Original Article

Validation of commercially available EIA kit for measurement of feline plasma kisspeptin

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Abstract

Circulating kisspeptin is described to be related with the reproductive system. A study of plasma kisspeptin levels, thus, will provide the better understanding of reproductive endocrinology in the domestic cat. The present study aimed (i) to validate the only available commercial kisspeptin EIA kit which is currently used in humans to be used in domestic cats and (ii) to determine and compare the plasma kisspeptin levels at different reproductive stages. The intra-assay coefficient of variation (CV) was 11.13%. The inter-assay CV ranged from 5.00 – 8.25%. The parallel graphs represented the homology between human kisspeptin and feline endogenous kisspeptin. The recovery rate ranged from 78% - 106%. To compare the plasma kisspeptin levels among reproductive status, plasma samples were collected from the prepubertal female cats (n = 6) and sexually mature female cats with an inactive (n = 6) and follicular staged (n = 6). Although no statistically significant difference in the plasma kisspeptin concentration between reproductive stages was observed, there was a tendency that the plasma kisspeptin levels were higher in the prepuberal cats compared with pubertal cats. In conclusion, the commercial EIA kit can be used to determine plasma kisspeptin in the domestic cats.

Keywords: ELISA, Enzyme immunoassay, feline, kisspeptin-10, Metastin, plasma

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Introduction

Kisspeptin has been considered to be the key regulator of reproductive system, mainly through controlling the secretion of a gonadotropin-releasing hormone (GnRH) at the hypothalamus *(Messager et al*., 2005). In response to GnRH, the pituitary gland releases the gonadotropins which subsequently control the gonadal functions. The mutation of kisspeptin or its receptor results in reproductive abnormalities e.g. delayed puberty and ovulatory failure in mice (Seminara *et al*., 2003; Lapatto *et al*., 2007). In addition to the hypothalamus, kisspeptin has also been identified in various reproduction-related tissues of rodents: ovary (Terao *et al*., 2004), placenta (Terao *et al*., 2004; Kinsey-Jones *et al*., 2014), uterus (Zhang *et al*., 2014) and testis (Salehi *et al*., 2015). Interestingly, the fluctuation of *Kiss1* expression was demonstrated in a cyclic-dependent manner in the rat ovary, with the highest level during the preovulatory period (Castellano *et al*., 2006).

For clinical study, growing evidence supports that kisspeptin can be used as the biological markers for diagnostics in reproductive diseases or the determination of reproductive stages. In humans, the concentration of plasma kisspeptin can indicate the risk of polycystic ovarian syndrome (PCOS) as the relatively high plasma kisspeptin has been observed in patients with a PCOS compared to healthy women (Chen *et al*., 2010). Moreover, the level of circulating kisspeptin is closely related to reproductive status in both human (Latif and Rafique, 2015) and animals (Ricu *et al*., 2012; Mondal *et al*., 2015). In non-pregnant cattle, the highest concentration of plasma kisspeptin has been observed prior to ovulation; therefore, it is assumed that kisspeptin might be used as an indicator for the pre-ovulatory period (Mondal *et al*., 2015). Interestingly, plasma kisspeptin levels are significantly higher in prepubertal female rats compared with pubertal female rats; consequently, plasma kisspeptin concentration can be used to identify the pubertal stage in rats (Ricu *et al*., 2012). Taking these studies into consideration, the evaluation of plasma kisspeptin levels might be able to specify precise reproductive stages.

Domestic cats have long been an important animal model for the study of the reproductive system in endangered wildcats. However, the investigation of kisspeptin in domestic cats has just begun. Recently, kisspeptin was identified in the hypothalamus and reproductive tracts of the female cats (Tanyapanyachon *et al*., 2018; Amelkina *et al*., 2019). In cat ovaries, the expression of kisspeptin encoding mRNA, *Kiss1*, has been reported to be related to reproductive stages, with an increased of *Kiss1* at follicular phase (Tanyapanyachon *et al*., 2018). Thus, it is worth investigating whether circulating kisspeptin in the cats can be used as a useful biological marker to specify the reproductive status of cats.

