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*Original Article*

# Effects of senktide on kisspeptin/neurokinin B/dynorphin peptide mRNA expression and luteinizing hormone secretion in fasted female goats: a pilot study

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## *Abstract*

This pilot study aimed to examine KNDy peptide mRNA (*Kiss1*/*Tac3*/*Pdyn*) expression and to determine the effects of peripheral neurokinin 3 receptor agonist (senktide) administration on the ARC *Kiss1* mRNA expression and LH secretion that was affected by fasting condition in intact female goats during the luteal phase of the estrus cycle. During the mid to late luteal phase of the estrus cycle, the goats were randomly divided into three groups; the fed group (n = 2), which was fed ad libitum throughout the experimental period, the fasted group (n = 2) and the fasted + senktide group (n = 2) which were fasted for 96 hours. Blood samples were collected every 10 mins for 7 consecutive hours on the last day of fasting. The brain was collected immediately after the cessation of blood sampling. Senktide (200 nmol) was intravenously administered 2 times, 1 hour after the beginning of blood collection and 30 minutes before brain collection. The serum LH concentration was measured using a competitive inhibition enzyme immunoassay. KNDy peptide mRNA expression was evaluated by RT- qPCR. The ARC *Kiss1* and *Pdyn* expressions level in both fasted and fasted + senktide groups were significantly lower than in the fed group ($p<0.05$), which corresponded to mean serum LH concentration. There was neither recovery of serum LH concentration nor an increase in *Kiss1* mRNA expression in the fasted + senktide group. This pilot study suggests that fasting conditions suppress serum LH concentration by decreasing ARC *Kiss1* mRNA expression. Moreover, peripheral senktide administration was unable to recover either *Kiss1* mRNA expression or serum LH concentration in fasted goats during the luteal phase of the estrus cycle.

**Keywords:** KNDy peptide, metabolic stress, luteinizing hormone, senktide, luteal phase

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## Introduction

In female goats, the mode of gonadotropin releasing hormone (GnRH) secretion is regulated by kisspeptin neuronal populations located in the pre-optic area (POA) and the arcuate nucleus of hypothalamus (ARC). Abundant evidence in several species has reported that kisspeptin neurons (Kiss1 neurons) located in the POA involve establishing GnRH/LH surges in response to the positive feedback action of estrogen and subsequent ovulation. Meanwhile, the ARC Kiss1 neurons that co-express two important neuropeptides including tachykinin neurokinin B (NKB) and endogenous opioid peptide dynorphin (Dyn), commonly known as KNDy neuron, play a crucial role in controlling episodic GnRH secretion (Goodman *et al*., 2007). These neurons reciprocally connect to each other and become a large neuronal network, in which each of the neuropeptides has a different effect on GnRH/LH secretion. NKB plays a kisspeptin stimulatory role via the neurokinin 3 receptor (NK3R), while Dyn plays a kisspeptin inhibitory role via the kappa opioid receptor (KOR) and kisspeptin serves as KNDy neuron output to drive the pulsatile GnRH secretion at GnRH neurons soma and its terminal.

Widely reported evidence has shown that KNDy peptide expression is associated with changes in circulating sex steroid hormones. The vast majority of KNDy neurons express receptors for gonadal steroid feedback including estrogen (ERα), progesterone (PR) and androgen (AR), while GnRH neurons do not. Expression of NKB in the ARC is suppressed by estrogen, which leads to a decreased kisspeptin secretion. In ewes and rodents, the removal of gonadal steroid hormones by gonadectomy increases NKB and kisspeptin secretion, meanwhile the reintroduction of estrogen decreases this expression (Moore *et al*., 2018). Likewise, the inhibitions of kisspeptin and GnRH/LH secretion from progesterone are mediated by Dyn. Progesterone treatment increased dynorphin concentration in CSF collected from the third ventricle and pre-prodynorphin mRNA expression in dynorphin neurons of OVX ewes (Foradori *et al*., 2005).

Moreover, metabolic signals are involved in the change of KNDy peptide expression level (Evans and Anderson, 2017). However, the specific substrates and their mechanisms remain controversial and need to be fully clarified. A subset of KNDy neurons has expressed various receptors for metabolic hormones, such as an obesity receptor (OBR, leptin receptor), a growth hormone secretagogue receptor (GHSR, ghrelin receptor), an insulin receptor (IR) and also other receptors for orexigenic/anorexigenic neurotransmitters such as agouti related peptide (AgRP), neuropeptide Y(NPY) and pro-opiomelanocortin (POMC)-derivative substances (Wahab *et al*., 2018). Hence, KNDy neuron has been suggested as a mediator neuron conveying metabolic signals to the reproductive system.

