

DETERMINATION OF OPTIMAL BREEDING TIME IN BITCHES BY USING VAGINAL CYTOLOGY WITH HAEMATOXYLIN AND EOSIN STAIN.

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ABSTRACT

Optimal breeding requires precise timing to enhance fertility. Cytology alone can't determine this timing, therefore vaginal smears from 08 bitches were collected during the proestrus, LH surge and ovulation periods. An LH surge corresponds to progesterone levels between 2-4 ng/ml, and ovulation is indicated by levels between 4-10 ng/ml in present study. Haematoxylin and Eosin stain was used to identify cornification in cells. During proestrus, there was a decrease in parabasal and intermediate cells, and an increase in anuclear superficial cells. Cornification index peaked during preovulatory (9.33 ± 3.36 %) and then a non significant ($p > 0.05$) increased in ovulatory stages (91.38 ± 1.60 %) and at ovulation (94.14 ± 1.06 %). Initially low progesterone (0.52 ± 0.12 ng/ml) levels in proestrus, it rose during proestrus the LH surge (2.49 ± 0.28 with range of 1.45 to 3.81 ng/ml) and reached at peaked (9.91 ± 1.65 with a range 5.62 to 20 ng/ml) at ovulation. It may conclude that there is no significant difference in the cornification index between the LH surge and ovulation and Hematoxylin and Eosin is sufficient to identify the cornified cells. At the ovulation stage, the cornification index and progesterone concentration are useful to decide the breeding time in bitches.

Keywords: Bitches, Estrous cycle, progesterone analysis, vaginal cytology, breeding, Chennai

INTRODUCTION

The reproductive processes such as ovulation, corpus luteum lifespan, pregnancy, and parturition are linked to the estrus cycle. Bitches are monoestrous, experiencing one or two estrus cycles per year, mainly proestrus (Proestrus (7–9 days; range 3–27 days), estrus (7–9 days; range 1–24 days). , Diestrus/Pregnancy (2 months) and Anestrus (2 to 10 months). The average interval of seven months between cycles. The duration and stages of these cycles vary between breeds (Olson et al., 1984a; Feldman and Nelson 1996). Compared to other domestic animals, they have longer estrus cycles with extended follicular and luteal phases. Proestrus and estrus each last approximately nine to ten days. Anoestrus, a period without visible estrogen activity, involves ovarian inactivity. The lifespan of the corpora lutea is similar or longer in non-pregnant individuals compared to pregnant ones, making mating timing crucial (Concannon, 1986). Additionally, prolonged anoestrus periods increase the cost of commercial dog breeding.

The vaginal epithelium in dogs and cats is influenced by the ovarian hormones, particularly estrogen (Roszel, 1977). During proestrus and estrus stage developing follicle secretes estrogen. As estrogen levels increase, the vaginal epithelium undergoes proliferation, differentiation, keratinization, and exfoliation of epithelial cells. Consequently, the epithelium transitions from a two- to four-layered cuboidal structure during anestrus to a stratified squamous epithelium comprising twenty to thirty cell layers by the end of proestrus. (Schutte, 1967; Roszel, 1977; Aydin et al., 2011; Wehrend et al., 2013; and Sharma and Sharma, 2016). Due to the increase in epithelial thickness, the cells in the luminal layers of the epithelium are increasingly removed from the blood supply, causing them to die and

exfoliate into the vaginal lumen and subsequently can be observed using vaginal cytology (Aydin et al., 2011).

Estimating ovulation in bitches is essential for managing reproduction, as it helps determine the optimal time for mating or insemination. Fertilization occurs 2 to 3 days after ovulation, so insemination should happen just before fertilization is possible. Knowing ovulation dates also helps predict whelping time, around 63 days later, aiding in diagnosing primary uterine inertia.

In veterinary practice, identifying the stage of the estrous cycle quickly is crucial, with vaginal cytology being the best tool. It is safe, quick, cost effective and accurate (Al-Abbadi, 2011). Meanwhile, cytology is delineated into two branches namely aspiration and exfoliative cytology.

According to Durrant et al. (2002), Stockard and Papanicolaou were the first to recognise the specific diagnostic value of vaginal smear (exfoliative cytology) in guinea pig using the same Papanicolaou stain. Various stains are used for diagnosing reproductive disorders and cancer in vaginal cytology, each with advantages and disadvantages. This study compares two stains to identify the optimal breeding time.

