

## Determination of the Stages of the Sexual Cycle of the Bitch by Direct Examination

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**Abstract:** The aim of this study was to determine the stages of the sexual cycle of the bitch by direct examination and also to assess the reliability of this new technique by comparing it with the classical staining techniques used in bitches. Forty mixed-breed bitches, of different ages and sexually mature were used in this study. A total of 120 vaginal smear samples were collected using a cotton swab, three from each bitch. The collected samples were air dried and coded. One of the prepared samples from each bitch was stained with May-Grunwald Giemsa and the second sample was stained with Papanicolaou. The third sample was left unstained for direct examination. The stages of the sexual cycle were determined using all of the samples. The researcher who evaluated the samples did not have information about the coding system. The evaluation was made blindly and the results were compared after determination of the stages of the sexual cycle from all of the samples. The sexual cycle stages determined with the May-Grunwald Giemsa and Papanicolaou techniques were completely consistent with each other. However, when the direct examination technique was compared with the classical staining techniques, there was a significant difference in the proestrus, diestrus and anestrus stages of the cycle ( $p < 0.05$ ) while there was no significant difference ( $p > 0.05$ ) in the estrus stage of the cycle. In conclusion, it was found that when determining the stages of the sexual cycle of the bitch by vaginal cytology, the direct examination technique was reliable only in detecting the estrus stage of the cycle.

**Key words:** Bitch, sexual cycle, vaginal smear, vaginal cytology, direct examination, Turkey

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### INTRODUCTION

Bitches usually experience one or two sexual cycles a year. Only one estrus is observed in each mating season, regardless of whether there is mating or not. Estrus can be seen in all seasons of the year, though it is more common in spring and autumn (Arthur *et al.*, 1989; Johnston *et al.*, 2001). The sexual cycle of bitches is composed of proestrus, estrus, diestrus and anestrus stages. The stages of the cycle of the bitch are long in comparison with those of other domestic animals. The interestrus interval in the bitch is 5-11 months (7 months on average). However, the duration of the sexual cycle and the individual stages of the sexual cycle vary within the same breed and from breed to breed (Olson *et al.*, 1984a; Feldman and Nelson, 1996).

One of the important issues in canine reproduction is the determination of the stages of the sexual cycle. The stages of sexual cycle can be determined by looking at clinical, endoscopic, endocrinological and cytological changes (Feldman and Nelson, 1996; Johnston *et al.*, 2001). One of these methods, vaginal cytology is preferred in practice because it is an easy and reliable method

(Schutte, 1967; Christie *et al.*, 1972; Olson *et al.*, 1984a). The vaginal epithelium is one of the target tissues for ovarian hormones (Thrall and Olson, 1999; Henson, 2001). The increased concentration of estrogen during the proestrus and early estrus periods causes the vaginal wall to thicken as a result of a rapid increase in the number of cell layers in the mucous membrane of the vagina. As a result of this thickening, the cells in the luminal layer of the vagina move away from their blood supply and because they do not have enough blood flow, these cells die and exfoliate into the vaginal fluid (Schutte, 1967; Feldman and Nelson, 1996; Thrall and Olson, 1999). The exfoliated vaginal epithelial cells are named according to the morphology of the cells. The variations in cell morphology include increases in the size of the cell, changes in its shape, observation of keratin precursors in the cell and degeneration of the nucleus (pyknosis). Different cell types represent the stages of cell death (Feldman and Nelson, 1996; Johnston *et al.*, 2001). Such changes in the cell can be determined easily by the use of vaginal cytology. Vaginal cytology gives an approximate but reliable indirect reflection of the estrogen concentration. This reflection continues even when the

concentration of estrogen decreases to basal levels (Wright, 1990; Feldman and Nelson, 1996). Therefore, vaginal cytology is a diagnostic method that is useful in determining the stage of the reproductive cycle (Olson *et al.*, 1984a; Post, 1985; Wright and Parry, 1989; Mestre *et al.*, 1990; England, 1992) and in the diagnosis of some pathological conditions (Olson *et al.*, 1984b; Wright and Parry, 1989; Wright, 1990; Erunal-Maral *et al.*, 2000) which vary according to the cell types present and the proportions of the different cell types.

Classical staining techniques, using stains such as May-Grunwald Giemsa, Papanicolaou and new methylene blue are used in vaginal cytology in the microscopic assessment of cellular changes (Olson *et al.*, 1984a; Post, 1985; Hiemstra *et al.*, 2001; Yener *et al.*, 2007). A new technique has been introduced in recent years in which samples of vaginal smear taken from rats are examined directly without staining (Marcondes *et al.*, 2002; Yener *et al.*, 2007). The aim of this study was to determine the stages of the sexual cycle in the bitch by the direct examination technique and also to assess the reliability of this new technique by comparing it with classical staining techniques.