Many expression and functional studies have indicated that the ARC Kiss1 neurons are sensitive to different forms of metabolic stress known to perturb puberty and gonadotropin secretion. For instance, several studies have illustrated that female animals under acute or chronic food-deprivation display a significant decrease in the hypothalamic *Kiss1* (the gene encoding kisspeptin) mRNA expression (Backholer *et al*., 2010;Ladyman and Woodside, 2014;Wang *et al*., 2016), coupled with a significant decrease in serum LH concentration. Ladyman and Woodside (2014) reported that exogenous kisspeptin administration was able to ameliorate gonadal suppression in rats subjected to fasting. This result further supports the functional relevance of suppressed kisspeptin secretion in the inhibition of the hypothalamic pituitary gland (HPG) function in the presence of a negative energy balance. Moreover, *Tac3* (the gene encoding NKB) and *Pdyn* (the gene encoding pre-prodynorphin) are possibly altered by stress signals. It is demonstrated that stress-like levels of cortisol significantly increased *Pdyn* mRNA expression in ewes during the follicular phase of estrus cycle (Ralph *et al*., 2016). Furthermore, fasting for 48 hours decreased *Tac2* (gene encoding neurokinin B in rats) and *Tac3r* (gene encoding NK3R) mRNA expression in rats (Navarro *et al*., 2012).

Senktide, which is abbreviated for selective neurokinin B receptor peptide, is an analogue of the amino acid fragment which is initially synthesized to study the action of different tachykinins on multiple receptors expressed in the guinea pig ileum (Wormser *et al*., 1986). Since the reproductive role of NKB was first highlighted in 2009 by Topaloglu *et al*., they have discovered that patients who have mutations in *Tac3* and *Tac3r* develop hypogonadotropic hypogonadism and several later studies have revealed the beneficial use of senktide on increasing LH secretion and successfully induce ovulation in several animal species (Endo and Tanaka, 2014, 2015;Ramaswamy *et al*., 2010). However, the effects of senktide on reproductive performance were influenced by the hormone milieu and need to be fully clarified. Thus, this pilot study aimed to examine the KNDy peptide mRNA (*Kiss1*/*Tac3*/*Pdyn*) expression and to determine the effects of peripheral senktide administration on the ARC *Kiss1* mRNA expression and LH secretion that was affected by fasting conditions in intact female goats during the luteal phase of the estrus cycle.

## Materials and Methods

***Animals:*** All procedures were approved by Kasetsart University's Institutional Animal Care and Use Committee (ACKU63-VET-026).

Six female, mixed-breed goats (12.83±1.07 months of age, 26.22±1.60 kg in body weight) that had never been bred were used in this study. All the goats were confirmed to be clinically healthy and had normal estrus cycle before attending the experiments. They were raised in paddocks with sheltered areas within the natural photoperiod at the Kasetsart University Kamphaeng Saen Campus between July 2019 and October 2019.

To minimize the disturbing effects of sex steroids on KNDy peptide mRNA expression without employing ovariectomy or ovariohysterectomy (OVX), all the experiments were performed when the goats were in the luteal phase of the estrus cycle. Before the experimental period, all the goats were normally fed a

diet based on corn silage (approximately 3 kg/goat/day) and additional 16% protein concentrate supplement (approximately 150 g/goat/day). The amounts of feed were formulated according to the Nutrient Requirements of Small Ruminants (2007). Pangola hay, clean water and mineral salt were available ad libitum. Estrus signs, including standing heat, swollen vulva and clear vaginal discharge were checked daily using a buck. The day that a goat showed the onset of estrus signs was recorded as Day 1. On Day 11, transrectal ovarian ultrasonography was performed to access the presence of corpus luteum (CL) in order to confirm that the goats had already entered the luteal phase of the estrus cycle. All the experimental procedures were initiated when the goats entered the luteal phase of the estrus cycle. The goats were weighed and randomly assigned to three groups; (1) the fed group (n = 2) in which goats were fed ad libitum throughout the experiment period, (2) the fasted group (n = 2) and (3) the fasted + senktide group (n=2). Both fasted and fasted + senktide groups were fed in a manner similar to the fed group before the experiment period but they were fasted for 4 days (96 hours) at the onset of the experiment (Day 12).

