Slov Vet Res 2018; 55 (Suppl 20): 125–35
DOI 10.26873/SVR-637-2018

Original Research Article

PREDICTING THE OPTIMAL TIME OF BREEDING AND THE POSSIBLE APPROACHES FOR TREATMENT OF SOME ESTRUS CYCLE ABNORMALITIES IN BITCHES

Faten M. Labib, Hassan H. Mansour, Atef B. Mahdy, Asmaa A. Abdallah*

Department of Theriogenology, Faculty of Veterinary medicine, Zagazig University, Egypt

*Corresponding author, E-mail: asmaa.abdallah2016@yahoo.com

Abstract: Infertility problem is a common syndrome among bitches and its causes are numerous, but the apparent most frequent cause is the mating at incorrect time. The aim of this study was to avoid the possible causes of infertility in bitches by predicting the optimal time of breeding and try to treat disorders in estrus cycles. A total number of 18 bitches of German shepherd breed were used in this study for monitoring phases of estrus cycle and predicting the optimal time of breeding. Those bitches examined by vaginal cytology, progesterone assay, measurement of vaginal PH, vaginal endoscopy and ultrasonographic examination of ovaries from proestrus to diestrus. By vaginal cytology, cornified cells first appeared on Day 4 from the beginning of proestrus, then increased till reached to 85% on Day 12 which is considered as the ovulation day. Measuring of progesterone concentration at the beginning of proestrus was very low (less than 1 ng/ml) then increased in 13 bitches only out of 18 bitches which used in this study till reach to 6.5 ± 1.37 ng/ml within ranges (4.8 to 8.3 ng/ml) on Day 12 from the beginning of proestrus. The other five bitches showed disorders in normal sequence of estrus cycle which was discovered by progesterone assay, vaginoscopy and ultrasonography of ovaries. Abnormalities in estrus cycle were recorded; two cases of anovulation and three bitches of persistent proestrus. As a trial for correcting the deviated estrus cycle, we injected hCG hormone in two anovulatory bitches for occurrence of ovulation, and also used methergine drug in cases of persistent proestrus as a trial for stoppage of continuous bloody discharge. The optimal time of breeding in 13 ovulatory bitches was within 2 to 3 days after ovulation day. Pregnancy rate after breeding by 20 days was recorded by ultrasonographic examination was 92.3 % (12/13 bitches). When applying these applications, we concluded that progesterone hormone assay was the accurate method for predicting the ovulation time and achieving higher pregnancy rate , and also can judge on estrus cycle if normal or abnormal.

Key words: bitch; bvulation; brogesterone assay; bersistent proestrus; hCG administration

Introduction

Estrus cycle in bitches is unusual and differs than in other domestic animals; include four stages as proestrus, estrus, diestrus and anestrum. Estrus period is long with average 9 days in most bitches. Female dog accepts male for such long period and the poor relationship

Received: August 2018
Accepted for publication: September 2018

between behavioural characteristic and time of ovulation in the bitch can lead to difficulties in identifying the optimal mating time. Anestrum period is very long within a range of 4 to 10 months (1). Fertility in the bitch has a great socio-economic importance. The most common cause of infertility in the bitch is mating at the incorrect time **(**1), so determining the optimal time of breeding is considered the most important aim of routine breeding management (2). The character of spontaneous ovulation and long estrus period lead to difficulty in determining the suitable time of mating and lead to apparent infertility in the bitch (3). Previous studies discussed clinical assessments for estimating the time of insemination as visualization of physical and behavioral signs during estrus cycle, vaginal cytology, and examination of cervico-vaginal secretion and measurement of reproductive hormones (4,5). Progesterone assay has been a valuable tool for following the reproductive events in bitches, and also rise of serum progesterone before ovulation due to the luteinization of preovulatory follicles is one of the distinctive features in canine reproduction (6). Another valuable tool in breeding management program was a vaginal cytology, it can help to demarcate the estrus cycle stages under the effect of reproductive hormones which play role in morphological changes in vaginal epithelium, also is available and inexpensive (4). Another clinical assessment used for predicting ovulation time was ultrasound as mentioned by Renton et al. **(**7) who compared endocrine changes and ultrasound as means for determining ovulation in the bitch and improve fertility. The other workers used assessment of turgidity of vulva and record the changes in vaginal mucosa as the way for identifying the ovulation time (8,9). The vaginal pH in bitch changes during different stages of estrus cycle under the effect of steroid hormones, but very limited studies measure pH as a tool for identifying ovulation time (10,11). "The development of a rapid and reliable method for predicting ovulation in dogs would have a large impact on canine reproduction", as improving the fertility, lowering the cost especially if used frozen semen and more benefit in embryo transfer (12). Following up measurement of serum progesterone concentration from beginning of proestrus till end of estrus period, not only to accurately detect the insemination time and predict parturition date, but also to identify unusual estrus cycles which is mainly caused by ovarian dysfunction (13). This study aimed to improve fertility and find the best accurate facilities for breeding management in bitches throughout comparing the clinical assessments as vaginal cytology, progesterone assay, and measurement of vaginal pH, vaginal endoscopy and ultrasonographic examination of ovaries, and use them in predicting the optimal time of breeding. Also recording abnormalities in estrus cycle and try to treat them.