Therefore, the present study aimed (i) to validate the commercially available kisspeptin enzyme immunoassay (EIA) kit to be used in the domestic cats and (ii) to determine and compare the plasma kisspeptin levels in the domestic cat at different reproductive stages.

Materials and Methods

Animals: The study was conducted with ethics approval and performed under the license of Chulalongkorn University of Animal Care and Use (reference number 1831015), with care to comply with the 3R concept.

The plasma samples were obtained from client-owned female cats presented for ovariohysterectomy at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. All samples were collected from cats kept in the Bangkok area and the perimeter. Cat owners were informed about thr protocol and signed an agreement to allow the participation of the cats. As a routine pre-operative check-up, all cats received a general physical examination, basic hematological test and biochemical analysis of their blood.

Plasma samples from 42 cats (n = 24 for analytical validation of EIA, n = 18 for the determination of plasma kisspeptin) were included. For analytical validation of EIA, the samples from female cats (≥10 months to ≤5 years, weighing 2.3 – 4.5 kg) with inactive (n = 12), follicular (n = 6) and luteal (n = 6) stages were used. Their ages were obtained by history taking. For comparison of plasma kisspeptin levels different reproductive stages, plasma samples from prepubertal (≥3 to ≤4 months; weighing 1.27 – 1.64 kg; n = 6), sexually mature cats at the inactive stage (≥7 months to ≤2 years, weighing 2.1 – 2.9 kg, n = 6) and follicular stage (≥7 months to ≤2 years, weighing 2.1 – 2.6 kg, n = 6) were investigated.

The inactive stage was determined from no evidence of follicles ≥ 2 mm in diameter and no evidence of corpora lutea (CL) in both ovaries (Uchikura e*t al*., 2011). The follicular stage was classified by the presence of follicles ≥ 3 mm in diameter in one or both ovaries with 40 – 60% superficial cells in vaginal cytology (Shille *et al*., 1979). The vaginal epithelial cells were examined at a magnificationx100. The superficial cells were determined as stated in a previous report (Kanca e*t al*., 2014). The luteal stage was determined by the presence of CL in one or both ovaries with blood progesterone ≥2 ng/ml (Shille and Stabenfeldt, 1979). Progesterone level was evaluated by an automated immunoassay system, Tosoh ST AIA-Pack PROG assay (Tosoh, Tokyo, Japan), with an assay range of 0.1 – 40 ng/ml

Blood Collection: Blood samples were obtained prior to ovariohysterectomy. To minimize the possible effects of circadian rhythm on kisspeptin (Kriegsfeld, 2013), the blood collection was performed between at 08:00 - 11:00 a.m..

Whole blood (3 ml) was collected from the cephalic vein of each cat and placed in a tube containing K3-EDTA (Greiner Bio-One GmbH, Kremsmünster, Austria). The blood was further centrifuged at 2,000 g for 20 min at 4°c to separate the plasma. The plasma was then transferred to 1.5 ml polypropylene eppendorf tubes and stored at -20°C until the extraction process.

Sample preparation for the measurement of kisspeptin: The plasma (1 ml) from each cat was acidified with

buffer A (1 ml) (RK-BA-1, Phoenix Pharmaceuticals Inc, California, United States) and centrifuged at 6,000 g for 20 minutes at 4℃. The solution was then loaded into a sep-column containing 200 mg of C-18 (RK-SEPCOL-1, Phoenix Pharmaceuticals Inc, California, United States) which had previously been equilibrated by buffer B (1 ml) (RK-BB-1, Phoenix Pharmaceuticals Inc, California, United States) once and buffer A (3 ml), respectively. The sep-column was washed with buffer A (3 ml) and the solution was discarded. The sep-column was eluted by buffer B. The plasma rich eluant was collected into a polystyrene tube (HCG-1100-TPS, Biomed Thailand) and frozen at -20℃ until the drying process. The lyophilizer (77535-01, Labconco, Germany) was used to evaporate and dry the peptide rich solution to a peptide rich pellet. The pellet was then stored at -80℃ until kisseptin measurement.

