RESEARCH ARTICLE

Studies on Salivary Fern Pattern during Estrous Cycle in Gir Cow and Jaffrabadi Buffalo

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ABSTRACT

Bovine saliva changes its biochemical composition and biophysical properties due to the variations in sex steroid levels during the estrous cycle. As a consequence of estrogen rise, saliva is produced in larger amounts at estrus - a stage also characterized by an increase in mucus crystallization when observed under light microscopy. This study was aimed to provide information on the shape and size of the crystallization of saliva and estrual mucus during entire estrous cycle in Gir cows and Jaffrabadi buffaloes (n=12 each) and compare micrometry of their salivary crystallization patterns (SCP). The types of crystallization discriminated were: branch-like, fern-like, fir-like, and a mixture of these. Branched patterns were observed more prominent in both saliva and mucus during the peak estrus in both species. Score 3 of salivary and cervical mucus fern pattern was considered as the complete fern pattern at peak of the estrus activity. Typical spur-like branching of SCP was the predominant in the cows, while in buffaloes the leaf shape of branching was observed more. Based on SCP scale 1-3, the cows exhibited a longer estrus phase than buffaloes, also the pattern scale 2 and 3 were significantly (p<0.05) higher in cows than buffaloes. The SCP patterns in both species showed difference in onset of estrus phase but its micrometry revealed no significant difference among the two species.

Key words: Crystallization, Fern pattern, Gir cow, Jaffrabadi buffalo, Saliva.

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INTRODUCTION

The diagnosis of estrus is the key point and always a challenge for farmers and handlers to get animal pregnant for production. Lots of research has been done and many traditional layman techniques are popular for this. Invasive techniques include the estimation of the hormones from the blood or plasma for estrus diagnosis. Levels of LH and estrogen are considered for the evidence of heat in the animals. But non-invasive methods are very less popular for sure diagnosis of heat period, such as infrared thermography for estrus detection in animals (Riaz *et al.*, 2023), fern pattern in cervical mucus of bovine (Cortes *et al.*, 2014) and vaginal cytology in bitch (Kustritz, 2020). Saliva has been demonstrated to be a promising bodily fluid for early detection of diseases, and salivary diagnostics has exhibited tremendous potential in clinical applications (Lee and Wong, 2009). Saliva is a biological secretion from the oral cavity that has the property of crystallization and this produces a typical pattern that may be helpful for the diagnosis of heat periods in all mammals and also as stress marker in pigs (Prims *et al.*, 2024).

Crystallization is made possible because of NaCl and mucin present in the saliva (Kumar *et al.*, 2024). Different scientists have shown different salivary crystallization patterns (SCP) in Murrah buffalo (Devi *et al.*, 2016) and used in further diagnosis and confirmation of early estrus period with synchronization protocol in sheep (Goncalves *et al.*, 2020;

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Sangeetha and Rameshkumar, 2015), pregnancy diagnosis in cattle (Skalova *et al.*, 2013) and buffaloes (Kandiel *et al.*, 2017; Ravinder *et al.*, 2016), tool for timing of insemination in Sahiwal cows (Chavan *et al.*, 2023), fertile period in women (Priya *et al.*, 2020), mating time in bitch (Pardo-Carmona *et al.*, 2010), and libido level in male (Ramachandran *et al.*, 2024). With this background we conducted this study to observe the salivary crystallization patterns (SCP) of cows and buffaloes

during the estrous cycle and to compare micrometry of salivary fern patterns among two species.

MATERIALS AND METHODS

Experimental Animals and Identification of Estrus

This study was carried out on *Bos indicus* (Gir cows) and *Bubalus bubalis* (Jaffrabadi buffaloes) breeds maintained at Cattle Breeding Farm, Junagadh, Gujarat, India. Twelve sexually matured non-pregnant female bovines from each species were randomly selected for the present study. The experimental animals were fed with a conventional diet (cultivated forage crops, rice straw, green fodder) and water *ad libitum.*

The estrus was determined with the help of the conventional estrus behavioural signs in females such as vaginal swelling, frequent intermittent urination, restlessness, Flehmen's reaction, mounting, male sniffing with female vulva and fern pattern (saliva and cervico-vaginal fluid). The length of the estrous cycle was assessed by observing a saliva smear in order to know the phase of the estrus. Salivary smears were performed and analyzed by light microscope at 100X.