*Experimental protocol*

***Effects of senktide on pulsatile LH hormone secretion during the luteal phase of intact female goats induced metabolic stress conditions by fasting:*** On the last day of fasting or Day 15, a blood sample (2.5 ml) was collected via a jugular catheter at 10 minute intervals for 7 consecutive hours (90-96 hours after fasting). Both fed and fasted groups were slowly administered with normal saline, while the fasted + senktide group was slowly administered with 200 nmol of senktide (Sigma-Aldrich, Inc., USA) dissolved in 5 mL of normal saline containing 0.25 % dimethyl sulfoxide over 5 minutes through the jugular vein at 90 hours after fasting. The administration protocol was determined according to a previous study (Endo and Tanaka, 2014). The dose of senktide was determined in accordance with the concentration which was able to stimulate LH secretion in goats with anestrus symptoms (Endo and Tanaka, 2014) and to rapidly increase LH secretion after each injection in feed-restricted goats (Endo and Tanaka, 2015). All blood samples were allowed to coagulate in blood collection tubes with a coagulation activator at room temperature and then were centrifuged at 2000×g for 10 minutes to obtain the serum. Separated serum was stored at -20°C until measurement for LH.

Blood samples which were collected for a third time were used to measure serum NEFA, BHBA and glucose concentration by the enzymatic colorimetric method using Randox NEFA assay, Randox D-3 Hydroxybutyrate (Ranbut) assay (Randox Laboratories Ltd., UK) and Erba Glucose Reagent (Collateral Medical Pvt Ltd, Inc., India), respectively, following the manufacturer's instructions. The optical density of the sample obtained after enzymatic colorimetric reaction was then determined by eppendorf Biospectrometer® (Eppendorf Co., Ltd., Germany).

Serum LH concentration was measured using the competitive inhibition enzyme immunoassay method according to Browns *et al.*,'s report (Brown, Walker and Steinman, 2003). The variation within assays, determined by repeated analysis of samples using control samples with a concentration of 1200 pg/mL (low control, 30% binding) and a concentration of 400 pg/mL (high control), was 4.82%. The variation between assays, determined by repeat analysis of the same control sample in several assays, was 5.86%.

***Kisspeptin/Neurokinin B/Dynorphin (KNDy) peptide mRNA expression and the effect of senktide on the ARC Kiss1 mRNA expression during the luteal phase of intact female goats induced metabolic stress condition through fasting:*** To determine the effects of peripheral senktide administration on the ARC *Kiss1* mRNA expression, a second senktide was administered at 30 minutes prior to the cessation of blood sampling with a protocol similar to the first senktide injection. After the blood sampling was done, the goats were weighed and then euthanized by the injection of sodium thiopental (25 mg/kg) followed by $MgSO_4$ through the jugular vein in accordance with the AVMA Guidelines for the Euthanasia of Animals (Leary *et al.*, 2013). Brain samples covering the hypothalamus with a size of approximately 3-4 cubic centimeters were immediately collected and preserved with Killik O.C.T. compound embedding medium (Bio-Optica Milano S.p.A., Italy) and kept at -80°C. Twenty micrometer thickness brain samples covering the arcuate nucleus according to Zuccolilli's report (Zuccolilli, Hayashi and Mori, 1995) were cut in coronal planes at -20°C using Leica CM1850 cryostat (Leica Biosystems Inc., USA). One brain section from every three cut sections was gathered and embedded on to the positive charge glass slides. All of the slides were divided into 8 series (12 brain sections/series) and kept at -80°C until RNA extraction was conducted. Each brain series was equivalent to one RNA sample.