EIA: The plasma rich pellet was reconstituted to plasma rich solution by adding 1X buffer (125 µl) provided by the EIA kit. The concentration of plasma rich solution was 8 times which resulted in a detectable range of the commercial EIA kit. The assay was performed following the instruction of the manufacturer. The absorbance was measured at 450 nm. The kisspeptin levels were quantified by four parameter logistic functions (Magellan data analysis software V6.00). The measured concentrations were then divided by 8 to give plasma concentration.

Assay validation: Intra-assay variability was determined by the coefficient of variability (CV) of a measure of endogenous kisspeptin of pooled plasma which was run in duplicate 7 times within the same assay. These pooled plasma samples were obtained from cats which were categorized as being at different reproductive stages: inactive (n = 5), follicular (n = 5) and luteal (n = 5) stages. The CV was then calculated: CV = (SD/mean)*100.

Inter-assay was determined by the CV of a measurement of standard kisspeptin provided by EIA kit at three different concentrations which were run in duplicate in three separate assays. The CV was calculated: CV = (SD/mean)*100. Such standard kisspeptin is known to cross-react with human kisspeptin-10/metastin (45-54).

To study the parallelism, the plasma from cats at the inactive (n = 1), follicular (n = 1) and luteal stages (n = 1) were pooled. These pooled plasma samples were diluted serially two fold in assay buffer (1:1(neat), 1:2, 1:4 and 1:8) and run in duplicate along with a standard curve. The standard curve was plotted by the known concentrations of standard kisspeptin.

For the recovery test, the pooled plasma samples from cats at the inactive stage (n = 6) were spiked with an equal amount of 10, 1, 0.1, 0.05 and 0.025 ng/ml of standard kisspeptin. Additionally, the pooled samples were analyzed in the absence of spiked standard kisspeptin to evaluate the endogenous feline kisspeptin. The endogenous feline kisspeptin was then subtracted from each spiked pool sample. The amount expected was calculated: each concentration of standard kisspeptin/2. The amount observed was calculated: each concentration from each spiked pool sample – endogenous feline kisspeptin. The percentage of the recovery was determined: (amount observed/ amount expected)*100. The percentage of recovery was set between 80% – 120%.

Measurement of plasma kisspeptin in different reproductive stages: The plasma obtained from prepubertal cats (n = 6), sexually mature cats with inactive (n = 6) and follicular (n = 6) stages was investigated for plasma kisspeptin levels by the validated commercial EIA kit following the instructions of the manufacturer. Briefly, rich peptide solution (50 µl) from each cat was added into each well of the kit in duplicate. Then, the samples were incubated with primary antibody (25 µl) and biotinylated peptide (25 µl) at room temperature for 2 hours. Then, the contents were discarded and each well was washed with the assay buffer (350 µl). The immunoreactivity was visualized by incubating with streptavidin-horseradish peroxidase (100 µl) and substrate TMB (100 µl). This reaction was terminated by adding 2 N HCl (100 µl). The concentration of plasma kisspeptin was conducted as mentioned above.

Statistical analyses: All data were statistically analyzed by IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The mean and standard error (SEM) were calculated. A one-way analysis of varience (ANOVA) and LSD test was performed to compare the differences of means of plasma kisspeptin levels at the different reproductive stages of the cats.

Results

Validation of commercial kisspeptin EIA kit: The intra-assay CV determined using rich plasma solution containing 0.057 ng/ml of endogenous feline kisspeptin was 11.13%. The inter-assay CV determined using standard kisspeptin provided by the company, containing 0.1, 1 and 10 ng/ml were 5.00, 4.11 and 8.25%, respectively. The mean ± SEM inter-assay was 5.78 ± 1.25.

The feline endogenous kisspeptin with increasing dilution tended to be parallel to the standard curve (Fig. 1). Additionally, 1:1 (neat) is the dilution of rich plasma solution that gave the 50% binding (Fig. 1). Therefore, neat is the suitable dilution for the measurement of cat kisspeptin using this EIA.

