บทบาทของคิสเปบทินต่อแกนการทำงานทางการสืบพันธุ์ระหว่างไฮโปทาลามัส ต่อมใต้สมอง และรังไข่ในแม่กระบือ

นางสาวธัชฎาพร ไชยคุณ

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THE ROLE OF KISSPEPTIN IN THE HYPOTHALAMIC PITUITARY OVARIAN AXIS IN BUFFALO COW REPRODUCTION

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ธัชฎาพร ไชยคุณ : บทบาทของคิสเปบทินต่อแกนการทำงานทางการสืบพันธุ์ระหว่างไฮโปทาลามัส ต่อมใต้สมอง และรังไข่ในแม่กระบือ (THE ROLE OF KISSPEPTIN IN THE HYPOTHALAMIC PITUITARY OVARIAN AXIS IN BUFFALO COW REPRODUCTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. น.สพ. ดร.ศิริวัฒน์ ทรวดทรง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. สพ.ญ. ดร.พงษ์ศิวะ โสตถิพันธุ์, 119 หน้า.

คิสเปบทินเป็นโปรตีนที่สร้างจากเซลล์ประสาทโดยผลิตจากยีน *Kiss1* ในกลุ่มเซลล์ประสาทไฮโปทาลามัสบริเวณพรีออพติค (พีโอเอ) และอาร์คูเอท นิวเคลียส (เออาร์ซี) ซึ่งพบว่ามีส่วนในการควบคุมของแกนการทำงานส่วนไฮโปทาลามัส ต่อมใต้สมอง และอวัยวะสืบพันธุ์ในสัตว์เลี้ยงลูกด้วยนม ถึงแม้ว่ามีการศึกษาหลายงานที่เกี่ยวข้องกับคิสเปบทิน ซึ่งเป็นหัวข้อหลักในงานวิจัยทางระบบสืบพันธุ์ในช่วงทศวรรษที่ผ่านมา แต่ยังไม่มีการศึกษาในกระบือ การศึกษานี้เพื่อสำรวจบทบาทของคิสเปบทินต่อแกนการทำงานของไฮโปทาลามัส ต่อมใต้สมอง และรังไข่ในแม่กระบือที่มีวงรอบการเป็นสัด การวิจัยภายนอกตัวสัตว์พบหลักฐานของ *Kiss1* mRNA โดยการใช้เทคนิคอินซิตู ไฮบริไดเซชัน และพบโปรตีนคิสเปบทินด้วยการใช้เทคนิคอิมมูโนฮิสโตเคมิสทรี ในกลุ่มเซลล์ประสาทไฮโปทาลามัส (พีโอเอ และ เออาร์ซี) ของกระบือ นอกจากนี้ยังตรวจพบลักษณะการพบร่วมกันระหว่างตัวรับคิสเปบทินและเซลล์ประสาทโกนาโดทรอปิน รีลิซซิง ฮอร์โมน (จีเอ็นอาร์เอช) ด้วยเทคนิคดับเบิล อิมมูโนฟลูออเรสเซนต์ ในบริเวณเดียวกัน ทั้งนี้ยังพบตัวรับเอสโตรเจนอัลฟา และตัวรับโปรเจสเตอโรนในบริเวณพีโอเอ และ เออาร์ซี เช่นเดียวกันกับการพบตัวรับจีเอ็นอาร์เอชที่บริเวณต่อมใต้สมองส่วนหน้าซึ่งตรวจด้วยวิธีอิมมูโนฮิสโตเคมีสทรี การวิจัยภายในตัวสัตว์ถึงการตอบสนองของลูติไนซิงฮอร์โมนต่อการให้สารคิสเปบทิน-10 เข้าทางเส้นเลือดจำนวนหนึ่งครั้งพบว่าให้ผลตอบสนองที่ไม่แน่นอนในแม่กระบือที่อยู่ในระยะลูเตียล และพบว่ามีการตอบสนองน้อยกว่าการตอบสนองต่อการให้สารจีเอ็นอาร์เอชเข้าทางกล้ามเนื้อ ผลเหล่านี้แนะนำได้ว่าคิสเปบทีนมีความสัมพันธ์กับการสืบพันธุ์ในแม่กระบือ การศึกษานี้บ่งบอกถึงคิสเปบทินอาจมีความเกี่ยวข้องกับแกนการทำงานของไฮโปทาลามัส ต่อมใต้สมอง และรังไข่ และอาจส่งผลกระทบต่อการทำงานของระบบสืบพันธุ์ในแม่กระบือ

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THUCHADAPORN CHAIKHUN: THE ROLE OF KISSPEPTIN IN THE HYPOTHALAMIC PITUITARY OVARIAN AXIS IN BUFFALO COW REPRODUCTION. ADVISOR: SIRIWAT SUADSONG, D.V.M., Ph.D., CO-ADVISOR: PONGSIWA SOTTHIBANDHU, D.V.M., Ph.D., 119 pp.

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 mammals. Although in the last decade there have been many studies done on kisspeptin, and it remains a major topic in reproductive research, no studies have been done on kisspeptin in buffalo. This study investigated the role of kisspeptin in the hypothalamic pituitary ovarian axis in the cycling buffalo cows. *In vitro* research found evidence of *Kiss1* mRNA using the in situ hybridization technique and kisspeptin protein using the immunohistochemistry technique in the hypothalamic nuclei (POA and ARC) of buffaloes. Moreover, structural interactions were found between kisspeptin receptors and gonadotropin releasing hormone (GnRH) neurons as revealed by double immunofluorescent in the same areas. Also, the localization and distribution of estrogen receptors and progesterone receptors in the POA and ARC as well as GnRH receptors in the anterior pituitary glands were detected by immunohistochemistry. In an *in vivo* study, the luteinizing hormone response to a single intravenous injection of kisspeptin-10 showed a variable response in early luteal phase buffalo cows and was less than the usual response to a GnRH intramuscular injection. These results suggest that kisspeptin is related to buffalo cow reproduction. This study indicates that kisspeptin may be involved in the HPO axis and may influence reproductive functions in buffalo cows.

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Chapter I

Introduction

Importance and rationale

In the last two decades, there has been a continuous decrease in the buffalo population in South-East Asia, especially swamp buffalo in Thailand (from 1.7 to 1.2 million heads between the years 1991 and 2011) (Department of Livestock Development, 2012). With a -12% annual growth rate during 1992-2002 (FAO Regional office for Asia and Pacific, 2003) Thailand has the lowest annual growth rate in South-East Asia. This is a serious problem.

The limitations of productive performance in buffalo, specifically swamp buffalo include many unique features such as; inherent late maturity, a prolonged intercalving interval, decreased ovarian function (especially in summer), poor estrus, and difficulties detecting estrus which causes problems in predicting the time of ovulation for artificial insemination (de Araujo Berber et al., 2002; Neglia et al., 2003; Presicce et al., 2003; Campanile et al., 2005; De Rensis et al., 2005; Paul and Prakash, 2005; Chaikhun et al., 2010)

The long intercalving period is one of the main problems in buffalo reproductive efficiency, thus lengthening their non-productive life. An intercalving interval of 501-600 days has been reported in conventional farming (Chantalakhana et al., 1981) and 486.2±75.02 days in intensive farming (Chaikhun et al., 2012) in swamp buffaloes and 360-480 days in river buffaloes (Barkawi et al., 1998; Campo et al., 2002), depending on the region where buffalos were raised, the calving season, environment and management (Fischer and Bodhipaksha, 1992; Barile, 2005).

Postpartum resumption of ovarian cyclicity is a critical point in the reproductive life of buffalo cows and its failure can lead to their becoming anestrous buffaloes. There are many factors affecting postpartum resumption of ovarian activity and postpartum anestrus in buffalo that are similar to those in cattle, but the

mechanisms involved in each of these factors (such as the physiological recovery of the pituitary gland from the effects of high placenta estrogen concentration in blood circulation breed, parity, nutritional status, milk yield, suckling, season of calving, biostimulation of bull effect, heat stress, uterine health and health status) are still unclear in relation to buffalo (Barile, 2005; El-Wishy, 2007a; Qureshi and Ahmad, 2008). All these factors affect the hypothalamic pituitary ovarian (HPO) axis in different parts and mechanisms.

Previous studies have been done on the efficiency of gonadotropin releasing hormone (GnRH) for stimulating ovarian resumption and controlling ovulation in postpartum buffaloes. The results were mainly positive - indicating that single GnRH administration can: stimulate both the pulse and amplitude of luteinizing hormone (LH) release from the pituitary (only in the follicular phase) to resume ovulation (Suthikrai, 1994), reduce the period between calving and the first progesterone increasing period (Aboul-Ela et al., 1983), increase the conception rate in postpartum buffaloes (Nasir et al., 1986), and induce ovarian function in anestrous animals (Singh et al., 1984).

At the present time, research on neuroendocrinology in humans (de Roux et al., 2003; Seminara et al., 2003) has discovered that GnRH secretion is mainly regulated by kisspeptin functions in the hypothalamus. *KISS1* gene produces the neuropeptide kisspeptin in different lengths of amino acid (10-54). G protein- coupled receptor (GPR54 or KISS1R) is the strongly cognate receptor of kisspeptin (Gottsch et al., 2004; Hashizume et al., 2010). The kisspeptin-GPR54 signaling plays a pivotal role in the control of the HPO axis which is necessary for pubertal activation and reproductive functions such as ovarian resumption, follicular development, ovulation and steroidogenesis in female mammals (Roseweir and Millar, 2009; Tsukamura and Maeda, 2011). Several studies of the distribution and function of kisspeptin and GnRH have been conducted in mammals such as mice (Gottsch et al., 2004), hamsters (Revel et al., 2006), rats (Irwig et al., 2004; Smith et al., 2006b), horses (Decourt et al., 2008), pigs (Tomikawa et al., 2010), monkeys, humans (Rometo et al., 2007) and sheep (Estrada et al., 2006; Smith et al., 2007). Since variations have been found among species due to differences in their anatomy and physiology (Colledge, 2009),

and since no study has been done in buffaloes, the exact nature of how these processes function in buffalo is unknown.

Based on the evidence from previous reports, I am interested in studying the role of kisspeptin in the hypothalamic pituitary ovarian axis in buffalo cow reproduction with the goal of increasing both our knowledge of, and our ability to improve, buffalo reproductive performance.

Research questions

1. Can *Kiss1* mRNA be detected in the POA (preoptic area) and ARC (arcuate nucleus) hypothalamic nuclei of buffalo cows (in both the follicular phase and the luteal phase of the estrous cycle), and if so, what its protein localization and distribution?
2. Are there any relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha, progesterone receptors in the POA and ARC hypothalamic nuclei, and GnRH receptors in the pituitary gland?
3. How do the effects of the administration of kisspeptin-10 and those of GnRH in term of the characteristics of LH secretion in luteal phase?

Objectives of study

1. To detect the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle.
2. To determine if there are any relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha, progesterone receptors in the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland.
3. To compare the effects of the administration of kisspeptin-10 and those of GnRH on the characteristics of LH secretion in luteal phase. Also, to determine if progesterone may plays a role in the feedback control of kisspeptin on the hypothalamic level.

Hypothesis

Kisspeptin plays a key role in the hypothalamic pituitary ovarian axis in buffalo cows reproduction.

1. Kisspeptin does exist in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle.
2. There are the relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland which are depended on the area of neuronal ganglions and phase of estrous cycle.
3. The administration of kisspeptin-10 has different effects on the releasing of LH, compared to the administration of GnRH in luteal phase. Also, progesterone may plays a role in the feedback control of kisspeptin on the hypothalamic level.

Scope of study

This project has been reviewed and approved by the Certification of Institutional Animal Care and Use Committee (IACUC) in accordance with Chulalongkorn University Animal Care and Use Committee regulations and policies governing the care and use of laboratory animals. The Animal use protocol and the approval number is 13310007. This review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

The *in vitro* part of this study: The brains were collected from 6 cycling buffaloes (n=3 in follicular phase and n=3 in luteal phase) and processed for paraffin blocks. Four-micron paraffin sections of the POA and ARC hypothalamic nuclei were prepared for:

1. Chromogenic in situ hybridization using a *Kiss1*cRNA probe designed from the ovine kisspeptin gene sequence (GenBank accession no. DQ059506). The expression, localization, and distribution of *Kiss1* mRNA in the POA and ARC hypothalamic nuclei were detected through in situ hybridization.
2. Immunohistochemistry for kisspeptin, kisspeptin receptors, GnRH neurons,

estrogen receptors alpha, progesterone receptors in the POA and ARC hypothalamic nuclei, and GnRH receptors in the pituitary glands. The immunohistochemical reactions of each antibody were identified and analyzed for localization and distribution. For each buffalo, single labeling was calculated as a percentage of the total number of immunoreactive cells of each antibody in the POA and ARC hypothalamic nucleus and then averaged across animals to calculate a mean (±SEM). A comparison of the average number of immunoreactive cells in the POA and ARC was made within groups (follicular phase or luteal phase) and between groups (follicular phase and luteal phase) by Student's t-test ($P<0.05$). For double labeling, the number of each type of immunoreactive cells with co-localization or non-co-localization was calculated as a percentage of the total number and then averaged across animals to calculate a mean (±SEM). Characterization of co-expression and non-co-expression were described.

The *in vivo* part of this study: Six healthy postpartum buffalo cows were selected for this study. There were 3 treatments in this study: 1. Kp-10 treatment 2. GnRH treatment and 3. Distilled water treatment. All animals were used by repeated measurement experimental design. Each treatment in this study used the same animals in the luteal phase of estrous cycle (day 10 after onset of estrus, using estrus synchronization) with an estrous cycle interval waiting period. Blood was collected every 15 min interval between -2 hr and 3 hr (time=0 hr; injective time) and every 30 min after that for the next 3 hr (total experimental time was 8 hr). The LH concentration of the plasma samples was measured by a commercial test kit enzymatic immunoassay. All data in each treatment group was presented as the mean ± SEM. Peak-shaped responses were observed in the concentrations of LH. The maximum LH concentration in each treatment was observed and calculated among serial samples during the time length of peak concentration as well as the timing of the samples containing them. The area under curve (AUC) of the LH response curves, as linear trapezoidal summation between successive pairs of concentration and time, were calculated. The AUCs of LH in each treatment group were analyzed by repeated measures ANOVA and the comparison of each sampling time between treatment groups were determined by Student's t-test ($P<0.05$).

The possible role of kisspeptin in the hypothalamic pituitary ovarian axis in buffalo cow reproduction, suggested by this study, is described and discussed.

Definition of words, symbols and abbreviations

ADP	Anterodorsal preoptic area
AR	Androgen receptor
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
AUC	Area under curve
Buffalo	The buffalo in this study is domestic buffalo (*Bubalus bubalis*) as swamp type (chromosome 2n=50), which was identified by characteristic of phenotype.
cRNA	Complementary ribonucleic acid
DMN	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
ERα	Estrogen receptor α
FSH	Follicle-stimulating hormones
GH	Growth hormone
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
GPR	G protein- coupled receptor
HPO axis	Hypothalamic- pituitary- ovarian axis
HPG axis	Hypothalamic- pituitary- gonadal axis
IHC	Immumohistochemistry
ISH	In situ hybridization
i.v.	Intravenous
KISS1/ KISS1	Human kisspeptin gene/protein
Kiss1/ Kiss1	Animal kisspeptin gene/protein
KISS1R/ KISS1R	Human kisspeptin receptor1 gene/protein
Kiss1r/ Kiss1r	Animal kisspeptin receptor1 gene/protein

Kp-10	Kisspeptin10, ten amino acid C-terminus peptide of kisspeptin (the shortest form) is the potency to activate its receptors
LH	Luteinizing hormone
MBH	Mediobasal hypothalamus
ME	Median eminence
mRNA	Messenger ribonucleic acid
µg/kg	Microgram/ kilogram
OVX	Ovariectomy
PBS	Phosphate buffer saline
PI	Pituitary gland
pmol/kg b.w.	Picomol/ kilogram of body weight
POA	Preoptic area
PR	Progesterone receptor
PRL	Prolactin releasing hormone
RNA	Ribonucleic acid
SEM	Standard error of mean
SD	Standard deviation

Expected advantages of this study

1. The *in vitro* part of this study of kisspeptin role in the HPO axis in buffalo cow (in cooperation with other hormones such as GnRH, estrogen and progesterone) should provide fundamental knowledge concerning buffalo reproduction which can be used for further studies.
2. The *in vivo* part of this study of the comparative effects of intravenous administration of Kp-10 and GnRH on the characteristic secretion of LH in luteal phase should provide optional data for field application and may help improve the reproductive performance of buffalo. Also it should furnish knowledge that can be applied to different breeds of buffalo in various regions around the world.

Chapter II

Literature Review

Research ideas and theories

Despite the fact that the buffalo (*Bubalus bubalis*) is a common domestic animal used for meat and milk supply, this species has many 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. In addition, the long intercalving period of buffalo cows is another main problem affecting their reproductive efficiency (De Rensis et al., 2005; Paul and Prakash, 2005; Chaikhun et al., 2012). Both of these reproductive issues might be related to the HPO axis, in terms of the tonic center and surge center functions in the hypothalamus (Maeda et al., 2010).

The hypothalamus is a specialized ventral portion of the brain consisting of groups of nerve cell bodies called hypothalamic nuclei. The two main groups of nuclei within the hypothalamus that influence reproduction are the surge center and the tonic center. Neurons in these regions secrete GnRH. Neurons in the paraventricular nucleus secrete oxytocin. It is important to understand that each hypothalamic nucleus has a different function and is stimulated by different sets of conditions (Maeda et al., 2010). Research in sheep has indicated that, in this species, the POA functions as a GnRH surge center and that the ARC area functions as a GnRH tonic center (Clarke et al., 2009). The POA and ARC are thus believed to be the main hypothalamic nuclei through which kisspeptin regulates puberty and reproductive functions in the HPO axis in sheep and possibly other ruminants (Estrada et al., 2006; Franceschini et al., 2006).

Recent research on neuroendocrinology in humans has discovered that GnRH secretion is also mainly regulated by kisspeptin functions in the hypothalamus (de Roux et al., 2003; Seminara et al., 2003). The *Kiss1* gene produces the neuropeptide

kisspeptin in different lengths of amino acid (10-54). G protein- coupled receptor 54 (GPR54 or Kiss1r) is the strongly cognate receptor of kisspeptin (Gottsch et al., 2004; Messager et al., 2005; Hashizume et al., 2010). Kisspeptin-GPR54 signaling plays a pivotal role in the control of the HPO axis by initiating GnRH release which is necessary for pubertal activation and reproductive functions such as oocyte maturation, follicular development, ovulation and steroidogenesis in female mammals (Han et al., 2005; Roseweir and Millar, 2009; Tsukamura and Maeda, 2011).

Several studies on the distribution and function of kisspeptin have been conducted in mammals such as mice (Gottsch et al., 2004), hamsters (Revel et al., 2006), rats (Irwig et al., 2004), horses (Decourt et al., 2008), pigs (Tomikawa et al., 2010), monkeys, humans (Rometo et al., 2007; George et al., 2012), sheep (Estrada et al., 2006; Franceschini et al., 2006; Caraty et al., 2007; Smith et al., 2007), cattle (Kadokawa et al., 2008a; Kadokawa et al., 2008b; Whitlock et al., 2008) and goats (Hashizume et al., 2010; Saito et al., 2012; Tanaka et al., 2012). In situ hybridization (ISH) and immunohistochemistry (IHC) are the main techniques used in kisspeptin research. ISH is a hybridization type that utilizes a labeled complementary DNA or RNA strand (i.e., probe) to identifiy, in an area or section of tissue (in situ), the location of a particular DNA or RNA sequence. RNA ISH, which is used in the majority of kisspeptin research, is useful for determining and localizing, within sections of tissue or whole mounts, mRNA and other transcripts for gene expression (Jin and Lloyd, 1997). This is different from IHC, which is usually used to localize and detect antigens (e.g., proteins) in tissue sections by taking advantage of the fact that specific antibodies bind to specific antigens in biological tissues (Ramos-Vara, 2005). In our study, therefore, ISH was used to detect kisspeptin gene expression and IHC for kisspeptin protein localization, respectively.

In the rodent hypothalamus, *Kiss1* expression has been localized in the AVPV, ARC and ADP (Smith et al., 2006a), but this has rarely been found in primates (Rometo et al., 2007). Variations of kisspeptin localization in hypothalamic nuclei have been found among mammal species, presumably due to differences in their anatomy and physiology (Colledge, 2009). In the ewe, for example, kisspeptin immunoreactive neurons have been found predominantly in ARC and POA

(Franceschini et al., 2006; Pompolo et al., 2006). Other kisspeptin studies done on sheep found that kisspeptin neurons in the POA and ARC hypothalamic nuclei are the key feedback regulator of estrogen for GnRH/ LH release (Estrada et al., 2006; Smith, 2008). However, there are still many questions regarding the role of kisspeptin in the regulation of buffalo reproduction and basic research on their neuroanatomy still remains to be done.

Literature Review

1. Buffalo biodata

1.1 General information

The buffalo is a mammal in the family Bovidae which is divided into wild and domestic buffaloes. The scientific name of domestic buffalo (water buffalo) is *Bubalus bubalis*. There are two types of domestic buffalo; swamp type and river type which have differences in genetics, morphology, geographic distribution and commercial use (Borghese and Mazzi, 2005)

1.2 Estrous cycle

The estrous cycles in swamp buffalo are 20-34 days (Chantalakhana, 1981), 21.5 days (Kanai and Shimizu, 1983). The differences and variations in the estrous cycles of buffalo depend on the individual's follicular waves and their follicular development pattern. The duration of the estrous cycle is related to the duration of the luteal phase and the number of follicular waves. If the buffalo presents 2 follicular waves or 3 waves, the estrous cycle might be 21 or 24 days respectively (Baruselli et al., 1997). Although there are many more follicular wave studies in river buffalo than in swamp buffalo, overall there has been much more research done on cattle than on buffalo (Baruselli et al., 1997; Presicce et al., 2003; Promdireg et al., 2004; Yindee et al., 2011). Another study in peripartum swamp buffaloes reported that the first ovulation after calving was 39±13.5 (21-59) days without estrous signs. It is possible that the hormonal imbalance could have occurred after calving, so the

effective service should apply in the 2nd estrous cycle or 1-2 months after calving under good management (Yindee et al., 2011).

There are four stages of the estrous cycle: proestrus, estrus, metestrus and diestrus. Estrous signs in swamp buffalo have rarely been observed. Basically, the characteristics and behavior of female swamp buffaloes have been noticed to be the same as those of cattle but seem to be more difficult to detect. The study in the different types of ovarian status detection found that ovarian function diagnosis by plasma progesterone assay and rectal palpation had an overall accuracy of 86% and 81% respectively. Although the plasma progesterone assay was more reliable in this experiment, rectal palpation was recommended for detecting the stage of estrous cycle under field conditions as it provides an immediate assessment of the reproductive status of the buffalo (Jainudeen, 1983).

Previous study of hormonal levels during estrous cycle in swamp buffaloes reported that the LH, progesterone and estrogen secretory patterns (which were detected by radioimmunoassay) were fundamentally similar to those in cattle, except for the fact that the actual progesterone concentration during the luteal phase was low in buffalo. During 4 days before estrus, progesterone concentration decreased rapidly, then LH and estrogen showed a sustained increase in concentration. Preovulatory LH surge was initiated in association with the estrous behavior onset and lasted for about 12 hr, then ovulation occurred approximately 30 hr after the peak of LH (Kanai and Shimizu, 1983). In postpartum swamp buffalo, the plasma progesterone concentration during estrus (d0) was low (0.24 ng/ml). Then it increased to 0.42 ng/ ml by d6 and reached the peak of 1.51 ng/ml on d15. After that it decreased to the basal level of 0.20 ng/ml on d22 (Jainudeen et al., 1981). The progesterone level of swamp buffalo in the luteal phase, especially d14-16, was a lower mean value of 1.51-2.6 ng/ml compared to the value of 4.0-4.26 ng/ml in Murrah buffaloes (Kumud, 1999). Anestous buffaloes were detected to have a low progesterone level of 0.16 ng/ml throughout the period of study (about 21 days) (Chau et al., 1983). The 17-beta estradiol (E2) level increased to a peak of 0.077±0.035 ng/ml on the day before estrus. It constantly reached this peak for 4 days then it sharply decreased to the basal level of 0.036 ng/ml. According to the

results, the progesterone level is lower while the estradiol level is higher than those found in cattle (Bodhipaksha et al., 1978).