The ARC region (Figure 1) was selected for RNA extraction using Rneasy® Mini Kit (Qiagen, Germany). The purity and amount of RNA were measured using NANODROP 2000 spectrophotometer (ThermoFisher Scientific, USA). A total of 900 ng of RNA was then converted to cDNA immediately using RevertAid Fisrt Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) following the manufacturer's instructions and stored at -20°C until quantitative reverse transcription PCR (RT-qPCR) was initiated. RT-qPCR was performed using the CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, Inc., USA) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., USA). Each sample was treated in triplicate with 15 µl reaction volume. PCR cycling conditions were 95°C for 10 minutes, 40 cycles of 90°C for 10 seconds, 60°C for 20 seconds and 72°C for 30 seconds and 72°C for 5 minutes. The negative (no template) controls were run for each reaction. Specific primer pairs for *Kiss1*, *Tac3*, *Pdyn* and *ACTB* amplification were referenced from previous publications or newly designed based on the Capra hircus kisspeptin (NM_001285710.2), neurokinin B (AB499062), pre-prodynorphin (AB499063) and β-actin (DQ845171) mRNA sequence, respectively, published

in the NCBI database, using Primer3 software version 4.0.0 (Table 1). The quantitative cycle (Cq) obtained from RT-qPCR was analyzed by relative quantification. This method determined the changes in steady-state mRNA expression levels across multiple samples and expressed them relative to the levels of reference gene (β-actin), which was calculated by a normalized expression (ΔΔCq) method using Bio-Rad CFX Masetro 1.1 (Bio- Rad Laboratories, Inc., USA).

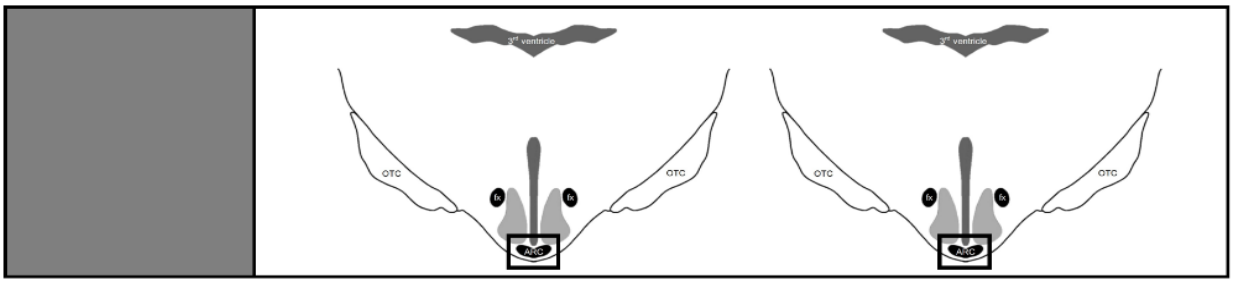


**Figure 1** The ARC region in the square selected for RNA extraction.

**Table 1** Specific primers used for *Kiss1*, *Tac3*, *Pdyn* and *ACTB* mRNA amplification.

| Gene name | Primer sequence (5′ – 3′) | Product size (bp) | Amplification efficiency (%) | References |
|---|---|---|---|---|
| *Kiss1* | **F*:** TCACCTGGCGAGAGTTTAGC<br>**R*:** CTGTGGTTCTAGGATTCTCC | 354 | 90.3 | -<br>- |
| *Tac3* | **F:** ATGCGGAGCACCCTGCTGTT<br>**R*:** CATTCCACACTTGGAGGGTA | 444 | 105.7 | (Wakabayashi *et al.*, 2010)<br>- |
| *Pdyn* | **F:** TGTGCTGTGAAGACCCAGGAA<br>**R*:** CCGAGTGACCACCTTGAACTG | 157 | 80.9 | (Wakabayashi *et al.*, 2010)<br>- |
| *ACTB* | **F:** TGCCCTGAGGCTCTCTTCCA<br>**R:** TGCGGATGTCGACGTCACA | 103 | 96.3 | (Zhang *et al.*, 2013) |

*The specific primers were designed in this study.

***Statistical analysis:*** Normalized relative expressions of GOI between the fed group, the fasted group and the fasted + senktide group are shown as the geometric mean in the bar charts. Error bars are expressed as mean ± $2^{[\log \text{ transformed expression} \pm (1 \times \text{SEM (log transformed expression)})]}$. The significance of differences in normalized relative expression of each GOI was assessed by one-way ANOVA followed by the Tukey's HSD test using Bio-Rad CFX Masetro 1.1 (Bio-Rad Laboratories, Inc., USA).

Hormonal data is presented as mean ± SEM. Mean serum LH concentration between three experimental groups analyzed using one-way ANOVA followed by the LSD test. To examine the effect of senktide on serum LH concentration, the area under the curve with respect to ground ($AUC_G$) of the LH response during 60 minutes pre-treatment period was compared with that during the 60 minutes post-treatment period according to Pruessner *et al.*,'s report.