The mean ± SEM percentage of recovery was 84.8 ±5.31. The recovery of the amount expected (x-axis) versus observed (y-axis) kisspeptin concentration was shown by linear regression (Fig. 2) and gives the equation, y = 1.0618x – 0.0385. The percentage of recovery is shown in Table 1.

Feline plasma kisspeptin profile at different reproductive stages: The plasma concentrations of the cats at prepuberal, inactive and follicular stages were 7.91 ± 0.53, 6.87 ± 0.53 and 6.25 ± 0.64 pg/ml, respectively. No significant differences in plasma kisspeptin were observed among groups (Fig. 3).

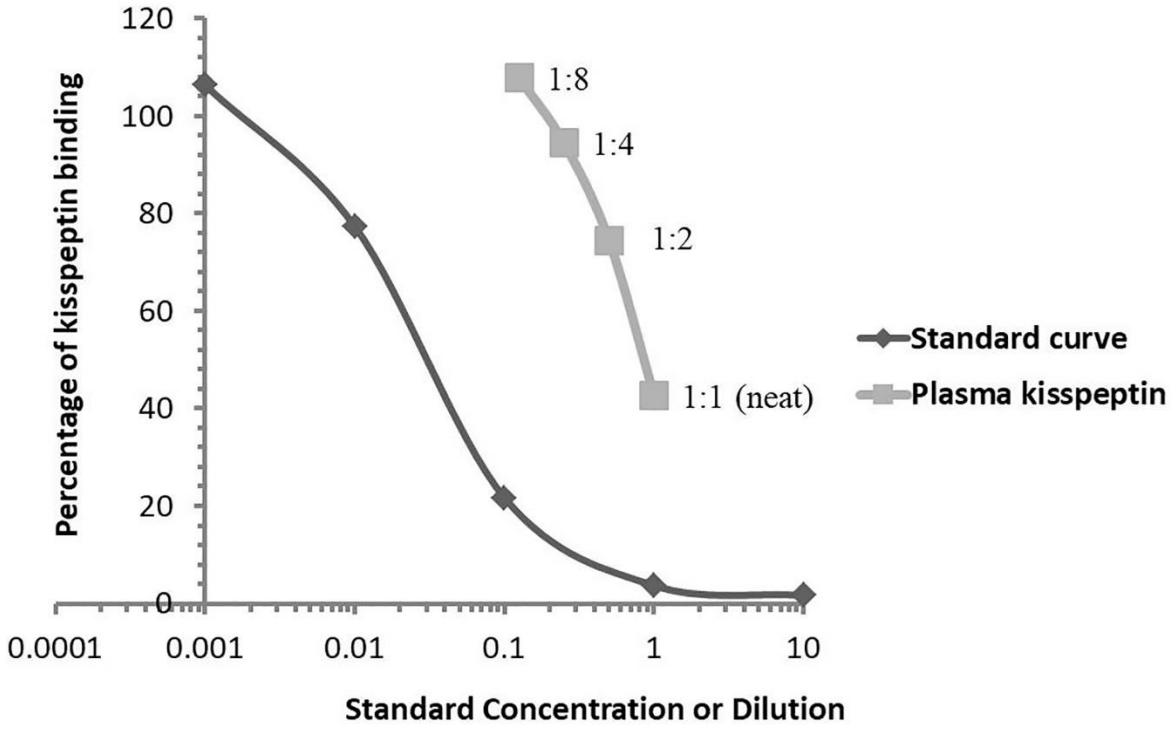

Figure 1 Parallelism between standard kisspeptin and two fold diluted cat plasma containing endogenous kisspeptin. Standards of kisspeptin concentrations ranged from 0.001 to 10 ng/ml per well.