2. Postpartum resumption of ovarian cyclicity and postpartum anestrus

2.1 The postpartum resumption of ovarian cyclicity

Timing of uterine involution was approximately 30-45 days in dairy buffalo (Presicce et al., 2005; El-Wishy, 2007b). In dairy buffaloes, the first ovulation after calving is detected by rectal palpation and progesterone analysis occurring between 28-71 and 24-55 days, respectively and postpartum estrus presenting between 44-87 days (El-Wishy, 2007a). In Mediterranean buffaloes, the calving to first postpartum ovulation in primiparous and pluriparous buffalo cows was 25.5 (range: 16-46) and 15.5 (range: 8-20) days, respectively (Presicce et al., 2005). Studies from India, Pakistan and have reported that 34-49% of buffaloes presented estrus during the first 90 days after calving while 31-42% remained anestrus for a period longer than 150 days (El-Wishy, 2007a).

In swamp buffaloes both postpartum ovulation and estrus are more delayed than in dairy buffaloes. A study on the follicular dynamics after parturition found that the first ovulation after calving was 39.8±13.5 days (range 21-59, n=16) and the estrous signs were more present in the second and subsequent postpartum ovulations (Yindee et al., 2011).

Test have shown blood progesterone concentration to be at baseline levels within 24 hours from calving (<1ng/ml) (Presicce et al., 2005). Availability of releasable FSH during and after calving is not believed to be crucial for beginning the first estrous cycle after calving (El-Wishy, 2007b). On one hand, low serum levels of LH were noted in postpartum buffaloes with no previous background of ovulation or estrus within 4 months after parturition (0.6-1.1 ng/ml). Also, between 3 and 90 days postpartum there were no major changes in the basal LH levels between milked and suckled anestrus Murrah buffaloes (El-Wishy, 2007b). On the other hand, one study found that basal plasma LH levels in the 2-3 weeks after calving had an inverse relationship to the first postpartum ovulation interval, in which the concentrations

also were significantly higher in buffaloes presenting estrus than in anestrus animals. Similarly, A study noted that a factor limiting the development and maturation of dominant follicles could be the restoration of pulsatile LH releasing (Manik et al., 2002). Using exogenous GnRH to stimulate pituitary gonadotropin was recommended on day 20 and day 30 postpartum in dairy and swamp buffaloes, respectively (El-Wishy, 2007b). The plasma levels of estradiol-17β dropped dramatically over the first 24-72 hours after parturition, then stayed in basal level between 2 and 7 days after calving. In acyclic buffaloes the pronounced fluctuations of total estrogen, between 38-61 pg/ml (level during estrus 63 pg/ml), most likely are related to waves of follicular growth and atresia (El-Wishy, 2007b).

The initial postpartum ovulation is mainly followed by one or more short estrus cycles (<18 days). Following short cycles, extended anovulatory anestrous periods have been found to occur, caused by a prolonged inter-luteal phase. Additionally, about 25% of the buffaloes had long anestrus periods due to the cessation of cyclicity (true anestrus) for 3 or more weeks and about 8-11% of the buffaloes had luteal activity that extended for at least 28 days or more (El-Wishy, 2007a). In Thailand, 93.3% (n=14/15) of postpartum cows showed short ovarian cycles (10.4±1.51 days). Around 66.7% of the buffaloes in this study still showed short estrus cycles (11.5±2.38 days) during the second to third ovulation postpartum. Moreover, it was demonstrated that the follicular dynamic in swamp buffaloes developed in an irregular wave pattern (Yindee et al., 2011).

2.2 The factors associated with re-initiation of the ovarian cycle

There are many factors affecting postpartum resumption of ovarian activity and postpartum anestrus in buffaloes that are similar to those in cattle but the mechanisms involved in each of these factors (such as breed, parity, nutritional status, milk yield, suckling, season of calving, biostimulation of bull effect, heat stress, uterine health and health status) are still unclear in relation to buffaloes (Barile, 2005; El-Wishy, 2007a; Qureshi and Ahmad, 2008). The physiological recovery of the pituitary gland from the effects of high placenta estrogen concentration in blood circulation is the main factor. In cattle, (on which there have been many more

studies done than buffalo) it has been reported that the pulsatile secretion of luteinizing hormone (LH) in blood circulation is reduced by the decreasing of follicular wave activity and the reduced prominence of dominant follicles during pregnancy. Also during late gestation, placenta estrogen inhibits the synthesis of the LH molecule in gonadotrophs in both the beta subunit and some alpha subunits. At parturition, LH concentration is low but not FSH. In particular, mRNA expression of the LH beta subunit is low (Montiel and Ahuja, 2005).

2.3 Ovarian activity and luteinizing hormonal (LH) change during the estrous cycle

In cycling buffalo, during the estrous cycle, ovarian activity presents a continual change of follicular dynamics. First a single follicle is selected, which then becomes larger in size and begin to dominate the other follicles. Following this initial dominance phase, the dominant follicle then either becomes atretic or ovulates, depending on whether the dominance phase is or is not associated with luteolysis (Taneja et al., 1996). In the follicular phase, LH levels and follicular growth are hightest when the progesterone levels are lowest. The interval from estrogen peak to LH peak has been demonstrated to be 14.8 h and the duration of LH peak to be 4.0 hr. LH peak values of 20- 40 ng/ml have been reported on the day of estrus (Kanai and Shimizu, 1986). The interval between the onset of estrus and the LH surge has been found to be 8 hr by (Kumar et al., 1991). In swamp buffaloes, Kanai and Shimizu (1986) reported that a LH surge presented in association with estrous signs and lasted for 7-12 hr. Peak LH concentrations of 61-126 ng/ml were observed 4-15 hr after the onset of estrus whereas the interval between LH peak and the end of estrus was much less variable. Although a direct positive feedback effect of estrogen on LH secretion has not been demonstrated, the occurrence of an estrogen peak (which is produced from the dominant follicle) before the pre-ovulatory LH surge and a positive correlation between estrogen and LH during the 24 hr period before the LH surge indicate a role of estrogen in mediating LH release (Kanai and Shimizu, 1986). In the luteal phase at the time of high levels of progesterone, the LH levels remain at a baseline which fluctuates around 0.72-3.0 ng/ml (Mondal et al., 2007).

The follicles are still growing until the dominance phase but do not continue to ovulation because the high level of progesterone inhibits the hypothalamus from producing the GnRH which causes LH and FSH releasing from the anterior pituitary (Agriculture and Consumer Protection, 1991). The relationship between the ovarian cycle and hormonal changes during the estrous cycle on HPO axis are shown in figure 1.

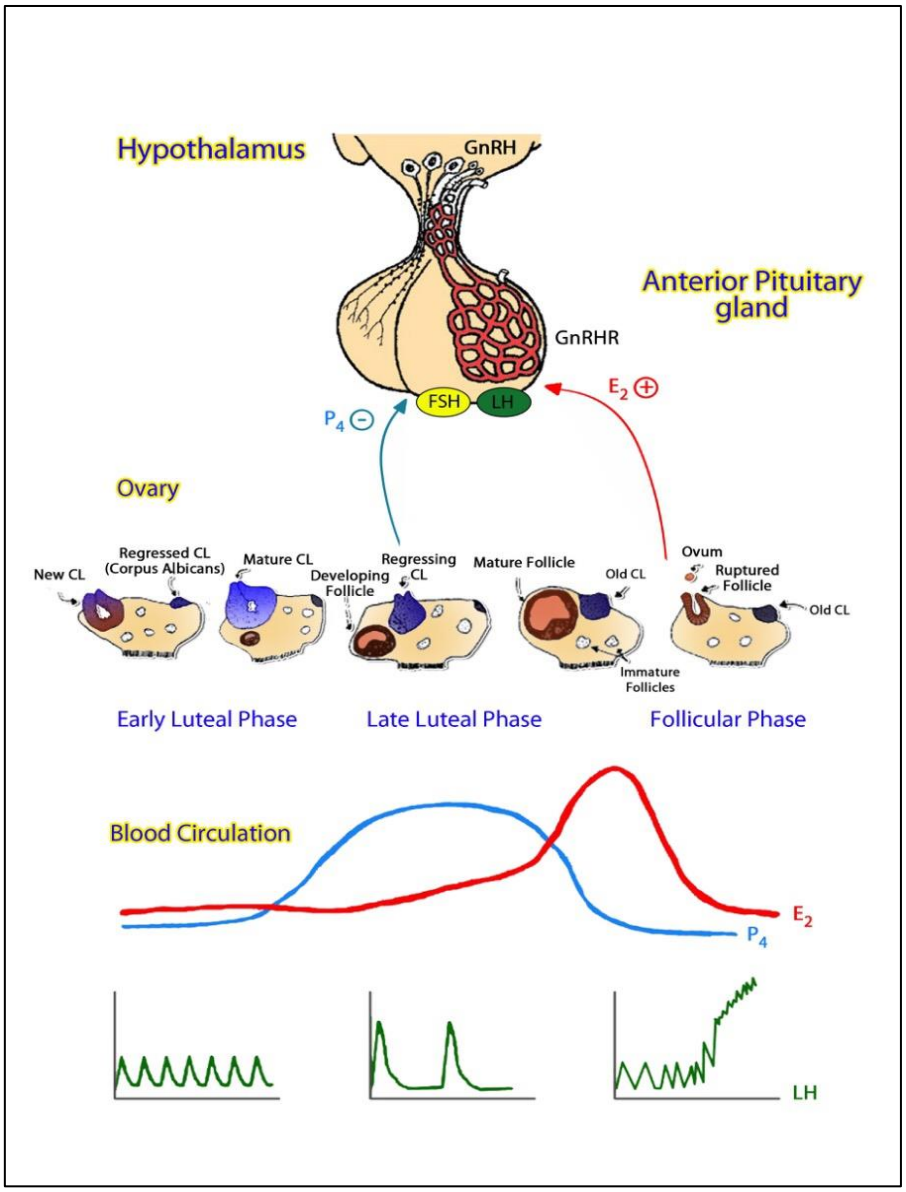

Figure 1 HPO axis is the main partway of reproductive function. In buffalo, HPO axis acts similar to cattle. The ovarian steroids show the feedback on GnRH releasing in different phases of the estrous cycle. During the luteal phase, progesterone (P4) plays negative feedback on GnRH neurons in the hypothalamus and causes LH

release in basal level and the follicle from the 1st follicular wave can not ovulate. However, in the late luteal phase luteolysis is occurred and the 2nd follicular wave – a follicle can grow and become dominant in the late luteal phase from FSH effect. Estrogen (E2) in that dominant follicle shows the positive feedback to GnRH which stimulates LH amplitude and frequency and is called l LH surge. The dominant follicle is ovulated after that. If there are any defects or deficiencies or imbalances in any part of the HPO axis, it will lead to reproductive problems (Kanai and Shimizu, 1986; Agriculture and Consumer Protection, 1991; Promdireg et al., 2004).

3. The control of ovulation by GnRH

3.1 Single GnRH application for control of ovulation and fertility in buffalo

There are many studies on the effect of GnRH on the reproductive functions (especially ovulation control) in buffalo which present different results, depending on the GnRH protocols and animal status. Singh et al. (1984) have studied the application of 250 μg GnRH for inducing ovulation and increasing fertility in 25 anestrus Murrah buffaloes, which showed a response rate of 14%. Another study in Murrah buffaloes found that injection of 250 μg GnRH on the day of insemination can increased the conception rate (Rao and Rao, 1984). Aboul-Ela et al. (1983) have examined the changes in the levels of progesterone concentration after injecting 6 or 12 μg GnRH in 15 days postpartum buffaloes. The results showed that GnRH administration can reduce the period between calving and the first increase in progesterone release, especially when using a high dose of GnRH. When Pattabiraman et al. (1986) used 500 μg GnRH in anestrus and 20, 25 and 30 days postpartum cows, the anestrus cows presented estrus cycles 80% of the time with 11-30 days of cycle, and 16.7% of the postpartum cows showed a 10-22 day estrous cycle. Nasir et al. (1986) reported that 14 days postpartum buffaloes showed faster uterine involution and ovarian function as well as an increased first service conception rate with GnRH dosages of both 100 and 250 μg (with no detectable

differences between the two dosages). Suthikrai (1994) did research on ovarian resumption and the changing pattern of plasma progesterone, estradiol-17β and LH profiles caused by GnRH in postpartum swamp buffaloes. Also the same study reported that a 250 μg dose of GnRH during the follicular phase stimulated both pulse and amplitude of LH release from the pituitary and led to a resumption of ovulation in buffaloes.

4. The role of kisspeptin signaling in reproduction

4.1 What's kisspeptin?

Kisspeptin (syn. metastin) was discovered in 1996 by Lee and others who identified the KISS1 gene for suppressing metastasis in human malignant melanoma (Lee et al., 1996). KISS1 was named from the home of the famous Hershey chocolate Kiss, Hershey, Pennsylvania, USA, which was the location of this gene's discovery (Gottsch et al., 2004). Kisspeptin are peptide hormones which encode a 145- amino acid peptide that produces various lengths (10-54) of biologically active peptide (Gottsch et al., 2004; Hashizume et al., 2010). The larger peptides contain some variability between species. Whereas the 10 amino acid C-terminus peptide is well maintained it also has the potency to activate its receptors (Lee et al., 1996; Muir et al., 2001; Adachi et al., 2007; 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; Adachi et al., 2007; 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 GnRH neurons and reproductive function 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).

4.2 Kisspeptin 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 of anatomy (Colledge, 2008; Colledge, 2009). ISH and IHC are the main techniques used in researching kisspeptin, GPR54 and GnRH gene or protein expression and localization. ISH is a hybridization type that utilizes a labeled complementary DNA or RNA strand (i.e., probe) to identifiy, in an area or section of tissue (in situ), the location of a particular DNA or RNA sequence. ISH can also use two or more probes, labeled with radioactivity or other non-radioactive labels, to simultaneously detect two or more transcripts (Jin and Lloyd, 1997). RNA ISH, which is used in the majority of kisspeptin research, is useful for determining and localizing, within sections of tissue or whole mounts, mRNA and other transcripts. This is different from IHC, which usually detects antigens (e.g., proteins) in tissue sections by taking advantage of the fact that specific antibodies bind to specific antigens in biological tissues (Ramos-Vara, 2005). Many kisspeptin, GPR54 and GnRH distribution studies have been done in mammals such as mouse (Gottsch et al., 2004), hamsters (Revel et al., 2006), rats (Irwig et al., 2004; Smith et al., 2006b), horses (Decourt et al., 2008), pigs (Tomikawa et al., 2010), monkeys, humans (Rometo et al., 2007) and sheep (Estrada et al., 2006; Smith et al., 2007). The information in this review is divided according to the 3 main species of mammals involved in kisspeptin studies: ruminants, rodents and primates (table 1).

In the rodent's hypothalamus, Kiss1 expression has been localized by ISH in the AVPV (Smith et al., 2006a) and has been found to display a sex difference (with more kisspeptin cell bodies in female because it is depend on exposure to testoseterone during perinatal development), but this has rarely been found in primates (Rometo et al., 2007). In ewes and monkeys, Kiss1 neurons were found in the ARC and POA regions with a greater cell density in ARC, especially in primates (Estrada et al., 2006; Smith et al., 2007).

Several anti-kisspeptin antibodies 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. It has thus been suggested that anti-protein antibodies should be validated for specificity (Colledge, 2009). A monoclonal antibody excited against an amidated rat kisspeptin sequence found kisspeptin neurons in the ARC and AVPV of adult female rat (Kinoshita et al., 2005; Adachi et al., 2007). In adult mice, using an antibody raised against an amidated mouse Kp-10 sequence detected many kisspeptin-immunoreactive cells (Kp-ir) in the ARC with lower numbers in the APVP and dorsomedial nucleus (DMN) (Clarkson and Herbison, 2006). In sheep, Kp-ir cells have been mainly found in the ARC, POA and DMN which are the same areas in the ARC and POA of monkeys (Franceschini et al., 2006; Rometo et al., 2007).

In sheep, GnRH cell bodies are presented both in the POA and in the ARC. Most of GnRH cell bodies in primates are identified lateral to the ARC in the ventral hypothalamic tract of the MBH, but they rarely are found in POA. Also, although GnRH cell bodies are scattered as a continuum from the medial septum and diagonal band of Broca to the medial POA, they are rarely found in the ARC areas in rodents (Colledge, 2009).

Table 1 The locations of Kisspeptin and GnRH neuronal cell bodies in the hypothalamus which were detected by in situ hybridization and/or immunohistochemistry. Abbreviations: POA,preoptic area; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; ME, median eminence; DMN, dorsomedial nucleus; ADP, anterodorsal preoptic area; PI, pituitary gland (Colledge, 2009; Smith, 2009; d'Anglemont de Tassigny and Colledge, 2010).

Animal	Neuron type	Location	
		ISH	IHC
Sheep	Kiss1/kisspeptin	POA, ARC	POA, ARC, ME, DMN
	GnRH		POA, ARC
Rodent	Kiss1/kisspeptin	AVPV, ARC, ADP	AVPV, ARC
	GnRH		POA
Primate	Kiss1/kisspeptin	AVPV, POA, ARC	POA, ARC
	GnRH		Lateral of ARC, POA

4.3 Connections and actions of kisspeptin on GnRH neurons

The hypothesis that kisspeptin neurons stimulate GnRH neurons directly in some mammalian species, has been proved by many immunofluorescence data reports. In adult female rats (Kinoshita et al., 2005) and mice (Clarkson and Herbison, 2006), Kp-ir fibres in POA are associated with GnRH neuron cell bodies. In female sheep, 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 ME (Franceschini et al., 2006; Pompolo et al., 2006) and it is thought that these terminals might be causative for the kisspeptin that has been

identified in the ewe hypophyseal portal blood (Smith et al., 2008) and in the female monkey stalk-ME (Keen et al., 2008). In mares, Kp-ir fibres and GnRH fibres were found in the POA, ARC nucleus and ME but there were numerous appositions with GnRH fibres in the ME (Decourt et al., 2008). From 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). For example, kisspeptin is also released in a pulsatile type in the stalk-ME in monkeys and this release increases at puberty, suggesting that kisspeptin can directly stimulate at the level of ME (Keen et al., 2008). Interestingly, a study in mice found no kisspeptin fibre co-location with GnRH cell bodies in pre-puberty, but kisspeptin co-localization with GnRH cell bodies gradually increased during the pubertal period in both male and female mice (Clarkson and Herbison, 2006). Moreover, in primates and sheep, the kisspeptin cell bodies are located closer to the GnRH cell bodies than in rodents (Colledge, 2009).

Kisspeptin has been proven to directly initiate the process of sustained depolarization in GnRH neurons. This responsiveness of GnRH neurons to kisspeptin appears to change in relation to sexual development, increasing from 25% during the prepubertal period to more than 90% in adults. The cause of this increase is not completely understood, but Han et al. (2005) have suggested that this rise in GnRH neuron responsiveness is not related to GPR54 expression but instead to progressively increasing KISS1 gene expression. Kisspeptin induced GnRH neural depolarization, moreover, can be both prolonged and powerful– in studies on the POA region of mice it was found to continue up to 30 minutes after kisspeptin withdrawal, and to involve voltage ranges between 5mV to 22mV in up to 90% in the GnRH neurons in this region (Han et al., 2005; Liu et al., 2008). Since no difference between the sexes in the number of GnRH neurons was found, it is assumed that there is no sexual dimorphism in GPR54 expression by GnRH neurons (Liu et al., 2008).

Current- voltage relationship research as well as studies in the field of pharmacology suggest that inwardly rectifying potassium channel (K_{ir}) inhibition and sodium-dependent non-selective cation channel (NSCC) activation induce the

depolarization process. A study reporting that the inhibition of K_{ir} by intracellular caesium or extracellular barium (Ba^{2+}) caused a reduction of about 50% in the number of GnRH neurons responding to kisspeptin, appears to be related to this (Liu et al., 2008; Zhang et al., 2008), as does the finding that this same intracellular cesium or extracellular Ba^{2+} inhibition of K_{ir} had a strong effect on the reversal potential at hyperpolarized potentials (Zhang et al., 2008). One NSCC activity study indicates that the transient receptor potential cation (TRPC) channel family members might create these ion channels. Also subunit composition of individual GnRH neurons may affect TRPC channel activity (Zhang et al., 2008). A study by Kotani et al. (2001) found that kisspeptin binding to GPR54 couples to G protein $G_{q/11}$ to activate phosphalipase C (PLC) and increase intracellular Ins(1,4,5)P3 and Ca^{2+} release, arahidonic acid release, and activation of extracellular signal- related kinase 1/2 (ERK 1/2) and p38 mitogen-activated protein kinase (MAPK) pathways. This finding may indicate that this same signaling pathway is involved in kisspeptin-stimulated GnRH secretion (Castellano et al., 2006). The full details of this process by which the GnRH membrane is depolarized (including all the various messengers and channels) is not, however, completely understood (Colledge, 2009). In addition, it is possible that GnRH neurons maybe influenced indirectly by kisspeptin's synaptic effect on other neurons in the hypothalamus that express GPR54 (Herbison et al., 2010).

4.4 Action of kisspeptin on reproductive function

4.4.1 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., 2012).

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 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 POA and ME), then GnRH awake the reproductive axis and bring about pubertal maturation via hypophyseal portal circulation to stimulate the production and release of the gonadotropins such as LH and FSH (Kadokawa et al., 2008a; Roseweir and Millar, 2009). GnRH releasing is regulated by gonadal steroid feedback action (Tsukamura and Maeda, 2011).

4.4.2 Kisspeptin mechanisms in the 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 have found that OVX animals and estrogen replacement can effect kisspeptin expression in different regions of the hypothalamus which 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 the pulsatile, basal GnRH/LH releasing modes. The model is most well developed in the 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 affecting the 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 the 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

in which estrogen responsive cells in the AVPV project 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), but the major site of sex steroid negative feedback action is the mediobasal hypothalamus (Caraty et al., 1998). Furthermore, the positive feedback effect of estrogen is also seen in the ARC (specifically, in the caudal region) which increases the 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, demonstating 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 the different responses. Three types of estrogen feedback have been found to be significant 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 chronic effects of constant estrogen levels, which induce negative feedback, from the more intense increases in estrogen during the late follicular phase of the estrous cycle, which initiate the switch to transient positive feedback (Smith, 2009).

In earlier research, sex steroids were not found to have a regulatory effect on Kiss1 mRNA expression in the POA. However, one study doubled the number of POA tissue sections and reported that chronic estrogen replacement following OVX increased *Kiss1* mRNA expression and kisspeptin proteins in the POA. In addition, of the cells expressing kisspeptin, one half co-expressed ERα. It is possible that

kisspeptin cells in the POA of sheep may take part in the E-induced preovulatory surge of LH (similar to the process found in rodents in AVPV neurons), even though it is the action of estrogen in the ARC at the mediobasal hypothalamus, not the POA, that induces the GNRH/LH surge, If so, the positive feedback control of estrogen mostly likely indirectly sitimulates the sheep POA kisspeptin neurons (Smith, 2009). An illustration of the role of kisspeptin in reproductive function in rodents and ewes is presented in figure2.

