수의학석사학위논문

Timing of fertile period for successful pregnancy in American Bully dogs

아메리칸 불리 품종의 교배 적기 판단을 위한 임상적 지표 연구

2017년 8월

서울대학교 대학원
수의학과 임상수의학 전공
한 상 은

Timing of fertile period for successful pregnancy in American Bully dogs

아메리칸 불리 품종의 교배 적기 판단을 위한 임상적 지표 연구

지도교수 장 구

이 논문을 수의학 석사학위논문으로 제출함

2017 년 4 월

서울대학교 대학원

수의학과 임상수의학 전공

한 상 은

한상은의 석사학위논문을 인준함

2017 년 6 월

위 원 장	이 병 천	(인)
부위원장	장 구	(인)
위 원	조 증 희	(인)

Timing of fertile period for successful pregnancy in American Bully dogs

by Sangeun Hahn

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF THE MASTER OF SCIENCE

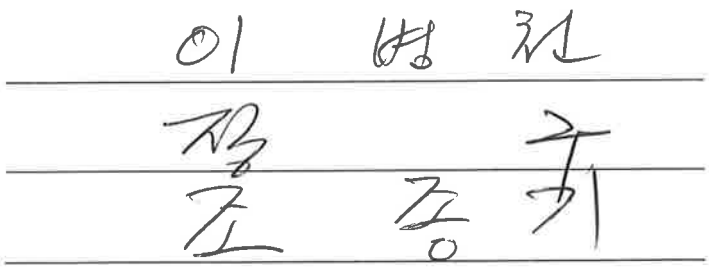
in

Veterinary Clinical Sciences

Department of Veterinary Medicine, Graduate School

Seoul National University

We accept this thesis as confirming to the required standard



Seoul National University

June 2017

Timing of fertile period for successful pregnancy in American Bully dogs

Sang eun Hahn

(Supervisor: Professor Goo Jang, D.V.M., Ph.D.)

Veterinary Clinical Sciences

Department of Veterinary Medicine, Graduate School

Seoul National University

ABSTRACT

Determination of the timing of the estrus cycle is essential for fertile mating. There are physiological variations among breeds, between bitches, and between cycles of the same bitch [1, 2]. If serial monitoring and many tools applied, the exact moment of ovulation could be pinpointed. However, it leads to time and costly

difficulties. Progesterone concentrations during estrus cycles follow a specific pattern, and it is largely used in the timing of the fertile period. Although it has a similar pattern in general, it is likely that breed-specific differences exist.

The aim of this study was to investigate the way of timing the fertile period of successful pregnancy in American Bully dogs based on vaginal cytology and progesterone assay with minimized cost. To identify the empirical relations among reproductive characteristics, we performed statistical analyses on data from proestrus-to-estrus 27 American Bully dogs referred for 7 months. We found the significant correlations between the cyclic changes of vaginal cytology and progesterone assay. The relationship of serum progesterone concentrations with the days from the vaginal discharge onset was analyzed through linear regression assay.

In conclusion, we addressed two standards in the timing fertile period with a minimal number of progesterone assays in the breeding management of American Bully dogs. We were able to provide some reproductive traits of American Bully dogs.

Keywords: American Bully, Canine breeding, Litter size, Fertile period, Progesterone assay, Vaginal cytology

Student Number: 2015-21843

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREIVIATIONS

h	hour
mL	milliliter
ng	nanogram
pg	picogram
iv	intravenous
p	Probability
SD	standard deviation
SEM	standard error of the mean
r	correlation coefficient
r^2	coefficient of regression
CL	Corpus Luteum
LH	Luteinizing Hormone
FSH	Follicular Stimulating Hormone
P4	Progesterone
N:C ratio	Nuclear to cytoplasmic ratio
ELISA	Enzyme Linked Immunosorbent assay
RIA	Radioimmunoassay

PUBLICATION LISTS

PUBLISHED PAPER

Eun Jung Park, Seok-Hee Lee, Young-Kwang Jo, **Sang-Eun Hahn**, Do-Min Go, Su-Hyung Lee, Byeong-Chun Lee and Goo Jang, Coincidence of Persistent Mullerian duct syndrome and testicular tumors in dogs, BMC Veterinary Research, 2 June 2017.

ABSTRACTS AND PRESENTATIONS

Sang-Eun Hahn, Soo-Young Yum, Song-Jeon Lee, Choong-Il Lee, Hee-Soo Kim, Hyeong-Jong Kim, Woo-Jae Choi, Ji-Hyun Lee, Woo-Sung Lee, Goo Jang. Gene editing platform in Cattle, The 16thInternationalSymposiumonDevelopmentalBiotechnology, 2016.

Sang-Eun Hahn, Soo-Young Yum, Song-Jeon Lee, Choong-Il Lee, Hee-Soo Kim, Hyeong-Jong Kim, Woo-Jae Choi, Ji-Hyun Lee, Woo-Sung Lee, Goo Jang. Production of Cas9-Expressing Cattle using DNA transposon, 43rdAnnual Conference of the IETS, 2017

Sanghee Lee, WooJae Choi, **SangEun Hahn**, Choongil Lee, JiHyun Lee, SooYoung Yum and Goo Jang. Criteria for Short-term storage at 4 Degree C in Bovine Embryo, Korean Society of Animal Reproduction, 2015.

WooJae Choi, JiHyun Lee, SangHee Lee, SooYoung Yum, **Sangeun Han**, Choongil Lee, ByeongChun Lee, SungKi Kim and Goo Jang. Pregnancy after Transferring Bovine Embryos Derived Chemically Defined Medium, Korean Society of Veterinary Clinics, 2015.

Young-Kwang Jo, Seok-Hee Lee, **Sang-Eun Hahn**, Byeong-Chun Lee, Goo Jang. Interuterine Artificial Insemination with abnormally enlarged uterine horn in a dog, Korean Society of Veterinary Clinics, 2015.

SeokHee Lee, EunJung Park, JoonHo Moon, YoungKwang Jo, **SangEun Han**, ByeongChun Lee and Goo Jang, Tumors in testes of Dogs, Korean Society of Veterinary Clinics, 2015.

LITERATURE REVIEW

1. The canine estrous cycle

The classical canine estrous cycle is classified as monoestrus, polyovulatory, non-seasonal and found to be unique in several aspects since Heape first described the sexual season of bitch [3, 4]. The unique features entail that the follicular and luteal phases are longer than those found in most other domestic animals; the anestrus occurs independent of the seasons: preovulatory increase in peripheral progesterone [5].

The stages of the canine estrous cycle consist of proestrus, estrus, diestrus, and anestrus. The detailed description of each phase would be discussed subsequently.