Material and methods

Study design

A total of 18 German shepherd bitches with different weights ranged from 10 – 20 Kg and with age ranged from 9 months to 3 years. These bitches were housed at the clinic of veterinary hospital, Faculty of Veterinary Medicine, Zagazig University, Egypt from January to July, 2018. The bitches were fed on a balanced diet and provided with water *ad libitum* (14). These bitches were examined from the beginning of proestrus (The first day of serosanguinous vaginal discharges and vulval edema) till the beginning of diestrus period (identified by bitch not accept male dogs and sharp decrease in cornified epithelium cells percent) for predicting the optimal time of breeding by vaginal cytology, progesterone assay, vaginal pH measurement, vaginal endoscopy and ultrasonographic examination of ovaries and recording the phases of estrus cycle if normal or abnormal. All procedures involved bitches has been carried out after approval from Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC/2/F/42/2018).

Vaginal cytology

Vaginal smear was applied day after day from first onset of proestrus till the beginning of diestrus. It was carried out by using a sterile

vaginal swab, which was introduced into the vulva upward cranially to avoid clitorial fossa and was forced into anterior vagina**.**

The swab was rotated inside the anterior vagina, rolled on clean glass slide and then fixed by methyl alcohol for one minute. The slide was stained with modified wrights Giemsa stain for five minute, rinsed by a few drops of water and then dried by filter paper (4). Finally, the slide was examined under high power of microscope (X 400) to determine estrus cycle stages and detect the vaginal cytology cornification index.

Cornification index = Total number of cornified vaginal cells / Total number of vaginal cells in smear × 100.

Measurement of progesterone hormone

Blood samples were withdrawn from bitches day after day from the beginning of proestrus till the beginning of diestrus period. They were collected from cephalic vein and then centrifuged at 1000 round per 15 minutes at room temperature to obtain a serum sample (6). Serum samples were sent to ULTRA lab, Sharkia governorate at the same day of collection for measuring progesterone hormone by chemiluminescence assay (CA) as a quantitative assay, and the results were expressed as ng/ml.

Measuring of vaginal pH

The vaginal pH was measured using pH strip (Gomhoria Company, Egypt) within a range from 4 to 9. This was done by insertion of the pH paper into the vagina by the aid of a glass speculum. The pH paper was left in contact with the vaginal wall and the vaginal secretion for at least 30 seconds (11). The readings were recorded by comparing the changes in the color of pH strip with the appropriate table strip.

Vaginal endoscopy

Vaginal examinations were performed day after day using 1.5 meter long and 8.2 mm diameter rigid endoscope (USA). It was inserted into the vagina, following the natural anatomy of the tract. The moving images appeared on screen were recorded and saved to a memory card. These procedures were applied in the non-sedated, standing bitch but in viscous bitch 0.5-1 ml of 2% xylazine solution was injected intramuscular as a sedative. Vaginoscopic assessment is based upon observation of the mucosal fold contours and profiles, the color of the mucosa and of any fluid present as well as the changes in vagina from the beginning of proestrus to diestrus onset.

Ultrasonographic examination of ovaries

Bitches' ovaries were examined by ultrasound (Mylab esaote, the Netherlands) day after day from proestrus onset till beginning of diestrus. Bitches were secured at lateral recumbancy during examination and examined by curved transducer with frequency 7.5 MHZ or by linear transducer with frequency 8 MHZ. The kidney was considered as a guide during ovarian examination. Ovaries were monitored throughout cycle to record any newly appeared structures and to detect the time of ovulation.

Recording disorders in estrus cycle and dealing with them

By applying the previous clinical methods especially progesterone assay and vaginal endoscopy for judging the status of estrus cycle (normal or abnormal), disorders were recorded as persistent proestrus and anovulation. Methergine (1cm/20Kg BW for three days intravenous) was used as a hemostatic drug for treating persistent proestrus, however, hCG hormone (250 IU/ bitch for once time intramuscular) was used as a trial for helping the occurrence of ovulation in anovulatory bitches.

Laparotomy operation was carried out for one anovulatory bitch which not respond for hCG treatment under general anesthesia as Thiopental sodium 2.5% with a dose of 20 mg/kg BW for exploration of disturbance causing abnormalities in estrus cycle. During laparotomy, 2 uterine horns, 2 fallopian tubes, 2 ovaries were removed. Pieces of right and left ovaries, fallopian tubes, and uteri from bitch with estrus cycle abnormalities were collected, fixed in 20 % buffered neutral formalin (N.B.F.), dehydrated in series of ascending

grade of ethanol followed by clearing in Xylol (three changes) and then embedded in paraffin. Using rotary microtome, sections of 4-6 µm thickness, then slides were stained with Hematoxyline and Eosin (H&E). All stained sections were examined with standard light microscope and then photographed (15).

Statistical analysis

The date characterizing the duration of estrus cycle phases, vaginal PH and progesterone assay was analyzed using the SPSS program, version 22. The average progesterone concentration for cyclic ovulatory bitches and sampling day were calculated as the mean ± SD for normal ovulatory bitches. Analyses were performed using bar chart. The values of vaginal pH were expressed as the mean ± SD and represented in bar chart.

Results

These clinical assessments applied on 18 bitches of German shephered breed with spontaneous normal proestrus, mean ± SD of their proestrus was 10.2± 0.05 days, mean ± SD of their estrus was 8.04 ± 0.12 days and diestrus started between 19 and 20 days after the onset of the proestrus.