## *Results*

Fasting for four days caused an average 1.7 kg of body weight loss (n=4). Meanwhile, the fed group (n = 2) had approximately ± 0.3 kg of body weight change. On the last day of fasting, the mean serum non-esterified fatty acid (NEFA) concentration in both the fasted and the fasted + senktide groups (n = 4, 1.421 mM) were much higher than in the fed group (n = 2, 0.052 mM), which was consistent to the mean serum β-hydroxybutyric (BHBA) concentration between the fed group (0.356 mM) and both the fasted and the fasted + senktide groups (0.616 mM). Similarly, the mean serum glucose concentration in the fed group (73.761 mg/dL) appeared to be higher than in both the fasted and the fasted + senktide groups (56.280 mg/dL).

***Effects of senktide on pulsatile LH hormone secretion during the luteal phase of intact female goats induced metabolic stress condition by fasting:*** The mean serum LH concentration in the fed group was significantly higher (0.595 ± 0.013 ng/mL) than in both the fasted (0.357 ± 0.013 ng/mL) and the fasted + senktide groups (0.354 ± 0.009) ($p<0.05$). Meanwhile, no significant difference between serum LH concentration in the fasted and the fasted + senktide group was observed (Figure 2). The $AUC_G$ during 60 minutes after the first senktide administration period appeared to be not different from that 60 minutes before senktide administration (Figure 3). In addition, repeated senktide injection (30 minutes before the cessation of blood sampling) was unable to suddenly enhance LH concentration which was similar to the first senktide injection (Figure 4). Consequently, senktide was unable to recover fasting-induced LH suppression in the fasted + senktide group.

***Kisspeptin/Neurokinin B/Dynorphin (KNDy) peptide mRNA expressions and the effect of senktide on the ARC Kiss1 mRNA expression during the luteal phase of intact female goats induced metabolic stress condition by fasting:*** The mRNA expression levels of *Kiss1*, *Tac3* and *Pdyn* normalized with *ACTB* of brain samples obtained from both the fasted and the fasted + senktide groups were expressed as an increase or decrease relative to the fed group (control) and are shown in Figure 5. Our findings illustrate that there were no differences in the *Kiss1*, *Tac3* and *Pdyn* expression between the the fasted group and the fasted

+ senktide group. However, the normalized relative *Kiss1* and *Pdyn* expression in both the fasted and the fasted + senktide groups were significantly lower than in the fed group ($p$<0.05), while no significant difference in normalized *Tac3* expression between the three experimental groups was observed.

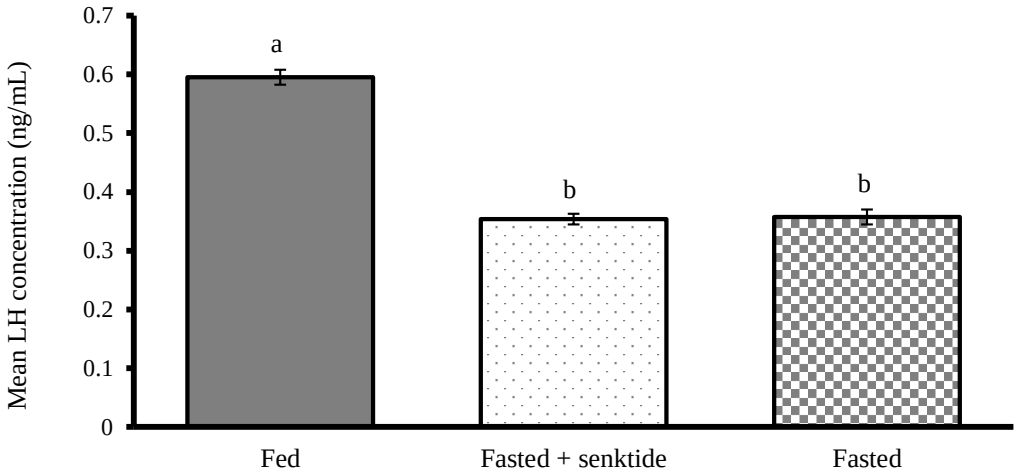


**Figure 2** Mean serum LH concentration in the fed group, the fasted group and the fasted + senktide group. Data is expressed as mean ± SE. Different letters indicate a statistical difference ($p$<0.05).