Salivary Ferning

The saliva samples were collected daily before the meal in the morning between 8 to 10 h using a clean dry finger placed sublingually. The tip of the tongue was pressed to the palate. A drop of non-foamy saliva sample was consequently smeared on a glass slide and air-dried at room temperature. The samples were microscopically assessed at magnification 100X and 400X. Crystals were classified according to a system adapted by Haberova (2010) as none, dotted, branch-like, fir-like, fern-like, mixed branch- and fir-like, mixed branch- and fern-like, mixed fir- and fern-like, mixed branch- fir- and fern-like, or atypical. The fern formation was graded as per the method described by Roland (1971). Only one general type of crystallization was described in one sample. The entire area of each saliva sample on glass slide was examined. Similarly, cervical mucus was also collected during the peak time of the estrus before AI by gentle aspiration from the cervix and observed under the microscope. Crystallization of saliva and cervical mucus was observed and eventually photographed within 10 min after visualization. After completion of sample collection, and smear making, the observed fern patterns were measured under a light microscope using the Image-J software. The ferning pattern was scaled as per Gnanamuthu and Rameshkumar (2015) from 1-3, with PS1 = no visible ferning (random and unconnected dots), PS2 = partial ferning (a combination of dots and ferns), and PS3 = full or peak ferning (complete fern-like patterns). The observations of the salivary crystallization pattern were validated by using a simple microscope as per the technique of Surla *et al.* (2021) in Murrah buffaloes. The micrometrical data obtained was analyzed by student t-test to compare the different micrometry patterns in SCP of cows and buffaloes.

RESULTS AND DISCUSSION

### Salivary and Cervical Mucus Crystallization Patterns during Estrous Cycle

The saliva, collected at the whole length of estrous cycle (day 21) from each cow and buffalo, was smeared and observed under a simple student microscope. The types of crystallization discriminated were: branch-like, fern-like, fir-like, and a mixture of these. The duration of the normal estrous cycle in both species was between 18 to 24 days with an average of 21 days. Branch-like patterns were observed more prominent during the peak period of the estrus in both species. The salivary and cervical mucus fern pattern
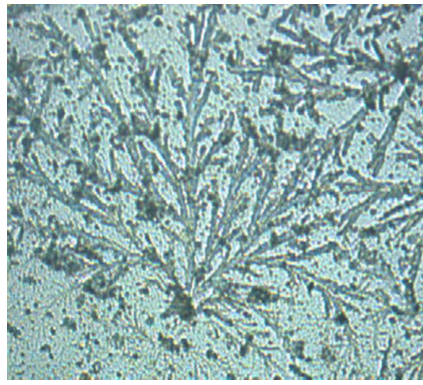

Fig. 1: Crystallization of saliva in branch-like fern pattern in cows during peak estrus (100X) (scale 3)
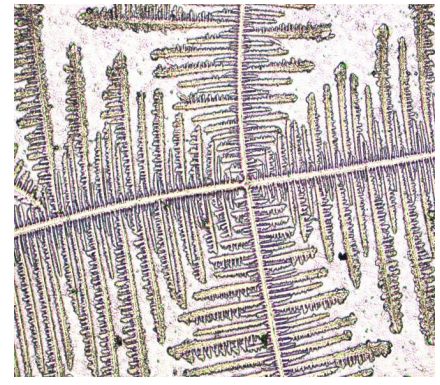

Fig. 2: Crystallization of saliva in branch-like fern pattern showing leave shape in buffalo during peak estrus (100X) (scale 3)
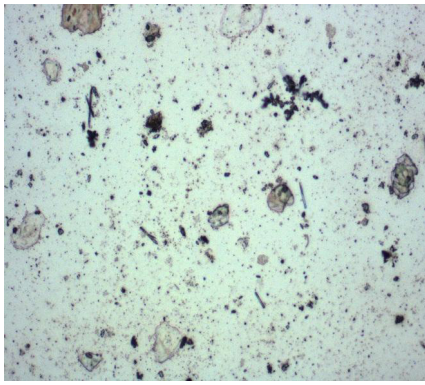
Fig. 3: PS1 = no visible ferning (random and unconnected dots) (40X)

Fig. 4: PS2= partial ferning (a combination of dots and partial ferns) (40X)

Fig. 5: PS3 = full or peak ferning (complete fern-like patterns) (40X)

was compared within both species. Score 3 of salivary and cervical mucus fern pattern was considered as the complete fern pattern and a peak of the estrus period in the cycle, ideal for breeding the cow or buffalo. The difference between the SCP in both species was observed in that typical spur-like branching (Fig. 1) was the predominant in the cows, but in buffaloes the leaf shape branching was observed more prominently (Fig. 2). Salivary crystallization patterns observed in animals for scale 1, 2 and 3 are depicted in Figures 3 to 5.

The crystallization pattern of estrual cervical mucus (Fig. 6 & 7) observed was similar to that of saliva. During estrus the fern pattern of saliva and cervical mucus appeared akin to each other, signifying utility of salivary fern pattern in estrus detection in bovines. The cervical mucus crystallization pattern has been described at different phases of estrus in cattle by Cortes *et al.* (2014). Khanoria *et al.* (2022) showed different CVM patterns and their correlations with conception rate in bovines.