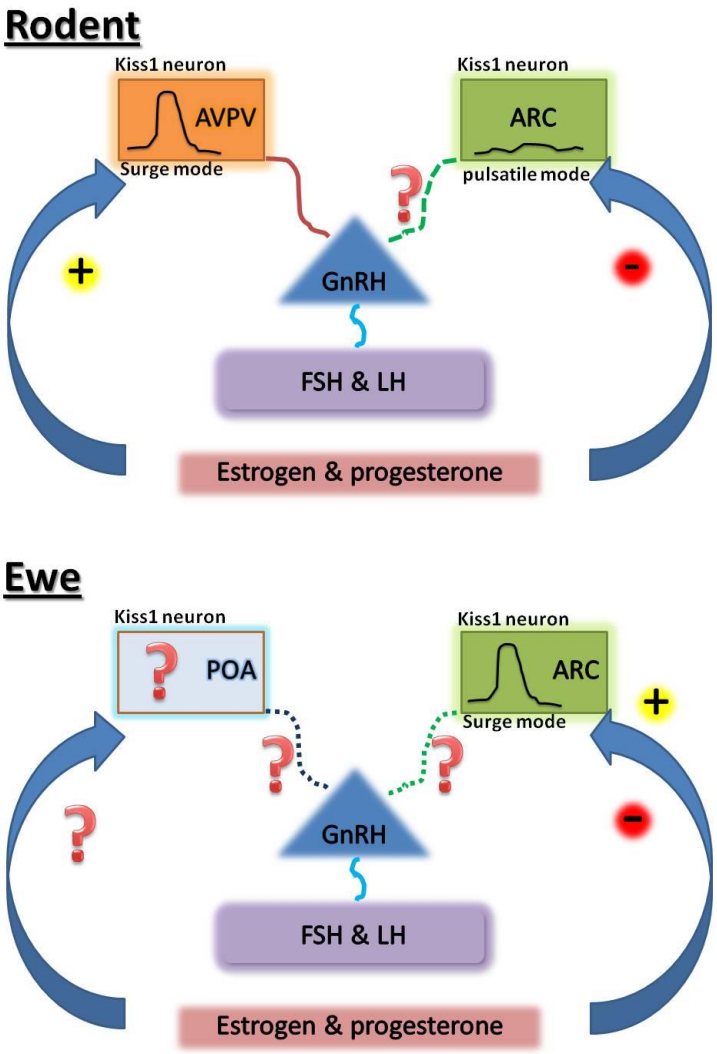

Figure 2 The positive feedback and negative feedback regulation of ovarian steroids in kisspeptin neurons and GnRH/LH stimulation. In the rodent, estrogen induces the AVPV kisspeptin expression as positive feedback regulation which stimulates the GnRH/LH surge mode directly. In the ARC area, kisspeptin neurons are blocked as negative feedback by estrogen which indirectly stimulates the GnRH neurons, inducing the GnRH/LH release in basal mode. In the ewe, estrogen effects the ARC kisspeptin neurons in both negative and positive feedback controls which activate the GnRH/LH sure mode. The kisspeptin neurons in the POA region also are identified, but it is unclear what their role is in the regulation of estrogen and GnRH neurons. Though kisspeptin -ir cells have been detected on GnRH neurons, their exact origin is still unknown (Smith, 2009).

4.5 Kisspeptin: reproductive research in ruminants

In ruminants, kisspeptin has been studied mainly in sheep and to some extent in goat and cattle, but not in buffalo.

In ewes, kisspeptin- immunoreactive cells are located in the POA and the ARC. In ruminants the dynamics involving kisspeptin cells in the POA is not fully understood yet, however in the ARC of ewes kisspeptin expression has been found to increase just before the preovulatory GnRH/LH surge. The "surge center" in ewes is located in the mediobasal hypothalamus for GnRH/LH releasing and GnRH neurons expressing GPR54. The rapid increase in LH secretion stimulated by peripheral administration of Kiss1 peptide to OVX ewes appears to reflect a direct action on the hypothalamus (Arreguin-Arevalo et al., 2007). Also, kisspeptin cells in the ARC express ERα and PR. This evidence indicates that kisspeptin may play a role in the steroid feedback control mechanism for GnRH secretion in ruminants (Estrada et al., 2006; Smith, 2009; Hashizume et al., 2010). Since sheep are seasonal (short-day) breeders their reproductive activity is activated by the photoperiodic hormone melatonin. During the non-breeding season (anestrus), GnRH secretion is decreased by both steroid-independent and steriod dependent mechanisms (Robinson et al., 1985; Barrell et al., 1992; Smith et al., 2007; Smith, 2009). Interestingly, the estrogen effects on Kiss1 mRNA and kisspeptin protein expression in the ARC are greater during the non-breeding season in ewes (Smith et al., 2008). Therefore, kisspeptin cells appear to be main candidates for facilitating the change in the feedback effect of estrogen (Smith, 2009). Furthermore, a seasonal alteration in Kiss1 expression in the ARC of OVX ewes intensely indicates that kisspeptin is involved in the control of seasonal changes in reproductive function (Smith et al., 2007).

In cattle, kisspeptin stimulated LH and, in addition, GH in OVX cows which were injected with Kp-10 in different doses. Importantly, this study found that the dose of Kp-10 used (100 pmol/kg b.w. or 0.13 µg/kg b.w.) to provide maximal LH response was 1/20th of the dose used in preputeal cattle and the rapid onset and also short duration of the LH response presented in this study is similar to that observed in pubertal gilts and adult ewes, which contrast to the response in prepubertal cattle (Whitlock et al., 2008). Also *in vitro* research indicates

kisspeptin, in ruminants, is related to the release of gonadotropin, GH and PRL (Hashizume et al., 2010). One study has indicated that Kp-10 treatment stimulated LH secretion from anterior pituitary cells in cattle (Ezzat et al., 2010).

In OVX goats, the peripheral infusion of Kp-10 stimulates GnRH neurosecretion into hypophyseal portal circulation and the action of kisspeptin on LH releasing is mediated by GnRH (Tanaka et al., 2012). Another study found that kisspeptin stimulated LH and FSH releasing but not GH and PRL releasing during the luteal phase in female goats, and that the releasing effect of LH and FSH from i.v. kisspeptin administration is less potent than that of i.v. GnRH administration (Hashizume et al., 2010). Additionally, other studies in the neuroendocrinology of ruminants with a multiple-unit activity have used an electrophysiological technique for monitoring the neural activity of GnRH pulse generation and were able to evaluate the factors effecting the GnRH pulse (for example; male pheromones, fasting, hypoglycemia and gonadal steroids- Okamura and Ohkura (2007)). Saito et al. (2012) found that in pre-pubertal as opposed to post-pubertal male goats the LH releasing response to Kp-10 is greater and that Kp-10, as well as GnRH, was able to initiate the release of testosterone. Additionally, in post-pubertal male goats the negative feedback control by testicular steroids of GnRH secretion increased.

Several *in vivo* studies report the maximum LH-releasing effect of Kp-10 i.v. injection) doses were observed at 0.54-0.65 μg/kg b.w. in OVX ewes (Caraty et al., 2007), 1 μg/kg b.w. in luteal phase goats (Hashizume et al., 2010), 0.13 μg/kg b.w. in OVX cows (Whitlock et al., 2008) and 4.76 μg/kg b.w. and in prepubertal heifers (Kadokawa et al., 2008a).

Chapter III

Methodology

Research pattern

Experimental research

Duration of study

Between November 2010 and May 2016 (5 years 6 months)

Year no./activities	1	2	3	4	5	6
Review literature	X	X	X			
Research planning		X	X			
Research performing			X	X		
Data analysis			X	X	X	
Thesis writing and publication			X	X	X	X
Thesis defense						X

Places of study

1. Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
2. Research and Development Centre for Livestock Production Technology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand
3. Private buffalo farm, Phayao, Thailand
4. Murrah Farm, Chacheungsao, Thailand

5. Faculty of Veterinary Medicine, Mahanakorn University of Technology, Nongchok, Bangkok, Thailand

Animals

Animal ethic

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).

Animals for part I and part II

The brains were collected from 6 cycling buffaloes (age 4-7 years old and body condition score 3-4 out of 5) from commercial slaughter houses. All of the animals presented a corpus luteum and/ or follicle in their ovaries which were detected by rectal palpation before slathering (Fig. 3). The heads were perfused with 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. 4). The brains were then removed and the hypothalamic and pituitary glands were collected and fixed in the same fixative for 24 hr (Fig. 5-6). The samples were prepared for paraffin blocks and stored at room temperature (RT).

All brains were identified according to the phase of their estrous cycle (Ali et al., 2003; Tienthai et al., 2008).

Luteal phase brains (n=3; cow number 1, 2, 3) were determined by the fact that the buffalo do not show estrous signs such as vulva edema, cleared vaginal discharge and a protruding corpus luteum present in the ovary which was detected by observation and rectal palpation before slaughter. Then confirmation was done by means of the postmortem ovarian morphology (Fig. 5).

Follicular phase brains (n=3; cow number 4, 5, 6) were determined according to the buffalo's estrous signs such as vulva edema, cleared vaginal discharge and a more than 1 cm of diameter of follicle present in the ovary which was detected by

observation and rectal palpation before slaughter. Then confirmation was done by means of the postmortem ovarian morphology (Fig. 6).

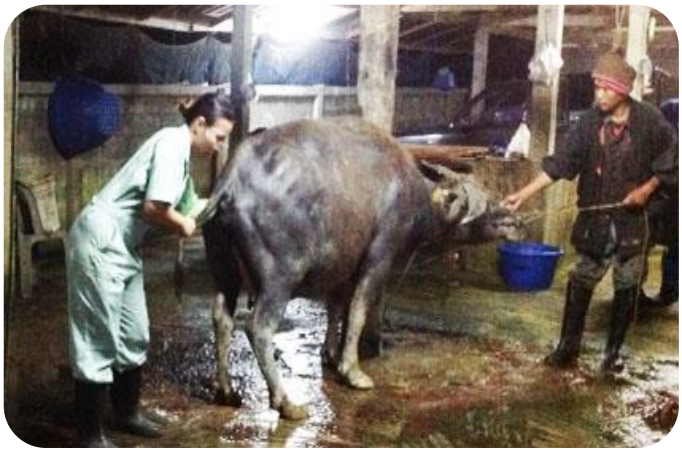

Figure 3 The buffalo cow is identified the stage of estrous cycle by rectal palpation.

Figure 4 The head is perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) into a common carotid artery.

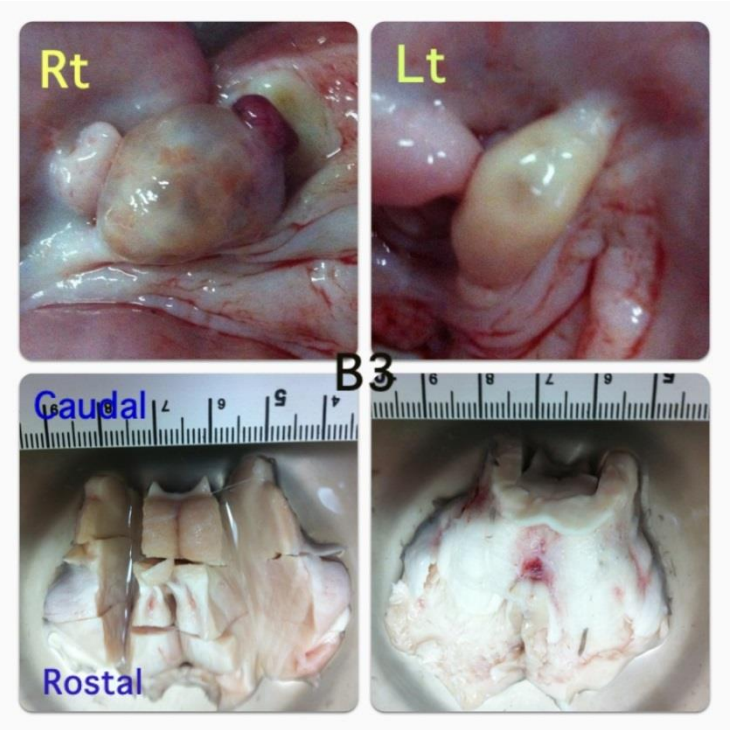

Figure 5 The postmortem hypothalamus and ovarian morphology from luteal phase cow. There is an active corpus luteum in the right ovary.

Figure 6 The postmortem hypothalamus, pituitary gland and ovarian morphology from follicular phase cow. There is a dominant follicle in the left ovary.

Animals for part III

Six healthy postpartum buffalo cows which have the age between 4-6 years (using dental age estimation technique; Moran (1992)) and day opened ≥45 days were selected for this study. Each treatment in this study used the same animals in the luteal phase of estrous cycle (day 10 after onset of estrus, using estrus synchronization) with an estrous cycle interval waiting period.

In waiting period (without experimental activity), the buffaloes were freed feeding by conventional grassing in the fields and bathing in the natural pounds during 8.00 am to 5 pm (Fig. 7A, 7B). The animals were housed in individual stable during the blood collecting time on the experimental day (7 am – 5 pm). They were fed fresh grass, hay and water ad libitum (Fig. 7C, 7D).

Figure 7 Buffaloes are fed by grassing in the field freely during waiting period (A, B). In the experimental period, the buffalo stay in their section in the stable. Cut and carry feeding system is applied by the farm manager (C, D).

Materials

Anatomical details of hypothalamus in buffalo

Since no anatomical references are available for the anatomical structure of the POA and ARC hypothalamic nuclei of buffaloes these were determined in our study by standard cresyl violet staining of the adjacent hypothalamic sections - a technique previously used in sheep (anatomical sources: Haines (2003)). The histological location of the POA and ARC areas were determined by hematoxylin and eosin staining on the sample slides.

The random samples of POA and ARC hypothalamic nuclei of each buffalo, used for ISH and IHC in part I and part II, were serial sample slides, each one taken from tissue adjacent to the other.

Part I: Detection of the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle

In situ hybridization of *Kiss1* mRNA

The ISH protocol was modified following 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. The main 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 are ruminants and seasonal (short-day) breeders similar to sheep (Borghese and Mazzi, 2005; Mondal et al., 2007). 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. 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 rehydrated sections were immersed in a citrate buffer (Bio-Optica, Milano, Italy) (pH 6.0) and autoclaved for 10 min at 121 ℃. 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 with a hybridization cocktail (Hybridization cocktail 50% formamide, Amresco, Ohio, USA) for 1 hr at RT. A complimentary RNA probe was heated at 95 ℃ for 5 min and then diluted with a hybridization cocktail. The hybridization was done at 45 ℃ 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 stringent washing was conducted at 45 ℃. Next, the sections were equilibrated in a 0.1 M Tris-HCl buffer in 150 mM NaCl (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 kisspeptin immunohistochemical 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 shown a species reactivity in ovine, rat and mouse but a very low level of reactivity with human kisspeptin10. In addition, its' specificity has been proven not 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 in immunostaining) (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. 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 Kp-ir (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 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 Axiovision software (Axiolab, Zeiss, Oberkochen, Germany).

Controls and specificity

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 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.

Part II: Determination of the possible relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland

The optimum condition of immunohistochemistry for detection of kisspeptin receptors, GnRH neurons in hypothalamus and GnRH receptors in pituitary were discovered by the standard immunohistochemistry.

Double label immunohistochemistry for KISS1R and GnRH in the POA and ARC hypothalamic nuclei

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 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. 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 Axiovision 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 (Zerani et al., 2012) and it has been shown to have cross reactivity in rats, mice, hamsters, sheep and monkeys (El-Majdoubi and Weiner, 2002; Pompolo et al., 2003). 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.

Single label immunohistochemistry for estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei

The sections from POA and ARC hypothalamic nuclei were prepared at 4 microns for standard single label immunohistochemistry study. Paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethyl alcohol. Antigen retrieval in citrate buffer (pH 6.0) was done for 10 min at 121 °C by autoclaving. 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 goat serum (catalog number X0907, Dako, Glostrup, Denmark) for 20 min at RT. Then the sections were incubated with 1:150 dilution of a rabbit anti- human ERα polyclonal antibody (ERα (H-184), catalog number sc-7207, Santa Cruz Biotechnology, Texas, USA) and 1:250 dilution of a rabbit anti- human PR polyclonal antibody (PR (C-19), catalog number sc-538, Santa Cruz Biotechnology, Texas, USA) at 4 °C, overnight (16 hr). After that, 1:250 dilution of a polyclonal goat anti-rabbit immunoglobulins/biotinylated (catalog number E0432, Dako, Glostrup, Denmark) 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). In each step, the sections were washed in 10 mM phosphate buffer saline (PBS; Bio-Optica, Milano, Italy) (pH 7.2). The Nissl counter- stained by heamatoxilin was applied on the sample slides. ERα and PR -ir cells were identified under light microscope (Axiolab, Zeiss, Oberkochen, Germany), with a single observer counting the number of ERα and PR -ir cells found in the 100 mm^2 area per slide from each of the buffalo cow's POA and ARC hypothalamic nuclei. The images were captured by Axiovision software (Axiolab, Zeiss, Oberkochen, Germany).

Controls and specificity

This ERα antibody has shown a species reactivity in bovine, equine, canine, porcine, rat, mouse and human confirmed by the company product data sheet. For the PR antibody, it is recommended for detection of PR of bovine, equine, canine, porcine, avian, mouse, rat and human. Protein alignment of buffalo ERα showed 99% and 85% amino acid homology with ovine and human, respectively. For the PR antibody, it is recommended for detection of PR of bovine, equine, canine, porcine, avian, mouse, rat and human. Homology of the PR amino acid between buffalo and ovine and human are 53% and 77%, respectively. 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.

Single label immunohistochemistry for GnRH receptors in the pituitary gland

Serial paraffin sections from each block of pituitary gland samples were prepared at 4 microns for GnRHR immunohistochemical study. The sections were deparaffinized and rehydrated in a graded series of ethyl alcohol. Antigen retrieval was done by 800 w in microwave for 10 min. Following this step, the endogenous peroxidase activity was blocked by incubating in 1% hydrogen peroxide in methanol for 30 min at room temperature. The non- specific binding was blocked using 10% normal horse serum (Gibco®) for 20 min at room temperature. The sections then were incubated with the 1:100 goat polyclonal anti-human GnRH receptor antibody (catalog no. sc-8682, Santa Cruz®) at 4 °C, overnight (16 hr). After that, a biotinylated link (LSAB+System-HRP, catalog no.K0679, Dako®) was used at room temperature for an hour. The sections then were incubated with streptavidine horseradish peroxidase (LSAB+System-HRP, catalog no.K0679, Dako®) at room temperature for 50 min. In the final step, 3, 3'-diaminobenzidine (DAB, catalog no. K3467, Dako®), a chromogen, was added to visualize bound enzyme (brown colour). In each step, the sections were washed in 10 mM phosphate buffer saline (PBS; pH 7.2). Then counter- stained by

hematoxylin, was applied on the sample slides. After that the GnRHR-ir cells were identified under light microscope. The images were captured by Axiovision software (Axiolab, Zeiss, Oberkochen, Germany).

Controls and specificity

Positive control reaction was prepared using sheep pituitary gland, and negative control reaction was conducted using PBS instead of primary antibody application.

Part III Comparative study on the effects of administration of kisspeptin-10 and GnRH on the characteristics of LH secretion in luteal phase

There were 3 treatments in this study: 1. Kp-10 treatment 2. GnRH treatment and 3. Distilled water treatment. All animals were used by repeated measurement experimental design. Initially, the cows were synchronized their estrous phase before each treatment was performed.

Estrous synchronization

The animals were synchronized of the estrous cycle by 2 ml prostaglandin F2α (PF2α) intramusculary (500 µg Cloprostenol, Estrumate, Merck Animal Health, USA) when the corpus luteum presents on the ovary, which was detected by ultrasounography. Estrous signs were detected by observation twice a day after PF2α administration. The early luteal phase (day 10 after onset of estrus) was confirmed by rectal palpation for ovarian structure which presents an early corpus luteum and without dominant follicle. Also the plasma progesterone concentration on the experimental day of all cows was analyzed by radioimmunoassay (RIA) which was >1 ng/ml.

Treatment application

***1. Kp-10 treatment*:** Animals were received a single intravenous injection (at jugular vein) of kisspeptin-10 (human metastin 45-54 (YNWNSFGLRF-NH2), 4389-V2, Peptide Institute Inc., Osaka, Japan) on 1,000 pmol/kg b.w. or 1.3 µg/kg b.w. a dose dissolved in 2 ml distilled water. This dosage is the optimal dose from preliminary study which had been titrated form 100, 200, 500, 1,000 and 2,000 pmol/kg b.w. The Kp-10 dose started at 100 pmol/kg b.w. or 0.13 µg/kg b.w. a dose which has been proven to stimulate LH secretion after single i.v. administration in cattle (Whitlock et al., 2008). The details for dosage calculation and cost are presented in the appendix.

***2. GnRH treatment*:** The cows were administrated a single intramuscular injection of 10 µg GnRH (Buserelin, Receptal®, Intervet, Netherlands).

***3. Distilled water treatment*:** Buffaloes were applied a single intravenous injection (at jugular vein) of 2 ml distilled water for injection.

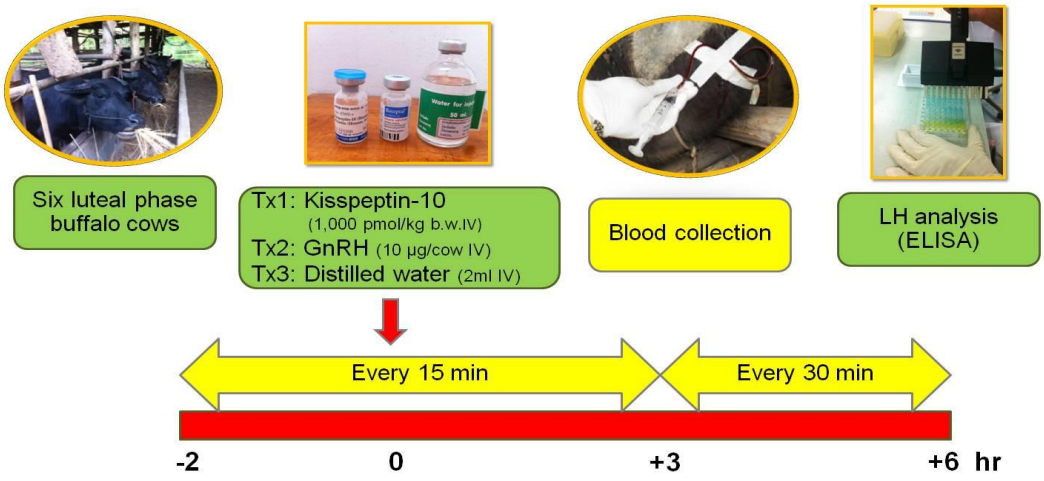

Figure 8 The illustration of the protocol in our part III, in vivo experiment.

Blood sample collection

On the experimental day at 7.00 am, the buffaloes were fitted with an indwelling jugular vein catheter (Fig. 9). Blood was collected into a 4 ml tube (Heparin; anticoagulation) and placed on ice every 15 min intervals between -2 hr and 3 hr (time=0 hr ; injective time) and every 30 min after that for the next 3 hr (total

experimental time was 8 hr). After centrifugation at 3000 x g for 5 min, plasma was harvested and stored at -20 ^{o}C until the analysis of plasma LH and progesterone concentration.

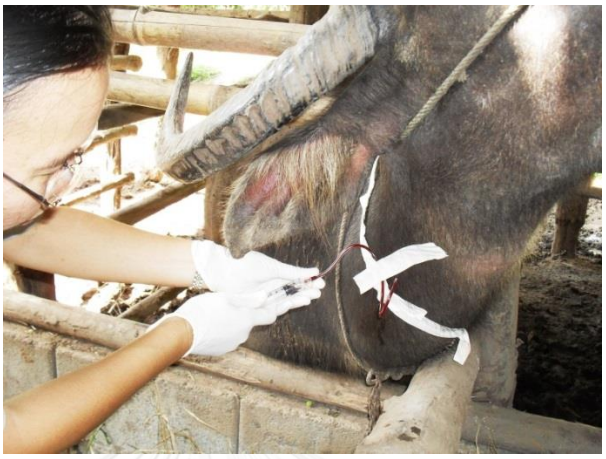

Figure 9 The buffalo is restrained and fitted with an indwelling jugular vein catheter during experimental period.

Plasma luteinizing hormone (LH) and progesterone analysis

For treatment response assessment, plasma samples were measured the LH concentration by a commercial test kit enzymatic immunoassay (EIA) (LH DETECT for Buffalo, Repropharm, France), followed the kit's instruction. The LH intra- and inter-assay coefficients of variation were 32.16% and 39.53%, respectively and sensitivity was 0.25 ng/ml.