Proestrus is the stage where the bitch first exhibits physical and behavioral changes of heat. Physical changes are turgid swelling of the vulva and passage of serosanguineous vulvar discharge. This discharge arises in the uterus and should not be frankly hemorrhagic or have a foul odor. Male dogs will be attracted to proestrus bitches, but will not be accepted [6]. Proestrus occurs in response to serum estradiol increases from 5 to 15 pg/mL initially, to reach peaks of 40-120 pg/mL [7]. It averages 9 days. It can be observed as external estrogenization like vulvar swelling, serosanguineous vulvar discharge. Proestrus ends with the onset of receptive behavior typically within a

day of the preovulatory LH surge, which represents the endocrinological termination (*ibid*).

Estrus is characterized by male receptive and seeking behavior and lasts for 5-10 days. The classic signs include softening of the vulva and change in color of vulvar discharge from serosanguineous to straw-colored at estrus onset. However, the length of this phase and proestrus may vary within and among breeds. This makes it difficult to predict the length of proestrus or estrus of a dog from 1 day to 20 days [8]. Estrus is initiated by the synergistic rise in progesterone resulting from the LH surge. Because the most common cause of breeding failure is improper ovulation timing, tests based on these endocrinological and physiological changes are important[9].

Diestrus begins when bitch suddenly refuses to breed. It is the phase of progesterone dominance following estrus, and ends when serum progesterone concentration returns to basal levels (<1.0 ng/mL). A dramatic shift on vaginal cytology can be observed as superficial cells disappear. The duration averages 56 to 58 days in pregnancy and 60 to 100 days in the non pregnant bitch [8].

Anestrus is the time when the uterus involutes. Underlying hormonal changes during this stage are characterized by increasing frequency and amplitude of gonadotropin secretion, mediated by dopamine. There are no specific physical or behavioral changes. Like

the other phases, the duration of anestrus varies, but averages 4.5 months. The average bitch begins a new proestrus every 7 months [6].

2. Assessment of ovulation timing

In order to achieve a successful pregnancy, the day ovulation will occur should be accurately determined. There are several different techniques to assess the optimal time for mating, including physical examination, measurements of plasma hormone concentration, examination of exfoliative vaginal cells, and etc.

2.1. Observation of behavioral signs and physical changes

Once proestrus begins, a bitch attracts male dogs, but she does not accept them until she is in estrus. Standing heat means the bitch's willingness to accept copulation, and is caused by the combination of a peak and following the decline of estrogen with preovulatory progesterone elevation[9]. Throughout proestrus and estrus, the vulva and perineal tissues become enlarged and edematous. Rising estrogen levels during proestrus cause the vulva to be swollen and turgid, and vaginal discharge to be hemorrhagic. As estrogen decreases with concomitant progesterone increasing, the vulva becomes soft and pliable; the vaginal discharge gets scant and straw colored [9, 10].

2.2. Measurement of reproductive hormones

The female reproductive cycle is under control of a series of hormonal events. An estrogen elevation initiates the proestrus. A subsequent decline in estrogen and slight increase in progesterone commences estrus. The increase in plasma progesterone is the result of preovulatory luteinization, and the low ratio of estrogen to progesterone causes the LH surge. Most ovulations occur between 24 and 72 hours after LH peak [11].

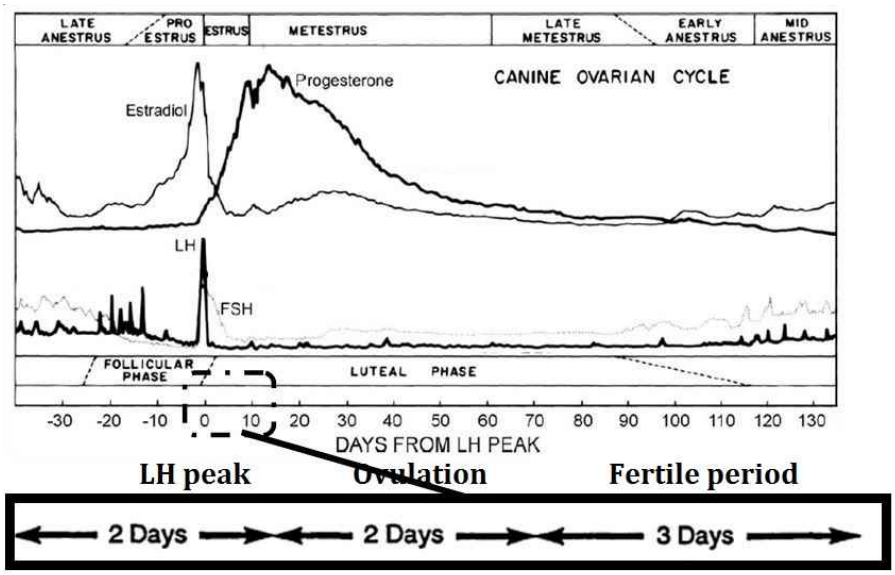


Figure 1. The illustration of reproductive hormonal change in canine estrus cycle with the fertile period marked.

Reconstructed from the figure published on Concannon, 2011. A late proestrus estradiol peak is noted 1-2 days prior to the preovulatory LH peak. The estrus phase is characterized by transition of falling estradiol and increasing progesterone concentrations. Progesterone concentration reaches the peak values in early metestrus around day 20-30 and shows a slow decline over a 4-8 week period after the peak [7].

Ovulation generally occurs 48 hours after the serum LH surge. Oocytes must undergo a second meiotic division of 48-72 hours before fertilization can take place. The fertilizable ova remain viable in the oviduct for 2-3

days. Thus, the fertile period begins 4-5 days after the LH surge, and terminates 6-8 days after the LH surge [9].

2.2.1. Luteinizing hormone (LH)

Luteinizing hormone (LH) is the hormone that stimulates ovulation. It is secreted as a single large peak, with high concentrations persisting for about 24 to 48 hours. Serum LH concentrations of greater than 1 ng/mL precede ovulation by 2 to 3 days [6]. Therefore, measurement of the peripheral LH concentration provides an accurate method for determining the optimal time for mating. Although there is no commercial LH enzyme-linked immunosorbent assay (ELISA) kit, radioimmunoassay, which is the only reliable method, is often expensive and not generally available to the practitioners [11].

The role of pituitary LH is less obvious and remains controversial to some extent. It has been suggested that if LH is required to maintain Corpus Luteum (CL) function, its role would be the stimulation of progesterone production by the CL and it is not necessary for pregnancy maintenance [12]. The basal level of LH is 0.4-1.5 ng/mL and the peak level is 5-40 ng/mL [7].

2.2.2. Estrogen

The role of estradiol 17β in the regulation of the canine corpus luteum (CL) has not been clearly identified. The origin of the observed increase in the estradiol from some researchers like Hadley is also inconclusive, yet assumed to originate from the CL. It can be possible that estradiol forms part of the luteotrophic complex in association with prolactin and LH in dogs as in other species [12].

