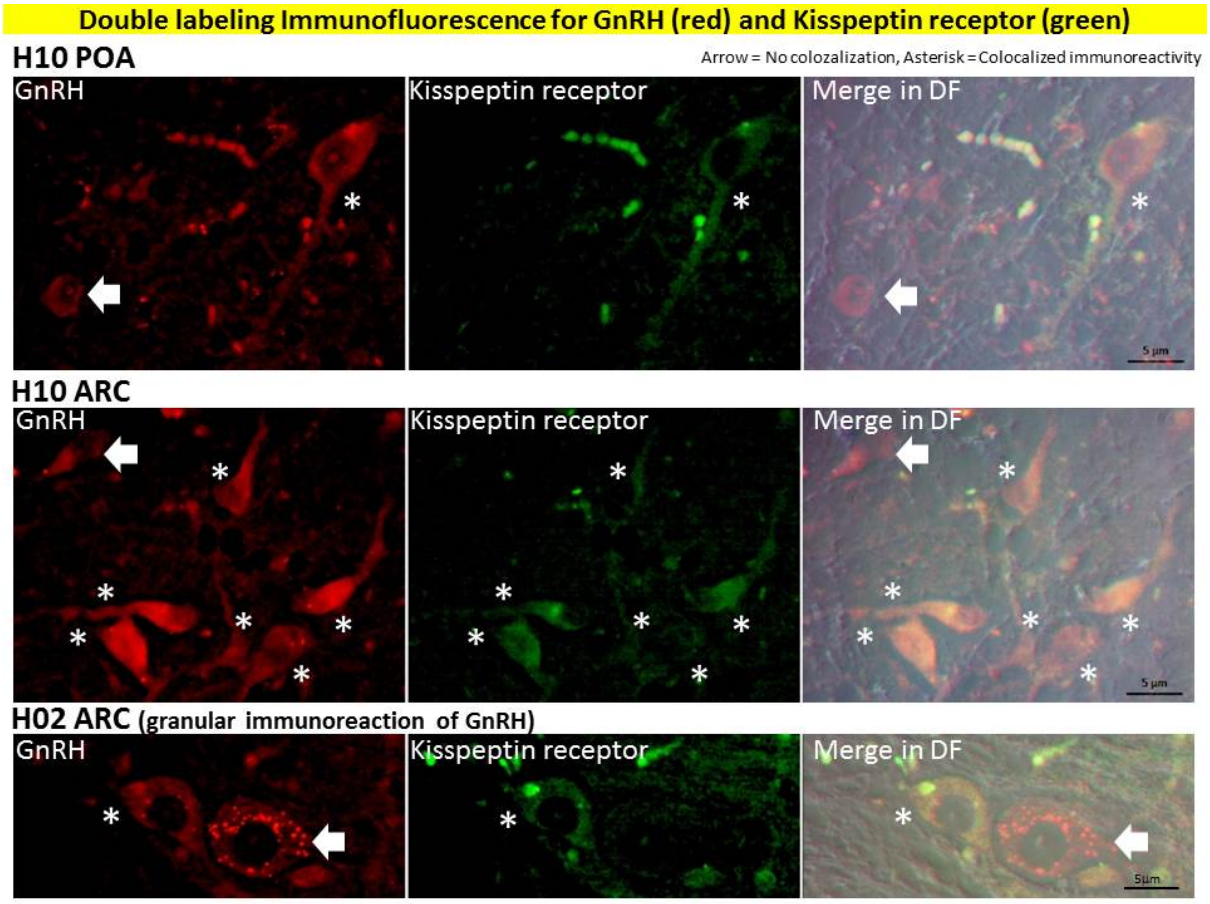

Fig. 1: The KISS1R immunoreactions are co-localized with GnRH neurons in the POA and ARC hypothalamic nuclei (asterisks). However, some neurons in both hypothalamic nuclei express only GnRH reactions (non co-localized) some of which appear in a granular formation (arrows), scale bar 5 µm.