Vaginal smear from along estrus cycle

It revealed the appearance of superficial cornified cells on day 4 from the beginning of proestrus and the cornification index was 10%. This cornification index continued to increase till reached 85% on Day 12 from the beginning of proestrus (Figure 1A and B), and then slowly decreased from days 13 to 15. After Day 15, cornification index sharply decreased till reach 30% on Day 20 from the beginning of proestrus, and then continued to decrease until completely disappeared on Day 24 from the beginning of proestrus (Figure 1B).

Progesterone concentration along estrus cycle

The progesterone concentration was low (< 1ng/ml) at the beginning of proestrus then continued in low values till the beginning of estrus that reached 1.55± 0.155 ng/ml. Two days after the beginning of estrus, progesterone concentration was 6.5 ± 1.37 ng/ ml with a range of (4.8 to 8.3 ng/ml) then continued to increase with progression of days to diestrus (Figure 2).

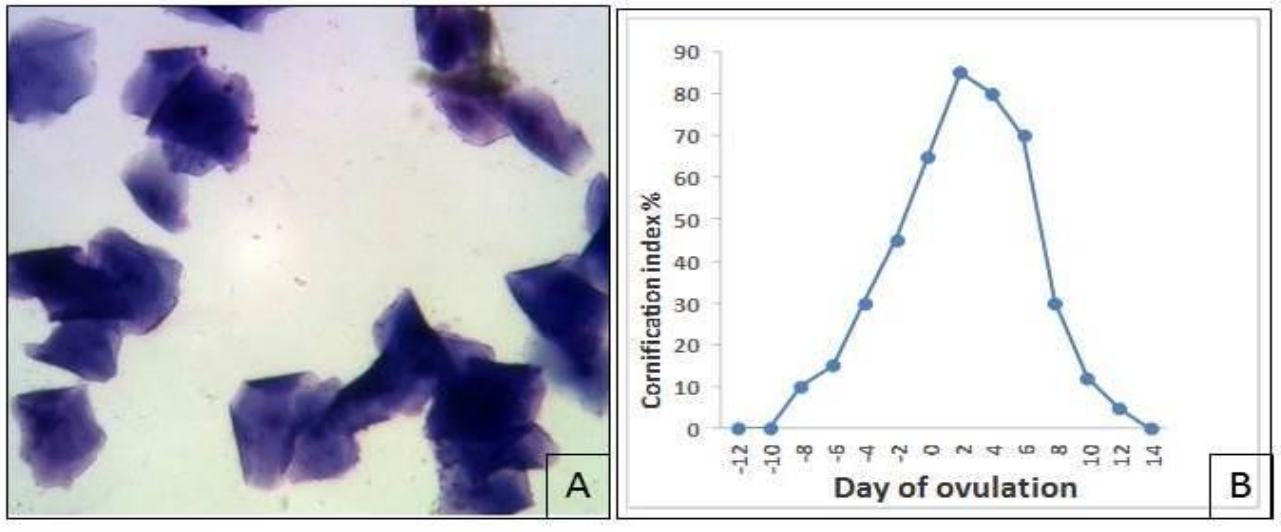

Figure 1: (A) Vaginal smear during estrus of German shepherd bitches showing high percent of cornnified angulated cells; take more staining with Geimsa stain (X400). (B) Cornification index curve from the beginning of proestrus till the beginning of diestrus in German shepherd bitches