Measurement of plasma estrogen has no merits over LH due to its variable value within bitches and rapid decreasing one day before the LH surge [11]. During proestrus, developing ovarian follicles produce estrogen from 2-10 pg/mL over a 10 to 14 day period. Estrogen then peaks reaching 50-120 pg/mL, approximately 2-3 days before estrus, followed by a rapid decline just before the LH surge [9].

2.2.3. Progesterone

Plasma progesterone concentrations increase from the end of proestrus to peak approximately 20-30 day after the LH surge, and then slowly decline to basal concentrations by 60-70 day in both pregnant and non-pregnant animals. Continuous availability of progesterone is required for initiation and maintenance of pregnancy. In dogs, plasma progesterone concentration should exceed 2 ng/mL to maintain pregnancy [12].

Monitoring the initial rise of Progesterone is an excellent way of predicting ovulation. By measuring the progesterone in a sequential manner and interpret the concentration, it is possible to predict the timing of reproductive events – LH peak, ovulation, and the fertile period. Plasma progesterone concentration begins to increase rapidly from baseline approximately 2 days before ovulation, during the LH surge. This rapid increase is distinct from the very slow rise over the previous week. Since the initial rise in progesterone is progressive, it is necessary to collect blood samples every second or third day, unlike the daily regime required to detect the LH surge [2].

Among several laboratory methods for progesterone, radioimmunoassay (RIA) is considered the reference method, however, it has limited accessibility [13]. Instead, there are two types of

commercial ELISA kits for the detection of plasma progesterone: quantitative and semi-quantitative.

Preovulatory follicular luteinization results in progesterone level elevation from baseline to 1-2 ng/mL during late proestrus. Progesterone level reaches 2-4 ng/mL two days before ovulation. The fertile period is assumed to be between 4 and 10 ng/mL of progesterone [6].

Some reports suggest that breeding had better commence one day after Progesterone concentration exceed 8 to 10 ng/mL, which are typically seen at the beginning of the fertilization period[2]. However, fertility for single mating is maximal from the LH surge until 5 days later and decreases over the next 3 days partly due to a cervical closure at day 7-8 from LH surge [14]. Progesterone concentration increases from under 2 ng/mL to under 10 ng/mL during this period.

2.3. Vaginal cytology

Vaginal cytology is the most useful clinical laboratory tool to understand and manage canine breeding. The principle of vaginal cytology is that vaginal epithelium changes from a bistratified cuboidal epithelium to a stratified squamous epithelium of greater than 30 cell layers with every estrous cycle, under estradiol's influence. The population of vaginal epithelial cells collected by vaginal swab varies in a characteristic manner throughout the estrous cycle according to this principle. In early proestrus, the number of cell layers increases and a gradual transition of cell morphology from cuboidal to squamous occurs. Through late proestrus and estrus, the components the stratified epithelial cell layers change from parabasal and small intermediate cells to large intermediate and keratinized cells. Late in estrus, the epithelium meets an influx of leukocytes and returns to a large proportion of parabasal and small intermediate cells because of the loss of estradiol as ovulation occurred[15, 16].

Cells seen in the smear consist of epithelial cells, leukocytes, erythrocytes, and bacteria. Epithelial cells have different names for their mature phase. Parabasal cells are small ovoid cells with a large nuclear to cytoplasmic ratio (N:C ratio) and the least mature form. Intermediate cells have a wide range of sizes from small to large, they become larger, more angular, flatten and have lower N:C ratio as they mature. Superficial or cornified cells are the fully mature

squamous epithelial cells with pyknotic nucleus. During anestrus, early proestrus and again in diestrus, a various number of leukocytes are present. Only in late proestrus and estrus, the multiple layers of stratified epithelium block leukocytes, almost neutrophils, migrating from the subepithelial vasculature. Erythrocytes are present during proestrus, estrus and early diestrus. The number of erythrocytes varied proportionately with the depth of color of the sanguineous discharge, therefore it is best to acknowledge only for their presence given that there is no reliable correlation made with the other important patterns in the epithelial cells. It is not unusual to see various kinds of bacteria because the vagina is not a sterile chamber. However, large numbers of bacteria along with reactive or degenerating neutrophils may be evidence for vaginitis [17].

The interpretation of the vaginal cytology smears is based on a differential count of parabasal, intermediate and superficial cells on a sequential basis. Examining a single random smear during one estrous cycle cannot give an exact information at what point the bitch is in [17]. Because the time of ovulation can be estimated retrospectively and the fully keratinized status may be maintained for several days. At the first days of estrus correspond to 80% or more of superficial cells on vaginal cytology, fertility test needs to be evaluated [18]. However, the vaginal cytology is not recommended for the sole method for detecting ovulation because of the low pregnancy rates [19].

2.4. Other diagnostic tools

Vaginal endoscopy can detect thickening in the vaginal mucosa of the mid to anterior vagina as vaginal cytology does. During proestrus, the mucosa appears edematous and pink. The hormonal switch from estrogen to progesterone reduces vaginal mucosal vascularity and edema, leading to progressively more wrinkled (crenulated) luminal surface. The maximal crenulation is noted to occur in the interval between ovulation and oocyte maturation [20].

Ultrasonography of ovarian follicles may be used to identify ovulation in dogs. Ovarian follicles grew to their largest sizes within one to two days after the LH peak. The data regarding ovarian follicular sonography have shown the correlation to the ovulation timing established by LH and progesterone assay [21].

Measurement of electrical conductivity of vaginal mucus has been studied. It was found that electrical resistance of vaginal mucosa increases as estrus approaches, and at some point during maximum electrical resistance plateau ovulation occurred. While it has not reliable correlation with the LH surge or the fertile period [9, 22].

INTRODUCTION

Timing of fertile period is key to increase the success rate of pregnancy in dogs. It is difficult to determine the optimal mating time because of the spontaneous ovulation characteristics of dog. There are many clinical assessments discussed for estimating the optimal window of breeding, such as observation of physical and behavioral signs, vaginal cytology, reproductive hormone measurements, vaginal endoscopy, ultrasonography, and examination of cervicovaginal secretion [9].

Of these methods, progesterone assay has been a valuable tool for assessing the reproductive events in bitches [6]. The preovulatory rise in serum progesterone due to preovulatory follicular luteinization is one of the unique features in canine reproduction. After LH surge, the serum progesterone rises from around 1 ng/mL during anestrus and early proestrus [7] to 4-10 ng/mL at ovulation [21, 23]. Generally Progesterone can be determined either by quantitative or semi-quantitative methods. Semi-quantitative progesterone assays allow for rapid house testing of canine blood samples, however, a subjective component cannot be excluded due to the nature of these tests [24]. Radioimmunoassay (RIA) or Enzyme immunoassay provide accurate and reliable results, but these have the disadvantage of high expense and long turn-around time [25].