The different SCP scales obtained in cows and buffaloes are compared in Table 1. Pattern scale 3 is considered the peak estrus period in both the species. A significant difference (p<0.01) was observed for pattern scale 1 and the sum of pattern scale 2 & 3 between the species. The days for fern pattern scale 1 (diesterus phase or non-estrus phase) were significantly longer with shorter days for fern pattern scale 2 and 3 (pro/metestrus and estrus phase) in buffaloes as compared to cows. Frequency for the days for the pattern scale 3 (typical estrual mucus/saliva fern pattern) observed was particularly higher in cattle compared to buffalo species, indicating that cows exhibit a significantly longer estrus phase than buffaloes. This may help to identify the best time of insemination of animals, being longer in cows than buffaloes. Even the period of pre- to post-estrus (PS2 & PS3) was also significantly longer in cows than buffaloes proving that the follicular phase or estrogenic activity in greater in cows compared to buffaloes.

Micrometry of Salivary Fern Pattern

The maximum ferning pattern was considered for the micrometry study, and the length of a branch from center, length of sub-branch, a width of sub-branch and the distance between the fern branch (µm) were also compared between both species (Table 2). There were no significant differences

Fig. 6: Crystallization of cervical mucus in branch-like fern pattern in cows during peak estrus (100X)
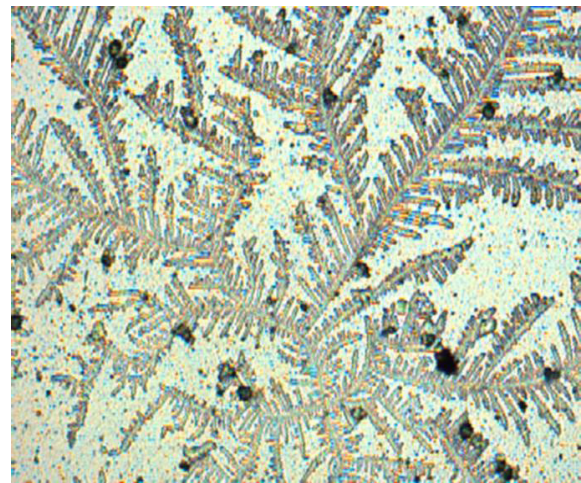
Fig. 7: Crystallization of cervical mucus in branch-like fern pattern showing leaf shape in buffalo during peak estrus (100X)

Table. 1: Pre- and post-estrus observation and frequency of different pattern scales of saliva in cows and buffaloes (Mean ± SEm, n=12)

Parameters	Cows	Buffaloes	T-test
Pattern scale 1 (days) -Diestrus	14.58±0.34	16.75± 0.18	**
Pattern scale 2 (days) -Pro/Met-estrus	4.50±0.31	3.00±0.25	*
Pattern scale 3 (days) - Estrus	1.92±0.15	1.25±0.75	*
Pattern observed during Pre- & post-estrus (PS2 & PS3) (days)	6.42±0.34	4.25±0.18	**

Significant difference at *$p<0.05$ and **$p<0.01$; PS- Pattern scale

Table. 2: Comparison of micrometry of salivary crystallization pattern in cows and buffaloes (Mean ± SEm, n=12)

Parameters	Cows	Buffaloes	T-test
Length of branch from center (µm)	976.32±54.02	960.57±51.27	NS
Length of sub-branch (µm)	298.55±18.88	279.69±11.93	NS
Width of sub-branch (µm)	101.79±4.64	92.28±8.92	NS
Distance b/w fern branch (µm)	65.98±3.17	65.04±5.55	NS

NS= non-significant difference

observed between the species. A similar way of micrometry was done earlier to compare induced and spontaneous estrus in HF cows (Bernardi *et al.*, 2016).

The estimation of total protein in saliva (Manasa Varra *et al.*, 2022) and determination of miRNA in saliva (Kumar *et al.*, 2022) helps to correlate and confirm the early estrus in the animals, although we did not analyze these parameters. The proposed system includes a hardware part, making a miniaturized microscopic device with high performance for image acquisition, and software able to analyze the images and display real-time results on human saliva samples (Covaciu *et al.*, 2020). This type of software system should be developed for studying fern patterns in animal species.

Conclusion

The salivary fern patterns, like cervical mucus patterns, observed were a typical sharp spur and flower shape, respectively, in Gir cows and Jaffrabadi buffaloes, and the complete fern pattern lasts longer during the estrus phase in Gir cows as compared to Jaffrabadi buffaloes. With this buffalo owners are advised to check the complete fern pattern of saliva at the time of the estrus phase at their doorstep by placing saliva on a glass slide, a flower-like fern pattern under sunlight or artificial light after drying is preliminary indicative of heat. However, further studies are required on larger scale to correlate these patterns with estrus behaviour and conception rate, and to design a farmers-friendly device for routine use in practice for estrus detection in bovines.

Acknowledgements

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