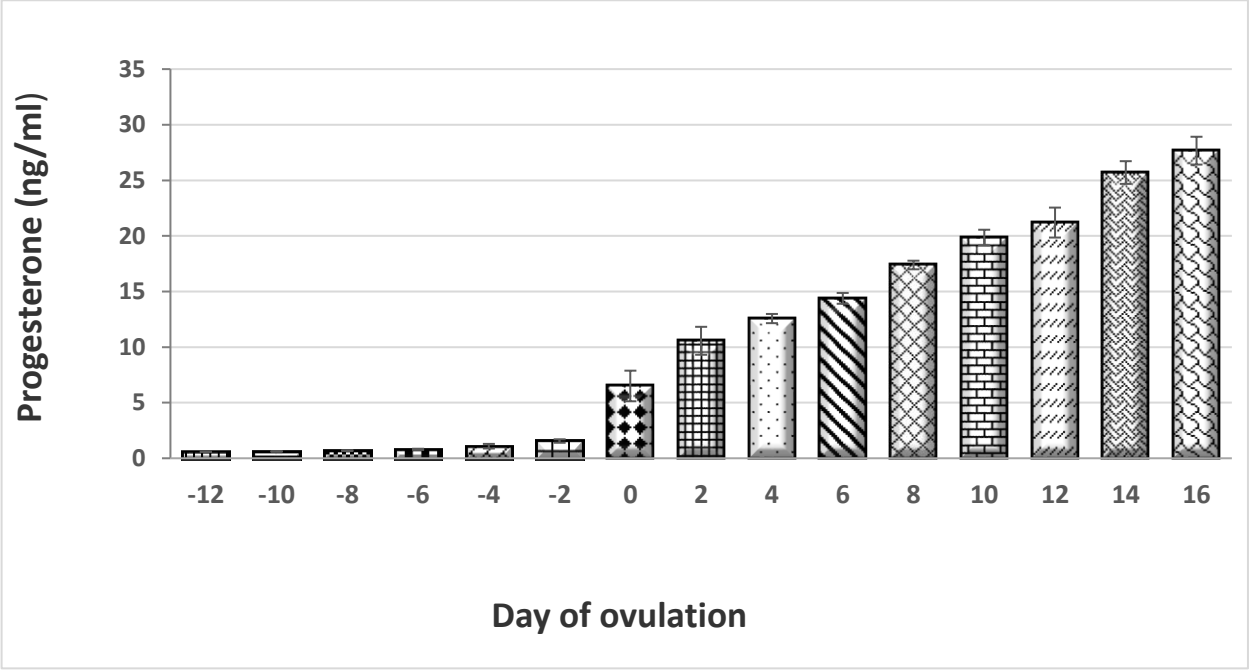

Figure 2: Bar chart showing mean ±SD of progesterone concentration from the beginning of proestrus till the beginning of diestrus in German shepherd bitches

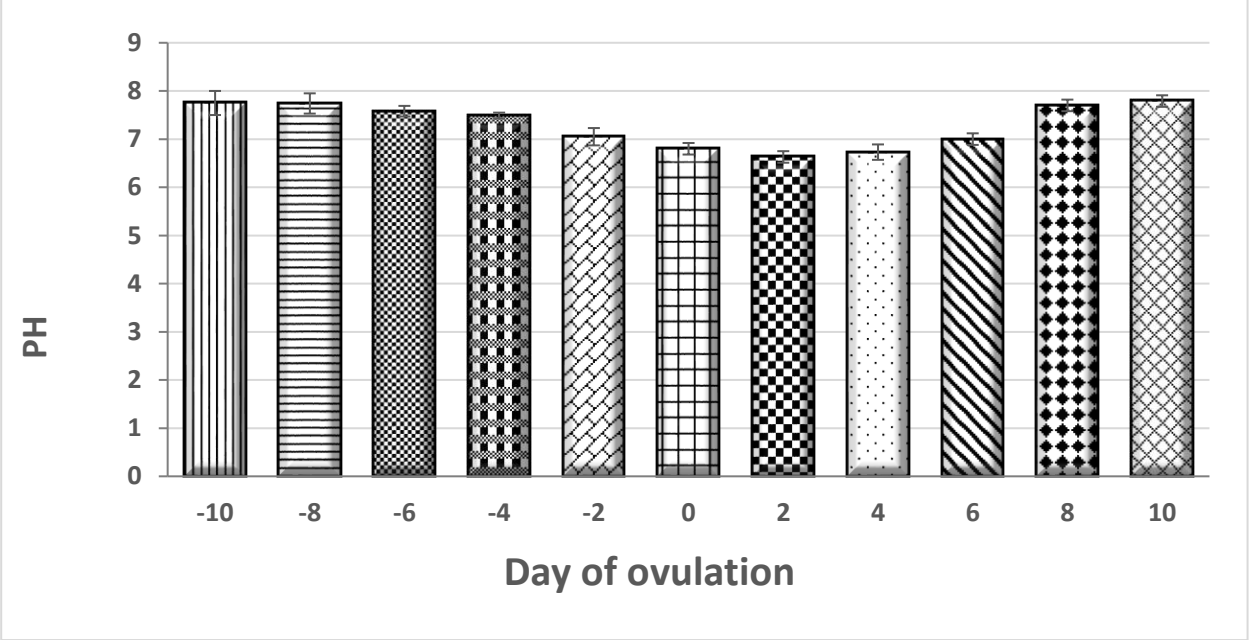

Figure 3: Bar chart Showing mean ± SD of vaginal pH from the beginning of proestrus to the beginning of diestrus in German shepherd bitches

The dynamic of vaginal pH during estrus cycle

Measuring of vaginal pH during proestrus and estrus shown that the pH of vaginal secretions at the second day of proestrus ranged from 7.3 to 8.2 (7.76±0.17) then continued to decrease till reach their low values at the last day of proestrus between 7.4 and 7.6 (7.49±0.06). The trend of a reduction of the vaginal pH continued during the first days of estrus as following; pH ranged from 6.8 to 7.3

(7.05±0.28), 6.5 and 7.1 (6.8±0.27) and 6.5 and 6.8 (6.63±0.12) on the first, third and fifth day of estrus, respectively. After the fifth day of estrus, pH values returned to increase till reach on Day 10 post ovulation to 7.79± 0.12 as illustrated in (Figure 3). The expected ovulation day was day 12 where pH ranged from 6.5 to 7.1 (6.8± 0.27).

Vaginal endoscopy findings

Vaginal endoscopy of bitches during proestrus, revealed that, the vaginal mucosa was edematous with swollen folds, take round shape and red color with oozing of bloody discharges from cervix (Figure 4A). During the first three days of estrus, the edema of mucosal folds decreased, the vaginal folds collapsed and appeared wrinkled, and the color changed into white. During the mid of estrus, mucosa became more crenulated, folds are flattened**.** During diestrus, mucosal folds appeared flat and covered with green mucoid discharges**.** The expected day of ovulation was decided when mucosal folds appeared wrinkled and mating occurred when more wrinkled.