MATERIAL AND METHODS

Total 08 bitches as clinical cases with history of pro-oestrus bleeding presented in the Department of Animal Reproduction Gynaecology and Obstetrics, Bombay Veterinary College, Parel, Mumbai-400 012 and private clinics located in Mumbai were selected. The details regarding their age, breed, past history of conception were recorded and date of vaginal discharge first noticed was noted down. Further, laboratory tests like vaginal cytology and progesterone estimation were performed.

Collection of vaginal smears : The vulva was cleaned using plain cotton and vulval lips were separated by two fingers. A sterile cotton-tipped swab stick was inserted and protected by a previously inserted speculum to collect a sample of vaginal cells. The swab was introduced at the dorsal commissure so as to avoid the clitoral fossa. Vaginal smears were collected from mid part of vagina when the swab at its full depth, vaginal mucosa was wiped with a combination of circular, twisting and back-and-forth motions. Due care was taken to ensure good contact of swab with the vaginal mucosa. The swab was then removed and rolled on clean glass slides and then smears were prepared. Intact cells were obtained during transfer by rolling the swab.

Staining techniques : slide of vaginal smear was fixed in fixative and stained using Hematoxylin and eosin stain mentioned by Bourne (1990). The swabs were collected on every alternate or two days interval up to the day till last mating was advised.

Classification of exfoliative cells in vaginal smears: Exfoliative cells in vaginal smears were classified as per Christie et al (1972) as Anuclear Superficial Cells, Superficial Cells, Intermediate Cells and Parabasal Cells. **Calculation of Cornification Index (CI) :** Cornification Index in percentage was calculated by adding anuclear superficial cells and superficial cells out of 100 cells counted in a smear.

Identification of stages of estrus cycle : In present study, pro-oestrus in bitches is described when <70% cornification index and oestrus is indicated by a degree of vaginal cornification of >70% as suggested by Romagnoli (2006).

Progesterone estimation: The blood was collected at each examination along with vaginal cytology in plain vials from pro-oestrus to oestrus. Serum was separated for progesterone estimation. The progesterone was estimated by Fully Automated Bi-Directional Inter-Faced Chemi Luminescent Immuno Assay method. In present study to indicate LH surge plasma progesterone concentration between 2 to 4 ng/ml and after 2-3 days progesterone level ≥ 4 to 10 ng/ml indicated ovulation was taken as reported by Wright (1990). Concannon et al. (1977) reported progesterone level 2.6 ± 0.3 ng/ml on LH surge and 1.5 ± 0.2 ng/ml eight hours before LH surge. The estimated concentrations of serum progesterone were used to advise mating in bitches.

Statistical analysis: The statistical analysis of the collected data in present study was undertaken as per the method described by Snedecor and Cochran (1967).

RESULTS

A total of 24 vaginal smears obtained from the bitches does (n=8) were evaluated to determine the proportion of different exfoliated vaginal cell types in the smear, relative to the proestrus, LH surge and ovulation stages of the oestrus cycle. It was found that, 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 (Plate 1 and 2). These cells remain almost the same in number during estrus and 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. These cells were identified equally by both the stain. In Hematoxylin and Eosin nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. The analysis of variance (ANOVA) for each of the cell types was conducted and the mean ($\pm$ SEM) values for the above stages were as shown in Table 1.

Table 1. Vaginal cytology comparison by two stains

Stage	Progesterone (ng/ml)	Parabasal cells	Intermediate cells	Superficial cells	Anuclear Superficial cells	Cornification Index.
Pro estrus	0.52 $\pm$ 0.12	1.44 $\pm$ 0.79	48.11 $\pm$ 3.23 ^a	43.11 $\pm$ 2.10	6.22 $\pm$ 1.61 ^a	49.33 $\pm$ 3.36 ^a
LH surge	2.49 $\pm$ 0.28	0.00 $\pm$ 0.00	8.63 $\pm$ 1.60 ^b	48.38 $\pm$ 4.10	43.00 $\pm$ 4.16 ^b	91.38 $\pm$ 1.60 ^b
Ovulation	9.91 $\pm$ 1.65	0.00 $\pm$ 0.00	5.86 $\pm$ 1.06 ^b	35.57 $\pm$ 4.86	58.57 $\pm$ 3.91 ^c	94.14 $\pm$ 1.06 ^b

Means with common superscript in column differ significantly (P>0.05).