Vaginal cytology is another valuable tool that can support a breeding program. It can be used to define the stage of the estrus

cycle because the vaginal epithelium undergoes morphological changes under estradiol and LH transition. Although vaginal cytology has advantages of low cost and availability, it alone is inadequate for accurate timing. Some bitches demonstrate poor cellular changes in the vaginal smear [26] and cornification peaks are variable 1 to 6 days before the LH surge [7]. Based on the accuracy of Progesterone concentration measurement with RIA and easy, but inaccurate aspects of vaginal cytology, we considered the timing of the fertile period in American Bully dogs with close monitoring of vaginal cytology and necessary times of progesterone RIA. To decide the optimal point of progesterone assay, the relationships between vaginal cytology and progesterone, and between vaginal discharge onset and progesterone were statistically analyzed.

Meanwhile, the American Bully dog has recently been accepted as a new breed and has been recognized by the United Kennel Club since 2013 [27]. There is limited reproductive information on this breed yet. Therefore, we hope this study may contribute to the breeding management in American Bully dogs.

Materials and Methods

1. Animals

Twenty-seven clients owned, proestrus to estrus American Bully dogs, presented to the referring hospital for breeding management to decide the mating date from June to December in 2016, became the subjects of study. Detailed information for each subject is described in the Table 1. Although their pedigree information lacked, the possibility of inbreeding cannot be excluded because of the small pool of American Bully dogs in South Korea. The pregnancy check-ups and delivery through cesarean section of subjects were done in local hospitals due to each owner's preference. Therefore, we gathered the pregnancy progress via contact to the owners afterwards. Litter size was defined as the number of puppies alive at birth.

The male American Bully dog is owned by a breeder. Its fertility, including clearance of *Brucella canis,* was examined in local hospital periodically. Mating was performed through vaginal insemination with fresh semen by the breeder, except for one dog (a) which had surgical insemination.

Table 1. Subject's information

Practitioner	Patient index	Age (Mo.)	Order of estrus (No.)	Previous pregnancy history (No.)	P4 conc at the decision making (ng/mL)	Delivery outcome (litter size)	Etc.
I	A	27	4	2	6.15	3	
	B	38	5	1	6.2	3	
	C	24	2	1	4.41	7	vaginal prolapse
	D	24	3	0	3.46	3	
II	E	36	5	0	5.1	10	
	F	40	5	4	1.23	miscarriage	2 miscarriages in previous pregnancy
	G	48	5	2	2.95	9	Natural delivery
	H	24	5	0	4.4	7	
	I	30	4	0	6.2	1	
	J	38	4	1	1.03	10	
	K	38	4	1	2.57	9	
	L	23	4	1	6.58	5	vaginal prolapse
	M	27	4	2	6.8	5	
	N	24	3	0	3.52	9	
	O	24	2	0	3.9	5	
	P	18	2	1	4.41	5	
	Q	15	2	1	2.99	2	
	R	13	1	0	4.4	4	
	S	9	1	0	0.846	0	Slow rise in preovulatory serum P4 conc
	T	23	1	0	6.14	0	obese
	U	11	1	0	8.4	1	
	V	12	1	0	6.5	miscarriage	Miscarriage due to Car accident
	W	19	1	0	12.8	1	obese
	X	14	1	0	5.82	2	
	Y	9	1	0	6.1	5	
	Z	11	1	0	2.57	9	
	a	36	4	0	8.85	0	surgical AI, vaginal polyp and pyometra

Twenty seven American Bully dogs became the subjects of the study. The time of ovulation was estimated from serum progesterone concentration as reported by many studies [4, 14, 15]. The day when the progesterone (P4) level initially reached 4 ng/mL or more was regarded as the day of ovulation. Some of mating date decision were made earlier than 4 ng/mL because the clinical visits were available only on work days. Therefore, the mating was decided as two days after the P4 level of 1-2 ng/mL; one day after the P4 level of 2-4 ng/mL; the very day when the P4 level reaches 4 or higher. Additional mating was recommended two days after the initial mating.

Other information that may affect the fertility are described on the "Etc." column. Besides breeding determination, further check-ups and delivery were taken at local hospitals. Miscarriages were diagnosed in local hospital also.

Except for “a”, vaginal artificial insemination (AI) was done by one breeder who owns male American Bully dog. Except for “G”, patients delivered through Cesarean section.

The numbers of animals were differentially allocated according to statistical design.

1) Forty blood and vaginal smear samples of 20 bitches (E-X) were used to examine the correlations between serum progesterone concentration and vaginal cytology indices. Vaginal cytology indices are ‘cornified cell ratio’ and ‘cornification index’. To exclude the performer’s bias, these 20 bitches are selected because their assessments were conducted by one clinician.

2) Fifty blood and vaginal smear samples of 24 bitches (A-X) were used to categorize and analyze for the Chi-square test. Categorizing procedure was considered to redeem the inter-practitioners bias.

3) Thirty nine samples of 15 dogs were used in a linear regression assay to predict the progesterone concentration based on the day from the vaginal discharge onset. Subjects with single visits (n=6), or inconclusive detection on vaginal discharge (n=4), or possible reproductive disorders (n=2; S,T) were excluded. However, data of “a” were included in this analysis, in spite of its reproductive disease-pyometra and vaginal polyp- which were discovered in laparotomy, because it took daily progesterone assay. The subjects of this analysis were D, E, G, H, I, L, M, N, P, Q, X, W, a.

4) Blood samples, litter size and delivery histories of 22 successfully delivered subjects were used to analyze the relation between the progesterone concentration at the time of mating-decision making and litter size outcome.

2. Physical and Physiological examinations

Physical examination was performed according to routine protocol of the referral hospital. It included observation of vaginal discharge, vulvar enlargement and erythema. History taking from the owner included past reproductive history and the characteristics of the current estrus cycle, such as vaginal discharge, vulvar change, and male acceptance.

To study vaginal cytology, vaginal smears were taken using sterile swabs. The smears were rolled on a slide glass, stained with Diff-quik®stain (International Reagent Corp., Kobe, Japan), examined under a light microscope linked to a computer (IMTcam3, i-solution Inc., BC, Canada) and classified as described by Christie and others [28]. The clinician counted the numbers of superficial intermediated cells, superficial cells, and pyknotic to anuclear cells out of total epithelial cells from the vaginal smear slide capture shot at x100 optical magnification, with medium cellular density. Two factors were chosen to quantify vaginal cytology: cornified cell ratio [11] and cornification index [29]. Cornified cell ratio represents the ratio of the sum of superficial intermediate and superficial cells to total epithelial cells. Cornification index represents the ratio of the number of cornified pyknotic to anuclear cells to total epithelial cells.