Ultrasonographic results of ovaries

Repeated examination of ovaries using real time B mode ultrasound during proestrus showed the presence of spherical, anechoic follicles with thin walls. The diameter of these follicles was ranged from 2 to 3 mm at the beginning of proestrus and increased with progression of proestrus days till reach 8 to 10 mm at the end of this stage. Two days after the beginning of estrus phase, diameter of these follicles decreased with increasing in echogenicity of ovaries. These changes referred to the time of ovulation. After these changes by 3 days, corpus luteum appeared as echogenic structure with small anechoic cavity. The optimal time of breeding in 13 ovulatory cyclic bitches was within 2 to 3 days after the day of ovulation, pregnancy rate after 20 days from breeding was 92.3 % (12/13 bitches).

Abnormalities of estrus cycle (unusual estrus cycle in bitches)

Deviation from normal progesterone concentration has been recorded from the beginning of proestrus in 5 bitches out of 18 examined. Following up these bitches, it was found the following.

Persistent proestrus

By examination of 3 out of 5 bitches under investigation in current study, there was bloody vaginal discharge continuously for a period ranged from 35 to 40 days as in (Fig.4.B). These bitches accepted male and bred on Day 20 although of continuation of bloody discharge, low serum progesterone concentration (less than 1 ng/ml) till day 30. Later on, serum progesterone increased till reach 4.40 ng/ml. After day 40, bloody discharge stopped and progesterone concentration reached 23.7 ng/ml due to corpus luteum formation. Pregnancy diagnosis of those bitches after mating by one month, appear empty hypoechoic uterus without any pregnancy. To our knowledge, methergine drug (1cm/20 Kg BW for three days intravenous) was used for these bitches on Day 25 from the beginning of discharge as a trial for stoppage these continuous bloody discharges, but this was useless as bloody discharges not stopped and continued for days ranged from 35 to 40 from the beginning in those three bitches.

Anovulation

In the remaining 2 bitches out of the 5 with abnormal estrus cycle, progesterone measured at the beginning of proestrus was 0.878 ng/ml and estrogen was 73.8 pg/ml in both bitches. Four days later, progesterone and estrogen concentrations were 1.43 ng/ml and 41.16 pg/ml, respectively in one bitch. Later on, estrogen decreased into 32.79 pg/ml and progesterone decreased into 0.652 ng/ml in same bitch. Serial measurement of serum progesterone for one month with two days interval revealed a stationary concentration of less than 1ng/ml in spite of stoppage of serosanguinous discharge after 8 days from the beginning.

In the other bitch, estrogen concentration decreased gradually till reach 19.82 pg/ml and continued at this concentration along one month. However, progesterone concentration continued less than 1ng/ml from the appearance of serosanguinous discharge and for a period of

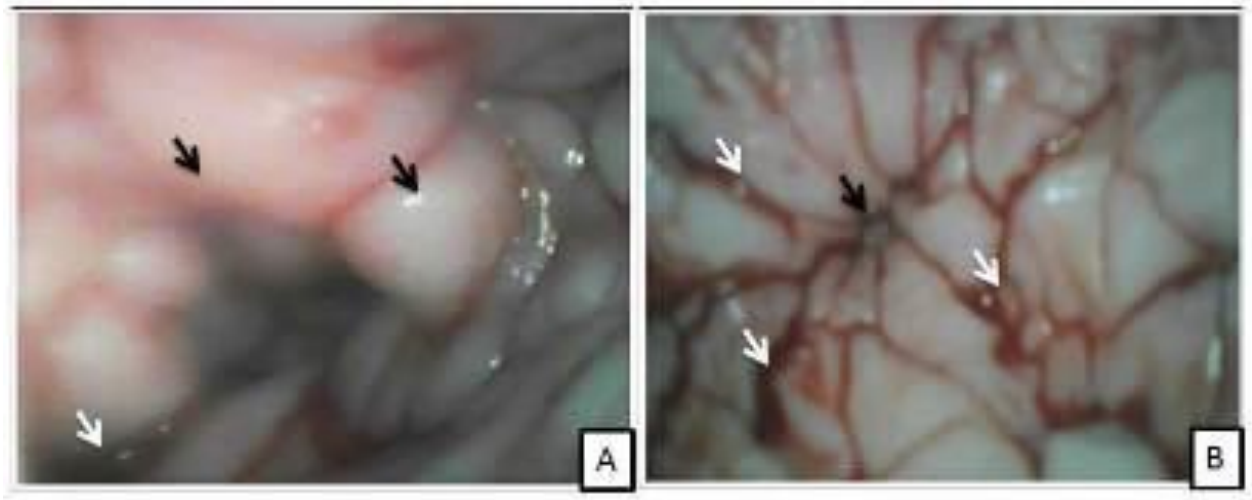

Figure 4: (A) Vaginal endoscopy showing edematous vaginal mucosal folds (black arrow) with presence of bloody discharge (white arrow) during late normal proestrus in German shepherd bitches. (B) Vaginal endoscopy showing opened cervix (black head), vaginal mucosa is fissured and covered with bloody discharge (white arrow) on Day 33 from the beginning of proestrus in abnormal persistent proestrus in German shepherd bitches

