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# Cervical mucus changes can predict ovulation time in Mithun

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ABSTRACT

The study aimed to assess the possibility of cervical mucus changes to predict ovulation time in Mithun. Experimental Mithuns (n=6) were estrus synchronized and cervical mucus was collected in different estrus phases from three consecutive estrous cycles (n=18). Cervical mucus colour, consistency, arborization pattern and cytology were evaluated. Plasma estradiol-17β (E2) and progesterone (P4) levels were determined during different phases of estrous cycles. Ovulation time and subsequent growth of the corpus luteum were determined using transrectal ultrasonography. Results indicated thick and moderate cloudy mucus with a typical arborization pattern in midestrus. Sticky caseous or white-coloured thick consistency cervical mucus was observed during metestrus in 66.66% (n=14) estrous cycles along with ovulation or ovulation occurring within the range of -3 to + 4.5 - +7.5 h after the appearance of white mucus. Cervical mucus cytology revealed percentages of polymorphonuclear neutrophils were significantly higher in metestrus. E2 concentrations reached the peak on the day of estrus and P4 concentrations were lower than 0.5 ng/mL. It was concluded that cervical mucus shows a typical arborization pattern in midestrus and the appearance of characteristic sticky caseous cervical mucus during metestrus can be used as a tool to predict ovulation time.

**Keywords:** Dominant follicle, Estrus, Estradiol-17β, Progesterone, Ultrasonography

Mithun (*Bos frontalis*) is a unique bovine species of the Northeastern hilly region of India that plays an important role in social, cultural and economic life and source of income for farmers (Ramesh *et al.* 2022). Presently, Mithun is reared under the free-range system with no scientific intervention. At a slow pace, farmers are shifting from a free-range system of Mithun rearing to a semi-intensive system and demand for the implementation of assisted reproductive technology (ART) is escalating (Joshi *et al*. 2020, Vikram *et al.* 2023). Maximizing reproductive performance is a crucial prerequisite for the success of the Mithun production system. To achieve this, a good understanding of the estrous cycle, estrus behavior, and accurate detection of estrus and insemination before ovulation are important factors (Hockey *et al*. 2010). In addition, fertilization rates and the percentage of good embryos are influenced by insemination at the ideal time before ovulation (Roelofs *et al*. 2006). In cattle, during the estrus, cervical mucus quality and quantity are under the control of the ovarian hormones and change from the onset of estrus until ovulation (*Tsiligianni et al.* 2001) and are used as an indicator of the estrous cycle stage or hormonal status (Benbia *et al.* 2011). Mithun exhibits a longer duration of estrus (Dhali *et al.* 2006, Ramesh *et al.* 2022) and there is no information documented on the cervical mucus changes and its association with the ovarian hormones from onset to end of estrus. Although transrectal ultrasonography can precisely assess the Mithun reproductive stage and identify ovulation (Ramesh *et al.* 2022), is expensive, needs trained technicians and is difficult to employ in field conditions. In different domestic animals, cervical mucus changes are routinely used to determine the best time to perform artificial insemination and ovulation time (Layek *et al.* 2013, Souza-Fabjan *et al.* 2021). However, in Mithun, the association between cervical mucus changes and ovulation time is yet to be demonstrated. Thus, it is hypothesized that the cervical mucus characteristics changes under the influence of ovarian hormones and can be used as a tool to predict the ovulation time in Mithun. Therefore, present study aim to determine the plasma estradiol-17β and progesterone levels during proestrus, estrus, and metestrus and to assess the possibility of cervical mucus changes to predict ovulation time in Mithun reared under a semi-intensive system.

## MATERIALS AND METHODS

*Experimental animals:* All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) (ethical code: NRCM/IAEC/2020(01)/07, Sl. No.

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07). The study was conducted at the Institute Mithun farm, ICAR–National Research Centre on Mithun. The Mithun cows (n=6; age=5-7 years; mean body weight=457. 62 kg), multiparous, non-lactating, body condition score of 3.5 on a 0-5 point scale were used in the study. Mithun cows that cycling with no reproductive abnormalities was included after a clinical examination of the genital tract. Experimental animals were fed according to ICAR (ICAR 2013) feeding standards (dry matter intake @2.5% of body weight). Cows received 9.45 kg (paddy straw (50%) + napier and congo signal grass (50%)), 2 kg concentrate fortified with the mineral mixture and salt. The crude protein (%) and crude fiber (%) were in the range of 8-9% and 28-30%, respectively. Experimental animals were maintained under a semi-intensive system and isomanagerial conditions and offered *ad lib.* drinking water.

*Estrus synchronization and estrus detection:* Transrectal ultrasonography (Honda, HS-2100V, Honda Electronics Co., Ltd, Japan) equipped with a 7.5 MHz probe was used to determine the presence of a corpus luteum (CL) on the ovaries of experimental Mithun cows. If a CL was present, the cows were synchronized with cloprostenol (500 µg i.m. Estrumate™, Vet Pharma Friesoythe GmbH) prostaglandin F2α analogue and were evaluated twice a day after 48-72 h by parading teaser bull and transrectal examination for the onset of estrus. The cervical mucus was evaluated in three consecutive estrous cycles, a total of 18 estrous cycles were studied. For validation, 10 random estrous cycles were considered from 10 different Mithuns without estrous synchronization.