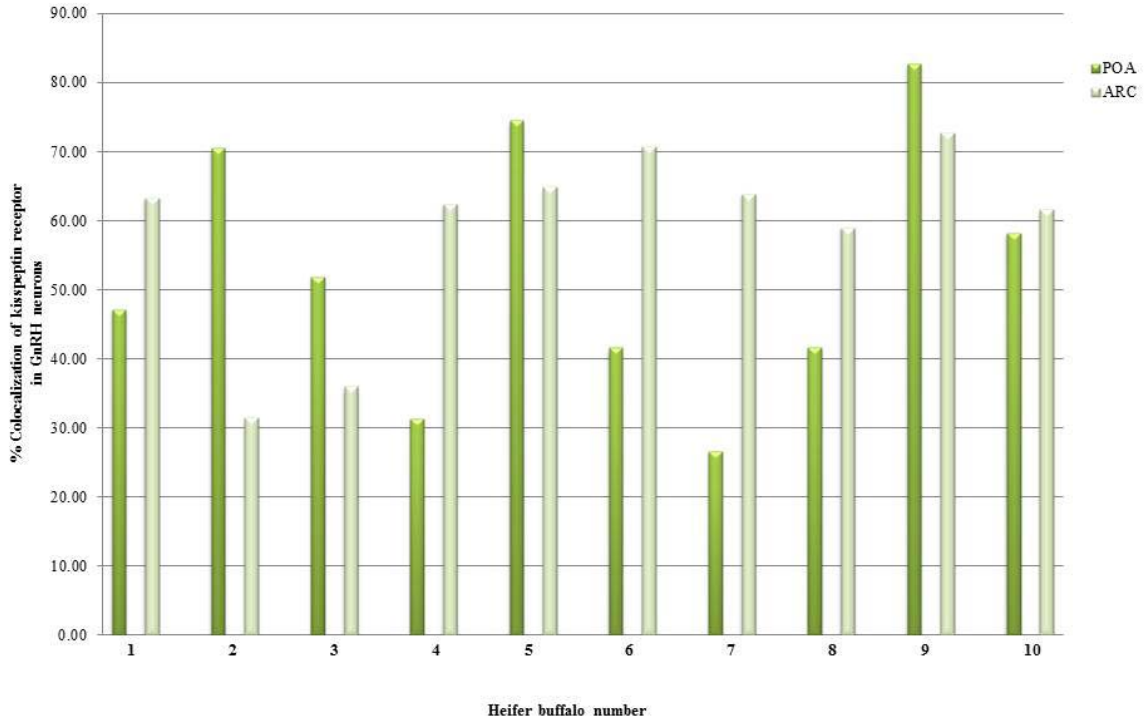

Fig. 2: The KISS1R-ir co-localized with GnRH-ir neuron populations (%) in the POA and ARC areas in pre-pubertal buffaloes.

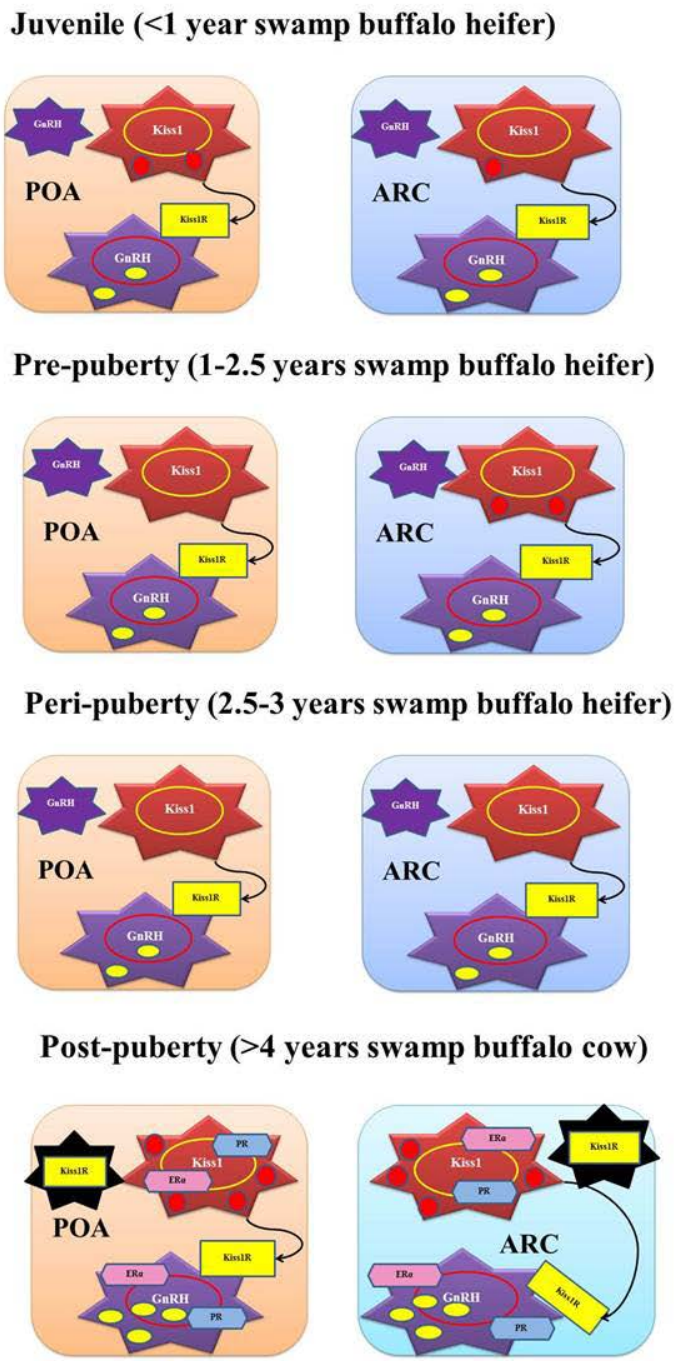

Fig. 3: Comparison of localization and distribution of *Kiss1* m-RNA, kisspeptin protein, kisspeptin receptor (KISS1R) and GnRH expressions in the POA and ARC hypothalamic nuclei in different age group samples of pre- and post- pubertal female swamp buffaloes.