For stage of estrous identification, plasma progesterone concentration was analyzed by radioimmunoassay (RIA) (Suthikrai, 1994). The progesterone intra- and inter-assay coefficients of variation were 7.7 and 13.9, respectively and sensitivity was 0.01 ng/ml.

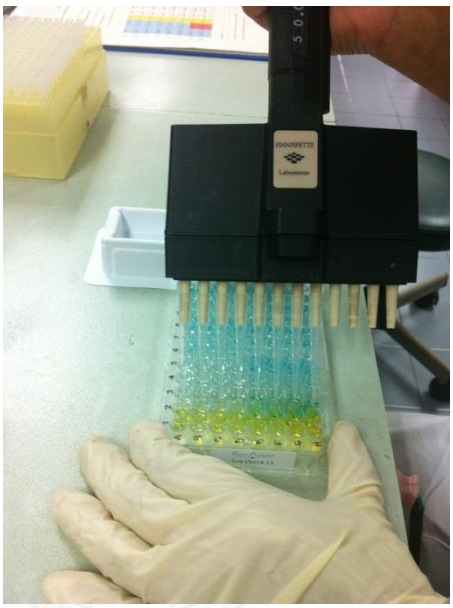

Figure 10 A commercial test kit enzymatic immunoassay for buffalo LH plasma concentration is performed in this study.

Statistical analysis

Part I: Detection of the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the preoptic area (POA) and arcuate (ARC) hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle

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 (±SEM). The comparison of the average number of kisspeptin-ir cells between the POA and ARC were analyzed by t-test ($P<0.05$). The distribution of kisspeptin-ir neurons was described.

Part II: Determination of the possible relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland

Double label immunohistochemistry for KISS1R and GnRH in the POA and ARC hypothalamic nuclei

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 (± SEM). 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.

Single label immunohistochemistry for estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei

The number of each type of immunoreactive neurons were calculated as a percentage of the total number and then were averaged across animals to calculate a mean (±SEM). A comparison between the average number of the ERα and PR -ir neurons in the POA and ARC was done by t-test ($P<0.05$). Characterizations of ERα and PR immunoreactions were described.

Single label immunohistochemistry for GnRH receptors in the pituitary gland

The number of GnRHR-ir cells in each part of pituitary gland was classified as minority (<30% of total cells), intermediate (30-70% of total cells) and majority (>70% of total cells). A comparison of the average number of immunoreactive cells in each part of pituitary gland was made within groups (follicular phase or luteal phase) and between groups (follicular phase and luteal phase) by a t-test ($P<0.05$).

Part III Comparative study on the effects of administration of kisspeptin-10 and GnRH on the characteristics of LH secretion in luteal phase buffalo

All data in each treatment group were presented as the mean ± SEM. The peak-shaped responses were observed in the concentrations of LH. The maximum LH concentration in each treatment were observed and calculated among serial samples during the time length of peak concentration as well as the timing of the samples containing them. The area under curve (AUC) of the LH response curves, as linear trapezoidal summation between successive pairs of concentration and time, were calculated. The AUCs of LH in each treatment groups were analyzed by repeated measures ANOVA and the comparison of each sampling time between treatment groups were determined by Student's t-test ($P<0.05$). The SAS System was used for statistical analysis.

Chapter IV

Results

Anatomical details of hypothalamus in buffalo

The buffalo hypothalamus was identified approximately 15.00 cm down from the skull bregma. In cross section, optic chiasma (OC) and mammary bodies were the main area for identification of POA and ARC, respectively. The POA was microscopically observed above the OC. The median part of the POA was seen close to the 3rd ventricle (3V) and the lateral part is shown in figure 11A and 12. The ARC area was identified above the 3V and in front of the mammary bodies (Fig. 11B and 12). In longitudinal section (Fig. 11C), the medial POA was found 2.5 mm above the OC. The supraoptic area (SON) was detected behind the OC. The ARC was found in a caudal part of the SON at 3.9 mm and was seen as a long area through the caudoventral part.

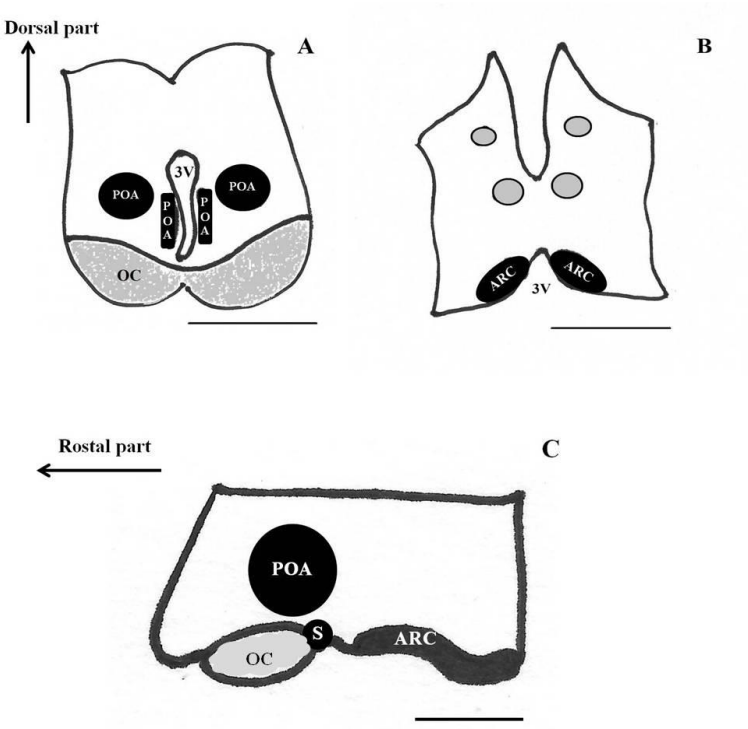

Figure 11 A drawing of the anatomical and histological location of the POA (Fig. 11A) and ARC (Fig. 11B) hypothalamic nuclei in buffaloes as identified on the sample slides in cross sections. The POA (black areas) is located above the optic chiasma (OC). The black rod shaped areas on either side of the 3rd ventricle (3V) are the median POA and the black oval shaped areas are the lateral POA. The ARC area (black areas) is located above the 3V and in front of the mammary bodies. The longitudinal section of buffalo hypothalamus (Fig. 11C) shows the medial POA (black area) located above the OC. The supraoptic area (S) is located next to the OC and has a slightly triangular shape. The ARC (thick curved black line area) is located from S trough the caudal ventral part. The grey areas are nerve tracts. Scale bar is 10 mm in A, B and C.

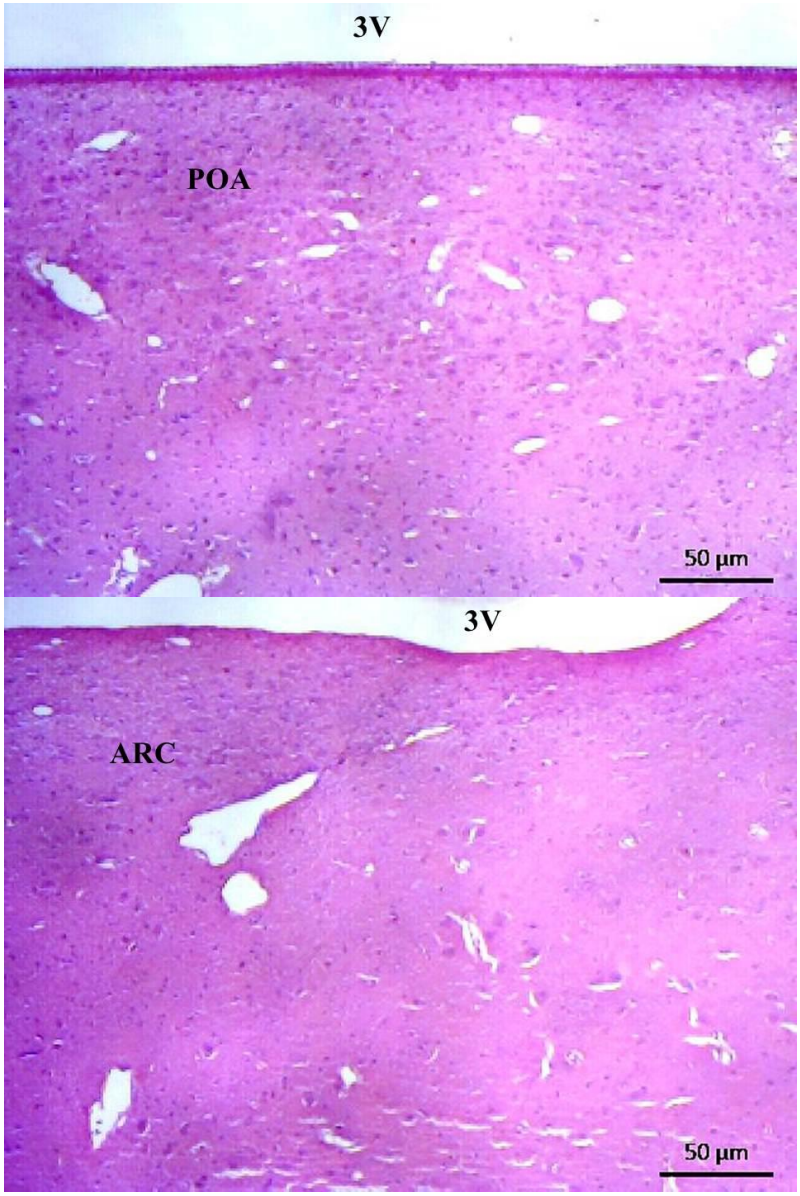

Figure 12 The histological appearances of the POA and ARC hypothalamic nuclei in buffaloes as identified on the sample slides in cross sections using the hematoxylin and eosin stain. Both POA and ARC area are located near the third ventricle (3V) Scale bar is 50 µm.

Part I: Detection of the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle

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 in both the buffalo POA (Fig. 13A and 13C) and ARC hypothalamic nuclei (Fig. 14A and 14C) of all samples. *Kiss1*mRNA was also found in some small neuronal cells (Fig. 13C) which were distinguished from glias by their vesicular nuclei. There was no signal of *Kiss1* mRNA in the buffalo POA and ARC sections in which the sense *Kiss1*cRNA probe was applied (Fig. 13B, 13D, 14B and 14D) and these were considered as negative control reactions. Positive control reactions were prepared using the ewe POA (Fig. 13E and 13F) and ARC (Fig. 14E and 14F) hypothalamic nuclei paraffin sections.

Immunohistochemistry of kisspeptin

The results showed reactions of kisspeptin located in the cytoplasm of the neuronal soma and some glias (Fig. 15A and 15B) in both the POA and ARC hypothalamic nuclei. In addition, kisspeptin proteins were also found in the cellular process of the POA and ARC neurons (Fig. 16B). Our immunohistochemical study of random samples also revealed a larger and denser population of Kp -ir neurons in the POA (79.8±2.5%) than in the ARC (62.5±4.5%) area ($P \leq 0.01$). The distribution pattern of kisspeptin-ir cells in the POA had a more scattered and widespread distribution of these cells throughout this area (Fig. 16A), as opposed to kisspeptin-ir cells in the ARC area which had a clumpy appearance (Fig. 16B). The negative control presented no non-specific reactions (Fig. 15C). The positive control for immunohistochemical reactions of kisspeptin proteins in the POA hypothalamic neurons in the ewe are shown in figure 15D.

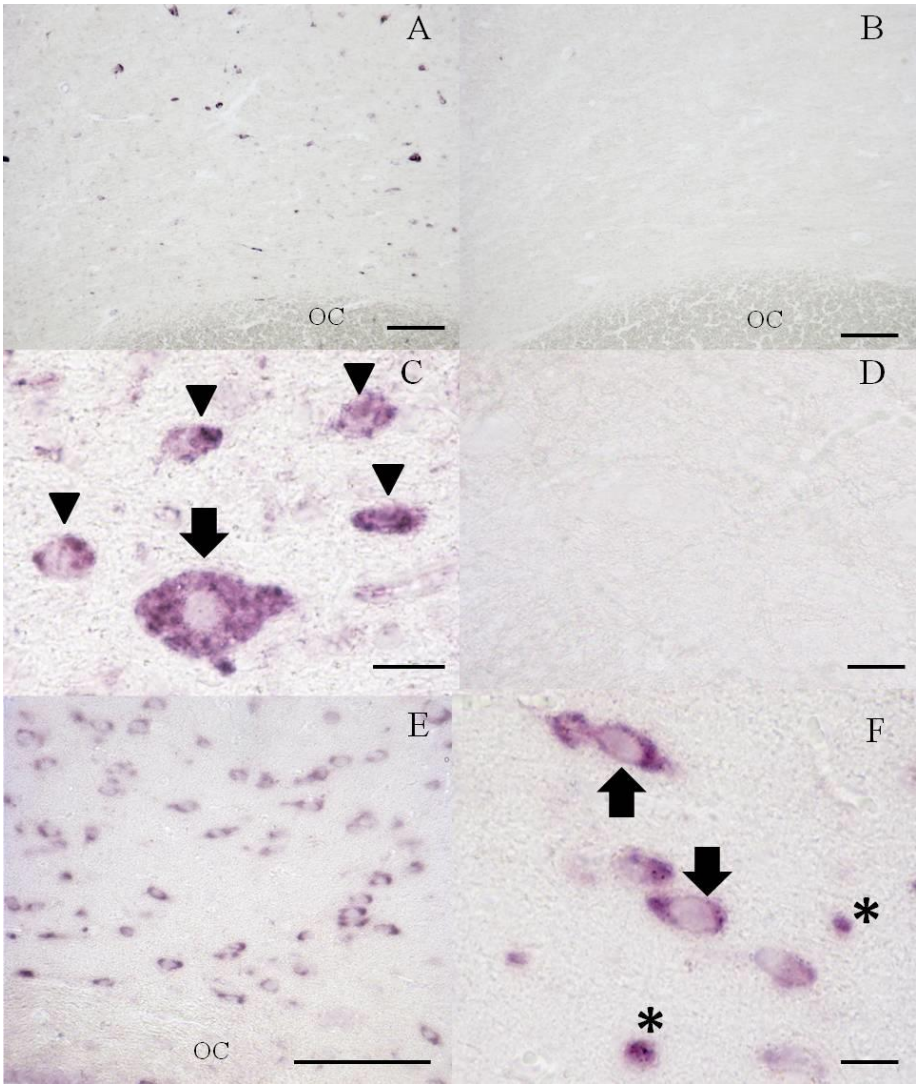

Figure 13 *Kiss1* mRNA in the POA, which were taken from the area beside the optic chiasma (OC), is visible in the anti-sense (positive result) of *Kiss1* mRNA samples (A and C) and is not expressed in the sense (negative result) of *Kiss1* mRNA samples (B, D). *Kiss1* mRNA expressions are localized in the cytoplasm of a neuron (full arrow) and some small neuronal cells (arrow heads) in C. In ewe (the positive control), *Kiss1* mRNA expressions are visible (E and F) in the cytoplasm of neurons (full arrows) and some glias which present dense nuclei (asterisks). Scale bar is 100 μm in A, B and E but it is 10 μm in C, D and F.

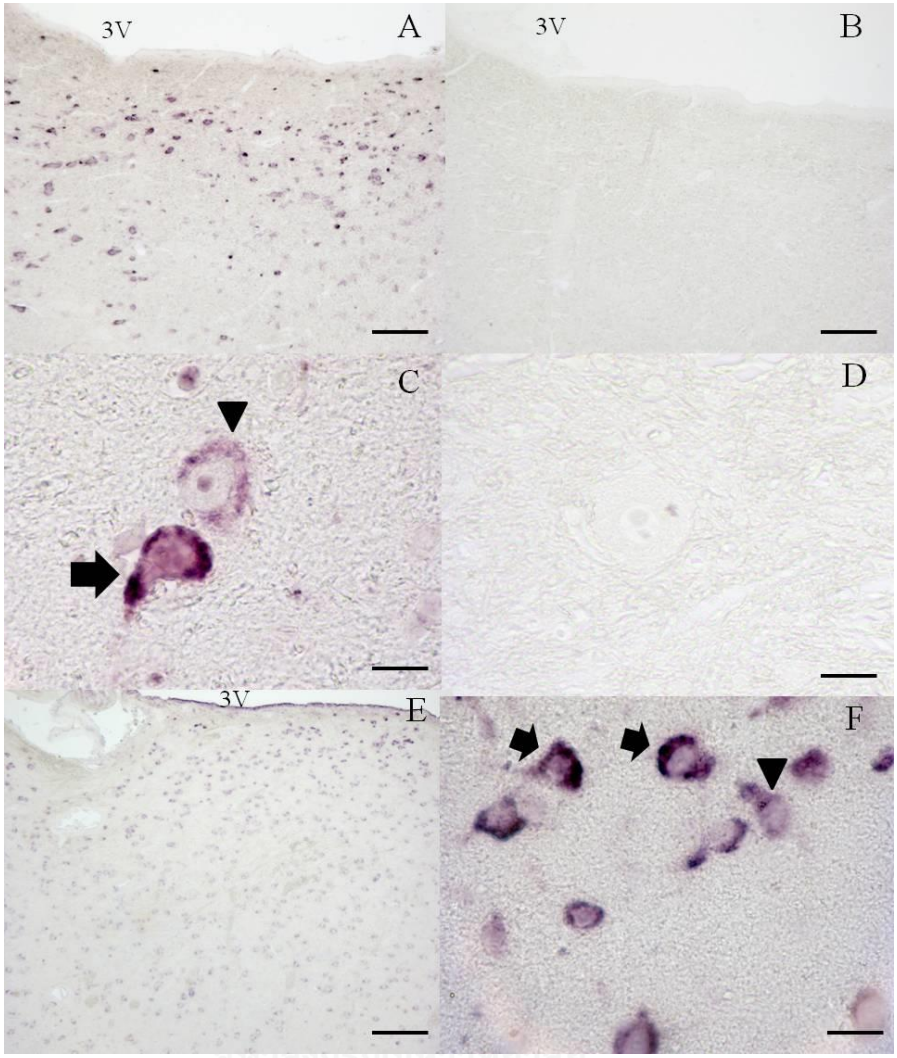

Figure 14 In the ARC samples, *Kiss1* mRNA is visible in the anti-sense (positive result) of *Kiss1* mRNA sample (A, C) but not expressed in the sense (negative result) of *Kiss1* mRNA sample (B, D). The *Kiss1* mRNA is localized in the cytoplasm of a neuron with a strong signal (full arrow) but a weak signal in another neuron (arrow head) in C. In ewe as the positive control sample, *Kiss1* mRNA shows a strongly visible signal in the cytoplasm of neurons (full arrows) and a weak signal in another neuron (arrow head) in E and F. Scale bar is 100 μm in A, B and E but it is 10 μm in C, D and F.

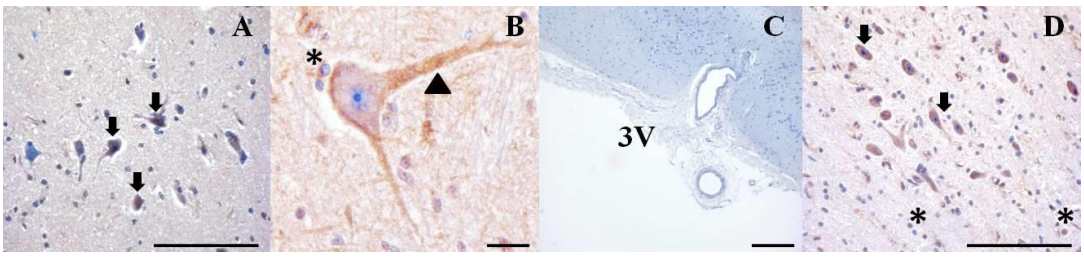

Figure 15 In hematoxylin counter- stained sections, the kisspeptin immunoreactions (full arrows, A) are located in the neuron cell bodies (in both the cytoplasm and axon) with a dendritic tree when visualized (arrow head, B). Some small neuronal cells present kisspeptin immunoreactions (asterisk, B). There is no reaction in the buffalo POA negative control slide (without primary antibody application, C). In the ewe POA sample (D) the kisspeptin immunoreactions in the neuron cell bodies (full arrows) and some small neuronal cells (asterisks) appear similar to those in the buffalo sample. Scale bar is 100 μm in A, C and D but it is 10 μm in B.

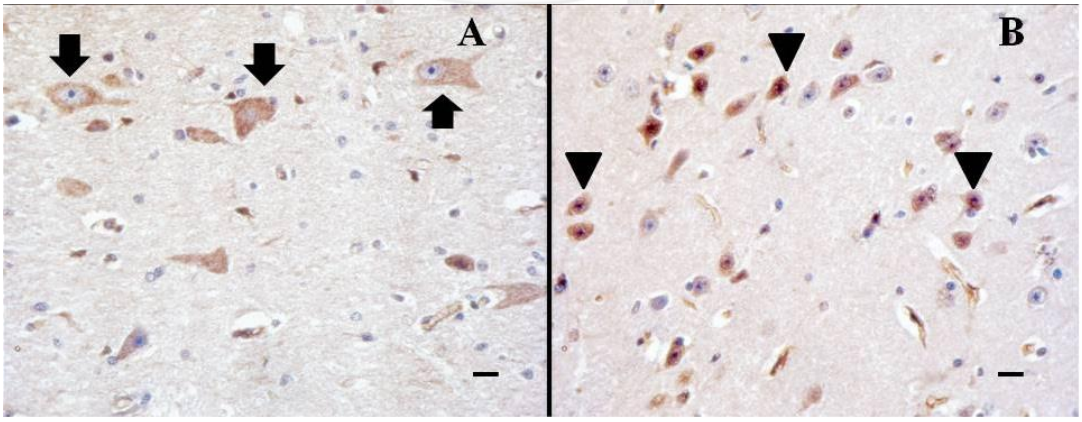

Figure 16 In the POA (A), the kisspeptin-ir neurons are larger (full arrows) and their distribution is more diffuse than in the ARC (B) hypothalamic nucleus neurons (arrow heads) in our studies' buffalo samples. Scale bar is 10 μm in both A and B.

Part II: Determination of the possible relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland

The percentage of each type of immunoreactive neuron in the POA and ARC hypothalamic nuclei of each buffalo cows (i.e. kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors are presented in the appendix.

Double label immunohistochemistry for KISS1R and GnRH in the POA and ARC hypothalamic nuclei

The KISS1R antibody in this study was validated by the detection of immunoreactivity in the cytoplasm of some neurons in the POA hypothalamic nucleus (positive control) of wild-type mice (Fig. 17e, 17f) but not in sections from *Kiss1r* KO mice (Fig. 17c, 17d). The positive controls for antibody and tissue specificity in the POA hypothalamic nucleus of ewe are shown in figure 18 (b1-b4). The pattern of immunoreactivity for KISS1R and GnRH in ewe and buffalo was found to be similar (Fig. 18, a1-a4, b1-b4). In both the buffalo POA and ARC areas, KISS1R-ir was detected in neuronal soma and some glia (Fig. 17a, 17b, 19a, 20a). GnRH-ir appeared to be granular in the cytoplasm of neuronal soma (Fig. 19b, 20b). The KISS1R-ir neuron population in the POA was the same as in the ARC (93±2 vs 93.2±2.4 %), and although the GnRH-ir neuron population in the POA (64.5±4.3%) trended lower than in the ARC (72.8±6.2%) – the difference was not statistically significant. Double label results showed that all observed GnRH-ir neurons were co-localized with KISS1R-ir with no difference in population between the follicular phase and the luteal phase cows in both hypothalamic nuclei (Fig. 21).