*Collection and examination of cervical mucus:* Cervical mucus was collected during early estrus, midestrus and metestrus before transrectal ultrasonography as previously described (Layek *et al.* 2013). Briefly, a sterile blue sheath (IMV technologies, France) was fitted to a universal artificial gun for mid-cervical-aspiration of cervical mucus by the recto-vaginal method. The cervical mucus aspirated was immediately transferred to the sterile test tube and the following parameters were recorded: color, consistency and arborization pattern. The cytological examination of Giemsa-stained dried cervical mucus smears was performed as previously described (Ahmadi *et al.* 2006) using a light microscope (MT5310H, Meiji Techno, Japan) at 40× magnification.

*Blood collection and estimation of estradiol-17β (E2) and progesterone (P4):* The single blood sample was collected during proestrus (day -3 to 0), estrus (day 0 to 2), and metestrus (end of estrus to complete development of CL) in three consecutive estrous cycles (n=18) by jugular vein puncture in heparinized 5 mL vacutainer tubes and kept immediately in 4°C after collection. The plasma was separated by centrifugation at 2000×*g* for 20 min at 4°C and stored at -20°C until assayed for hormones. The commercially available competitive ELISA kits of Estradiol-17β (E2) (Cat# 501890, Cayman Chemical, USA; analytical sensitivity: 20 pg/mL) and Progesterone (P4) (Cat# 582601, Cayman Chemical, USA; analytical sensitivity: 10 pg/mL) were used for hormone estimation in 96 well microplates using Multiskan™ FC microplate photometer (ThermoFisher Scientific, USA).

*Determination of ovulation time and growth of corpus luteum:* Ovaries were examined every 3rd hour by real-time transrectal ultrasound from mid-estrus (determined by arborization pattern) thereafter until ovulation as previously described by (Berg *et al.* 2020). The ovulation time, relative to cervical mucus, was recorded as midpoint between when the ultrasound detected the dominant follicle disappearing and its earlier detection with an intact follicle 3 h prior, i.e. 1.5 h before the ultrasound-determined ovulation time. The corpus luteum growth after ovulation was tracked until day 6.

*Artificial insemination and pregnancy diagnosis:* Mithun cows that were in estrus (detected by teaser Mithun bull and transrectal palpation) were selected for artificial insemination (AI). Estrus Mithun cows were artificially inseminated twice (morning and evening) during the mid-heat (confirmed by typical arborization pattern) with frozen-thawed semen collected by electro-ejaculation as described earlier (Nadaf *et al.* 2021). Pregnancy diagnosis was performed using real-time transrectal ultrasound (Honda, HS-2100V) with a 7.5 MHz probe on days 60-70 after insemination. The conception rate was defined as the number of cows pregnant at day 60-70 after repeated AI, divided by the total number of inseminations (n=60).

*Statistical analysis:* Statistical analysis was carried out using SPSS version 20, (IBM Corporation, Chicago, USA). The descriptive statistics were calculated for estrus duration. Repeated measures ANOVA was used to determine the statistical significance between the hormonal profiles and, epithelial cells and polymorphonuclear neutrophils during different phases of estrus. The results are presented as mean±SEM and considered statistically significant when $P<0.05$.

## RESULTS AND DISCUSSION

The behavioral expression of estrus was absent and the duration of estrus was in the range of 36-84 h with a mean±SEM of 52.77±2.82 h. Appearance of mucus at different stages of estrus in depicted in Fig. 1A. The cervical mucus discharge was scanty and at the early estrus, thin and clear cervical mucus was present with an atypical arborization pattern and absence of standing to be mounted. At midestrus thick and moderate cloudy mucus was present with a typical arborization pattern and standing estrus. The caseous or white-coloured thick consistency cervical mucus was observed during metestrus in 66.66% (n=14) estrous cycles along with ovulation or ovulation occurring within the range of -3 to + 4.5 - +7.5 h after the appearance of white mucus (Fig. 1B). It appeared as sticky mucus at the edge of the vulva in Mithun cows. The white discharge showed the absence of an arborization pattern and infiltration of polymorphonuclear neutrophils (PMN) cells (Fig. 1B and Fig. 2). In 33.33% (n=7) estrous cycles, the caseous thick discharge was absent and mucus dried up