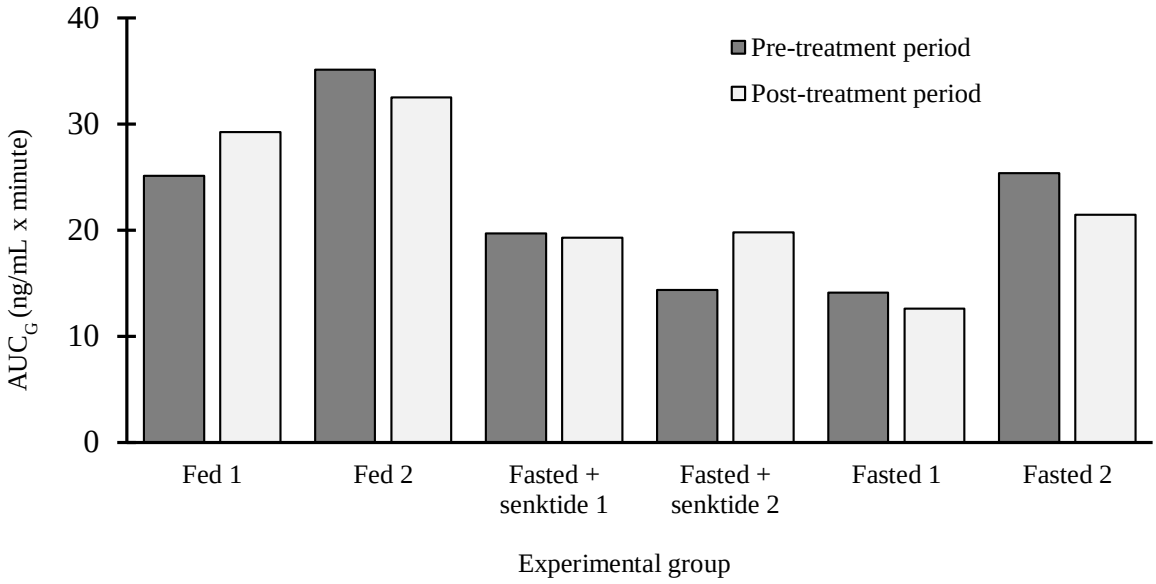


**Figure 3** The AUC$_G$ of LH response during pre- and post- treatment periods of the fed goats, the fasted goats and the fasted + senktide goats.