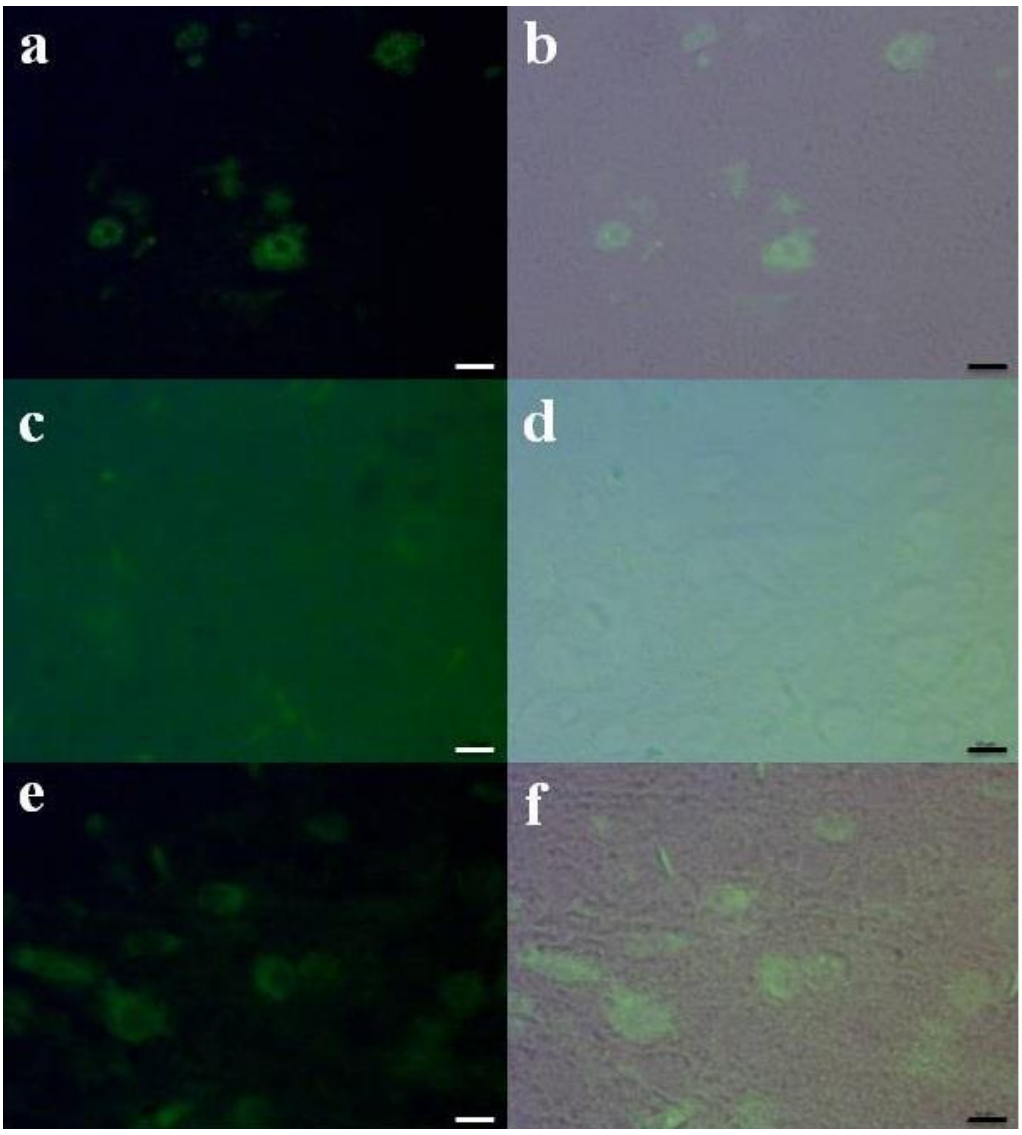

Figure 17 KISS1R antibody validation in the positive and negative control samples; buffalo, wild type and *Kiss1r* KO mice POA hypothalamic nuclei. KISS1R immunoreactivity was found in the POA of buffalo (a, b), wild type mice (b, c) but not in Kiss1r KO mice (e, f)**.** Scale bar = 10 μm.

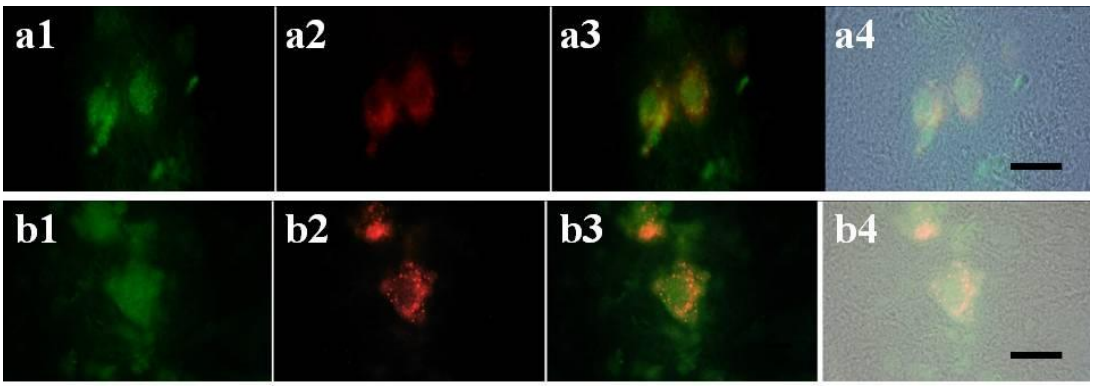

Figure 18 Immunoreactivity pattern for KISS1R and GnRH in the ewe and the buffalo. Both ewe (b1-b4) and buffalo (a1-a4) POA immunoreactivity for KISS1R (green, a1, b1) and GnRH (red, a2, b2) were similar. KISS1R-ir was detected in the neuronal soma and some glia (a1, b1). GnRH-ir appeared to be granular in the cytoplasm of neuronal soma (a2, b2). Co-localizations of KISS1R and GnRH-ir are shown (a3, b3). Combination of the double label images and differential interference contrast (DIC) images are shown (a4, b4). Scale bar = 10 μm.

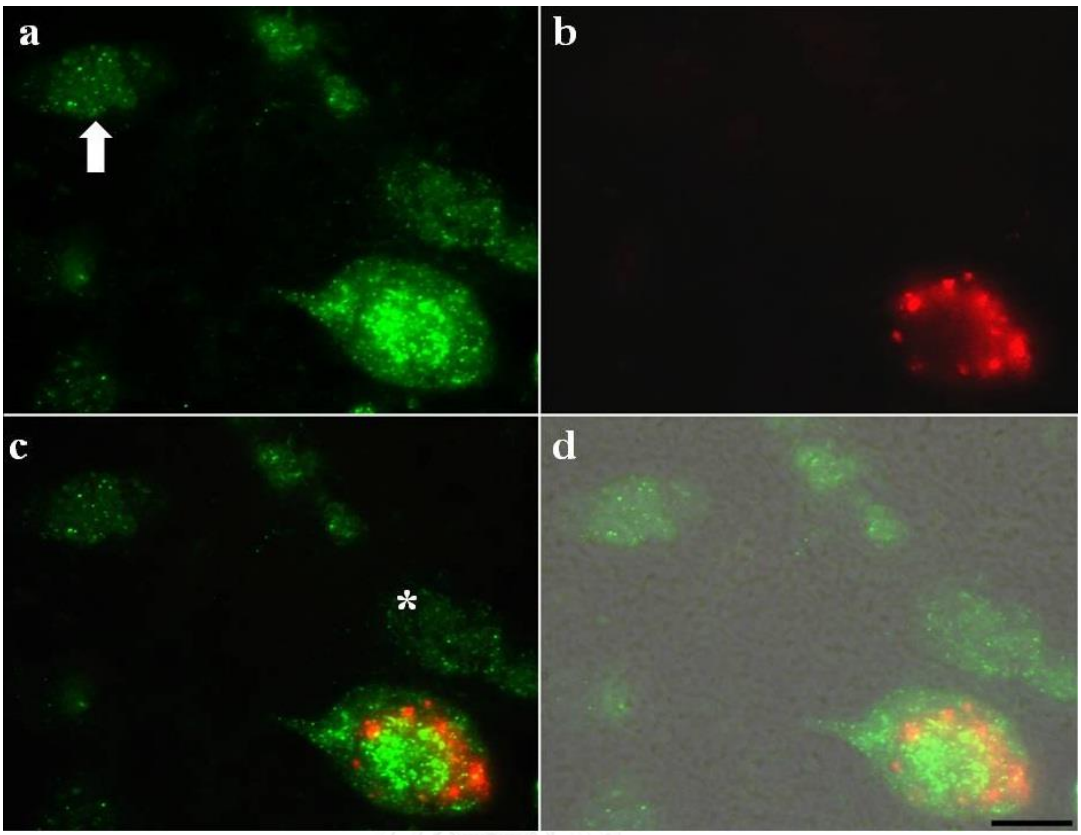

Figure 19 Double label immunohistochemistry for KISS1R and GnRH in the buffalo POA hypothalamic nucleus. KISS1R-ir was detected not only in the neuronal soma but also in glia cells (arrow in a). GnRH-ir appears as red granular formations in a neuron (b). Combination of the double label images and differential interference contrast (DIC) images are shown (c, d). Co-localized neurons are found in GnRH-ir neurons but some neurons show the KISS1R-ir without GnRH immunoreaction (asterisk in c). Scale bar = 10 μm.

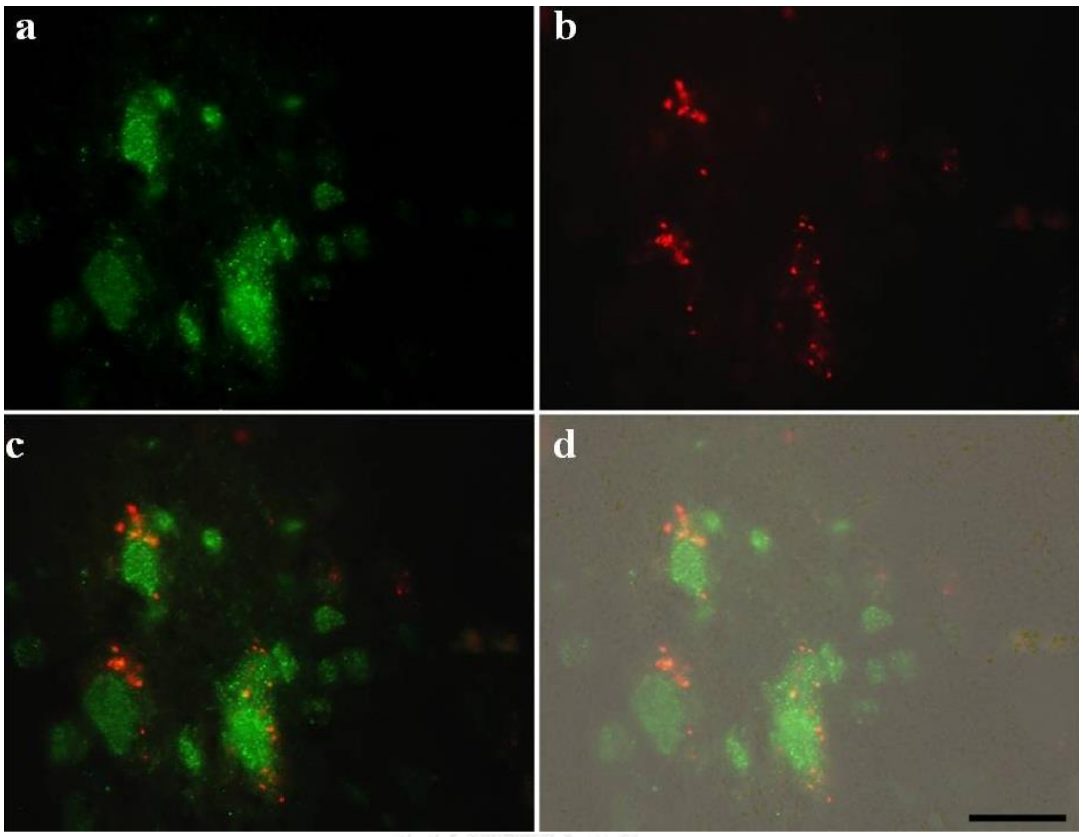

Figure 20 Double label immunohistochemistry for KISS1R and GnRH in the buffalo ARC hypothalamic nucleus. KISS1R-ir was detected in the neuronal soma in green color (a). GnRH-ir appears as red granular formations in the cytoplasm of neuronal soma (b). Combination of the double label images and DIC images are shown (c, d). Co-expressed neurons are presented in all GnRH -ir neurons (c, d). Scale bar = 10 μm.

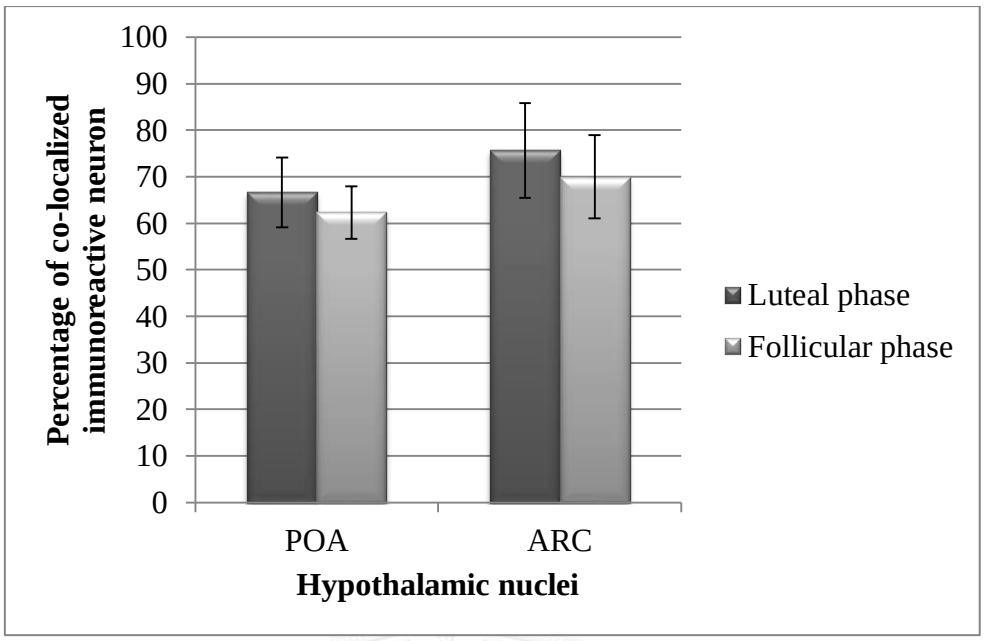

Figure 21 The percentage of double-labeled KISS1R and GnRH -ir neurons in estrous cycle cows. There was no difference in the co-localized KISS1R and GnRH -ir neuron populations in both the POA and ARC hypothalamic nuclei between buffalo cows in the follicular phase and luteal phase (P>0.05).

Single label immunohistochemistry for estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei

Both ERα (Fig. 22) and PR (Fig. 23) immunoreactions were found in the nucleus of some POA and ARC neurons, some small neuronal cells and some endothelial cells. The percentage of ERα and PR -ir neurons (mean±SEM) distributed in the POA (ERα; 76.17±4.06%, PR; 42.83±10.61%) were greater than in the ARC area (ERα; 51±6.85, PR; 25.33±5.46%) by a statistically significant percentage for ERα (P<0.01) but not for PR.

The percentages of ERα -ir neurons (POA; 76.33±15.04% vs 76±4.58%, ARC; 56±17.09% vs 46±18.36%) in both the follicular and luteal phase, respectively, (P>0.5) showed no statistically significant differences (Fig. 24) and were similar to the percentages of PR immunoreative neurons in the POA (follicular phase; 30.33±20.5% vs luteal phase; 55.33±28.29%) and ARC (follicular phase; 26±10.82% vs luteal phase;

24.67±18.15%). Remarkably, the percentage of PR -ir neurons in the POA area in luteal phase trends higher than follicular phase (Fig. 25).

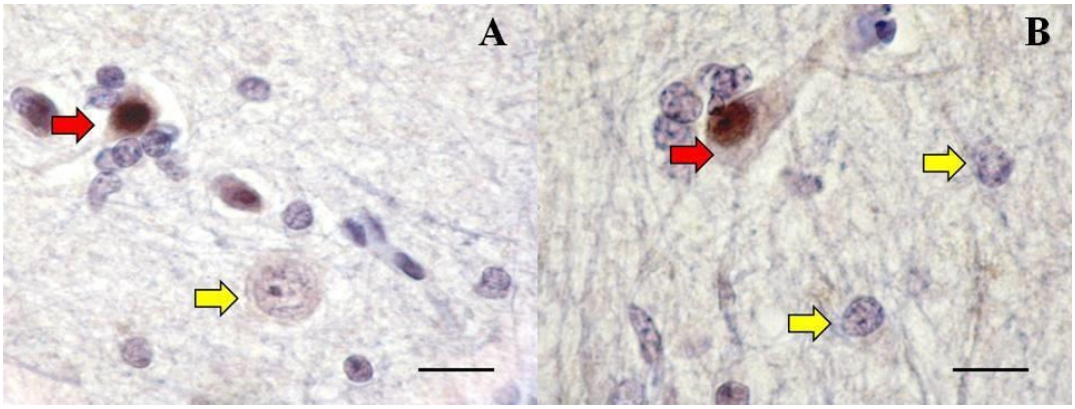

Figure 22 The hematoxylin counter- stained is applied on the sections. Expression of ERα immunoreactions in the POA (A) and ARC (B) neurons; ERα immunoreactions are presented in nucleus of some neurons (red arrows). In contrast, a few neurons express negative immunoreaction for ERα (yellow arrow). Scale bar is 10 μm.

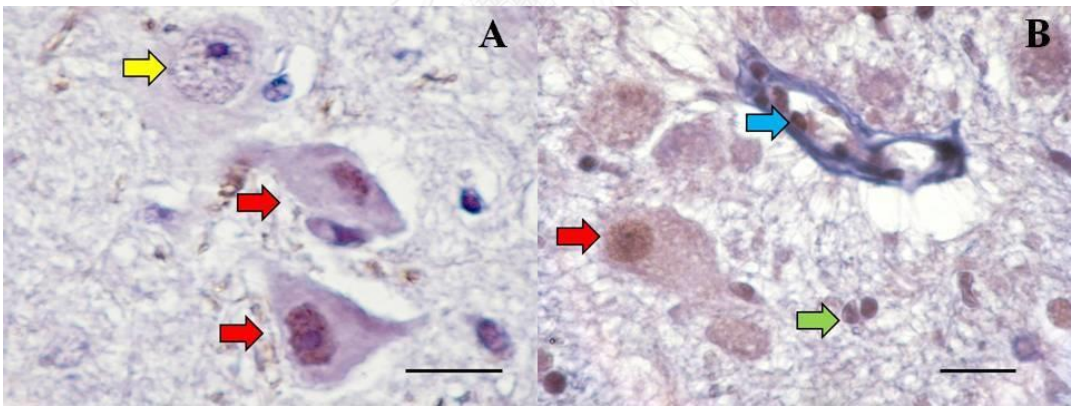

Figure 23 Expression of PR immunoreactions in the POA (A) and ARC (B) neurons; PR immunoreactions are presented in nucleus of some POA neurons (red arrows), some small neuronal cells (green arrow) and some endothelial cells (blue arrow). In contrast, a neuron expresses negative immunoreaction for PR (yellow arrow). The hematoxylin counter- stained is applied on the sections. Scale bar is 10 μm.

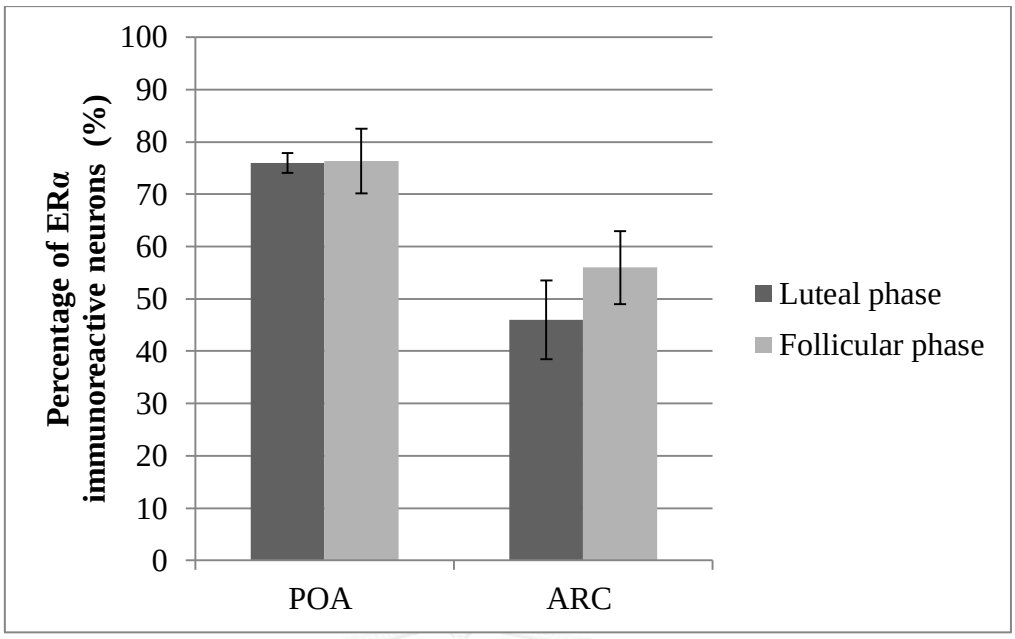

Figure 24 The percentages of ERα -ir neurons in the POA and ARC hypothalamic nuclei show no statistically significant differences in both the follicular and luteal phase (P>0.5).

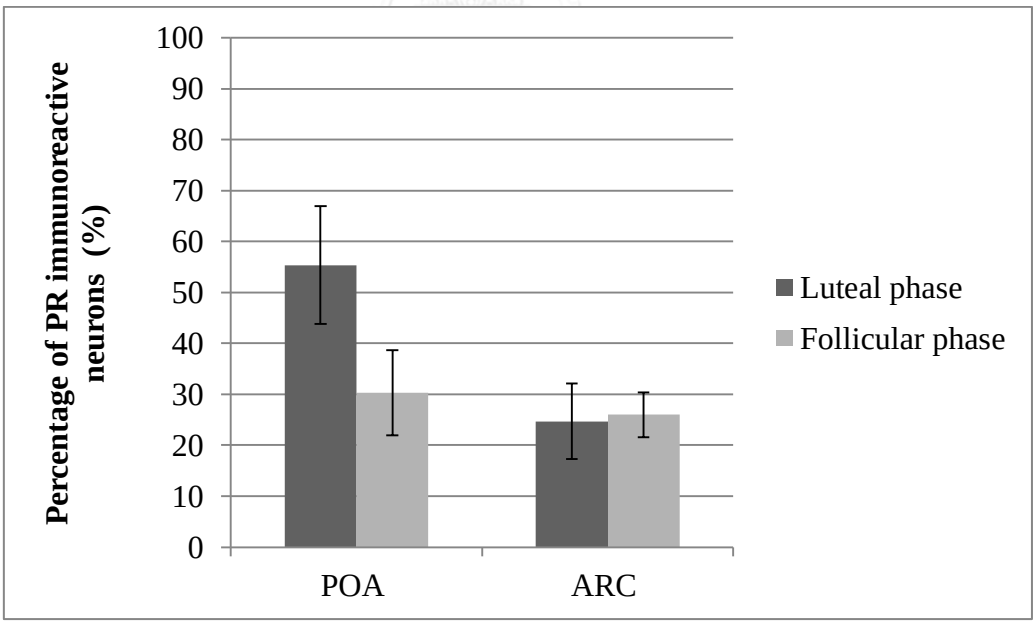

Figure 25 The percentages of PR -ir neurons in both of the POA and ARC hypothalamic nuclei show no statistically significant differences in both the follicular and luteal phase (P>0.5).

Single label immunohistochemistry for GnRH receptors in the pituitary gland

Histological section of buffalo pituitary gland for the pars distalis, pars intermedia and pars nervosa were identified by hematoxylin and eosin stain in figure 26.