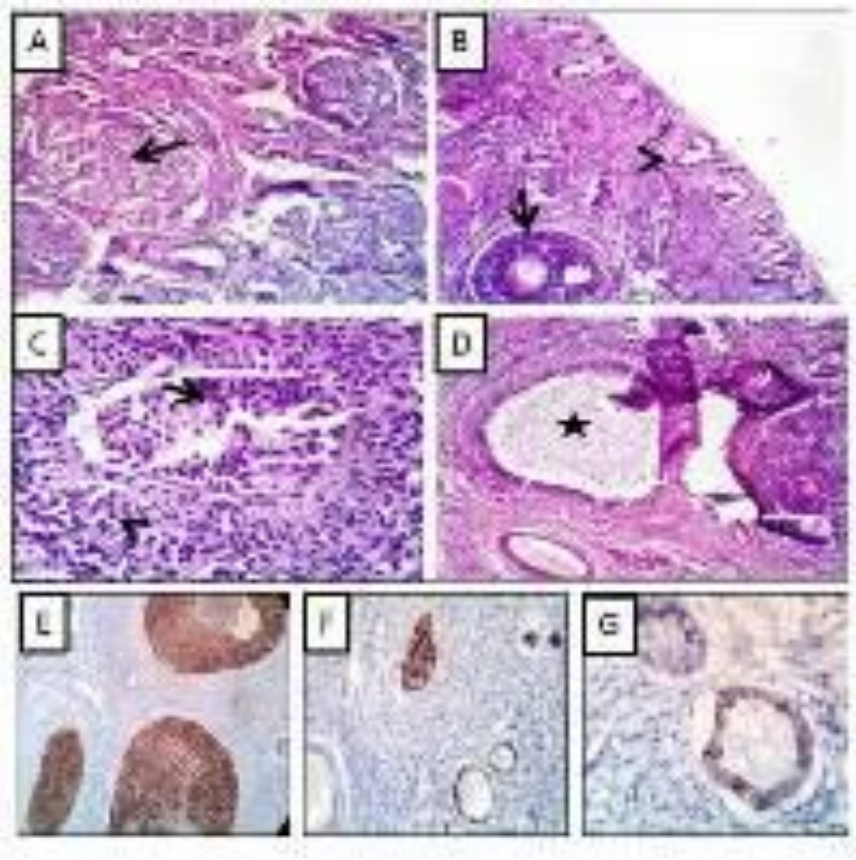

Figure 5: Photomicrograph of L. ovary in a case of anovulation in mongrel bitch (staining with H&E) showing: (A) dysmoplastic changes associated with the neoplastic growth (black arrow). (B&C) mass of globular structure with central radiating cells (black arrow) followed by compacted cell layer with large hyperchromatic nuclei (arrow head). (D) Cystic graffian follicle (star). In lower part of photo showing immunostaining with tumor markers as inhibin: Examined sections revealed diffuse reaction in mature graffian follicle granulosa cells (E) and focal positive reaction in tumor cells (F, G)

one month. In those two bitches, serosanguinous discharge stopped after 8 days, then bitches attracted to male and bred normally day after day for three times although ovulation not occur and progesterone not increase.

Pregnancy diagnosis of these bitches by ultrasonography after the last mating by 20 days revealed that the uterus was empty without pregnancy. The approach with those cases was by injection of hCG (250 IU/ bitch) once intramuscular. In one bitch, progesterone concentration increased after injection by 3 days and reached to 4.56 ng/ml, but in the other bitch serum progesterone did not changed or increased after injection. Histopathology applied on these genitalia after ovariohysterectomy to know the cause of anovulation and abnormal structure on ovaries. Histopathological alterations in this bitch clarified the presence two small follicles, follicular cyst and abnormal structure as suspected tumor on the two ovaries. The ovarian stroma of the right ovary appeared infiltrated by sheets, acini and trabecular structures of epithelial cells, some with dysplastic nuclear changes and dysmoplastic changes associated with the neoplastic growth. The ovarian follicles showed different developmental changes with regular structures. One of the graffian follicles was cystic and filled with fine granular esinophilic fluid; the granulosa cells of such cyst were atrophied. The Left ovary showed the same histomorphological changes beside a mass of globular structure with central radiating cells, followed by compacted cell layer with large hyperchromatic nuclei beside a group of infiltrating small epithelial cells with hyperchromatic nuclei. The Preliminary Diagnosis of such case was ovarian neoplasm with glandular differentiation, possibility of granulosa cell tumor. Immunostaining with tumor markers as inhibin showed the presence of granulosa cell tumor as in (Figure 5).

Discussion

The presence of variability in the day on which ovulation occurs among bitches play a role in difficulty to determine the optimal time of mating, so applying clinical assessments or finding methods for predicting ovulation time is the corner stone for controlling infertility's problems in bitches.

Monitoring the changes in vaginal epithelial cells during proestrus and estrus by vaginal cytology showed first appearance of cornified cells after beginning of proestrus by 4 days, then increased in percent with the progression in days of proestrus till reach 85% or more on the day of ovulation (Day 12 from beginning of proestrus). These results are comparable with previous observations and records **(**16-18). Our results also agreed with Moxon et al. **(**2) who reported a decrease in cornified cells percent post ovulation then followed by sharp decrease at the beginning of diestrus. Appearance of cornified cells within low percent on Day 4 from the beginning of proestrus attributed to increase in estrogen hormone concentration, and then reached a high concentration at the beginning of estrus where cornification index was 70%. Slowdown in cornification index after ovulation was caused by declining of estrogen concentration and increasing in progesterone concentration, and then disappeared completely at the beginning of diestrus due to high concentration of progesterone as a result of corpus luteum formation.

