# Study the Reproductive Pattern of Different Breeds Bitches in Iraq by estimation of Kisspeptin and detection ovulation time.

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## Abstract

This study was conducted to investigate the reproductive pattern of bitches by estimation the optimum time of ovulation and kisspeptin level. The study included 245 bitches (different breeds), from March/2019 to the end of February/2020.Canine ovulation timing kits was applied in order to determine the ovulation time. For estimation of Kisspeptin, blood collection of 80 samples allocated approximately 7 samples for each monthby using ELISA kit. The resultsrevealed the time of ovulation ranged from 2 to 5 days after the beginning of the estrus phase.The results showed that concentration of kisspeptin during different phases of the estrous cycle was (257.01±11.08, 262.93±9.15, 227.98±14.91 and 244.89±11.99) pg/mL for Proestrus, Estrus, Diestrus, and Anestrus respectively with no significant ($p>0.05$) differences among different estrous cycle phase. The concentration of kisspeptin in which distributed according to the season showed that it was (218.26±11.92, 254.12±10.09, 246.00±10.22, and 287.11±9.00) pg/ml for Spring, Summer, Autumn, and Winter, respectively and there was a significant difference ($p<0.05$) between Summer and Winter. As a conclusion, the ovulation timing kit was accurate and useful for detecting the time of ovulation and the season had a significant effect on the kisspeptin level.

***Keywords**: Bitches, Progesterone, Ovulation, Kisspeptin, Seasonality.*

## Introduction

Mating at the wrong time is the most common cause of infertility in a bitch [(1)], so deciding the optimum breeding time is regarded the most critical aim of routine breeding management [(2)]. The spontaneous ovulation characteristics and the long estrus cycle contribute to difficulties in deciding the appropriate mating time and contribute to noticeable infertility in the bitch [(3)].

The most commonly used method for the identification of ovulation is the measurement of circulating progesterone concentrations, as the luteinizing hormone (LH) spike is observed to be concomitant with the initial marked rise in circulating progesterone. In practice, the progesterone blood concentration of ~ 5 ng/ml is known to be predictive of ovulation [(4)].

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Kisspeptins are a family of peptides which are now well-established as essential reproductive regulators. They are essential for the growth of puberty and the preservation of reproductive processes. In particular, kisspeptin is a strong stimulator of the secretion of gonadotrophin, mainly through direct action on the secretion of gonadotrophin releasing hormone (GnRH) [(5, 6)].

In particular, KISS1 mRNA levels varied in rats during the oestrous cycle [(7)] and in response to gonadotropin stimulation, indicating that cyclical variation could be due to changes in FSH and/or LH levels, although Gaytanet al., [(8)] suggests that kisspeptin has a direct action in the ovaries that is gonadotropin-independent.

For canines, the condition is unclear. The bitch has a very lengthy and unusual oestrous cycle, followed by a 2-month dioestrus, with an average nine days of proestrus and nine days of oestrus, and an anoestrous

period that lasts 4-12 months [9]. This complex oestrous cycle makes it incredibly difficult for bitches to regulate their breeding. Ultimately, an increased understanding of the role of kisspeptins in ovary regulation will lead to improved methods of canine reproduction control. In view of these information, the aims of this study are: Detection of ovulation by assessment of progesterone concentration in blood and estimation of Kisspeptin through the complete calendar year.

## Materials and Methods

This study was carried out at Central Baghdad Veterinary Hospital/ Baghdad throughout a complete calendar year from March/2019 to the end of February/2020. The study included 245 cases.

The different breeds and ages from (7-132) months of bitches were carried out in this study, the different breeds of bitches were included {(German shepherd, Belgian Malianois, Terrier, Husky and other breeds (Pointer, Pekinese, Rottweiler, Doberman, Great Dane, Pitbull, Pug, Pomerian, Cane corso, Golden Retriever, Chow Chow, Cocker Spaniel and Caucasian)}.To achieve research goals diagnostic tools were performed. Blood sample was collected from the cephalic vein, and serum was obtained after centrifugation for 5 minutes at 6,000 rpm.

To regarding the determination of the breeding season canine ovulation timing kits (BioMetallics, Inc. USA) was applied in order to determine the ovulation time (by estimation of progesterone level) of studied bitches which can help in accomplishing the aims of the study.For estimation of Kisspeptin,blood Collection of eighty samples allocated approximately 7 samples for each month for assessment of Kisspeptin by ELISA kit (SunLong Biotech Co) in National Center for Drug Control and Research/ Ministry of Health and Environment to determine the levels of kisspeptin according to estrus phases, season, age and breeds.