## MATERIALS AND METHODS

**Animals:** A total of 40 sexually mature, healthy, mixed-breed bitches of varying ages that were kept in the Konya Metropolitan Municipality animal care and housing centre were used in the study. The bitches were kept in semi-open cages and were fed once a day with a balanced ration (Hill's Prescription Diet®). Study protocol was approved by the Ethics Committee of the Veterinary Faculty.

**Collection of vaginal smears:** A total of 120 vaginal smear samples were prepared from 40 bitches, three from each bitch. The samples were collected using the cotton swab technique. A sterile cotton-tipped swab, 10 cm long, was used. The cotton was moistened with two to three drops of sterile saline (0.9%). The lips of the vulva were gently separated. The cotton-tipped end of this swab was passed into the dorsal commissure of the vagina. The swab initially was pressed gently against the caudodorsal surface of the vaginal vault and then advanced in a craniodorsal direction, toward the vertebral column, until it passed over the ischial arch. The swab was then rotated through a complete revolution in each direction and withdrawn. The cotton tip was rolled from one end of a glass microscope slide to the other (Feldman and Nelson,

1996; Johnston *et al.*, 2001; Kustritz, 2006). Three different smears were prepared from each bitch using three different swabs. The prepared smears were air-dried and coded. Two of the three samples that were taken from each bitch were fixed at -10°C using cold gluteraldehyde-acetone (pH 4.8) for 3 min and then one was stained with Papanicolaou and the other with May-Grunwald Giemsa stain (Culling *et al.*, 1985). The other sample was left unfixed and unstained for direct examination.

**Evaluation of vaginal smears:** Evaluation of the vaginal smear samples was performed using a light microscope. The stages of the sexual cycle of the bitches were determined according to the cell types observed in the vaginal smear and the ratios between cell types. Determination of the stage of the sexual cycle was conducted in accordance with the following criteria.

**Proestrus:** All of the parabasal, intermediate and superficial cells are observed. Most of the cells are parabasal and intermediate cells. There are large numbers of erythrocytes. Neutrophils may be seen in the first few days but neutrophils cannot be found in the middle of proestrus.

**Estrus:** The background of the smear is fairly clear. There are only pyknotic nuclei and anuclear superficial cells (anuclear squames).

**Diestrus:** There are intermediate and parabasal cells. Large numbers of neutrophils are observed as soon as diestrus begins. As diestrus progresses, the number of neutrophils decreases. Moreover, metestrus and foam cells can be observed in this period.

**Anestrus:** There are very few cells. The cells that are found are parabasal and intermediate cells (Olson *et al.*, 1984a; Post, 1985; Feldman and Nelson, 1996; Johnston *et al.*, 2001).

The researcher who assessed the smears had no information about the coding system. The evaluation was made blindly and the results were compared after determination of the stage of the sexual cycle in all of the samples.

**Statistical analysis:** The  $\chi^2$ -test was used to evaluate the results obtained from the staining and the direct examination techniques (Minitab for Windows 12.1). Significance was accepted at a level of  $p < 0.05$ .

## RESULTS AND DISCUSSION

It took about 2-3 h to take vaginal smear samples from the bitches and to implement the staining procedures. For the direct examination technique, the vaginal smear sample was examined directly with a microscope without the need to wait for a procedure to be performed.

Unstained cells were observed more clearly under a light microscope with low illumination and without the use of the condenser lens. The proportional analysis of the types of cells observed in the vaginal

smear became easier using the 20X objective lens. On the other hand, characterization of the cell types was easier when the 40X objective lens was used. Vaginal smear samples belonging to the stages of the sexual cycle determined with different techniques are shown in Fig. 1.

The stages of the sexual cycle determined using the May-Grunwald Giemsa and Papanicolaou staining techniques were in total agreement with each other in all samples. It was determined with both techniques that eleven bitches were in proestrus, nine bitches in estrus, fourteen bitches in diestrus and six bitches in anestrus.