Blood sample (2mL) was collected from brachial vein venipuncture to SST tubes (Becton Dickinson, NJ, USA). Serum was prepared by centrifuged at 1660 g for 5 min at room temperature, and subsequently analyzed by using a DSL-3900 ACTIVE ® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., TX, USA).

The time of ovulation was estimated from serum progesterone concentration as reported by many studies [6, 21, 23, 30]. The day when the progesterone (P4) level initially reached 4 ng/mL or more was regarded as the day of ovulation. Some of mating date decision were made earlier than 4 ng/mL because the clinical visits were available only on work days. Therefore, the mating was decided as two days after the P4 level of 1-2 ng/mL; one day after the P4 level of 2-4 ng/mL; the very day when the P4 level reaches 4 or higher. Additional mating was recommended two days after the initial mating.

Figure 2. The pictures of physical examination and vaginal cytology

Edematous and erythematous vulva of American Bully dogs are represented. For vaginal cytology, a moistened swab was introduced into the vaginal vault. Rolling on a slide glass and stained in Diff-Quik method were followed.

3. Statistical analysis

IBM SPSS Statistics 23 software (SPSS Inc., Chicago, IL, USA) and GraphPad7 (GraphPad Software Inc., San Diego, CA, USA) were employed for statistical analysis.

To investigate the relation between vaginal cytology and serum progesterone concentration, these analyses was performed. Correlations between serum progesterone concentration and vaginal cytology indices were assessed by Pearson r calculation and Scatter plot. The trend among serum progesterone groups and vaginal cytology groups was analyzed through the Chi-square test from the cross-tabulation categorized according to standards described in the table 2 and 3.

To determine when the necessary time to examine progesterone is, these analyses were performed.

The relationship between vaginal discharge and serum progesterone concentration was assessed by linear regression assay. The relationship between serum progesterone concentration at the time of mating decision making and Litter size was assessed by Scatter plot and linear regression line.

The averages, standard deviations of litter size for different parous groups were calculated to demonstrate the feature of American Bully dogs' pregnancy.

Table 2. Standard for serum progesterone concentration[a]

Serum P4 concentration (ng/mL)	Interpretation
Less than 1	Anestrus or early proestrus
1 ~ 2	LH peak has not occurred
2 ~ 4	LH peak occurring
4 ~ 10	Ovulation occurring
Greater than 10	Ovulation has occurred

a) Reconstructed from the reference [6]

Table 3. Standard for Vaginal cytology[a]

Vaginal cytology	Interpretation
Cornified cell ratio is under 50%	Early proestrus
Cornified cell ratio is over 50% & Cornification index is under 80%	Late proestrus
Cornified cell ratio is over 50% & Cornification index is over 80%	Estrus

a) Cornified cell ratio represents the ratio of the sum of superficial intermediate cells and superficial cells to total cells. Cornification index represents the percentage ratio of the number of cornified anuclear cells to total number of epithelial cells. These concepts were introduced by Post [29] as “superficial cell index” and “karyopyknotic cell index” and were designated as “cornification index” by Hewitt and England [11].

A cornified cell ratio of over 50% indicates an abrupt change to diestrus cytology over 24 to 36 h [9, 18]. A cornification index exceeding 80% is proposed to be the optimal time for breeding by various studies [11, 31]

RESULTS

1. Correlations between serum progesterone concentration and vaginal indices

There was a significant positive correlation between serum progesterone concentration and cornified cell ratio (Pearson r = 0.501, $p<0.001$) and between serum progesterone concentration and cornification index (Pearson r = 0.613, $p< 0.001$). Through Fisher's z-test, we compared both correlation coefficients; however, the calculated z factor was not significant (z = 0.7, p = 0.242). Therefore, we performed a linear regression for both pairs to compare the slopes and goodness-of-fit. Cornification index was found to be a better standard for predicting the serum progesterone concentration based on the higher R^2 value and stronger r value. Nevertheless, those two factors were insufficient for estimation. Accordingly, to categorize vaginal cytology, both cornified cell ratio and cornification index were used (Figure 3).

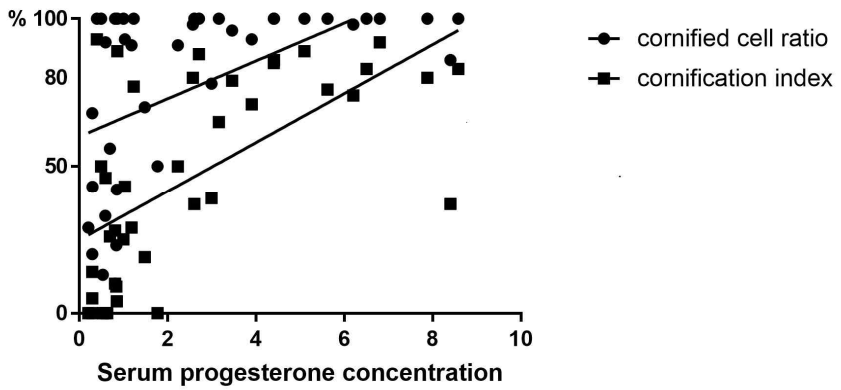


Figure 3. Scatter plot of serum progesterone concentration and vaginal cytology indices

The circle dots represent the cornified cell ratio, and the square dots represent the cornification index. The upper line indicates the linear regression of cornified cell ratio ($y = 6.405*X + 60.12$, $R^2=0.2515$, $p=0.001$), and the lower line indicates the linear regression of cornification index ($y=8.3*X+24.87$, $R^2=0.3764$, $p<0.001$).

Forty samples from 20 American Bully dogs were analyzed. Cornified cell ratio represents the ratio of the sum of superficial intermediate cells and superficial cells to total cells. Cornification index represents the percentage ratio of the number of cornified anuclear cells to total number of epithelial cells. Significant correlations were found between cornified cell ratio and progesterone concentration (Pearson r = 0.501) and between cornification index and progesterone concentration (Pearson r = 0.613). However, the low

R^2 value of each pair necessitated grouping analysis based on a combination of those two criteria.

2. Trend among serum progesterone groups and vaginal cytology groups

The calculated Pearson Chi-square value was 32.431 (df = 8; $p < 0.0001$). Since the χ^2 value exceeded $\chi_{2.005,\ df\ =\ 8} = 1.344$ and the p value rejected the null hypothesis, serum progesterone concentration may be positively correlated with the data obtained from vaginal cytology (Table 4).

Table 4. Grouping analysis of serum progesterone and vaginal cytology

Serum progesterone assay[a]	Vaginal cytology[b]			
	Early proestrus	Late proestrus	Estrus	Total
(A) Anestrus or early estrus	13	4	1	18
(B) LH peak has not occurred	6	0	2	8
(C) LH peak occurring	3	7	4	14
(D) Ovulation occurring	0	1	8	9
(E) Ovulation has occurred	0	0	1	1
Total	22	12	16	50

Fifty samples from 24 American Bully dogs were analyzed. For grouping analysis, data obtained from progesterone assay and vaginal cytology were coded according to specific criteria:

a) Standard for serum progesterone concentration (ng/mL), listed in Table 2.

b) Standard for vaginal cytology, listed in Table 3.