Measurement of progesterone concentration by chemiluminescence assay (CA) showed that progesterone was present in low values at the beginning of proestrus due to high estrogen concentration which secreted from follicles on ovaries, and then increased to 1.5 ng/ml at the beginning of estrus due to partial lutenization of follicular layers. By measuring progesterone, the expected ovulation time was on Day 12 from the beginning of proestrus as progesterone begun to increase within ranges of 4.8 to 8.3 ng/ml. These progesterone profiles for 13 cyclic ovulatory bitches only due to the other 5 bitches shown deviation in progesterone concentration than normal values. The current findings are in accordance with Johnston and Root, Concannon **(**19,20), who noticed that progesterone concentration at the day of ovulation ranged from 4 to 10 ng/ml due to complete luteinization of follicular layers. Consequently, the optimal time of breeding was

within 2 days after ovulation till the occurrence of maturation of primary oocyte into secondary oocyte which is fertilized by sperms (21).

The vaginal pH measurement is an important parameter to show certain biological patterns and also to determine the optimal time of insemination in the bitch. pH values during proestrus which were reported by Antonov et al. (11) Schulz (22) and Ross (23) were ranged from 6.9 to 8.2, then decreased toward the ovulation time within a range 6.5 to 6.8 and continued at these low values till insemination, then rose again on the way to diestrus onset, exactly analogous with our findings of PH value during ovulation time (6.8). However, Vaginal PH values were low during ovulation and in the days after wards when mating occurs to almost coincide with PH of seminal plasma and providing favorable conditions for survival of spermatozoa in the female reproductive tract for long period ranged from 4 to 6 days.

By using vaginal endoscopy for examination of mucosal folds, it was noticed that vaginal mucosal folds were edematous with bloody discharge during proestrus due to high concentration of estrogen hormone which caused water retention and diapedesis of erythrocytes. After that, mucosal folds were wrinkled and appeared white in color due to an abrupt withdrawal of the water retention effect of estrogen. These findings coincide with Lulich (24) and Xavier (25), who reported that the prominent vaginal endoscopic image during ovulation is wrinkled vaginal mucosa as a result of sharp decrease of estrogen. The optimal time of breeding was after appearance of more wrinkled vaginal mucosal folds as a confirmed result of ovulation occurrence.

The last method for examination was ultrasonographic of ovaries for following up the structures on it and progression of them to predict the ovulation time. Using real time B mode ultrasound at the first days of proestrus, it revealed the presence of small anechoic follicles within small diameters then with progression of days, these diameters increase till reach to 8–10 mm at the first day of estrus. These results were similar to Yeager and Concannon (26), who reported that proestrus stage is characterized by presence of small anechoic structures on ovaries. Identifying the ovulation moment by ultrasound is difficult due to presence of similarity in shape of follicles and corpus luteum to some extent as both contained retained follicular fluid. Repeated follow up of ovarian structures may help in identifying its structures. Our study revealed that changing ovarian structures from anechoic numerous structures with large diameter to hypoechoic smaller structure is considered the time of ovulation in parallel with the study of Renton et al. (7) that referred to formation of hypoechoic corpus luteum with small anechoic cavity post ovulation. In two bitches out of 18 bitches used in this study, serum progesterone concentrations did not elevate over 1.43 ng/ml, although, their entrance to the cytological estrus phase and accepting male dog. This was recorded anovulation where corpus luteum has no been formed and therefore progesterone was not elevated. The anovulatory cycles have been previously reported as uncommon **(**27). Meyers-wallen **(**13) stated that the possible causes of anovulation were either, the hypothalamus did not secret sufficient GnRH or pituitary gland did not secret enough LH or ovary did not well respond to these hormones due to problems in it. Interestingly, one German shepherd bitch responded to hCG administration, then after 3 days, progesterone concentration was elevated as may be the possible cause was insufficiency of LH secretion, but another bitch did not respond to treatment with hCG due to the presence of granulosa cell tumor which discovered by histopathological examination. There is no available literature regarding bitches with persistent proestrus that is characterized by continuous bloody discharge for a duration ranged from 35 to 40 days as reported in this investigation. Meyers-wallen (13) reported the occurrence of split heat which was defined as abnormally short period of proestrus or estrus accompanied by low serum progesterone value as in anovulatory cycle. Meyers-wallen (13) mentioned the persistent estrus for a period 2 months with normal proestrus period due to insufficient LH secretion. To our knowledge, this is the first study which referred to persistent proestrus. Secretion of bloody discharge conti-

nuously for a range 35 to 40 days may be caused by low LH surge, so follicles persist on ovaries and secret estrogen for that previous period which leads to continuous secretion of these discharges.

Conclusion

Progesterone assay and vaginal endoscopy are the accurate methods for predicting the optimal time of mating as progesterone hormones concentration increased only after ovulation and mating not occur except after ovulation, and also vaginoscopy can declare vaginal changes during ovulation. So following up cyclic bitches and applying clinical assessments, especially two cited above help detecting the optimal time of breeding and judging the estrus cycle)normal or abnormal) as well as achieving the high percent of fertility in bitches. The early interference in anovulatory cases with hCG administration enables us to correct this disorder and reduce cases of infertile bitches.