On perusal of Table 1 and fig 1. It was noticed that during proestrus, LH surge and at the time of ovulation the parabasal cells and intermediate cells were significantly decreased, whereas a significant rise in anuclear superficial cells were seen, whereas the superficial cells were higher during preovulatory and ovulatory stage and it decreases at the time of ovulation. **Progesterone concentration along estrus cycle :** The progesterone concentration was low (< 1ng/ml) at the beginning of proestrus (0.52 $\pm$ 0.12) then continued in low values till the beginning of estrus or during the LH surge that reached 2.49 $\pm$ 0.28ng/ml with range of (1.45 to 3.81 ng/ml). at the time of ovulation, progesterone concentration was 9.91 $\pm$ 1.65ng/ ml with a range of (5.62 to 20 ng/ml) then continued to increase with progression of days to diestrus. **Cornification index :** Monitoring the changes in vaginal epithelial cells during proestrus and estrus by vaginal cytology showed first appearance of cornified cells after beginning of proestrus was 49.33 $\pm$ 3.36 percent and then a non significant increased from 91.38 $\pm$ 1.60 to 94.14 $\pm$ 1.06 percent during LH surge and at the time of ovulation.

DISCUSSION

The optimal conception rate for natural mating in dogs is up to 7 days, starting just before the LH surge and ending with the degeneration of ova (Goodman, 2001). In modern dog rearing practices there is limited breeding opportunities during the fertile period, because the pet owner decides the mating timings. Most of the time matings are done outside the fertile window, which result in poor litter size and breeding failure (Concannon, 1986; Concannon et al., 1989). For better litter size and reproductive performance of bitch, mating should be done two to four days after ovulation Johnston et al. (1994). Fontbonne (2011) who reported 50% conception rate in French Kennel Club with

incidence of infertility as 40% to 80% due to mistimed mating. However, deciding the exact day of ovulation is challenging, and techniques like Vaginal Exfoliative Cytology are used to identify the fertile period. For this Vaginal Exfoliative Cytology (Linde and Karsslon, 1984; Olson et al., 1984; Wright and Perry, 1989), Vaginoscopy (Lindsey, 1984; Jeffocate and Lindsey, 1989) and plasma/serum progesterone concentration (Concannon, 1997) were used. Optimum time of mating and frequency of mating played a key role in improving the conception rate and litter size (Zoldag et. al., 1993; Johnston et. al., 1994).

The estrous cycle in bitches is identified by the changes and percentages of vaginal of epithelial cells in vaginal cytology (Siregar et al., 2011). In the study, during the proestrus stage, there was an increase in intermediate and superficial cells. Similar report was recorded by Kustritz (2012). During LH surge There is increase in anucleated superficial cells is likely due to higher estrogen levels from pre-ovulatory follicles, leading to increased uterine activity and vaginal mucosa changes (Zohara et al., 2014).

Increased estrogen also causes a rise in vaginal epithelial cell turnover, which leads to the progressive cornification seen in vaginal cytology (Concannon, 2011). During proestrus and estrus, vaginal cytology shows cornified cells appearing around 4 days after proestrus starts, reaching over 85% by ovulation (Post, 1985; Fontbonne, 2011 and Moxon et al. 2012). Cornification showed a moderate to strong positive correlation with receptivity near ovulation, exceeding 90%. Ovulation occurred when anuclear superficial cells surpassed 65%, higher than the 50% threshold reported by Allen, (2002), with cell pattern changes aligning with LH surge and ovulation phases. The present study results also agreed the above observations.

Measurement of progesterone concentration showed that progesterone was present in low values (0.52 ± 0.12 ng/ ml) at the proestrus due to high estrogen concentration which secreted from follicles on ovaries, and then increased to 2.49 ± 0.28 ng/ ml at the LH surge estrus due to partial lutenization of follicular layers. At ovulation it was 9.91 ± 1.65 ng/ ml, the current findings are in accordance with Johnston et al., (1994) and Concannon (2011), they noticed that progesterone concentration at the day of ovulation ranged from 4 to 10 ng/ml due to complete luteinization of follicular layers. Similarly the peak of keratinization or cornification occurs with the beginning of the increase in progesterone levels due to high progesterone from corpus luteum formation. This serum progesterone concentrations have been reported to increase gradually from basal values with the advancement of proestrus and exhibit a distinct, rapid, and detectable increase around the time of LH surge (Simpson et al., 1998; Concannon, 2011).