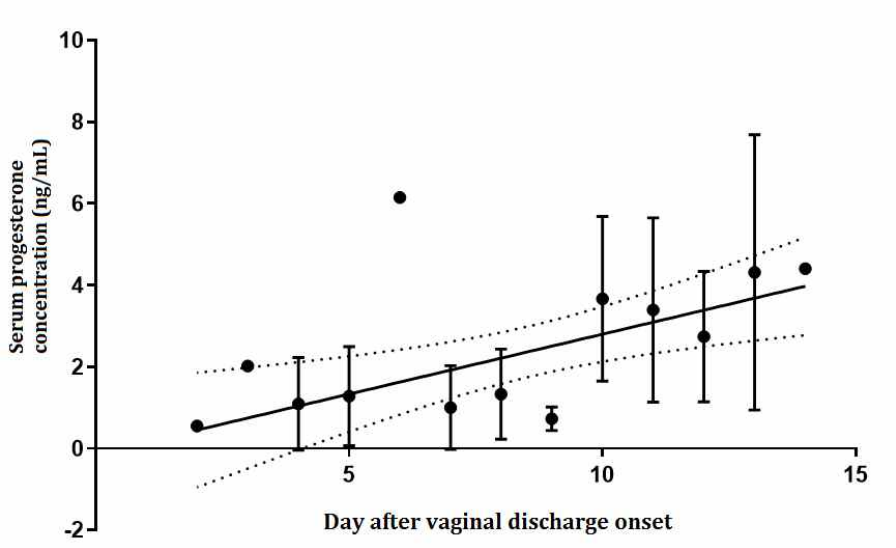
The intersection portions of progesterone concentration and vaginal cytology were counted and recorded in the table. A significant positive correlation between increasing progesterone concentration and vaginal cytological change was identified based on the Chi-square test.

3. Prediction of progesterone concentration based on the day from vaginal discharge onset

There was a significant regression relationship between the day after first vaginal discharge detection and serum progesterone concentration (R^2=0.2669,$p<0.05$), and it could be described by the following regression equation : serum P4 concentration (ng/mL) = 0.2936 * Day after vaginal discharge onset − 0.1385. Pearson's r was also significant ($r = 0.5167$, $p < 0.05$) (Figure 4).

Based on this equation, the day from the vaginal discharge onset at certain progesterone levels were calculated as follows: At 1 ng/mL, 3.88 days; at 2ng/mL, 7.28 days; at 4ng/mL, 14.10 days. During preovulatory luteinization, progesterone level rises above 1 ng/mL of anestrus to early proestrus [6, 7]. LH surge is assumed to occur, so ovulation seems to be imminent at 2-4 ng/mL of progesterone level [6, 13]. At 4 ng/mL or more, the time of ovulation is regarded as occurred [6, 21, 23, 30].

Figure 4. Linear regression between the day after vaginal discharge



detection and serum progesterone concentration

The solid line indicates the regression equation y = 0.2936*X − 0.1385. The two dotted lines indicate the 95% confidence intervals. Thirty-nine samples from 15 American Bully dogs were analyzed. Based on this equation, the day from the vaginal discharge onset at certain progesterone levels were calculated as follows: At 1 ng/mL, 3.88 days; at 2ng/mL, 7.28 days; at 4ng/mL, 14.10 days. During preovulatory luteinization, progesterone level rises above 1 ng/mL of anestrus to early proestrus [6, 7]. LH surge is assumed to occur, so ovulation seems to be imminent at 2-4 ng/mL of progesterone level [6, 13]. At 4 ng/mL or more, the time of ovulation is regarded as occurred [6, 21, 23, 30].

4. Litter size

There were significant regression relationships between the progesterone concentration at the time of decision making and Litter size both in the multiparous and primiparous groups (R^2=0.4243, $p<0.05$; R^2=0.3852, $p<0.05$) Decreasing trends were found in the both groups.(Figure 5)

Averages, standard deviations, correlations between serum progesterone level and litter size were calculated in total, multiparous, primiparous, and First ovulatory groups. Six of primiparous bitches were in their first estrus, on the other hand, another half failed in their previous estrus. However, Pearson's r were not significant in every category. The features of litter size of American Bully dogs were demonstrated in the values of the average and standard deviation. The average litter size of American Bully dogs was 5.23±3.09.

There was an increase in litter size between the ages. First ovulation groups (9-19 months old) had a smaller litter size (3.67±3.08) compared to multiparous group (15-48 months old, 5.80±3.33) or primiparous and multiple ovulation group (23-38 months old, 5.83±3.48). (Table 5)

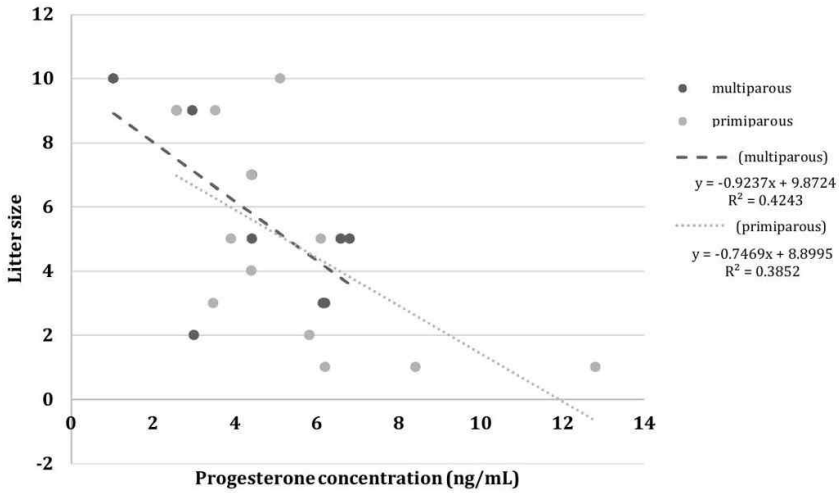


Figure 5. Scatter plot of serum progesterone concentration at the time of mating-decision making and litter size

Data of 10 multiparous bitches and 12 primiparous bitches marked respectively black and gray dots.

Linear regression lines and equations of data pairs are also expressed in the graph. The progesterone concentration represents the P4 level at the time of the last visit before mating. Significant decreasing trends are observed in both multiparous and primiparous bitches.