## Statistical Analysis

Data were expressed as the mean values, mean± standard error of the mean (SEM). The statistical significance of the differences among various groups was determined by one-way analysis of variance (ANOVA). Data analysis was done by using IBM SPSS (statistical package for social sciences) version 23. Differences were considered statistically significant for P-value less than 0.05.

## Results

### Progesterone test (Ovulation detection time)

Different concentrations of serum progesterone in studied animals were estimated according to the standard color of ovulation detection time.The results of the study showed the time of ovulation ranged from 2 to 5 days after the beginning of estrus phase and this result depending upon the color that appeared on the cup and compared it with the standard color of kit. When there was a bright color, it was advised the owner to reexamine after three days for detection the time of ovulation. The light blue refereed that the breeding should be done in 3-6 days, while the faint blue indicated that the mating should be in 1- 3 days. In comparison, the mating should occur immediately if the white color appeared. Also, this test was very useful for detecting the time of cesarean section in many cases in which it depends on the concentration of progesterone when it less than two ng/ml (light blue color). The caesarean section should be scheduled within 24 hours.

### Investigation of split heat in studied animals by application of ovulation detection time kit

One of the essential and useful application to ovulation detection time kit is used as a biological tool to help in diagnosis some fertility problem in bitches such as split heat. It was recorded five cases for bitches that suffering from split heat, these cases recorded of this study in bitches during their first estrous cycle. In this study, thirty-one test was made during the year. This result was obtained by measurement of progesterone level by using ovulation detection time kit, which refers to the occurrence of estrus behavior without ovulation.

### Investigation of kisspeptin in studied animals by application of ELIZA kit

The mean level (±SE) of kisspeptin was 250.36± 5.82 pg/mL. The results of kisspeptin in the serum of studied animals were formulated according to the

following parameters:

Concentration of kisspeptin during different phases of estrous cycle.

The results of the mean (±SE) of kisspeptin for Proestrus, Estrus, Diestrus and Anestrus were (257.01±11.08, 262.93±9.15, 227.98±14.91 and 244.89±11.99) pg/mL, *respectively*,(Table 1). When a comparison was made utilizing one way- ANOVA among different groups, it was demonstrated that there were no significant differences in kisspeptin level among different estrous cycle phase.

**Table (1): Serum concentration of kisspeptin in different phases of estrous cycle.**

| Phase | Phase of estrous cycle | Conc. of Kisspeptinpg/ml | Sig. |
|---|---|---|---|
| Proestrus | | 257.01±11.08NS | |
| | Estrus | 262.93±9.15 | 1.0 |
| | Diestrus | 227.98±14.91 | 0.714 |
| | Anestrus | 244.89±11.99 | 0.997 |
| Estrus | | 262.93±9.15 NS | |
| | Diestrus | 227.98±14.91 | 0.414 |
| | Anestrus | 244.89±11.99 | 0.918 |
| | Proestrus | 257.01±11.08 | 1.0 |
| Diestrus | | 227.98±14.91 NS | |
| | Anestrus | 244.89±11.99 | 0.988 |
| | Proestrus | 257.01±11.08 | 0.714 |
| | Estrus | 262.93±9.15 | 0.414 |
| Anestrus | | 244.89±11.99 NS | |
| | Proestrus | 257.01±11.08 | 0.997 |
| | Estrus | 262.93±9.15 | 0.918 |
| | Diestrus | 227.98±14.91 | 0.988 |

**- NS: Non-significant difference between the column.**

The results of kisspeptin (Table 2) were enrolled according to the season in which sample was collected. The mean of kisspeptin of Spring, Summer, Autumn and Winter was (218.26±11.92, 254.12±10.09, 246.00±10.22 and 287.11±9.00) pg/ml, *respectively*. The results showed there was a significant difference ($p$<0.05) in kisspeptinlevel between Summer and Winter. Moreover, there was a significant difference ($p$<0.05) between Winter and Autumn in kisspeptin level.

**Table (2): Seasonal changes in serum level of kisspeptin in studied animals of different reproductive status.**

| Season | Season | Kisspeptinpg/ml | Sig. |
|---|---|---|---|
| Season | Se | | |
| Spring | | 218.26±11.92* | |
| | Summer | 254.12±10.09 | 0.150 |
| | Autumn | 246.00±10.22 | 0.402 |
| | Winter | 287.11±9.00* | 0.000 |
| Summer | | 254.12±10.09 | |
| | Spring | 218.26±11.92 | 0.15 |
| | Autumn | 246.00±10.22 | 0.99 |
| | Winter | 287.11±9.00 | 0.11 |
| Autumn | | 246.00±10.22# | |
| | Spring | 218.26±11.92 | 0.40 |
| | Summer | 254.12±10.09 | 0.99 |
| | Winter | 287.11±9.00# | 0.027 |
| Winter | | 287.11±9.00*# | |
| | Spring | 218.26±11.92* | 0.000 |
| | Summer | 254.12±10.09 | 0.11 |
| | Autumn | 246.00±10.22# | 0.027 |

- *= Significantly different ($P$< 0.05) between spring and winter utilizing ANOVA.