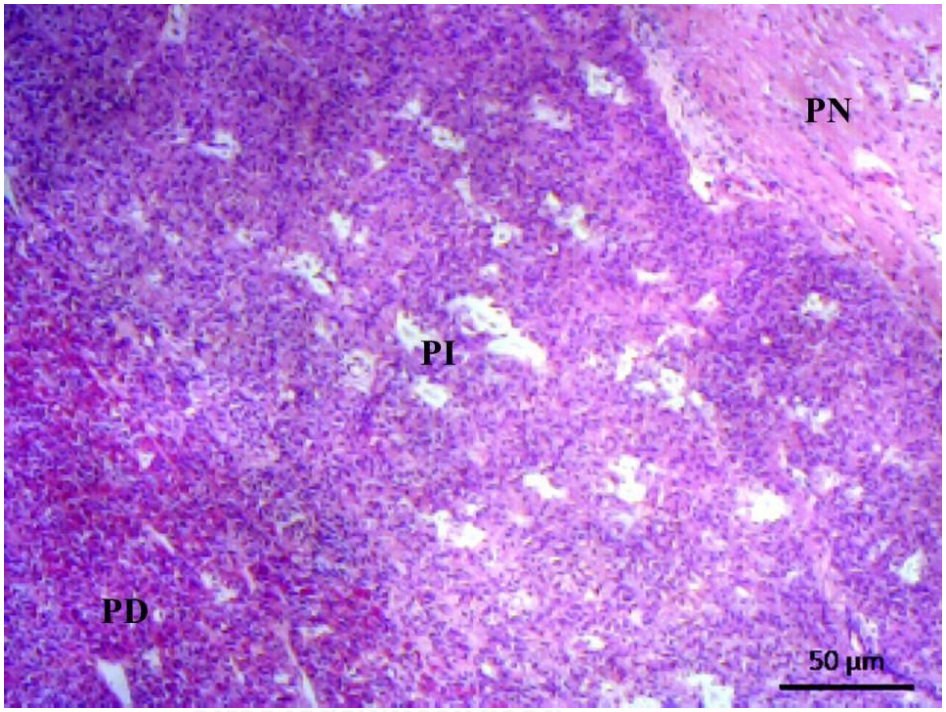

Figure 26 The histological appearances of pituitary gland of buffalo cow. There are 3 parts; pars distalis (PD), pars intermedia (PI) and pars nervosa (PN). Scale bar is 50 µm.

The results showed reactions of GnRHR located in the cytoplasm of subpopulations of cells in the pars distalis (Fig. 27A, 27C) and pars intermedia (Fig. 28), which might be regarded as gonadotrophs. In both the follicular and luteal phases, the GnRHR positive cells were found intensely accumulated in the proximal part of the pars distalis and pars intermedia and constituted the majority of cells in these areas. In contrast, in the distal part of both areas, only a few numbers of GnRHR positive cells were detected. No difference was found in this distribution between the follicular and luteal phases of the tested buffalo cows which were

>70% GnRHR-ir cells. The comparison between the positive GnRHR sample reactions and the negative control samples is shown in figure 27.

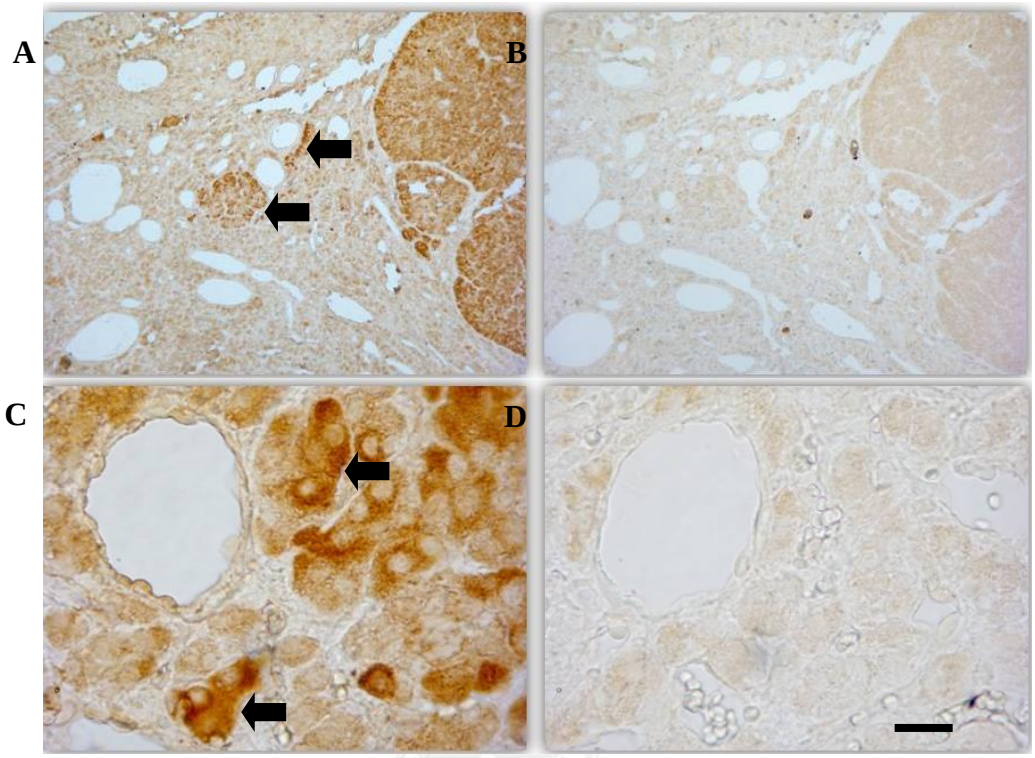

Figure 27 The GnRHR-ir cells (arrows) present in cytoplasm of subpopulations of cells in the pars distalis at low and high magnifications in A and C, respectively which compare to negative control in B and D. Scale bar= 20 μm.

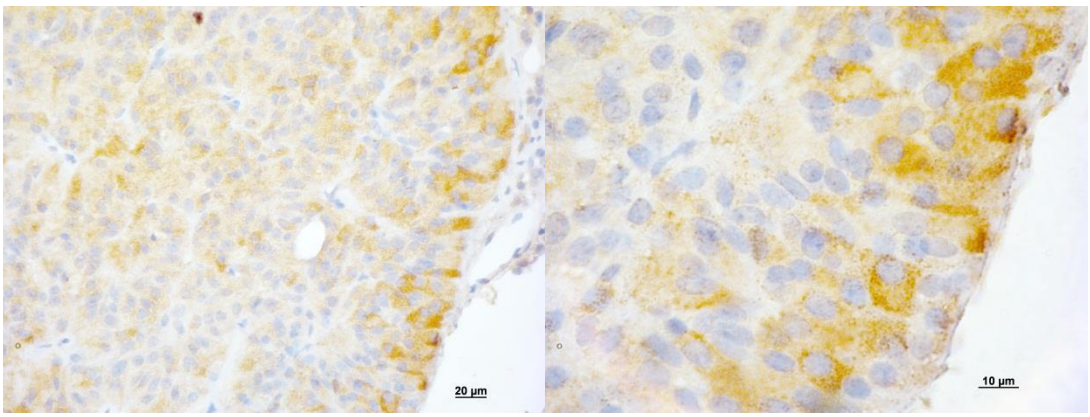

Figure 28 The GnRHR-ir cells (brown stain) present in cytoplasm of subpopulations of cells in the pars intermedia at low (40X) magnification on the left and high (100X) magnification on the right.

Part III Comparative study on the effects of administration of kisspeptin-10 and GnRH on the characteristics of LH secretion in luteal phase

The peak-shaped responses in the concentrations of LH before and after treatment in each cow were recorded and are presented in figure 29-34. Most of the cows had no LH response from Kp-10 injection and were similar to the control group which had no LH response to their distilled water injection. The average LH concentrations of all cows were 1.2±0.1 and 1.0±0 ng/ml in Kp-10 and control treatments, respectively. Interestingly, only the no.1 cow (Fig. 29) showed a spike peak of LH response at 165 min after Kp-10 treatment - about 5 times (5 ng/ml) of baseline LH response (1 ng/ml) – while the no.4 cow (Fig. 32) showed a fluctuating LH level between 1.5 – 3.5 ng/ml.

The average LH concentration of all GnRH treated cows was 9.8±2.8 ng/ml and the LH response curves of all cows showed the same bell shape. The LH level was mostly increasing at 15 min then reached a peak at 105-135 min and dropped to the baseline at 300 min after GnRH injection. The duration of the LH response to GnRH treatment was 3.58 hr (min=1.75 hr and max=4.75 hr) (Fig. 29B-34B). The

average AUC of the LH response curves after treatment in the GnRH group was 9 times that of Kp-10 group (min=4.7 times and max=18 times).

From table 2, the overall average of the pulse frequency of the Kp-10 group was 2.2±0.4 pulses with no statistical difference between the GnRH and control groups. However, the maximum LH concentration or peak LH value, the AUC of the LH response curve at peak time and during the 6 hr after treatment with GnRH, was significantly higher than in the Kp-10 and control groups ($P<0.05$).

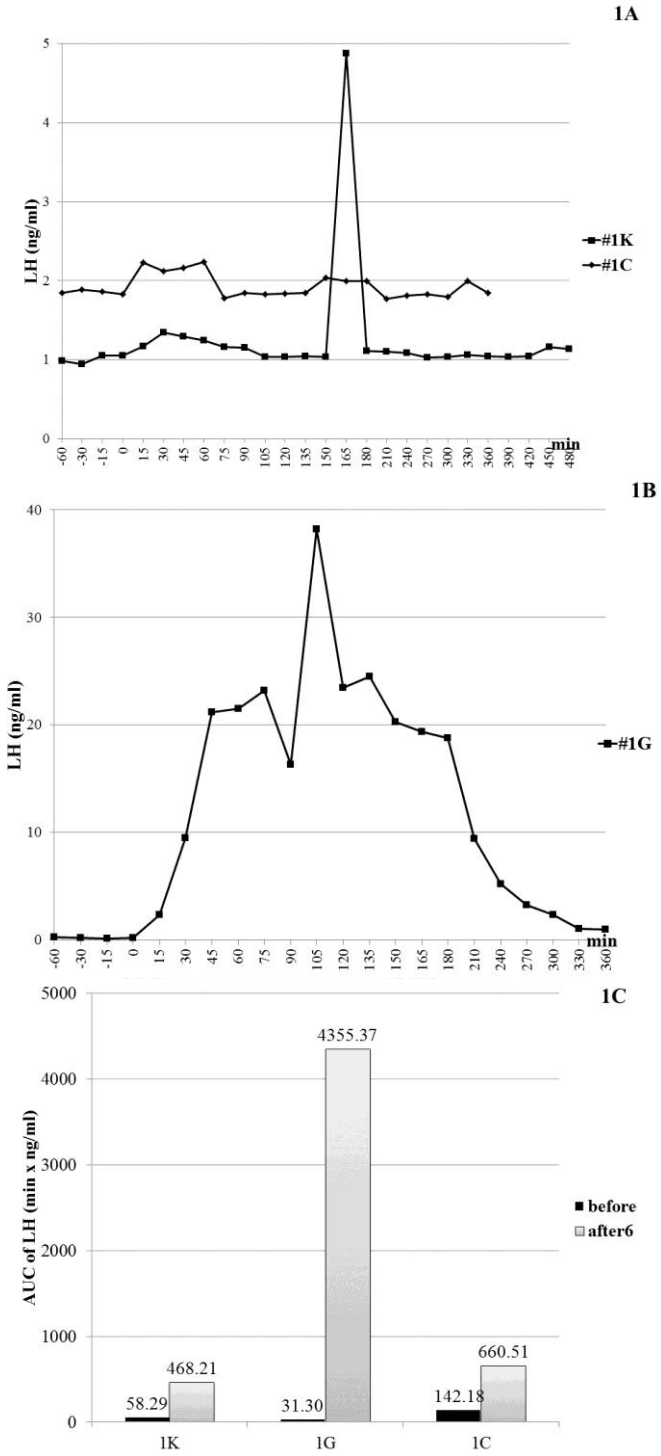

Figure 29 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 1 are shown in figure 1A, and after GnRH administration (G) in figure 1B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 1C.

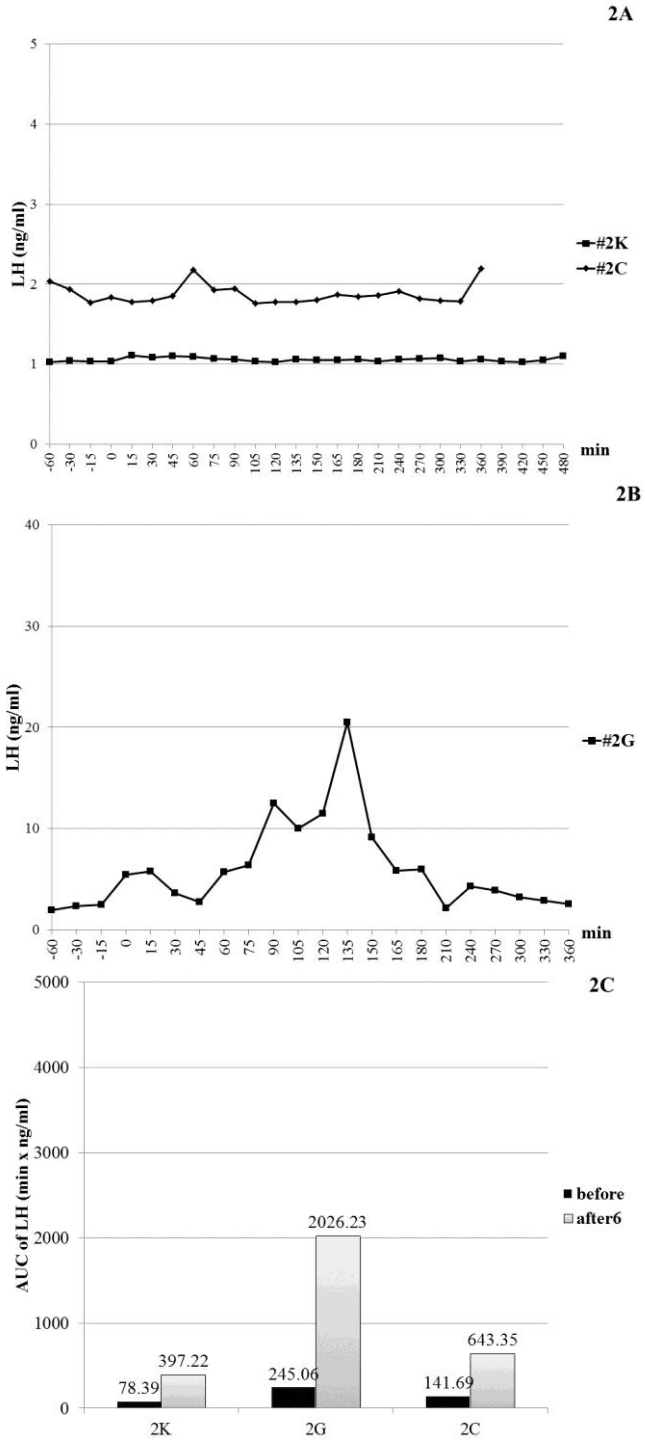

Figure 30 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 2 are shown in figure 2A, and after GnRH administration (G) in figure 2B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 2C.

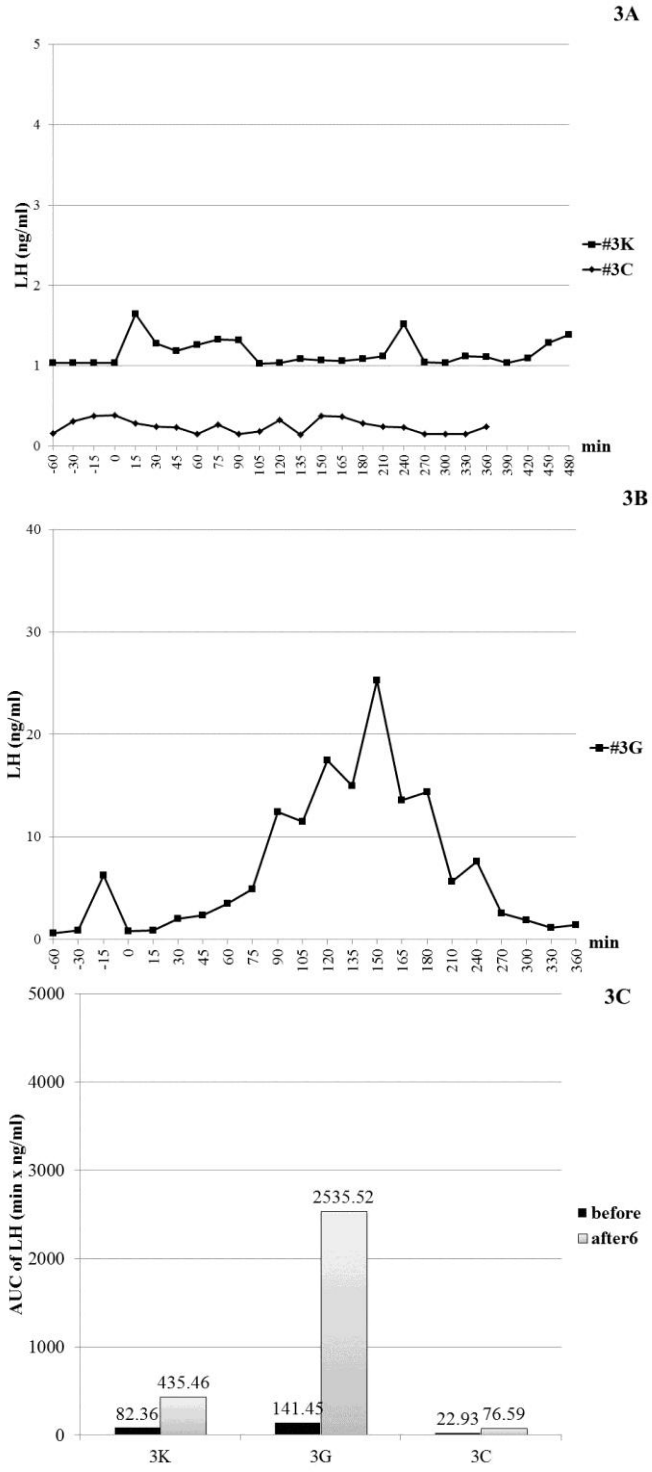

Figure 31 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 3 are shown in figure 3A, and after GnRH administration (G) in figure 3B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 3C.

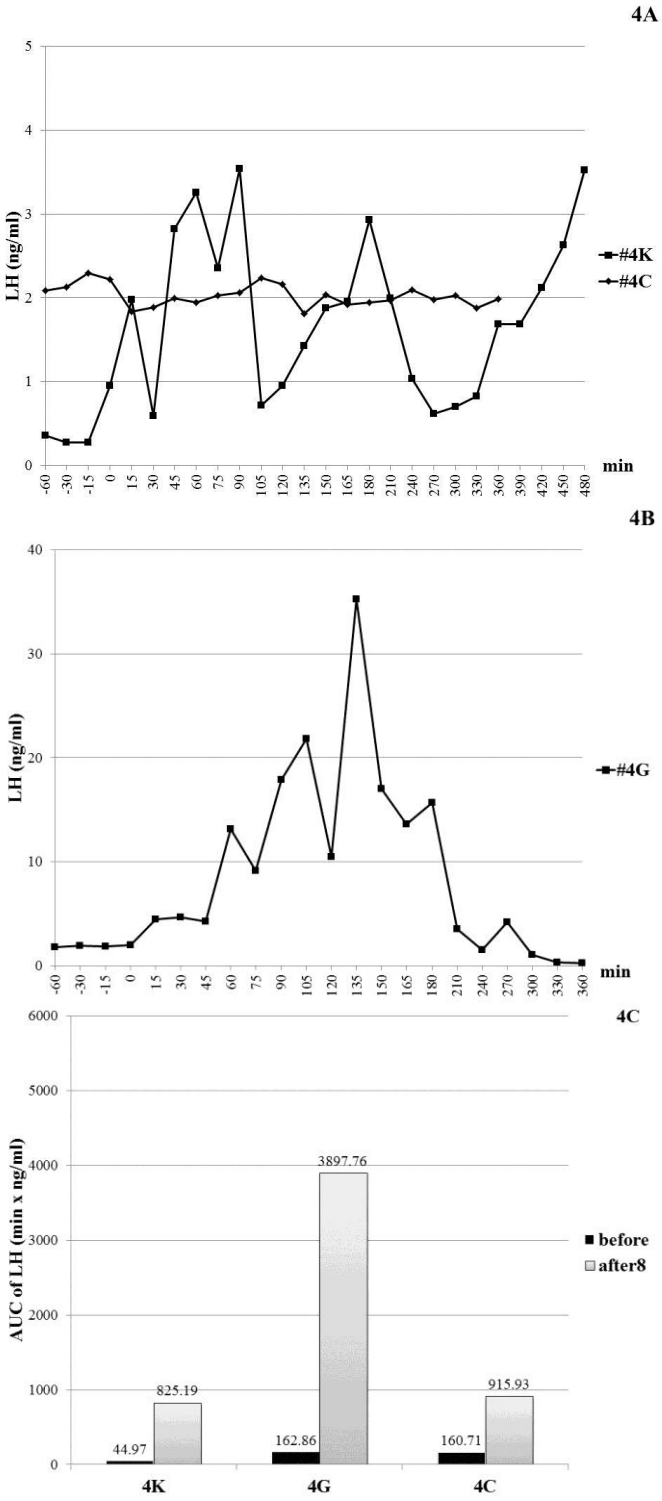

Figure 32 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 4 are shown in figure 4A, and after GnRH administration (G) in figure 4B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 4C.

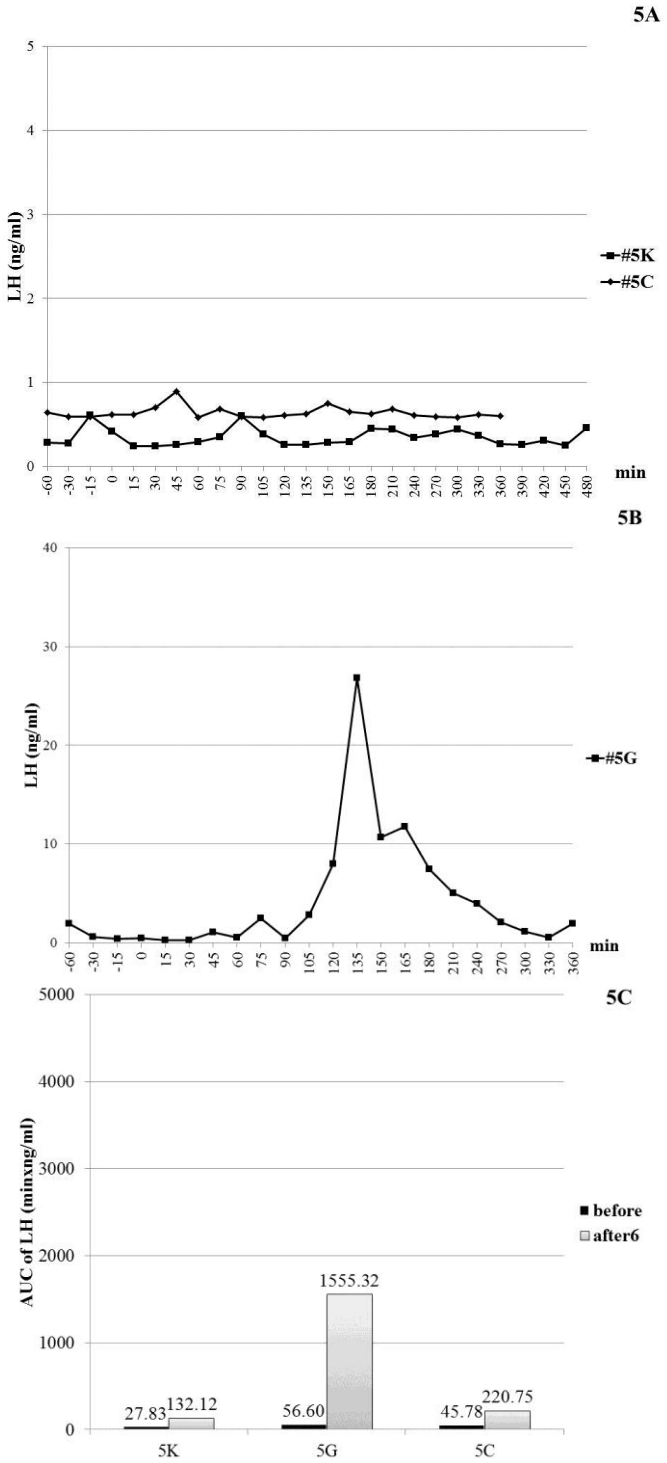

Figure 33 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 5 are shown in figure 5A, and after GnRH administration (G) in figure 5B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 5C.