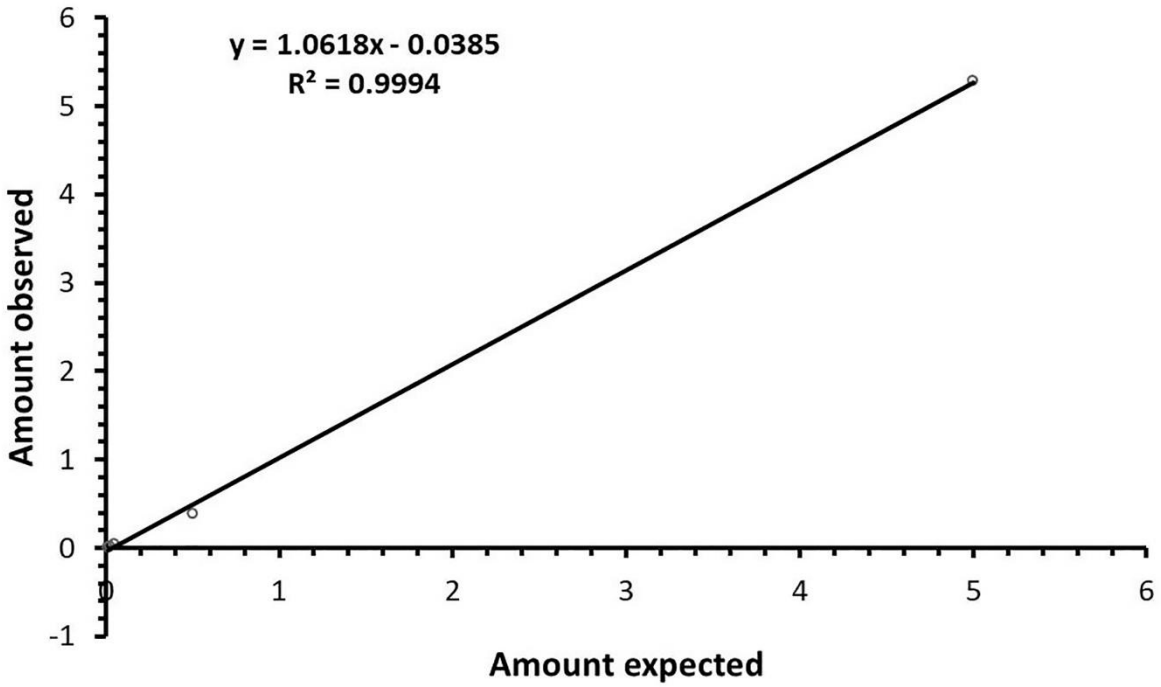

Figure 2 Linear regression of recovery of amount expected and measured in the assay.

Table 1 Recovery of spiked standard kisspeptin in pooled feline plasma

Amount expected (ng/ml)	%Recovery
5	106
0.5	78
0.05	80
0.025	80
0.0125	80

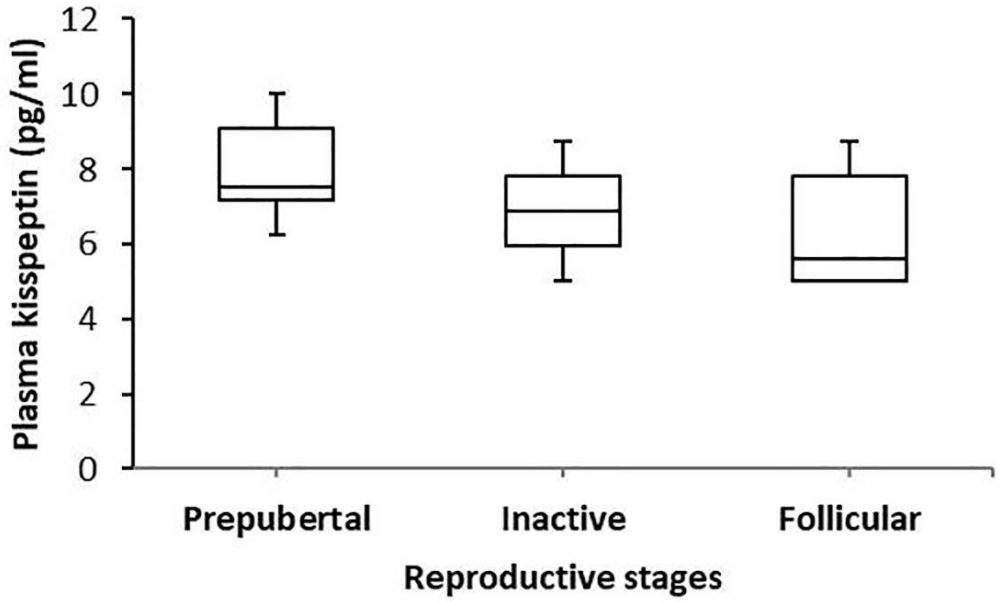

Figure 3 Plasma kisspeptin concentration (mean±SEM) during the prepubertal (n = 6), inactive (n = 6) and follicular (n = 6) stages of the cat.