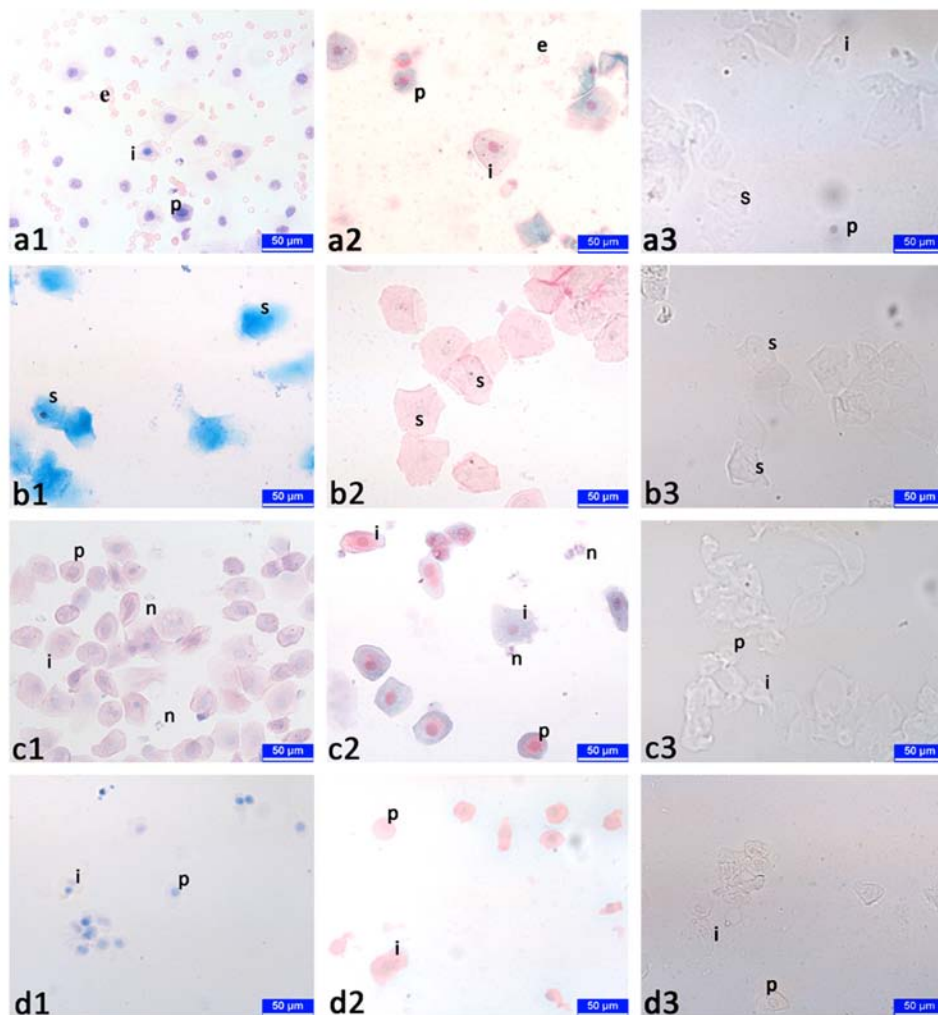


Fig. 1: Photomicrograph images of vaginal smears taken from bitches. a1-a3 indicate proestrus stages; b1-b3 indicate estrus stages; c1-c3 indicate diestrus stages; d1-d3 anestrus stages; a1-d1 refer to the samples that were stained using the May-Grunwald Giemsa staining technique; a2-d2 refer to the samples that were stained using the Papanicolaou staining technique; a3-d3 refer to the unstained samples that were left for direct examination. Parabasal cells are shown with the symbol: p, Intermediate cells: i, Superficial cells (nuclear and anuclear): s, Erythrocytes: e and Neutrophils: n

However, it was determined with the direct examination technique that nine bitches were in proestrus, nine bitches were in estrus, sixteen bitches in diestrus and six bitches in anestrus. When a comparison was made with the May-Grunwald Giemsa and Papanicolaou staining techniques, it was determined that, of the nine bitches that were determined to be in proestrus by the direct examination technique, five were in proestrus and four were diestrus; of the nine bitches that were determined to be in estrus, all were in estrus of the sixteen bitches that were determined to be in diestrus, seven were in diestrus, seven were in proestrus and two were in anestrus of the six bitches that were determined to be in anestrus, three were in anestrus and three were in diestrus. When the direct examination technique and the classical staining techniques were compared in terms of the sexual cycle stages determined, a statistically significant difference was found in the proestrus, diestrus and anestrus stages of the cycle ( $p < 0.05$ ) but no difference was determined ( $p > 0.05$ ) in the estrus stage of the cycle.

## DISCUSSION

Classical staining techniques have been used in many studies of vaginal cytology for the microscopic assessment of cellular changes (Linde and Karlsson, 1984; Post, 1985; Bouchard *et al.*, 1991; Hiemstra *et al.*, 2001). In recent years, a new technique has been introduced in which vaginal samples taken from rats are examined directly without being stained (Marcondes *et al.*, 2002; Yener *et al.*, 2007). However, the reliability of this technique has not been studied previously in bitches. In this study, the reliability of the direct examination technique was evaluated in comparison with the classical staining techniques in determining the stage of the sexual cycle in the bitch.