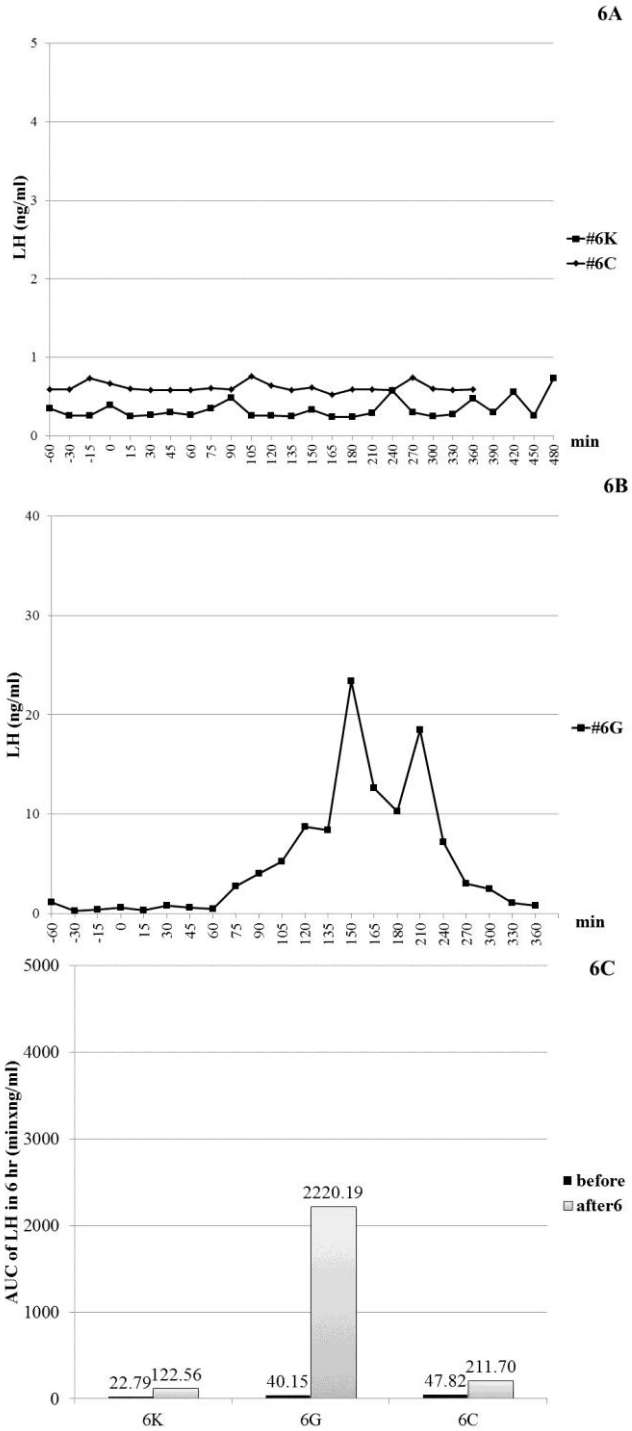

Figure 34 Change in plasma LH levels over time during kisspeptin-10 (K) and distilled water (C) administration to buffalo cow number 6 are shown in figure 6A, and after GnRH administration (G) in figure 6B. The change in AUC plasma LH is presented for each treatment group both before and after treatment in figure 6C.

Table 2 Data are shown as a mean±SEM in each parameter. The comparison of Kp-10, GnRH and distilled water (as a control) treatments were statistically analyzed by ANOVA (P=0.05).

Parameters for LH (Mean±SEM)/ treatment group	**Kisspeptin**	**GnRH**	**Distilled water**
Pulse in 6hr (pulse)	2.2±0.4[a]	1.3±0.3[a]	1.7±0.3[a]
Peak value-LH (ng/ml)	2.1±0.7[b]	28.2±2.8[a]	1.3±0.3[b]
Peak AUC (min x ng/ml)	133.0±34.6[b]	2493.1±438.2[a]	105.8±29.9[b]
AUC before treatment (min x ng/ml)	52.4±10.2[a]	112.9±34.6[a]	93.5±24.9[a]
AUC 6hr after treatment (min x ng/ml)	360.0±78.9[b]	2602.7±398.2[a]	416.6±112.5[b]

The numbers with superscript letters are significantly different from each other at $P < 0.05$.

Chapter V

Discussion

Part I: Detection of the localization of *Kiss1* mRNA and the distribution of kisspeptin protein in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle

The present study's detection and localization of *Kiss1* mRNA (using in situ hybridization technique with an ovine *Kiss1*cRNA probe) suggests the ability of buffalo POA and ARC hypothalamic nuclei to synthesize kisspeptin. Our reasons for using the ovine *Kiss1*cRNA probe for ISH and the ovine kisspeptin antibody for IHC (there was no reported research on the *Kiss1*sequence in buffalo in the year 2011, when our study began) were based on the following considerations. First, buffaloes like sheep are ruminants and their reproductive activities are similar (Robinson et al., 1985; Barrell et al., 1992; Smith et al., 2007; Smith, 2009). Second, similar results of ISH in both buffalo and ovine POA and ARC hypothalamic nuclei suggested the possibility that their *Kiss1* sequence exhibits cross-reactivity, and that this technique could be used to detect specific mRNA in specific cells (Yang et al., 1999). Third, the cross-reactivity of gene probes to the same mRNA in different animals (but still of the same ruminant type) suggested that this could make them sensitive and useful tools for the preliminary observation of *Kiss1* mRNA, in a manner similar to the way that the *Mycobacterium tuberculosis* probe has been used for detection of *Mycobacterium celatum* (a rare and newly found species in humans) (Butler et al., 1994). Phylogenetic analysis of kisspeptin in some ruminants, in addition, seemed consistent with this assumption (for example, the number of amino acids in ovine kisspeptin (53 amino acids) and its amino acid sequence are similar to other ruminants, such as cattle) (Tomikawa et al., 2010).

Interestingly, in 2014 (after our study was completed) three predicted buffalo *Kiss1* sequences were reported in a gene database (NCBI reference sequences: XM_006062321.1, XM_006062322.1 and XM_006062323.1, NCBIGenBank (2014)). In confirmation of our study's assumptions, 94% of the tested sequences showed significant alignments between the predicted buffalo and ovine *Kiss1* sequences when analyzed by BLAST 2.2.30+ program. Furthermore, as the ISH results of this study show, the cRNA probe of ovine *Kiss1* might be useful for the study of *Kiss1* in the POA and ARC areas of other ruminants, as well.

In previous studies, *Kiss1* mRNA localization in the POA and ARC was also found in sheep and confirmation of kisspeptin production in the POA and ARC area was later provided by immunohistochemistry tests (Estrada et al., 2006; Smith et al., 2007). The kisspeptin antibody in this study has been used in mares for kisspeptin-ir cell determination, proving that cross-reactivity can occur in other types of non-ruminants. This may be related to the fact that there is only one difference between the kisspeptin amino acid sequence found in horse and in sheep (Decourt et al., 2008). In addition, our study used the POA and ARC of sheep as a positive control for the *Kiss1* mRNA and kisspeptin-ir cells found in the same areas in the buffalo POA and ARC. Sheep POA and ARC samples were chosen as a positive control for the specificity of the probe and antibody.

The distribution of *Kiss1* mRNA in buffalo POA and ARC hypothalamic nuclei is similar, in most regards, to other animals. However, the areas of the hypothalamus which express *Kiss1* vary among different species of mammals; for example, *Kiss1* mRNA can be found in the POA and ARC of sheep, as a representative of ruminants (Goodman et al., 2007; Smith et al., 2007), in the ARC, ADP and AVPV of rodents (Gottsch et al., 2004; Irwig et al., 2004; Smith et al., 2005; Revel et al., 2006; Clarkson et al., 2009), in the POA and hypothalamic periventricular nucleus (PeN) of pigs (Tomikawa et al., 2010) and in the POA, ARC and AVPV of primates (Rometo et al., 2007). In relation to cattle, the only research that has been done concerns the distribution and localization of *Kiss1* and kisspeptin in the hypothalamus. *Kiss1* expression was found by qRT-PCR in sulking and weaning Brahman cow hypothalami. The expression within the ventral posterior area (including the ARC) was greater than

the expression within the anterior area (including the POA) (Husna et al., 2012). These interspecies variations in *Kiss1* distribution might be due to factors related to differences between seasonal/ non-seasonal animal species, 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, 2009; Liu et al., 2014; Cui et al., 2015)

In addition to the finding of *Kiss1* mRNA, the results of immunohistochemistry testing for kisspeptin in buffalo POA and ARC areas provides evidence of protein synthesized from *Kiss1* mRNA, as well as kisspeptin. Kisspeptin neurons in buffalo can also be detected in both the POA and ARC and are similar in their distribution to that of *Kiss1* mRNA in these areas (the distribution of both in the POA had a more scattered and widespread pattern, as opposed to their distribution in the ARC, which had a clumpy appearance). The variations in kisspeptin neuron distribution in the hypothalamic nuclei have been described in previous studies. The kisspeptin neurons reside in rodent AVPV and ARC (Irwig et al., 2004; Smith et al., 2006a; Clarkson et al., 2009), in sheep ARC, POA and dorsomedial nucleus (DMN) (Estrada et al., 2006; Franceschini et al., 2006), in horses ARC and DMN (Decourt et al., 2008) and in primates POA and ARC (Rometo et al., 2007). This variation in kisspeptin protein distribution might not be only dependant on *Kiss1* mRNA distribution but also on a possible difference in role of kisspeptin and its active mode in each species.

Interestingly, in contrast to other mammals, the population of kisspeptin-ir cells in buffalo POA is higher in concentration than in the ARC area. In mice (Clarkson et al., 2009), rats (Irwig et al., 2004; Smith et al., 2006b), hamsters (Revel et al., 2006), pigs (Tomikawa et al., 2010), mares (Decourt et al., 2008), sheep (Franceschini et al., 2006), primates and humans (Rometo et al., 2007), the number of kisspeptin neurons in the ARC is greater than in the POA. It is possible that the ARC in buffalo may be more highly developed than in other mammals in order to facilitate the fast-response of GnRH releasing to hypophysis and also on a possible difference in role of kisspeptin and its active mode, - which may account for this difference in kisspeptin neuron distribution.

In summary, the detection of *Kiss1* mRNA and kisspeptin protein in the hypothalamus of buffalo in this study provides fundamental data on kisspeptin and its relation to buffalo POA and ARC hypothalamic nuclei which, assuming buffalo is similar to other ruminants such as sheep, might be involved in HPO axis related reproductive functions. *Kiss1* mRNA was expressed in some neurons of both the POA and the ARC hypothalamic nuclei. Kisspeptin proteins were localized in subpopulations of neurons (in both neuronal soma and cellular processes) and some small neuronal cells. The kisspeptin-ir cell population in the POA was higher than in the ARC. However, the role of kisspeptin in the HPO axis in buffalo should be further explored for more knowledge on the possible relationships between kisspeptin neurons, GPR54 (kisspeptin receptors), GnRH neurons, sex steroid hormones (estrogen receptors alpha and progesterone receptors) in the POA and ARC hypothalamic nuclei, and GnRH receptors in the pituitary gland. This could provide basic knowledge that can be developed and applied to in vivo reproductive studies and reproductive management, in general, in the future.

Part II: Determination of the possible relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei and GnRH receptors in the pituitary gland

Double label immunohistochemistry for KISS1R and GnRH in the POA and ARC hypothalamic nuclei

This study provides information about the buffalo HPO axis; as well as the distribution, localization, co-localization, and mode of action of kisspeptin in controlling GnRH releasing. The co-localization of buffalo kisspeptin receptors (KISS1R) and the GnRH neurons in the POA and ARC found in this study is similar to previous findings in other mammals: many hypothalamic GnRH neurons co-express *Kiss1r* mRNA (77% in rat and 55-90% in mouse) (Irwig et al., 2004; Han et al., 2005). In this study, 93% of GnRH-ir neurons in the buffalo POA and ARC hypothalamic nuclei

contained KISS1R in their cells. This finding suggests a pathway of kisspeptin signaling in the buffalo: downstream of kisspeptin-expressing neurons to GnRH neurons as the target cells. This evidence suggests that GnRH neurons are influenced by kisspeptin directly: both in terms of controlling prolonged depolarization as well as stimulating their potential firing rate (Han et al., 2005; Quaynor et al., 2007). These effects have also been reported in ruminants such as sheep (Messager et al., 2005), cattle (Whitlock et al., 2008) and goats (Hashizume et al., 2010).

Interestingly, we found that the GnRH neuron population in the ARC of buffalos trended greater than in the POA. This suggests that buffalos are similar to sheep and primates in this distribution. In sheep, GnRH cell bodies are presented both in the POA and in the ARC. Most of GnRH cell bodies in primates are identified lateral to the ARC in the ventral hypothalamic tract of the mediobasal hypothalamus (MBH), but they rarely are found in POA. In rodents, controversely, GnRH cell bodies are scattered as a continuum from the medial septum and diagonal band of broca to the medial POA, but they are rarely found in the ARC area (Colledge, 2009). This information suggests the possibility that there are anatomical and functional correlations that depend on the distribution of GnRH-ir neurons and KISS1R-ir cells. A part of ARC called the median eminence (ME) is closely connected to the hypophysis. Our findings could be related to the ME and may suggest that this area presents a high sensitivity to GnRH release. This may explain the large amount of co-localized GnRH neurons and KISS1R-ir found in our studies of the ARC area. Previous studies have found GnRH cell bodies and kisspeptin-ir cell bodies in close proximity (Clarkson and Herbison, 2006; Decourt et al., 2008). Additionally, kisspeptin-ir fibres have been found to extend from the ARC into the external neurosecretory zone of the ME (Franceschini et al., 2006; Pompolo et al., 2006) and it is thought that these terminals might be causative for the kisspeptin that has been identified in ewe hypophyseal portal blood (Smith et al., 2008). This supports the concept that GnRH neurons are controlled directly by kisspeptin at the GnRH nerve terminals through axo-axonal non-synaptic interactions (Decourt et al., 2008; Uenoyama et al., 2011) in this area. A study by Backholer et al. (2010) supports our current findings. In this study kisspeptin cells in the POA were found to directly stimulate GnRH neurons, but

evidence suggested that GnRH neurons in the ARC might be activated by other cells via poly- transsynaptic regulatory mechanisms (Backholer et al., 2010).

As for the POA and ARC neurons which presented KISS1R-ir but no trace of GnRH, this could be explained by a possible un-related GnRH releasing function of kisspeptin. Kisspeptin might have an influence not only on the reproductive system through GnRH neurons but also on other protein-releasing neurons. One of the proteins involved in the metabolic system is the growth hormone (GH), which may be stimulated by kisspeptin in cattle (Whitlock et al., 2008). In terms of reproduction, GnRH neurons may be also stimulated by kisspeptin indirectly. Kisspeptin has been found to act on GnRH neurons by way of synaptic interaction from other KISS1R expressed neurons in the hypothalamus (Herbison et al., 2010).

The unexpected pattern of KISS1R localization in the buffalo POA and ARC areas and the presence of KISS1R-ir on some glia cells might suggest a kisspeptin-glia-hypothalamic vascular relationship. It is possible that glia cells might be non-neuronal cells which are related to GnRH release, especially the sub-type of glia cells called astrocytes. The concept of a “Neuro-gliao-vascular” relationship (Haydon and Carmignoto, 2006) might be the key to understanding this possible indirect stimulation of GnRH by kisspeptin through trans-synaptic regulatory mechanisms (Garcia-Segura et al., 2008). Our study found no relationship between the different stages of the estrous cycle and the number of KISS1R-ir in GnRH neurons in the buffalo cows. Research on co-expression, as well as the mechanisms involved in the relationship between sex steroid hormones (estrogen and progesterone) and GnRH and kisspeptin neurons in the POA and ARC areas have been done in other species (Gottsch et al., 2004; Franceschini et al., 2006). Similar studies of kisspeptin’s role in GnRH releasing in the follicular and luteal phases in buffalo cows should be conducted in the future. Also, although his study used the immunohistochemistry technique for qualification of localization and distribution of KISS1R-ir in GnRH neurons, confirmation of the KISS1R expression with other molecular techniques such as real-time quantitative polymerase chain reaction (qRT-PCR) and western blot analysis (which have been used in human granulose cell research) (Garcia-Ortega et al., 2014), could be considered in future studies.

Single label immunohistochemistry for estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei

Sex steroid hormones profoundly affect metabolic regulation in many species. ERα, which is abundantly distributed in several key brain regions, is not just involved in reproductive functions via the HPG axis – it is also involved in regulating energy homeostasis, control of food intake, energy expenditure, hypothalamic-pituitary- adrenal axis regulation and thermogenesis (Liu and Shi, 2015).

Sex steroid hormone receptors are usually located in the nucleus of neurons, however, some neurons present lighter cytoplasmic staining for immunoreactions (especially in the median eminence which is within the ARC hypothalamic nucleus) (Lehman et al., 1993) similar to the localization of both ERα and PR in this study. Previous research has reported the distribution of these receptors in not only the POA and ARC areas but also in several key brain regions (Liu and Shi, 2015). Lehman et al. (1993) reported that some glia cells also displayed both ERα and PR immunoreactions in ewes. Our study found immunoreactions of ERα and PR in some small neuronal cells. These might be glia cells which could be confirmed using a detection of glia marker technique in a further study. Moreover, there is research that has found these sex steroid receptors in endothelial cells; a finding similar to the present study. This evidence might imply that the estrogen and progesterone influence the central nervous system through the vascular system also.

Our study's finding that the percentage of ERα -ir neurons in the POA was higher than in the ARC in both phases of the estrous cycle may indicate that the POA functions as a surge center in the hypothalamus (through estrogen feedback control) in buffalo just as it does in many other animals (Gottsch et al., 2004; Estrada et al., 2006; Franceschini et al., 2006; Roseweir and Millar, 2009). However, the surge center might be controlled or inhibited by progesterone as well. This could be explained that the PR -ir neurons showed greater in luteal phase and lower in follicular phase, inducing the surge center activation in the POA and the LH surge which causes ovulation in the follicular phase. In the luteal phase, the neurons in the POA act in the mode of a tonic center and collaborate with other neurons in the ARC to

generate a constant LH tonic pulse for follicular development in buffalo, just as it does in many other animals.

Sex steroid hormones are not only produced from the gonads (ovary and testis) but are also secreted from the brain, adipose tissue, and the adrenal gland which act as paracrine to its receptors. Therefore, animal status (health and nutrition) and stress condition are also factors affecting ERα and PR expression. Regulation of ERα and PR expression in the hypothalamus due to dynamic endocrine status and changes in circulating steroid levels during the estrous cycle may be region-specific and specie difference (anatomy and function) (Liu and Shi, 2015). For example, the estrous cycle estrogen induced increase in progesterone responsive neurons in the ventromedial hypothalamus (ARC area) can account for the permissive influence of estrogen on progesterone facilitated reproductive behavior (Romano et al., 1989). Previous studies have indicated that fixation, antibody specificity and the antigen retrieval techniques used on samples are technical factors which can effect ERα and PR detection and expression (Lehman et al., 1993; Skinner et al., 2001).

This research points to the possibility of a relationship between sex steroid hormones (estrogen and progesterone) and some neurons in the POA and ARC hypothalamic nuclei. It suggests that they may be involved in kisspeptin and GnRH regulation in the HPO axis in buffalo – a finding similar to previous research studies in other mammals (Smith et al., 2005; Clarkson and Herbison, 2006; Adachi et al., 2007; Hashizume et al., 2010). Identification on type of neuron, especially kisspeptin and GnRH neurons in POA and ARC areas should be investigated by double labeling immunofluorescent in future studies.

Single label immunohistochemistry for GnRH receptors in the pituitary gland

This study is the first to report the distributions of GnRHR in the pituitary glands of buffalo cows. Although successful buffalo GnRHR gene detection has been reported (Konwar and Srivastava, 2005), GnRHR localization data in buffalo has never been presented in any reliable scientific publication or database. Generally, GnRHR is

expressed on the membrane of anterior pituitary gonadotroph, which relates to reproductive function (Rispoli and Nett, 2005). Interestingly, our study found GnRHR-ir cells located in the cytoplasm of subpopulations of cells in not only the pars distalis but also in the pars intermedia with an intense accumulation in the proximal part. In other animals, it has been found only in the pars distalis (Soga et al., 2005). No difference was found in this distribution between the follicular and luteal phases of the tested buffalo cows in this study. However, there are many studies in GnRHR density relating to both mRNA and numbers of GnRHR (which determines their ability to respond to GnRH). Research studies on gonadotrophs in sheep (Clarke et al., 1987; Gregg and Nett, 1989; Nett et al., 2002), cattle (Schoenemann et al., 1985) and laboratory animals (Bauer-Dantoin et al., 1995; Yasin et al., 1995) found that the density has been shown to increase during the follicular phase and is at its highest point just pre-ovulation. This evidence supports the long known fact that estrogen is the hormone regulating positive pituitary responsiveness to GnRH (Reeves et al., 1971), although the mechanism is still unclear. Factors affecting GnRHR gene expression in gonadotrophs are mainly GnRH, partially gonadal steroid hormones (estrogen and progesterone), and growth factors (inhibin and activin) (Rispoli and Nett, 2005). In our study, the distribution of GnRHR-ir cells was the same in both the follicular and luteal phases. This could be because LH pulse regulation in buffalo cows might not be involved at the anterior pituitary level. The control of LH releasing might be, instead, related to GnRH and/or kisspeptin regulation at the hypothalamus level. This assumes that the feedback control of estrogen in the follicular phase and progesterone in the luteal phase during the estrous cycle may also have its primary influence at the hypothalamus level rather than at the anterior pituitary level in buffalo cows. A study on the mutations of GnRHR in humans reported that it can cause hypogonadotropic hypogonadism leading to LH and FSH deficiency (de Roux, 2006). Therefore, it is possible that anestrous or infertile animals might also have this problem, which is an issue to be considered in further studies. Our present research suggests that, in buffalo cow, the target cells of the GnRH released by hypothalamic neurons are in the pituitary gland. These findings expose some of the mechanisms involved in the action of GnRH in the HPO axis, the

understanding of which is central to the enhancement of buffalo reproductive performance (which might be different from other animals). However, more studies on the mechanism of the HPO axis on pituitary responsiveness during the different stages of the estrous cycle should be done in the future.

Part III Comparative study on the effects of administration of kisspeptin-10 and GnRH on the characteristics of LH secretion in luteal phase buffalo

Synthetic kisspeptin administration has been done *in vivo* for gonadotropin hormones (LH and FSH) in several mammalian species such as rat (Irwig et al., 2004), mice (Gottsch et al., 2004), sheep (Caraty et al., 2007), cattle (Kadokawa et al., 2008b; Whitlock et al., 2008; Ezzat et al., 2009), monkeys (Shahab et al., 2005), humans (Dhillo et al., 2005; Shahab et al., 2005; Dhillo et al., 2007; Jayasena et al., 2010; George et al., 2012; Jayasena et al., 2013; Jayasena et al., 2014a; Jayasena et al., 2014b; MacLean et al., 2014) and, recently, in river type buffalo (Macedo et al., 2014). Our study is a novel report on kisspeptin's effect on LH secretion in swamp type buffalo.

Our study selected Kp-10 for the experiment. Kp-10 has been compared to longer peptide kisspeptin such as Kp54 in humans. Some of the advantages of Kp-10 are that its intravenous administration is very effective for gonadotropin stimulation, its shorter amino acid sequence make less expensive to synthesize, and it has no significant adverse effects for either acute or chronic use (Jayasena et al., 2015). Its plasma half-life, however, is one sixth as long as Kp54 (Jayasena et al., 2011).

Several *in vivo* studies report the maximum LH-releasing effect of Kp-10 (i.v. injection) doses were observed at 0.54-0.65 μg/kg b.w. in ovariectomized ewes (Caraty et al., 2007), 1 μg/kg b.w. in luteal phase goats (Hashizume et al., 2010), 0.13 μg/kg b.w. in ovariectomized cows (Whitlock et al., 2008) and 4.76 μg/kg b.w. and in pre-pubertal heifers (Kadokawa et al., 2008a). However, we used a high dose for the experiment, 10 times the dosage for ovariectomized cows which was 0.13 μg/kg b.w. (Whitlock et al., 2008).