Mating is recommended when the percentage of superficial or keratinized cells in vaginal smears exceeds 80% (Johnston et al., 2001; Feldman and Nelson, 2004; Concannon, 2011; England, 2013).). Above findings indicates that, identification and keratinised cells or cornification index >85% and progesterone level above 8 ng/ml is a important factor to decide proper time of breeding in bitches. For this reason in present study the two stains were utilised.

Exfoliative cytology is a minimally invasive method and relies on the presence of cells that are shed spontaneously from epithelial surfaces, using methods like scraping or swabbing (Ibrahim, 2004 Agarwal , 2005,). It helps identify various reproductive cycle stages and pathological lesions in canine and feline. The procedure is simple, painless, bloodless, and can be frequently repeated with minimal discomfort.

There is dearth of report on using Hematoxylin and eosin for vaginal cytology. A good stain must be clear enough to distinguish between different types of cells in the cytological field (Cora et al., 2015). A major problem with some of these stains has to do with durability of the stained slide to allow for later viewing of the slides. Generally, in the literature, Giemsa stain is one of the major stained used by veterinary practitioners for staging oestrus because of its ease to use and readily availability for haematology and other associate techniques in most laboratories (Barcia, 2007).

The hematoxylin and eosin is the most widely used histological stain. This is because Hematoxylin and eosin staining technique functions to recognize different types of tissues and their

morphological changes, particularly suitable for demonstration of nuclei which are the most important structures in every tissue. Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (plate 1 & 2). Haematoxylin and Eosin stains effectively displayed cytological changes accurately, with varying colors for cytoplasm and nuclei. Under estrogenic influence, superficial cells show pyknotic nuclear changes, which help in predicting the cycle stage and breeding time.

It may conclude that there is no significant difference in the cornification index between the LH surge and ovulation and Hematoxylin and Eosin is sufficient to identify the cornified cells. At the ovulation stage, the cornification index and progesterone concentration are considerably more effective compared to other stages.

REFERENCES

1. Agarwal SP.(2005). Role of diagnostic cytology. Manual for cytology in National Cancer Control Programme, Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India; . p 9-44.
2. AL-abbadi, M.A. (2011). Basics of cytology. Avicenna Journal of Medicine, 1 (1): 18-28.
3. Allen VJ. (2002). Polnyj kurs akusherstva i ginekologii sobak (2-e izd.) [Complete course in obstetrics and gynecology of dogs (2nd ed.)]. Akvarium LTD, Moskva. Pp. 126-134 (in Russian).
4. Aydın I, Sur E, Ozaydin T and Dinc DA. 2011. Determination of the stages of the sexual cycle of the bitch by direct examination. Journal of Animal and Veterinary Advances 10: 1962-1967.
5. Barcia, J.J. (2007). The Giemsa stain: its history and applications. International Journal of Surgical Pathology, 15 (3): 292-296.
6. Bourne, L. D. (1990) Gynaecological cytology. In : Theory and Practice Histological Techniques, 3rd ed. Edinburg: Churchill Livingstone : (460-467)
7. Choudhary, P., Sudhamani, S., Pandit, A. and Kiri, V. (2012). Comparison of modified ultrafast Papanicolaou stain with the standard rapid Papanicolaou stain in cytology of various organs. Journal of Cytology, 29 (4): 241-245.
8. Christensen, J. (1984) Reproduction in dog and cat. First ed. Bailliere Tindall, London : (1-54)
9. Christie DW, Bailey JB, Bell ET. Classification of cell types in vaginal smears during the canine oestrous cycle (1972). Br Vet J. Jun;128(6):301-10.
10. Concannon P. (2011). Reproductive cycles of the domestic bitch. Animal Reproduction Science, 124, (3–4): 200–210.
11. Concannon, P. W. (1986) Canine physiology of reproduction. A clinical approach to diagnosis and treatment. In Small Animal Reproduction and Fertility. 2nd Edn. Ed. J. J. Brukes, Lea and Febiger, Philadelphia : (23-77)
12. Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. Animal Reproduction Science, 124(3–4), 200–210.
13. Concannon, P. W. and D. H. Lein (1989) Hormonal and clinical correlates of ovarian cycle, ovulation, pseudopregnancy and pregnancy in dogs. Cited in Current Veterinary Therapy, Ed. X. Edited by Kirk, Small Animal Practice. Saunders. W.B. Saunders Co. Philadelphia, USA : (1269-1281).
14. Concannon, P. W., M. E. Powers, W. Holder and W. Hansel (1977) Pregnancy and parturition in the bitch. Biol Reprod. 16 : 517-526.