- #= Significantly different ($P$< 0.05) between autumn and winter utilizing ANOVA.

The results of kisspeptin were classified into two groups (A and B), group A involved bitch with age range from (8 - 59) months and group B involved bitch with age range from (60 - 108) months(Table 3). The results showed that the mean (±SE) level of kisspeptin of group A was 250.28± 5.57 pg/ml while the mean (±SE) level of kisspeptin of group B was 250.55± 16.26 pg/ml. No significant differences in kisspeptin level between the two groups were detected.

**Table (3): The concentration of kisspeptin according to the age of studied bitches.**

| Age | Kisspeptin (Mean ± SE) pg/mL |
|---|---|
| Group A (8 - 59) months | 250.28± 5.57NS |
| Group B (60- 108) months | 250.55± 16.26NS |

- NS: Non-significant

**Breed**

The animals were classified into five groups according to their breed including German Shepherd, Belgian Malinois, Husky, Terrier and the last group included many breeds (Pointer, Pekinese, Rottweiler, Doberman, Great Dane, Pitbull, Pug, Pomeranian, Cane Corso, Golden Retriever, Chow Chow, Cocker Spaniel and Caucasian). Table (4) showed that the mean of kisspeptin in German Shepherd, Belgian Malinois, Husky, Terrier and other breeds were (248.74 ± 11.53) pg/ml, (251.3 ± 10.76) pg/ml, (246.19 ± 10.1) pg/ml, (276.5216 ± 16) pg/ml, and (241.6455 ± 6.9) pg/ml *respectively*. No significant differences in kisspeptin level were reported among different breed groups.

**Table (4): The concentration of kisspeptin in different breeds of the dog during different phases of estrous cycle and different season.**

| Breed | Breed | Kisspeptinpg/ml | Sig. |
|---|---|---|---|
| GSD | | 248.74±11.53NS | |
| | B | 251.30±10.76 | 1.0 |
| | H | 246.19±10.10 | 1.0 |
| | T | 276.52±16.02 | 0.80 |
| | O | 241.64±6.91 | 1.0 |
| B | | 251.30±10.76NS | |
| | GSD | 248.74±11.53 | 1.0 |
| | H | 246.19±10.10 | 1.0 |
| | T | 276.52±16.02 | 0.85 |
| | O | 241.64±6.91 | 0.996 |
| H | | 246.19±10.10NS | |
| | GSD | 251.30±10.76 | 1.0 |
| | B | 248.74±11.53 | 1.0 |
| | T | 276.52±16.02 | 0.689 |
| | O | 241.64±6.91 | 1.0 |

**Cont... Table (4): The concentration of kisspeptin in different breeds of the dog during different phases of estrous cycle and different season.**

| Breed | Breed | Kisspeptinpg/ml | Sig. |
|---|---|---|---|
| T | | 276.52±16.02NS | |
| | GSD | 248.74±11.53 | 0.80 |
| | B | 251.30±10.76 | 0.85 |
| | H | 246.19±10.10 | 0.689 |
| | O | 241.64±6.91 | 0.48 |
| O | | 241.64±6.91NS | |
| | GSD | 248.74±11.53 | 1.0 |
| | B | 251.30±10.76 | 0.996 |
| | H | 246.19±10.10 | 1.0 |
| | T | 276.52±16.02 | 0.484 |

**- NS: Non significant ($p$>0.05) between the column.**

**- GSD= German Shephard), B=Belgian Malinois, T=Terrier, H=Husky and O=Others breeds (Pointer, Pekinese, Rottweiler, Doberman, Great Dane, Pitbull, Pug, Pomeranian, Cane Corso, Golden Retriever, Chow Chow, Cocker Spaniel and Caucasian).**

## Discussion

Using ovulation detection time kit for estimation the concentration of progesterone was very effectiveness for scheduling the time for mating in which this considered as the most cause of infertility in bitch [1, 10]. In this study the ovulation was detecting after 2-5 days from the beginning of estrus which indicated that the LH surge occurred in the late of proestrus phase or after 1-2 days after the onset of estrus. These results were in agreement with some studies [10- 15]. The assessment of progesterone levels considered as the best methods for detection the ovulation in bitches [2].