Many studies have researched the efficiency of kisspeptin administration for stimulating gonadotropin responses. These experiments controlled the animal sex steroid condition by using ovariectomized and pre-pubertal animals with or without sex steroid supplements. The results appear to reflect a direct action on the hypothalamus. For example, in OVX goats, the peripheral infusion of kisspeptin-10 stimulates GnRH neurosecretion into hypophyseal portal circulation and the action of kisspeptin on LH releasing is mediated by GnRH (Tanaka et al., 2012). Similar experiments have been done in OVX ewes (Arreguin-Arevalo et al., 2007; Caraty et al., 2007), OVX cows (Whitlock et al., 2008), pre-pubertal cattle heifers and male calves (Kadokawa et al., 2008a; Ezzat et al., 2009) and the OVX river type buffalo (Macedo et al., 2014). Also, some studies found that kisspeptin cells in the ARC express ERα and PR. This evidence indicates that kisspeptin may play a role in the steroid feedback control mechanism for GnRH secretion in ruminants (Estrada et al., 2006; Smith, 2009; Hashizume et al., 2010).

It is well known that steroid hormones fluctuate across the female estrous cycle and feedback from gonads regulate the HPG axis. Kisspeptin neurons express ERα, PR and AR, and hence have the potential to relay feedback effects on the GnRH neuron (Hashizume et al., 2010). Kisspeptin appears to be the main candidate for facilitating the feedback effect of estrogen (Smith, 2009). Generally estrogen presents in a high concentration during proestrus and acts as the positive feedback to kisspeptin functions in the GnRH/LH surge response. Our study focused on the administration of kisspeptin in luteal phase buffalo and proved that the sex steroid hormones, especially progesterone, may relate to the feedback control of kisspeptin and GnRH functions via HPO axis. It is already known that progesterone strongly inhibits pulsatile GnRH/LH releasing and also blocks the positive feedback effect of raised estrogen levels on GnRH/LH secretion (Skinner et al., 2001). Progesterone, however, may also act as the negative feedback to kisspeptin's function in LH releasing in luteal phase buffalo.

The Kp-10 injected luteal phase swamp buffalo cows had no LH response in the 6 hr after administration and the average LH concentration of all cows was

1.2±0.1 ng/ml. This is a similar LH range found in most luteal phase buffalo (high levels of progesterone) - the LH levels remain at a baseline which fluctuates around 0.72-3.0 ng/ml (Mondal et al., 2007).

Many previous studies have proved that GnRH administration activates gonadotropin hormones, especially LH, more than Kp-10 does, which is the same result found by our experiment. For example, GnRH can induce levels of AUC serum LH 3 times higher than Kp-10 at the same dosage at 1.0 nmol/kg/h (34.06± 5.18 vs 10.81±1.73 IU/l) in healthy men (Jayasena et al., 2015). Similarly, a study in male rats found a greater LH response to GnRH compared with the same dosage of Kp-10 using i.v. administration (Tovar et al., 2006). Another study in OVX river buffaloes reported that the amplitude of the LH peak is higher with GnRH (11.72 ± 1.73 ng/ml) than with Kp (7.89 ± 0.70 ng/ml; P = 0.0132) using intramuscular administration. In the non-breeding season (low melatonin secretion), however, kisspeptin has been observed to induces a short LH peak, which might be related to the reduced receptivity of GnRH containing neurons during this season (Macedo et al., 2014).

There is a possible reasons why the LH response to kisspeptin is significantly lower than GnRH. The first possibility involves the different physiological action of GnRH and kisspeptin on gonadotropins. Kisspeptin acts on GnRH secretion on the hypothalamus level and then GnRH stimulates gonadotropin hormones on the pituitary level. But GnRH acts on the pituitary level directly. This difference is in accord with the finding of GnRHR expression in the pituitary gland. Therefore, GnRH can stimulate LH response in the pituitary gland directly. Conversely, Kp-10 may not be able to activate GnRH/LH releasing on the hypothalamic level because of a negative feedback effect of progesterone in the luteal phase of the estrous cycle. The processes involved in kisspeptin's actions are more physiological and less direct than the primarily pituitary and gonadal stimulation invoked by GnRH or GnRH analogues and other gonadatropin products (Jayasena et al., 2015). This may be related to the fact that kisspeptin receptor cells are found in many tissues throughout the body (Lee et al., 1996; Muir et al., 2001; Adachi et al., 2007; Mead et al., 2007; Richard et al., 2008; Roseweir and Millar, 2009) – not in just a few areas (such as the hypothalamus, ovaries, and the pituitary) , as are GnRH receptor cells

(Rispoli and Nett, 2005). In order to improve the efficiency of LH response in buffalo, research into the production of a buffalo kisspeptin agent should be considered.

In conclusion, our study is the first report to compare the effects of the administration of Kp-10 and GnRH on the characteristics of LH secretion in luteal phase buffalo cows. Administration of Kp-10 and DW (control) intravenously was associated with similar levels of LH secretion in luteal phase buffalo cows, but GnRH intramuscular injection in conventional dosage for ovulation stimulation was more potent. This study provides important information suggesting that kisspeptin may play a role in the steroid feedback control mechanism in the HPG axis in buffalo cow reproduction. Although kisspeptin, especially Kp-10, may not at present be a good option for solving infertility problems in the field - it is possible that kisspeptin related fertility disorders may be treated with kisspeptin administration successfully in the future. Practical applications of kisspeptin for buffalo, however, may require further research into, and production of, a species specific buffalo kisspeptin agent to be truly effective.

Chapter VI

General discussion

General discussion

Evidence of mRNA signaling for *Kiss1* and its protein on kisspeptin-expressing neurons in the POA and ARC of buffalo was reported recently (Chaikhun et al., 2016). The signals for *Kiss1* mRNA and the localization of kisspeptin proteins were detected in the cytoplasm of the POA and ARC neuronal soma and some glias. Kisspeptin proteins were also found in the cellular process of the POA and ARC neurons. The population of Kp-ir neurons distributed in the POA (79.8±2.5%) was greater than in the ARC area (62.5±4.5%) ($P \leq 0.01$). This study provides evidence of *Kiss1* mRNA and kisspeptin protein in the hypothalamus of buffalo.

Kisspeptin is not only necessary for controlling reproductive functions throughout life in many animal species, it also key for pubertal activation of gonadotropin releasing hormone neurons in the early stages of animal life (Decourt et al., 2008; Clarke et al., 2009; Tomikawa et al., 2010; Cortes et al., 2015). The recent extended study was designed to explore the differences in location of kisspeptin in the POA and ARC hypothalamic nuclei in pre- and post- pubertal female buffaloes (Chaikhun et al., 2016). Hypothalami were collected from 10 buffalo heifers (age between 6 months and 3 years) after which the same protocol from the previous study in buffalo cows was applied. The results showed kisspeptin reactions located in the cytoplasm of neurons in the positive control and in some samples. Interestingly, the kisspeptin reactions showed mostly in the POA in the 6-12 months of age group, which suggests that the POA might be a main area related to kisspeptin expression. However, the 12-30 months old heifers presented kisspeptin reactions only in the ARC area which suggests that kisspeptin is also involved in this area. Surprisingly, all of the heifers up to 30 months of age showed a weak expression of kisspeptin in both areas. There are two possible reasons for this; this age period may

involve a temporary stop of kisspeptin production before the onset of puberty, or kisspeptin is produced but goes to other hypothalamic nuclei. To confirm and clarify these results however, in situ hybridization for *Kiss1* mRNA in the POA and ARC areas, along with detection using an immunoflorescent technique should be undertaken. Interestingly the cow samples in our study had a larger and denser population of Kp -ir neurons in the POA than in the ARC area. This is in contrast to other mammals (Decourt et al., 2008; Clarke et al., 2009; Tomikawa et al., 2010). It is possible that kisspeptin in the POA and ARC hypothalamic nuclei of buffalo may have a different role in its active mode, which may account for this difference in kisspeptin neuron distribution. Our study found dramatic differences in kisspeptin localization between pre- and post-pubertal female buffaloes. This different distribution of kisspeptin in pre-pubertal buffalo may suggest that kisspeptin's function is developmentally dependent and changes as buffalo age. The post-pubertal buffalos, on the other hand, had the same localization of kisspeptin in all age groups in both the follicular and luteal phases without any differences. Our findings suggest that the ARC area in pre-pubertal buffalo may be the main area of kisspeptin's action during this developmental period. The POA, alternatively, could be the major area controlling the reproductive cycle in post-pubertal buffalo.

The expression of KISS1R by GnRH neurons in the POA and ARC hypothalamic nuclei by double-labeling immunohistochemistry was investigated. In both the POA and ARC areas, KISS1R-ir was detected in the nucleus and cytoplasm of neuronal soma and some glia. GnRH-ir was found as granular formations in the cytoplasm of neuronal soma. The KISS1R-ir neuron population in the POA was the same as in the ARC (93%), however, the GnRH-ir neuron population in the POA (64.5±4.3%) trended lower than in the ARC (72.8±6.2%) ($P>0.05$). Double labeling showed that all observed GnRH-ir neurons were co-localized with KISS1R immunoreactions. 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 buffalo. Moreover, our extended studies of both pre- and post-pubertal female buffaloes found the target cells of kisspeptin in 3 parts of the pituitary gland: the pars distalis, pars intermedia and pars nervosa. However, the pre-pubertal buffaloes

showed more KISS1R -ir intensity than post- pubertal buffaloes (Chaikhun-Marcou et al., 2015b; Chaikhun-Marcou et al., 2015a). This finding suggests that kisspeptin might be involved not only reproductive functions but also other pituitary functions as has been found in other animals studies (Ramaswamy et al., 2009; Luque et al., 2011).

The localization and the distribution of the ERα and the PR in the POA and ARC areas of buffalo cows were determined by immunohistochemistry technique in this study (Chaikhun-Marcou et al., 2014a). Both ERα and PR immunoreactions were found in the nucleus of some POA and ARC neurons and some glias. The percentage of ERα and PR -ir neurons distributed in the POA (ERα; 76.2±4.1%, PR; 42.8±10.6%) were greater than in the ARC area (ERα; 51±6.8%, PR; 25.33±5.5%) by a statistically significant percentage for ERα ($P<0.01$) but not for PR. The percentages of ERα and PR -ir neurons in both the follicular and luteal phase showed no statistically significant differences. This research points to the possibility of a relationship between sex steroid hormones and some neurons in the POA and ARC hypothalamic nuclei and suggests that they may be involved in kisspeptin and GnRH regulation in the HPO axis in buffalo. Our studies's finding that the percentage of ERα -ir neurons in the POA was higher than in the ARC in both phases of the estrous cycle may indicate that the POA functions as a surge center in the hypothalamus (through estrogen feedback control) in buffalo just as it does in many other animals (Estrada et al., 2006; Smith, 2009; Hashizume et al., 2010).

This thesis's research points to the possibility of a relationship between sex steroid hormones (estrogen and progesterone) and some neurons in the POA and ARC hypothalamic nuclei. The identification of the types of neurons, especially kisspeptin and GnRH neurons in the POA and ARC areas, should be investigated by double labeling immunofluorescence in further studies. Therefore, the aim of our next extended study was the detection of ERα and PR in kisspeptin (Polnok et al., 2015) and GnRH (Hanrin et al., 2015) neurons in the POA and ARC areas by immunofluorescence technique in the same cycling buffalo samples. The results indicated that ovarian sex steroid hormones may control kisspeptin and GnRH neurons directly - impacting the reproductive system via the HPO axis in fertile

buffalo cows. Our findings suggest that they may be involved in kisspeptin and GnRH regulation in the HPO axis in buffalo – a finding similar to previous research studies in other mammals (Smith et al., 2005; Clarkson and Herbison, 2006; Adachi et al., 2007; Hashizume et al., 2010).

In addition, a report on the presence of GnRH receptors (GnRHR) in the par distalis and pars intermedia of buffalo pituitary glands was also previously published (Chaikhun et al., 2013a). The results showed distributions of GnRHR located in the cytoplasm of subpopulations of cells in the pars distalis and pars intermedia, which might be regarded as gonadotrophs. In both the follicular and luteal phases, the GnRHR positive cells were found intensely concentrated in the proximal part of the pars distalis and pars intermedia. In contrast, in the distal part of both areas, only a few numbers of GnRHR positive cells were detected. This study is the first to report the distribution of GnRHR in pituitary glands of the buffalo cows. No difference was found in this distribution between the follicular and luteal phases of the test cows. This research suggests that the target cells of the GnRH released by hypothalamic neurons are in the pituitary gland. Our findings expose some of the mechanisms involved in the action of GnRH in the HPO axis, the understanding of which is central to the enhancement of buffalo reproductive performance.

In vivo studies have been done on kisspeptin's effect on luteinizing hormone (LH) response in luteal phase swamp buffalo cows (Chaikhun-Marcou et al., 2014b). Administration of Kp-10 and DW (control) intravenously was associated with similar levels of LH secretion in luteal phase buffalo cows, but GnRH intramuscular injection in conventional dosages was more potent for ovulation stimulation. This study provides important information suggesting that kisspeptin may play a role in the steroid feedback control mechanism in the HPO axis in buffalo cow reproduction.

In this current study, the role of kisspeptin in buffalo and its relation to reproduction and the hypothalamic- pituitary- ovarian axis was explored. A diagram, inspired by our research results, of a proposed buffalo kisspeptin pathway in the POA and ARC hypothalamic nuclei via kisspeptin neurons - GnRH neurons – the pituitary – and the ovaries, a "buffalo kisspeptin HPO axis", is presented in Fig. 35.

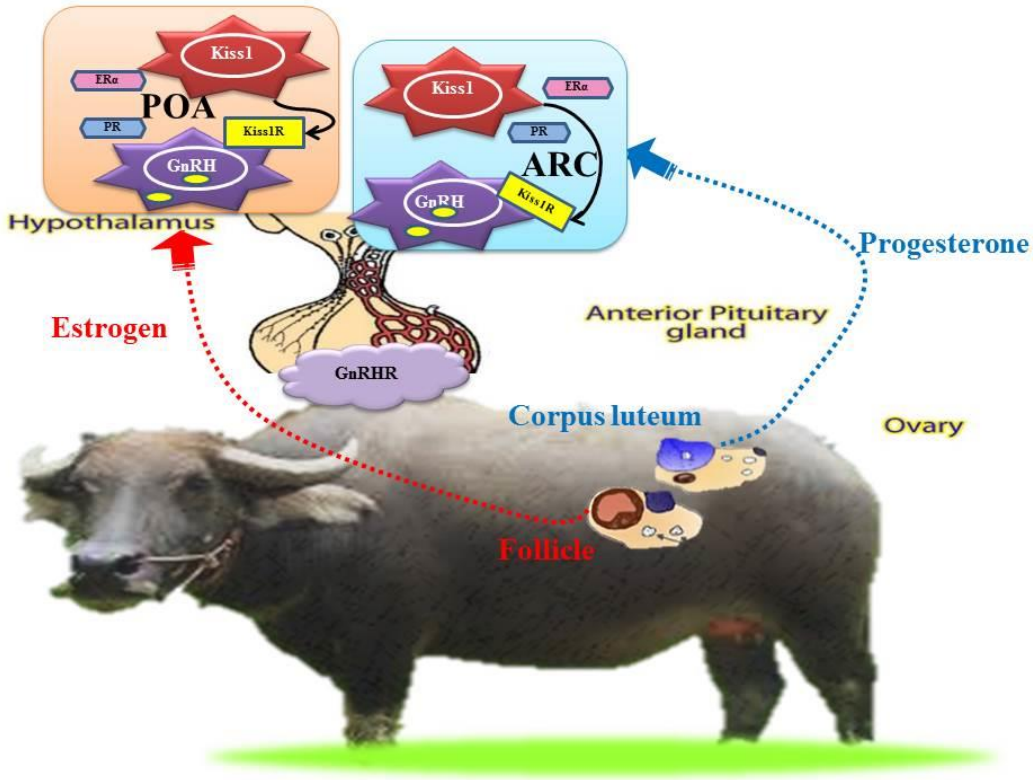

Figure 35 Schematic diagram representing the possible role of kisspeptin controlling in the HPO axis in cycling buffaloes.

Possible applications and suggestions

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, horses and river type buffaloes) 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 kisspeptin mechanism and physiology in buffalo could provide more information and help increase the potential applications of kisspeptin in the future (Chaikhun et al., 2013b).

Also, although this study used the immunohistochemistry technique for the identification of, localization of, and distribution of KISS1R-ir in GnRH neurons, estrogen receptors alpha, progesterone receptors in the POA and ARC hypothalamic nuclei, and GnRH receptors in the pituitary gland, confirmation of these expressions with other molecular techniques such as real-time quantitative polymerase chain reaction (qRT-PCR) and western blot analysis should be considered in future studies.

Although kisspeptin, especially Kp-10, may not at present be a good option for solving infertility problems in the field - it is possible that kisspeptin related fertility disorders may be treated with kisspeptin administration successfully in the future. For example, kisspeptin might be an alternative treatment for infertility problems such as hypothalamic amenorrhea (Jayasena et al., 2010), egg maturation for in vitro fertilization (Jayasena et al., 2014a), ovarian hyperstimulation syndrome (Jayasena et al., 2015) and delayed initiation of puberty (Shahab et al., 2005). Practical applications of kisspeptin for buffalo, however, may require further research into, and production of, a species specific buffalo kisspeptin agent to be truly effective.

These studies present preliminary information. They will hopefully help lay the groundwork for a further understanding of the role of kisspeptin in buffalo and its relation to reproduction and the HPO axis. Our findings indicate that further research into the clinical application of kisspeptin administration in buffalo may offer an alternative way to improve buffalo reproductive performance. Kisspeptin could prove to be useful in farm breeding management and infertility treatments due to its ability to influence the HPO axis.

Conclusion

Kisspeptin plays a key role in the hypothalamic – pituitary - ovarian axis in buffalo cow reproduction. Kisspeptin has been found to exist in the POA and ARC hypothalamic nuclei of buffalo cows in both the follicular phase and the luteal phase of the estrous cycle. There are relationships between kisspeptin neurons, kisspeptin receptors, GnRH neurons, estrogen receptors alpha and progesterone receptors in the POA and ARC hypothalamic nuclei, and GnRH receptors in the pituitary gland. The percentage of ERα -ir neurons in the POA was higher than in the ARC in both phases of the estrous cycle may indicate that the POA functions as a surge center in the hypothalamus (through estrogen feedback control) in buffalo. The administration of Kp-10 has different effects on the releasing of LH, compared to the administration of GnRH in luteal phase cows. These results suggest that kisspeptin is related to buffalo cow reproduction via the HPO axis but research needs to be done on increasing the efficiency of the *in vivo* response to kisspeptin in the future.

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APPENDICES

APPENDIX I

The percentage in each type of immunoreactive neurons such as kisspeptin neurons, kisspeptin receptors (KISS1R), GnRH neurons, estrogen receptors alpha (ERα) and progesterone receptors (PR) in the POA and ARC hypothalamic nuclei in each cow

Cow ID/ parameter	1L		2L		3L		4F		5F		6F	
	POA	ARC	POA	ARC	POA	ARC	POA	ARC	POA	ARC	POA	ARC
Kisspeptin	72	70	72	50	85	57	82	74	85	73	83	51
KISS1R	90	82	98	95	91	94	86	96	97	96	96	97
GnRH	54	56	80	90	66	81	73	52	60	80	54	78
Co-localized KISS1R+ GnRH	54	56	80	90	66	81	73	52	60	80	54	78
ERα	71	54	77	25	80	59	59	40	84	74	86	54
PR	39	32	39	4	88	38	18	29	54	35	19	14

APPENDIX II

Dosage and cost of kisspeptin-10

General product information

1 vial of kisspeptin-10 contain title compound = 129 µg/mL (0.1 mM)

1 vial has about 5 ml (4.1 ml distilled water) so total title compound = 645 µg

Estimated price (product+shipping+costom) = 3,000 THB/vial (cost of a vial = 7,200 yen and shipping cost = 10,000 yen/batch)

Dosage 0.13 µg/kg (130 ng/kg) b.w. or 100 pmol for preliminary trial

(Whitlock et al., 2008-in the OVX adult cattle cows)

Buffalo estimated weight = 400 kg/cow

Need 0.13 µg x 400 kg = 52 µg

1 vial can be used for 645 µg /52 µg = 12 buffaloes

So a buffalo need 52 µg = 52/129 = 0.4 ml

Injection of kisspeptin-10 + distilled water 1.6 ml, the total volume should be 2 ml i.v.

Cost per dose/cow 3,000 baht/12 cows = 250 baht

Dosage 1.3 µg/kg (1,300 ng/kg) b.w. or 1,000 pmol for the experiment

Buffalo estimated weight = 400 kg/cow

Need 1.3 µg x 400 kg = 520 µg

1 vial can be used for 645 µg /520 µg = 1.2 buffaloes

So a buffalo need 52 µg = 520/129 = 4 ml i.v.

Cost per dose/cow 3,000 baht/1.2 cows = 2,500 baht

APPENDIX III

List of publications and conferences

Publications

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. Accepted.

Conferences

Oral presentations

1. **Chaikhun, T.,** Sotthibandhu, P. and Suadsong, S. 2013. Localization of GnRH receptors in buffalo cow pituitary gland in follicular and luteal phases. In proceeding of The 10th World Buffalo Congress and The 7th Asian Buffalo Congress, Hilton Phuket Arcadia Resort and Spa, Karon, Phuket, Thaialnd, 6th-8th May, 2013. Page 39.

2. **Chaikhun-Marcou, T.**, Sotthibandhu, P. and Suadsong, S. 2013. Kisspeptin: New Focus in Animal Reproduction Research. In proceeding of the RGJ Seminar Series

XCIX (99th) “Innovative Reproductive Technology for Wildlife”, Kaokeaw Zoo, Sriracha, Chonburi, Thailand, 20th November, 2013.

3. **Chaikhun- Marcou, T.,** Sotthibandhu, P. and Suadsong, S. 2014. Structural Interactions between Kisspeptin Receptors and GnRH neurons in Preoptic Area and Arcuate Hypothalamic Nuclei in Cycling Buffaloes (Bubalus bubalis) as Revealed by Double Immunofluorescent. In proceeding of the 11th Internation Symposium on GnRH, Salzburg, Austria, Febuary 9-11, 2014 (CD).

4. **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.

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 *(Bubalus bubalis).* In: Proceeding of 5th SAADC 2015 on October 27-30, 2015. Dusit Thani Pattaya Hotel, Thailand. Page 132.

Poster presentations

1.**Chaikhun- Marcou, T.,** Sotthibandhu, P. and Suadsong, S. 2014. Comparative effects of administration of kisspeptin-10 and GnRH on LH secretion in buffalo cows. In proceeding of the World Congress of Reproductive Biology 2014, Edinburgh International Conference Centre (EICC), Edinburgh, Scotland, United Kingdom, 2nd-4th September, 2014.

VITA

Miss Thuchadaporn Chaikhun was born in Phayao, Thailand, on July 15th, 1980. She graduated with a Doctor of Veterinary Medicine from the faculty of Veterinary Medicine, Chiangmai University in 2005. In 2008, she got a Master of Science (Theriogenology) degree from the faculty of Veterinary Science, Chulalongkorn University. In 2010, she began her Ph.D. studies under the Thailand Research Fund (Research and Researchers for Industries), Veterinary Science, Chulalongkorn University Graduate Thesis Grant and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), at the Deparment of Obstetrics Gynaecology and Reproduction, the Faculty of Veterinary Science, Chulalongkorn University. She was a veterinarian at the Thai Dairy Development Co., Ltd. and worked on embryo transfer in cattle (planning, procedures, and collaborations with farms and co-workers) between 2005 and 2006. From 2007 to the present she has had a career as a lecturer at the Clinic for Obstetrics Gynaecology Andrology and Artificial insemination in Domestic Animals, the Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand. During this time she has also been a speaker and/or poster presenter at several international veterinary conventions and has published many articles in both national and international publications.