Table 5. Information and analysis of Litter size

	Total		Multiparous		Primiparous		First ovulation	
	P4	Litter size	P4	Litter size	P4	Litter size	P4	Litter size
Average	5.035	5.227	4.409	5.800	5.556	4.750	6.682	3.667
Variation	6.057	9.517	3.957	7.956	7.675	11.114	12.716	9.467
Observation	22		10		12		6	
r	-0.643		-0.651		-0.621		-0.783	
p-value	0.430		0.171		0.311		0.146	
significance	NS		NS		NS		NS	

Twenty two successfully delivered American bully bitches became the subjects. These subjects were categorized as “multiparous”, “primiparous”. “Primiparous” group was subdivided into “first ovulation” and “Primiparous & multiple - ovulation” based on their order of estrus.

The average and standard deviation for each group were calculated. The paired T test was performed between serum progesterone (P4) concentration at the time of decision making for mating and litter size for each group. There were no significant relationships between P4 and litter size. The average litter size of American Bully dogs was 5.23±3.09. There was an increase in litter size between the ages. First ovulation groups (9-19 months old) had a smaller litter size (3.67±3.08) compared to multiparous group (15-48 months old, 5.80±3.33) or primiparous and multiple ovulation group (23-38 months old, 5.83±3.48).

Discussion

The combination of vaginal cytology and progesterone assay is the most popular protocol in timing of the canine fertile period in the clinical field. The preferred means of accurate timing is progesterone assay because vaginal cytology is inadequate to suggest the optimal timing for mating or insemination [20]. However, progesterone assay, especially RIA, has disadvantages of high cost and inconvenience compared with vaginal cytology. Therefore, minimal but necessary times of progesterone assay are needed to propose the timing standards of the fertile period in American Bully dogs in combination of vaginal cytology and progesterone assay, which aims for minimal cost and successful pregnancy.

Quantitative assessments of vaginal cytology were done to determine when progesterone assay should be while monitoring cytological changes. To our best knowledge, there are limited studies on comparing the changes in vaginal cytology with serum progesterone concentration in bitches. Linde and Karlsson monitored progesterone and estradiol changes based on the day of maximum keratinization on a daily basis [32]. Bouchard and others proposed the ELISA standard curve of progesterone and matched it with LH peak, and whether superficial keratinization of vaginal epithelial cells exceed 80% [33]. These studies had limitations because they focused on keratinization only and did not link hormonal changes with cytological changes directly. Two indices, cornified cell ratio and cornification index, were adopted to assess the vaginal smears.

Cornification index, which represents pyknosis of vaginal epithelial cells, showed a stronger correlation with serum progesterone concentration than cornified cell ratio which represents keratinization of vaginal epithelial cells had. However, the low explanatory powers of those correlations necessitated analysis with a combination of the two indices. Grouping analysis demonstrated the effectiveness of using two indices to standardize vaginal cytology assessment. Accordingly, we propose that 50% of superficial keratinization and 80% of pyknosis of vaginal epithelial cells are the key conditions to perform progesterone assay.

The outlier cells on table 4 (A3, B3, C1) should be noted as the ground that another factor than vaginal cytology had to be considered to minimize the number of progesterone assay. To compensate this defect, a linear regression assay was performed between days from vaginal discharge onset and progesterone assay. Vaginal discharge in early proestrus is easy to detect for owners. The serosanguineous discharge observed passing through the vagina during proestrus usually clears during estrus, but can persist throughout estrus [34]. According to the analysis demonstrated in figure 3, it is wiser to make earlier decisions for mating based on progesterone concentration. Taking these into consideration, progesterone assay is recommended on day 7 and 14 from the vaginal discharge onset. Because the progesterone levels on these days, LH surge and ovulation are considered to be occurring respectively. However, the

heterogeneity of the linear regression curve on figure 4 weakens the independent value of the analysis. Therefore, it had to be considered together in breeding management of American Bully dogs that vaginal cytology, progesterone assay, and the date information of proestrus based on vaginal discharge detection.

In conclusion, we would like to propose two standards in the determination of the optimal time for mating in American Bully dogs as follows: a) progesterone assay is recommended at the condition of exceeding 50% of keratinization and 80% of pyknosis in vaginal cytology. b) Progesterone assay is recommended at the day 7 and 14 after vaginal discharge onset.

For subjects' information on table 1 and statistical analysis in table 5, some reproductive characteristics of American Bully dogs could be discussed. The first estrus cycle ranged from 9 to 23 months of age and the mode was 11 months. The litter size ranged from 1 to 11 and the average was 5.23±3.09. American Bully dogs seemed to ovulate non-seasonally given that even distribution of clinical visits and anecdotal comments from breeders, although this study had not managed to conduct year through research.

Some of the limitations of this study should be addressed. No dog was used repeatedly so inter estrus cycle difference in a dog could not be examined. Without LH assay or daily clinical examination, the

nature of ovulation in American Bully dogs was not discovered thoroughly. The small number of subjects for each statistical analysis may be the cause of low explanatory power and high heterogeneity.

REFERENCES

[1] Tsutsui T, Hori T, Kirihara N, Kawakami E, Concannon PW. Relation between mating or ovulation and the duration of gestation in dogs. Theriogenology.66:1706-8.

[2] England G, Concannon P. Determination of the optimal breeding time in the bitch: basic considerations. Recent advances in small animal reproduction. 2002:0602.

[3] Heape W. The" sexual season" of mammals and the relation of the" pro-oestrum" to menstruation. Quarterly Journal of Microscopical Science. 1900;44:1-70.

[4] Olson P, Nett T. Reproductive endocrinology and physiology of the bitch. Morrow D A Current therapy in theriogenology Saunders, Philadelphia, USA. 1986:453-7.

[5] Olson PN, Husted PW, Allen TA, Nett T. Reproductive endocrinology and physiology of the bitch and queen. The Veterinary clinics of North America Small animal practice. 1984;14:927.

[6] Kustritz MVR. Clinical canine and feline reproduction: evidence-based answers: John Wiley & Sons; 2011.

[7] Concannon PW. Reproductive cycles of the domestic bitch. Animal reproduction science. 2011;124:200-10.

[8] Feldman EC, Nelson RW, Reusch C, Scott-Moncrieff JC. Canine and feline endocrinology: Elsevier Health Sciences; 2014.

[9] Goodman M. Ovulation timing: Concepts and controversies. Veterinary Clinics of North America: Small Animal Practice. 2001;31:219-35.

[10] Tsutsui T. Gamete physiology and timing of ovulation and fertilization in dogs. Journal of reproduction and fertility Supplement. 1988;39:269-75.

[11] Hewitt D, England G. Assessment of optimal mating time in the bitch. In Practice. 2000;22:24-33.

[12] Verstegen-Onclin K, Verstegen J. Endocrinology of pregnancy in the dog: a review. Theriogenology. 2008;70:291-9.

[13] Rota A, Vannozzi I, Marianelli S, Gavazza A, Lubas G. Laboratory and Clinical Evaluation of a Feia Method for Canine Serum Progesterone Assay. Reproduction in domestic animals = Zuchthygiene. 2016;51:69-74.