Ovulation detection time kit also used for detection of split heat which defined as the length of proestrus or estrus were short than normal and accompanied by low level of serum progesterone which describe also as in anovulatory cycle [16]. In this study five cases were diagnosed as a split heat when measurement of progesterone value which indicated it was less than 1 ng/ml and reoccurrence of proestrus bleeding beyond 10-14 days after the end of estrus (acceptance for mating). This result was agreement with [17].

The mean concentration of kisspeptin was 250.36± 5.82 pg/mL during one year calendar. Kisspeptin considered as hormonal regulator for GnRH secretion [18]. In this study, Kisspeptin was measured according to different parameters. Firstly, according to the phase of estrous cycle in which sample was taken, the results showed that the mean of kisspeptin for Proestrus, Estrus, Diestrus and Anestrus were (257.01±11.08, 262.93±9.15, 227.98±14.91 and 244.89±11.99) pg/mL, *respectively*, in which there was no significant differences between each phase. Due to it a novel study for estimation kisspeptin there are few study describe the level of kisspeptin during different stages of estrous cycle. Cielesh et al.,[5] found kisspeptin and kisspeptin receptors in primordial follicles, granulosa cells, oocytes, and the corpus luteumduring pre-pubertal, anoestrus and cycling (proestrus, oestrus and dioestrus), with little variation in distribution of kisspeptin receptors across the reproductive cycle. Also Castellano et al.,[7]and Gaytan et al.,[8] found that kisspeptin and its receptors in the ovary of mice, rats, human and marmoset[5, 19]. According to Cielesh et

al.,[5]who suggested that distribution of kisspeptin and its receptors during different stages of estrous cycle may be to its role in development and survival of oocyte. Additionally, the presence of kisspeptin in granulosa cell or luteal cell in which it responsible for production of progesterone in chickens, human, mice and dogs [5, 19, 20], these evidences emphasize our result in which there no significant between estrous cycle phases and the unique feature of estrus in bitch due the luteinisation occur before ovulation [21].

According to the effect of season on kisspeptin levels, the samples were distributed depend on when it's collected during any season. Results showed that the concentration were (218.26±11.92, 254.12±10.09, 246.00±10.22 and 287.11±9.00) pg/ml for Spring, Summer, Autumn and Winter respectively. There was a significant difference ($p<0.05$) in kisspeptin level between Summer and Winter, additionally, there was a significant difference ($p<0.05$) between Winter and Autumn in kisspeptin level. There is little or no information that deals with the effect of season on kisspeptin levels in dog. From the results of previous study[22], Winter showed the highest percentage of estrus behavior when compare with other seasons and this may be in associated kisspeptin levels, also this result may attributed to decrease in the day length that effect on kisspeptin secretion. Heat may considered as a stress factor which have inhibitory effect on reproduction [23, 24], there is no study on the effect of heat directly on kisspeptin levels.

Many species of domestic animal have clear seasons of reproductive activity with polyoestrouscyclicity and sperm production coupled with periods of reproductive quiescence [25]. The idea that kisspeptin neurons are regulated by season/photoperiod was first mooted by Smith et al. [26], when they observed that the number of KISS1 mRNA-containing cells was higher in the arcuate nucleus in sheep during the breeding season than during the non-breeding season. The same result was also observed in Abadeh goat does [27] and in the mare [28], even though the mare is a long-day breeder, whilst sheep and goats are short-day breeders.

Chalivoix et al., [29] achieved that transferring ewes from an artificial long-day photoperiod to short days resulted in a higher number of kisspeptin neurons in the arcuate nucleus, while Wagner *et al*., [30] recorded that ewes housed in short day photoperiod had higher levels of *KISS1* mRNA in the arcuate nucleus compared with ewes housed in longer photoperiods.

The results of kisspeptin according the age which were classified into two groups [group A (8 - 59) months and group B (60 - 108) months] in which there was no significant differences between two groups There is no studies for comparing with our study, but this result may be attributed to that all samples collected from bitches after reach puberty in this study.

According to breeds of bitches the levels of kisspeptin were (248.74 ± 11.53) pg/ml, (251.3 ± 10.76) pg/ml, (246.19 ± 10.1) pg/ml, (276.5216 ± 16) pg/ml, and (241.6455 ± 6.9) pg/ml in German Shepherd, Belgian Malinois, Husky, Terrier and other breeds respectivelywith no significant. This result may be due the most breeds (German Shepherd, Belgian Malinois, Husky, Terrier) examined in this study considered as a non-seasonal pattern for estrous cycle (31-35).

It was concluded that the ovulation timing kit is the best method for detecting the optimum time for mating. Kisspeptin concentration affected by seasonal variation and there is a non-significant difference in the concentrations of kisspeptin concerning age, breed and phases of estrous cycle.

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