In the present study, the stages of the sexual cycle determined using the May-Grunwald Giemsa and Papanicolaou staining techniques were in total agreement with each other in all samples. However, when the direct examination technique and the classical staining techniques were compared in terms of the sexual cycle, a statistically significant difference was found in the proestrus, diestrus and anestrus stages of the cycle ( $p < 0.05$ ) but no difference was determined ( $p > 0.05$ ) in the estrus stage of the cycle. Unlike the studies conducted on rats (Marcondes *et al.*, 2002; Yener *et al.*, 2007) cellular changes could not be identified clearly in the unstained vaginal smear samples under light microscopy in this study. Observation of bloody vaginal discharge in bitches is an important indication of the beginning of proestrus.

Bloody discharge from the vagina is caused by diapedesis of erythrocytes through the endometrium. Therefore, the cytological indication of proestrus on a vaginal smear is the existence of an excess of erythrocytes among the parabasal and intermediate cells. Observation of numerous erythrocytes in a vaginal smear in the proestrus stage is important in distinguishing the proestrus stage from the diestrus and anestrus stages (Post, 1985; Feldman and Nelson, 1996; Thrall and Olson, 1999; Johnston *et al.*, 2001). On the other hand, observation of an excess of neutrophils in a vaginal smear taken from a bitch is a characteristic finding in the diestrus stage of the cycle. As diestrus progresses, the number of neutrophils decreases (Dore, 1978; Post, 1985). Common cells that are observed in vaginal smears in the proestrus, diestrus and anestrus stages of the cycle in bitches are parabasal and intermediate cells.

Therefore, observation of erythrocytes among parabasal and intermediate cells in vaginal cytology is a significant criterion for proestrus and observation of neutrophils is characteristic of diestrus (Post, 1985). Parabasal, intermediate and superficial cells were easily recognized using the May-Grunwald Giemsa and Papanicolaou staining techniques in the present study and erythrocytes and neutrophils were observed clearly among these cells in proestrus and diestrus, respectively (Fig. 1). Therefore, the stages of the sexual cycle could be determined accurately by both of the staining techniques. However, although parabasal, intermediate and superficial cells were observed by the direct examination technique, depending on the stage of the cycle, erythrocytes and neutrophils were not seen (Fig. 1). Accordingly, the stages of proestrus, diestrus and anestrus could not be distinguished from one another clearly in the present study using the direct examination technique.

Vaginal cytology remains constant in the estrus stage of the sexual cycle in bitches. A great majority of the cells observed in this period of the cycle are superficial cells. These cells remain almost the same in number during estrus. Superficial cells are the largest cells identified on vaginal cytology. These cells have sharp, flat and angular cytoplasmic borders. They have small, pyknotic, fading nuclei or no nuclei. They are also described as cornified cells in vaginal cytology (Olson *et al.*, 1984a; Post, 1985; Feldman and Nelson, 1996; Thrall and Olson, 1999). This typical appearance of superficial cells allows them to be recognized easily on vaginal cytology. In the present study, the estrus stage of the cycle identified using the classical staining techniques and the direct examination technique were in total agreement. Superficial cells could be determined easily by both the classical staining techniques and the direct

examination technique and the estrus stage could be distinguished from the other stages of the cycle (Fig. 1). The duration of estrus exhibits great variation among bitches and may be quite short in some individuals (Olson *et al.*, 1984a; Thrall and Olson, 1999). Therefore, the determination of whether a bitch is in estrus or not and the identification of the most suitable time for mating are important in terms of reproductive success (Linde and Karlsson, 1984; Olson *et al.*, 1984a; Post, 1985). The optimum mating time for a bitch occurs when a majority of the epithelial cells (>90%) that are seen on vaginal cytology are superficial cells (Olson *et al.*, 1984a; Thrall and Olson, 1999; Henson, 2001). Rapid and practical determination of the estrus stage of bitches using the direct examination technique may provide benefits in terms of reproduction.

In the present study, while the proportional analysis of the types of cells observed in the vaginal smear became easier using the 20X objective lens, characterization of the cell types was easier when the 40X objective lens was used. As reported in previous studies (Marcondes *et al.*, 2002; Yener *et al.*, 2007) unstained cells were observed more clearly under a light microscope with low illumination and a better contrast was achieved without the use of the condenser lens. It took about 2-3 h to take the vaginal smear samples from the bitches and implement the staining procedures. The vaginal smear sample was examined directly with a microscope in the direct examination technique without the need to wait for a staining procedure to be performed. Although, the direct examination technique has been reported to be a reliable, fast, practical and low-cost technique in determining the stages of the sexual cycle of rats (Marcondes *et al.*, 2002; Yener *et al.*, 2007), except for the estrus stage of the cycle it was inadequate for determination the stages of the sexual cycle in bitches in the present study.

### CONCLUSION

In this study, it was found that when determining the stage of the sexual cycle of the bitch by vaginal cytology, the direct examination technique was reliable only in detecting the estrus stage of the cycle. Rapid and practical determination of the estrus stage of bitches using the direct examination technique may provide benefits in terms of reproduction.

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