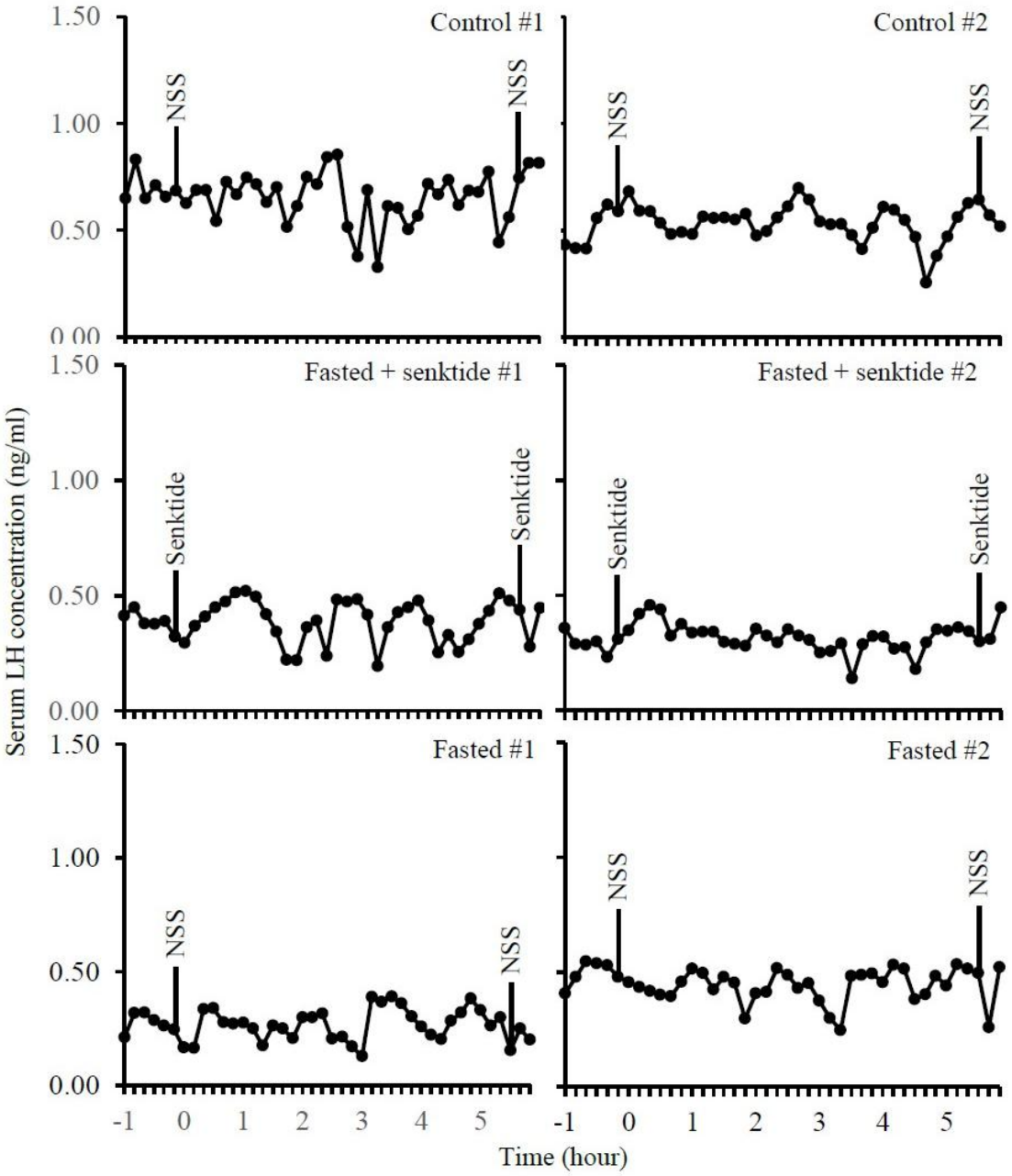


**Figure 4** Representative profiles of LH concentration in the fed group, the fasted group and the fasted + senktide group.

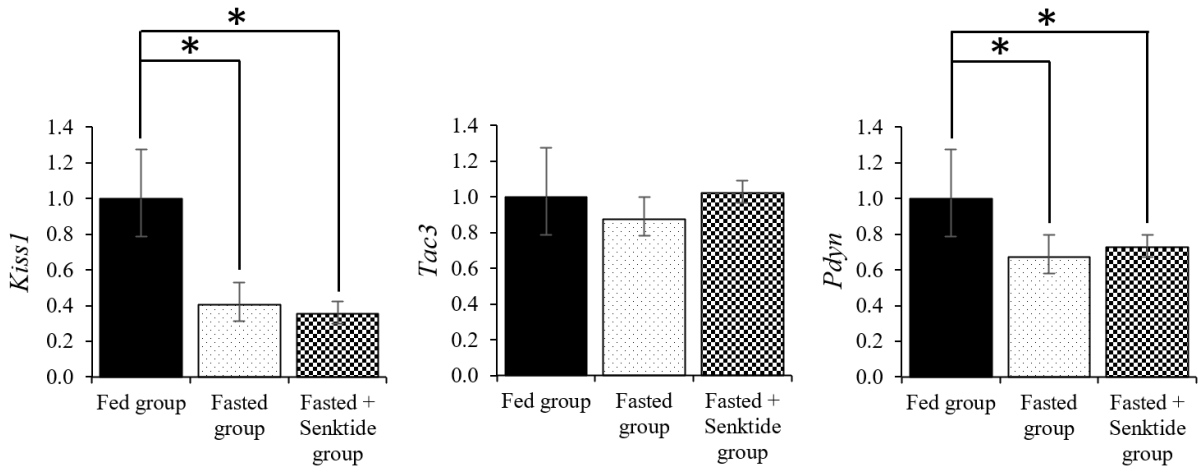


**Figure 5** The relative *Kiss1*, *Tac3* and *Pdyn* mRNA expressions in the ARC region in the fed group, the fasted group and the fasted + senktide group determined by RT-qPCR. Values were normalized by ACTB. Error bars were expressed as mean ± $2^{[\log \text{ transformed expression} \pm (1 \times \text{SEM (log transformed expression)})]}$. An asterisk represents a significant difference.