[14] Tsutsui T, Takahashi F, Hori T, Kawakami E, Concannon PW. Prolonged duration of fertility of dog ova. Reproduction in domestic animals = Zuchthygiene. 2009;44 Suppl 2:230-3.

[15] Holst P, Phemister R. Vaginal cytology. Canine and Feline Theriogenology Johnston SD, Kustritz MVR, Olson PNS, eds Philadelphia: Saunders. 2001:32-9.

[16] Moxon R, Copley D, England GC. Quality assurance of canine vaginal cytology: a preliminary study. Theriogenology. 2010;74:479-85.

[17] Holst P. vaginal cytology in the bitch. Philadelphia: Philadelphia : Saunders; 1980.

[18] Holst PA, Phemister RD. Onset of diestrus in the Beagle bitch: definition and significance. American journal of veterinary research. 1974;35:401-6.

[19] Okkens A, Dieleman S, Vogel F. Determination of the ovulation period in the dog a comparison of the rapid progesterone assay, vaginoscopy and vaginal cytology. Voorjaarsdagen 1985 Proceedings of the Netherlands Sm Anim Vet Ass1985. p. 26-7.

[20] Edward C. Feldman RWN. Canine and Feline Endocrinology and Reproduction. third edition ed: Elsevier Health Sciences; 2004.

[21] Hase M, Hori T, Kawakami E, Tsutsui T. Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. The Journal of veterinary medical science. 2000;62:243-8.

[22] Günzel A-R, Koivisto P, Fougner J. Electrical resistance of vaginal secretion in the bitch. Theriogenology. 1986;25:559-70.

[23] Johnston SD, Olson PN, Root MV. Clinical approach to infertility in the bitch. Seminars in veterinary medicine and surgery (small animal). 1994;9:2-6.

[24] Brugger N, Otzdorff C, Walter B, Hoffmann B, Braun J. Quantitative determination of progesterone (P4) in canine blood serum using an enzyme-linked fluorescence assay. Reproduction in domestic animals = Zuchthygiene. 2011;46:870-3.

[25] Chapwanya A, Clegg T, Stanley P, Vaughan L. Comparison of the Immulite and RIA assay methods for measuring peripheral blood progesterone levels in Greyhound bitches. Theriogenology. 2008;70:795-9.

[26] TSUTSUI T. Studies on the reproduction in the dog

III. Observations of vaginal smear in estrous cycle. The Japanese journal of animal reproduction. 1975;21:37-42.

[27] Club UK. American Bully Official UKC Breed Standard. 2013.

[28] Bell ET, Bailey JB, Christie DW. Studies on vaginal cytology during the canine oestrous cycle. Research in veterinary science. 1973;14:173-9.

[29] Post K. Canine Vaginal Cytology During the Estrous Cycle. The Canadian Veterinary Journal. 1985;26:101-4.

[30] Jang G, Kim MK, Oh HJ, Hossein MS, Fibrianto YH, Hong SG, et al. Birth of viable female dogs produced by somatic cell nuclear transfer. Theriogenology. 2007;67:941-7.

[31] Bouchard GF, Solorzano N, Concannon PW, Youngquist RS, Bierschwal CJ. Determination of ovulation time in bitches based on teasing, vaginal cytology, and elisa for progesterone. Theriogenology. 1991;35:603-11.

[32] LINDE C, KARLSSON I. The correlation between the cytology of the vaginal smear and the time of ovulation in the bitch. Journal of Small Animal Practice. 1984;25:77-82.

[33] Bouchard G, Solorzano N, Concannon P, Youngquist R, Bierschwal C. Determination of ovulation time in bitches based on teasing, vaginal cytology, and ELISA for progesterone. Theriogenology. 1991;35:603-11.

[34] Morrow DA. current therapy in theriogenology. In: Patricia N. Olson PWH, editor. Breeding management for optimal reproductive efficiency in the bitch and stud dog. 2 ed. Philadelphia, PA, USA: WB Saunders company; 1986. p. 463-8.

국문초록

아메리칸 불리 품종의 교배 적기 판단을 위한 임상적 지표 연구

한 상 은

(지도교수 : 장 구)

서울대학교 대학원
수의학과 임상수의학 전공

개의 번식에서 배란 시기를 정확히 파악하는 것은 교배 성공을 위해 필수적인 작업이다. 개는 개체 간이나 품종 내 혹은 심지어 한 개체 내에서도 발정 주기의 생체 리듬의 변이가 흔한 특수성을

갖고 있다. 연속적으로 번식 관련 호르몬과 번식 기계의 영상학적 측정을 수행한다면 배란이 언제 일어나는지 정확히 파악할 수 있지만, 이러한 방식은 실제 임상 환경에서는 비용적으로나 시간상으로 불가능하다. 따라서 이 연구에서는 아메리칸 불리 품종의 교배 적기를 파악하면서 경제적인 요소와 교배 성공률을 동시에 고려하였다.

배란 주기를 파악하는 방법은 여러 가지가 있으나, 그중 비교적 정확도가 높으면서도 접근성이 높은 방식은 황체 호르몬 측정이다. 질 도말 검사는 배란 시기를 정확히 짚어 내기에는 한계가 있으나 주기 파악에는 도움이 되고, 상대적으로 간편하고 저렴하다. 신체검사는 가정에서도 적절한 교육 후 쉽게 인지할 수 있는 요소로 구성되어 있다. 이 연구에서는 이러한 임상 지표 간의 연관성을 통계학적으로 분석하여 더욱 저렴한 검사 방법에 의존해 모니터링을 수행하고 필수적인 횟수의 황체 호르몬(Progesterone) 농도 측정으로 아메리칸 불리 품종의 교배 성공을 도모하고자 하였다.

개의 질 도말 검사와 관련된 연구는 임상적으로 활용하기 어려운 황체형성호르몬(LH)을 기준으로 제공된 것이 일반적이다. 본 연구에서는 더욱 활용도가 높은 황체 호르몬을 중심으로 수치화 한 질 도말 분석 자료를 연관 지어 유의미한 상관관계가 있음을 확인하였다 (cornified cell ratio – P4 concentration $r = 0.501$; cornification index – P4 concentration $r = 0.613$, $p \leq 0.001$). 또한, 신체검사 지표 중 하나인 발정 전기 분비물 관찰을

기준으로 황체 호르몬 농도 상승 패턴을 제공하여 보호자가 병원 내원 일을 놓치지 않도록 하는 쉬운 기준을 마련하였다.

아울러 이 연구는 2013년에 새로 등록된 품종인 아메리칸 불리 품종에 대한 생식학적 정보를 제공한다는 의의가 있다.

주요어 : 교배 적기, 발정 주기, 배란, 아메리칸 불리, 질 도말

학 번 : 2015-21843