15. Cora, M.C., Kooistra, L. and Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicologic Pathology*, 43 (6): 776-793.
16. Durrant, B., N. Czekala, M. Olson, A. Anderson, D. Amodeo, R. Campos-Morales, F. Gual-Sill and J. Ramos-Garza (2002) Papanicolaou staining of exfoliative vaginal epithelial cells facilitates the prediction of ovulation in the giant panda. *Theriogenology*. 57 : 1855-1864.
17. England, G. C. W. (2013). *Dog Breeding, Whelping and Puppy Care*. Wiley-Blackwell, Oxford.
18. Feldman, E. C. and R. W. Nelson (1996) *Canine and feline endocrinology and reproduction*. 2nd ed. W. B. Saunders, Philadelphia : (752-773)
19. Feldman, E. C., & Nelson, R. W. (2004). *Canine and Feline Endocrinology and Reproduction* (3rd ed.). St. Louis: Saunders.
20. Fontbonne, A. (2011). Infertility in bitches and queens: Recent advances. *Rev. Brasileiro. Reprodução Animal*, Belo Horizonte. 35(2): 202-209.
21. Goodman, M. (2001) Ovulation timing. Concepts and controversies. *Vet. Clin. North Small Anim. Pract.* 31(2) : 219-235.
22. Ibrahim ZA (2004). Comparison between Papanicolaou and May-Grunewald Giemsa stains in thyroid fine needle aspiration. *Faculty of medical laboratory sciences, University of Khartoum*; . p 1-35.
23. Izhar, S., Kaur, R. and Masih, K. (2014). Efficacy of rapid, economical, acetic acid, Papanicolaou stain in cervical smears as an alternative to conventional Papanicolaou stain. *Journal of Cytology*, 31 (3): 154-157.
24. Jeffcoat IA, Lindsay FEF. 1989. Ovulation detection and timing of insemination based on hormone concentrations,
25. Johnston, S. D., Root Kustritz, M. V., & Olson, P. N. S. (2001). *Canine and Feline Theriogenology*. Philadelphia: W.B. Saunders Company.
26. Johnston, S., Olson, P. and Root, M. (1994). Clinical approach to infertility in the bitch. *Seminars in Veterinary Medical Surgery*. 9: 2-6.
27. Kustritz MVR (2012) Managing the reproductive cycle in the bitch. *Vet. Clin.: Small Anim. Pract.* 42(3): 423-437.
28. Linde, C. and I. Karlsson (1984) The correlation between the cytology of the vaginal smear and the time of ovulation in the bitch. *J. Small Anim. Pract.* 25 : 77-82.
29. Moxon R, Batty H, Irons G and England GCW. 2012. Perioovulatory changes in the endoscopic appearance of the reproductive tract and teasing behavior in the bitch. *Theriogenology* 78: 1907-1916.
30. Olson, P. N., M. A. Thrall, P. M. Wykes, T. M. Nett and H. R. Sawyer (1984) Vaginal cytology. Part I. A useful tool for staging the canine estrous cycle. *Comp. Cont. Ed. Pract. Vet.* 6 : 288-298.
31. Post, K. (1985) Canine vaginal cytology during the oestrus cycle. *Can. Vet. J.* 26 : 101-104.
32. Romagnoli S (2006) Recent advances in canine female reproduction world congress wsava/fecava/csava 673-678.
33. Roszel, J.F., (1977). Normal canine vaginal cytology. *The Veterinary Clinics of North America* 7, 667-681.
34. Schutte, A. P. (1967) Canine vaginal cytology II cyclical changes. *J. Small Anim. Pract.* 8 : 307-311.
35. Sharma, M. and Sharma, N. (2016). Vaginal Cytology: An Historical Perspective on its Diagnostic Use. *Advances in Animal and Veterinary Sciences*, 4: 283-288.
36. Simmons J. The vaginal smear and its practical application. *Vet Med/ Small Anim Clinician*. 2006; 65:369-373.