Discussion

For the first time, kisspeptin was identified and measured in the plasma of the cats, using a commercially available EIA kit.

For validation of the commercially EIA kit to be used in domestic cats, an intra-assay precision test, inter-assay precision test, parallel test and recovery test were conducted. The intra- and inter-assay precision tests suggest the repeatability of an assay within the single assay and within the different assay, respectively. The parallelism reveals the considerable homology between the standard kisspeptin and feline kisspeptin. Consequently, the standard curve, plotted from the standard kisspeptin can be used to determine the concentration of plasma kisspeptin in cats. Furthermore, the recovery rates show little interference with the sample-specific matrix which is in the acceptable range (80% - 120%) (Andreasson *et al*., 2015). Taken together, the commercial kisspeptin EIA kit can be applied to measure plasma kisspeptin levels of domestic cats, as with humans (Torricelli et al., 2008; Logie *et al*., 2012; Kaya *et al*., 2015) and rats (Ricu *et al*., 2012).

With the validated EIA kit, the plasma concentration of kisspeptin in the domestic cat at different reproductive status was determined. Although no statistically significant difference in plasma kisspeptin concentration between the reproductive stages was observed, there was a tendency that the plasma kisspeptin levels were higher in prepuberal cats (7.91± 0.53 pg/ml) compared with pubertal cats at both inactive (6.87 ± 0.53 pg/ml) and follicular stages (6.25 ± 0.64 pg/ml). Our findings are in good agreement with previous studies of rats and humans where the plasma kisspeptin concentration at prepubertal period was higher compared to pubertal period (Ricu *et al*., 2012; Jayasena *et al*., 2014). In rats, the high concentration of circulating kisspeptin is assumed to be related with the elevation of sympathetic activity from ovary and celiac ganglion, which is characterized in the rats at prepubertal period (Ricu et al., 2012). In humans, it is suggested that the increased plasma kisspeptin levels could represent a drive from hypothalamic kisspeptin neurons to initiate the pubertal development (*Jayasena et al*., 2014). However, whether the cat plasma kisspeptin level is associated with the sympathetic nervous system or hypothalamic neurons remains unclear.

For pubertal cats, we found no changes in plasma kisspeptin levels between cats at the inactive and follicular stages. Our results are in contrast with the observation in the cows where a fluctuation of circulating kisspeptin was demonstrated during the reproductive cycle (Mondal *et al*., 2015). Therefore, plasma kisspeptin cannot be used as biological marker to distinguish the different reproductive statuses in domestic cats. It is possible that the circulating kisspeptin is not influenced by the sex steroid, estrogen, since the circulating estrogen is reported to be increased during the follicular phase in cats (Shille *et al*., 1979). Likewise, the circulating kisspeptin in human is not associated with estrogen (Katagiri *et al*., 2015).

In conclusion, the commercial EIA kit can be used to determine plasma kisspeptin in domestic cats.

Conflict of interest: The authors declare that they have no conflicts of interest.

Acknowledgements

This study was supported by the Royal Golden Jubilee PhD program (Grant number PHD/01882556). This work was financially supported by the 90th anniversary of Chulalongkorn University fund

(Ratchadaphiseksomphot Endowment Fund). We gratefully thank staff of the OGR unit at the small animal teaching hospital, Chulalongkorn University for providing facilities and samples. We appreciate the help of the staff at the Research Centre for Bioscience in Animal Production, Faculty of Veterinary Science, Chulalongkorn University in providing the facilities in the lyophilize process.

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