## *Discussion*

In the present study, four days of fasting in 4 goats resulted in approximately 1.7 kg body weight loss. All the fasted goats (n=4) showed a high NEFA and a low glucose concentration level compared to the fed goats (n=2). A mobilization of NEFA and decreased serum glucose concentration is the hallmark of a negative energy balance condition. NEFA is completely oxidized to establish acetyl CoA and it is then used to generate energy via the Krebs cycle. However, incomplete oxidation of NEFA which leads to the accumulation of ketones can take place. This process is an additional strategy to compensate for insufficient energy. Therefore, BHBA levels in all the fasted goats were likely higher than the fed goats.

To minimize the disturbing effects of sex steroids on KNDy peptide mRNA expressions without operating ovariectomy or ovariohysterectomy (OVX), we decided to use goats that were in the luteal phase (mid to late) of the estrus cycle because this period had the lowest hormonal fluctuation compared to the others. Under normal conditions during this period, the progesterone level was consistently high, while the estrogen level changed slightly (Fatet, Pellicer-Rubio and Leboeuf, 2011). We demonstrated that the ARC *Kiss1* and *Pdyn* mRNA expressions in female goats during the luteal phase of the estrus cycle subjected to fasting were significantly suppressed. However, this was inconsistent with the previous preliminary-work results. Ralph and co-workers found that stress-like levels of cortisol during the follicular phase of ewes significantly increased Dyn peptide mRNA expression levels (Ralph *et al*., 2016). Moreover, Lehman found an increase in the number of *Pdyn*-containing cells and the mRNA content per cell in the ARC of cortisol-treated ewes compared to the control (Lehman, unpublished observations). Such a contradiction is possibly due to (1) the differences between hormone milieu.

Dyn has been generally recognized to mediate progesterone negative feedback on GnRH release (Foradori *et al*., 2005; Goodman *et al*., 2004; Wakabayashi *et al*., 2010). Therefore, the influence of a large amount of progesterone together with the presence of a low level of estrogen in the luteal phase possibly lead to an increased *Pdyn* mRNA expression and then an increased dynorphin release.

In the present study, we proposed that the reduction of *Kiss1* led to decreased GnRH/LH secretion consequent to an insufficient steroid synthesis from the corpus luteum, resulting in a decreased progesterone level. Together with the possibility that a decrease in leptin or other factors inactivated theca interna in the follicles this contributed to the inhibition of luteal function and a reduction of progesterone levels (Al-Azraqi, 2007). Hence, we proposed that *Pdyn* was therefore decreased because of lower stimulatory factor. (2) Physiological stress responses may be stressor specific. These findings correspond with the previous study indicating feed-restriction suppress the ARC *Kiss1* and *Pdyn* expression in developing female rats (Majarune *et al*., 2019). In cases of metabolic stress induced by fasting, there were gut and adipose-derived hormones in addition to cortisol involved in KNDy peptide mRNA expression.

The dose of senktide was referred to a previous study in which 200 nmol of senktide was able to immediately increase serum LH concentration in anestrus Shiba goats after administration (Endo and Tanaka, 2014). Nevertheless, the exact reasons for anestrus remain unclear. In this study, neither the first nor the second senktide administration was able to increase LH concentration within 10-20 minutes (middle panels, Fig. 4). Noticeably, we found that under the influence of progesterone during the luteal phase of the estrus cycle of intact female goats, *Tac3* mRNA expression levels between 3 groups were not significantly different (Fig. 5), implying that *Tac3* mRNA expression may possibly not be influenced by fasting conditions. Meanwhile, there was a dramatic reduction in the ARC *Kiss1* mRNA expression level in both the fasted and the fasted + senktide groups which was probably considerably influenced by metabolic information. Thus, the activation of NKB-NK3R signaling may not overcome *Kiss1* suppression and recover fasting induced LH suppression. We suggest that a continuous senktide administration or raising the senktide concentration may be more useful. In conclusion, the reproductive performance was partially modulated by the KNDy neuron and we propose that under the influence of progesterone during the mid to late luteal phase of estrus cycle, metabolic signals had a dominant role in regulating the ARC *Kiss1* expression leading to a decrease in LH and progesterone secretion resulting in decreased *Pdyn* expression. Such an inhibitory signal on kisspeptin might potently regulate GnRH/LH secretion than NKB/NK3R signal. Hence, a single peripheral senktide administration was unable to recover the ARC *Kiss1* expression and LH secretion that were suppressed by fasting conditions.

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