37. Simpson G, England G and Harvey M. 1998. Manual of small animal reproduction and neonatology. British Small Animal Veterinary Association.
38. Simpson GM, England GCW, Harvey M. 2005. (Ed.). Rukovodstvo po reprodukcii i neonatologii sobak i koshek [Canine and Feline Reproduction and Neonatology Guide]. Sofion, Moskva, 280 pp. (in Russian).
39. Siregar, T. N., Melia, J., Rohaya, R., Thasmi, C. N., Masyitha, D., Wahyuni, S., Rosa, J., Nurhafni, N., Panjaitan, B., & Herrialfian. (2011). The use of vaginal cytology to determine the stage of the estrous cycle in bitches. Journal of Animal and Veterinary Advances, 10, 1962–1967.
40. Snedecor, G. W. and W. G. Cochran (1967) Statistical methods. 5th Ed. Oxford and IBH Publishing Co., New Delhi.
41. Wehrend A, Von Plato K, GoerickePesch S. Die exfoliative vaginalzytologie bei der hundin-indikationen, durchfuhrung,
42. Wright P and Parry B. (1989). Cytology of the canine reproductive system. Veterinary Clinics of North America. Small Animal Practice 19:851-874.
43. Wright, P. J. (1990) Practical aspects of estimation of the time of ovulation and insemination in the bitch. Aust. Vet. J. 68 : 10-13.
44. Zohara BF, Azizunnesa, Islam MF, Alam GS, Bari FY (2014). Exfoliative vaginal cytology and serum progesterone during the estrous cycle of indigenous ewes in Bangladesh. J. Embryo Trans. 29(2): 183-188.
45. Zoldag, L., Kecskemethy, S. and Nagy, P. (1993). Heat progesterone profiles of bitches with ovulation failure. Journal of Reproduction and Fertility. Supplement. 47: 561-565

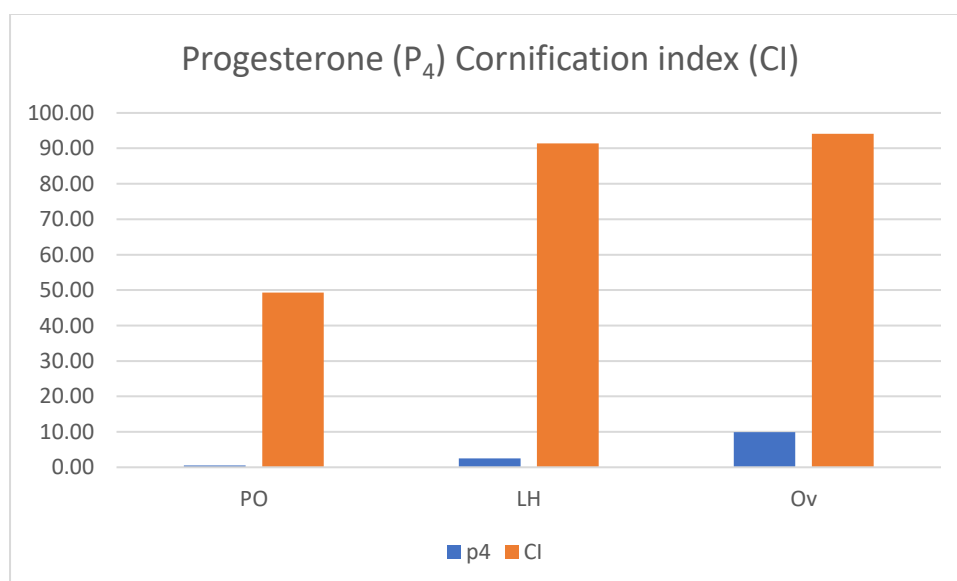


Fig 1. progesterone Vs Cornification index

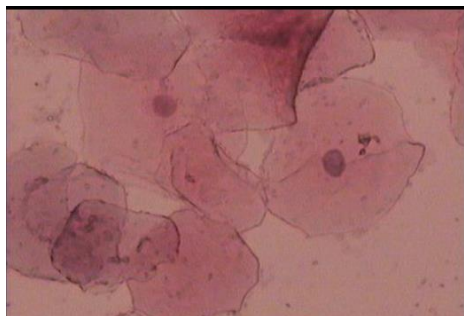


Plate-1 superficial cell



Plate-2 intermediate cell