Conflicts of interest

None of the authors have any conflict of interest to declare.

Acknowledgements

The authors would like to thank all staff members of Theriogenology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt for their contribution in completion of this study. We would like to express our gratefulness thanks to Prof. Dr. El Sayed Rashad El Attar, Department of Pathology, Faculty of Veterinary Medicine, Zagazig University for his help in histopathological work.

Reference

1. England G, Concannon PW. Determination of the optimal breeding time in the bitch: basic considerations. In: Recent Advances in Small Animal Reproduction, Concannon PW, England G, Verstegen J, Linde-Forsberg C (Eds.) , International Veterinary Information Service (www.ivis.org) , 2002.

2. Moxon R, Batty H, Irons G, England GC. Periovulatory changes in the endoscopic appearance of the reproductive tract and teasing behaviour in the bitch. Theriogenology 2012; 78: 1907–16.

3. Goodman M. Ovulation timing: concepts and controversies. Vet Clin Small Anim Pract 2001; 31 (2): 219–35.

4. Tsutsui T. Studies on the physiology of reproduction in the dog III, observations of vaginal smear in estrus cycle. Japanese Journal of Animal Reproduction 1975; 21: 37–42.

5. Johnston SD, Olson PN, Root MV. Clinical approach to infertility in the bitch.In Seminars in veterinary medicine and surgery (small animal) (USA) 1994.

6. Hahn SE, Jo YK, Jin YK, Jang G. Timing of fertile period for successful pregnancy in American Bully dogs. Theriogenology 2017; 104: 49–54.

7. Renton JP, Boyd JS, Harvey MJ, Ferguson JM, Nickson DA, Eckersall PD. Comparison of endocrine changes and ultrasound as means of identifying ovulation in the bitch. Res Vet Sci 1992; 53 (1): 74–9.

8. Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: plasma estrogen LH and progesterone. Biol Reprod 1975; 13 (1): 112–21.

9. Lindsay FE. The normal endoscopic appearance of the caudal reproductive tract of the cyclic and non-cyclic bitch: post uterine endoscopy. J Small Anim Pract 1983; 24 (1): 1–5.

10. De Oliveira C, Da Costa E, Da Silva J. The vaginal pH of healthy bitches during the oestrus cycle. Revista Brasileira Med Vet 1998; 20, 32–34.

11. Antonov A, Dineva J, Georgiev P. Dynamics of Vaginal pH in the Bitch during Proestrus and Estrus. Anim Vet Sci 2014; 2: 101–4.

12. Bouchard GF, Solorzan N, Concannon PW, Youngquist RS, and Bierschwal CJ. determination of ovulation time in bitches based on teasing, vaginal cytology, and ELIZA for progesterone. Theriogenology 1991; 35 (3): 603–11.

13. Meyers-Wallen VN. Unusual and abnormal canine estrous cycles. Theriogenology 2007; 68: 1205–10.

14. Kronfeld D, Donoghue S, Glickman L. Body condition and energy intakes of dogs in feral teaching hospital. Journal of Nutrition 1991; 121: 157–8.

15. Bancroft J, Gamble M. Theory and Practice of histological techniques 6th ed., Churchil, Livingstone, New York, 2008; Ch. 6, PP: 83–92.

16. Schutte AP. Canine vaginal cytology. II. cyclic changes. J Small Anim Pract 1967; 8 (6): 307–11. DSAY, F LINDSAY, F. E. F. (1983)

17. Post K. Canine vaginal cytology during the Estrous Cycle. Can Vet J 1985; 26 (3): 101–4.

18. Fontbonne A. Infertility in bitches and queens: recent advances. Rev Bras Reprod Anim 2010; 35: 202–9.

19. Johnston SD, Root MV. Serum progesterone timing of ovulation in the bitch. In: Proceedings of Society for Theriogenology. San Antonio. Montgomery (AL): Society for Theriogenology; 1995. P: 195–3.

20. Concannon PW . Reproductive cycles of the domestic bitch. Anim Repro Sci. 2011; 124 (3-4): 200–10.

21. Seki M, Watanabe N, Ishii K, Kinoshita YI, Aihara T, Takeiri S, Otoi T. Plasma progesterone profiles in beagle bitches with and without the whelping experience. Acta Vet Hung 2010; 58 (1): 117–24.

22. Schulz A. Evaluation of minimally-invasive methods to observe the cycle of the bitch in heat Ph.D. dissertation, Berlin: Freie Universität, 2002: 35–48.

23. Ross A. Investigations in the female dog's vaginal aerobic flora and pH, Master thesis, Dissertation, Berlin University, Germany, 2005.

24. Lulich J P. Endoscopic vaginoscopy in the dog. Theriogenology 2006; 66 (3): 588–91.

25. Xavier L. Video vaginoscopy of the canine vagina. Reprod Dom Anim 2016; 51 (1): 31-6.

26. Yeager AE, Concannon PW. Canine and feline reproduction. Small Animal Ultrasound (Ovaries). Philadelphia: Lippincott-Raven, 1996; 293-303.

27. Arbeiter K. Anovulatory ovarian cycles in do-gs. J Reprod Fertil. Supplement. 1993; 47: 453–6.