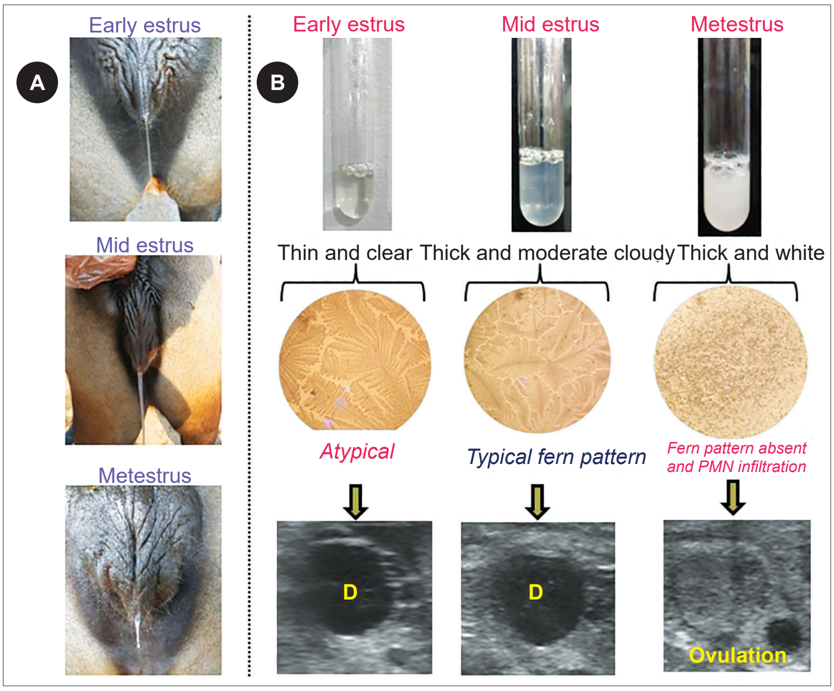


Fig. 1. A. Appearance of mucus at different phases of estrus immediately after transrectal examination in Mithun; B. Cervical mucus characteristics such as colour, consistency and arborization/fern pattern at different phases of estrus corresponding to dominant follicle status.

at the end of estrus. Subsequently, in validation, 70% (n=7) of estrous cycles exhibited caseous or white-coloured cervical mucus along with ovulation or ovulation occurring within the range of -3 to + 4.5 - +7.5 h after the appearance of white mucus.

The duration of estrus was longer in Mithun with no distinct visible signs of estrus (silent estrous) and agrees with an earlier study in Mithun with a mean duration of estrus of 67.2 h (Dhali *et al.* 2006). The longer duration of estrus is understood by the fact that the presence of a growing dominant follicle before reaching the maximum diameter is producing adequate E2 for early estrus onset and cervical mucus secretion in Mithun. In addition, concentrations of P4 were lower (<0.5 ng/mL) at the onset of estrus. The higher E2 and lower progesterone concentrations stimulate the early production of thin and transparent cervical mucus (Bernardi *et al.* 2016) along with vaginal congestion, and vulvar edema at the estrus onset in Mithun. Later, the cloudiness and viscosity of the mucus increased when E2 decreased and P4 increased towards the end of estrus. During the luteal phase, it becomes opaque, thick, viscous and scanty which is not favourable to sperm migration (Rutllant *et al.* 2005). The arborization pattern of cervical mucus in Mithun is under the control of E2 and P4. The arborization pattern was typical in midestrus and as the P4 increased the arborization diminished at the end of estrus. The crystallization in cervical mucus progresses under the influence of estrogen whereas progesterone diminishes the formation of the arborization pattern (Tsiligianni *et al.* 2001). Arborization pattern has a significant relation with the conception rates and is the best tool to determine the ideal time of insemination in Mithun (Nadaf *et al.* 2022). Higher conception rates were observed in buffaloes inseminated when cervical mucus had a typical arborization pattern as compared to the atypical pattern (Verma *et al.* 2014). The movement of spermatozoa is higher in typical arborization

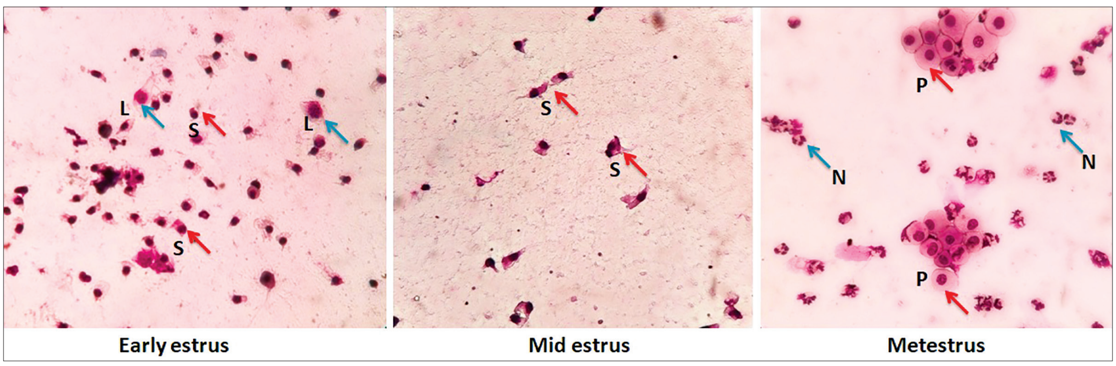


Fig. 2. Cytological examination of dried cervical mucus during different phases of estrus in Mithun (Giemsa stained); S. Superficial cells; L. Large intermediate cells; N. Polymorphonuclear neutrophil (PMN) cells; P. Parabasal cells.

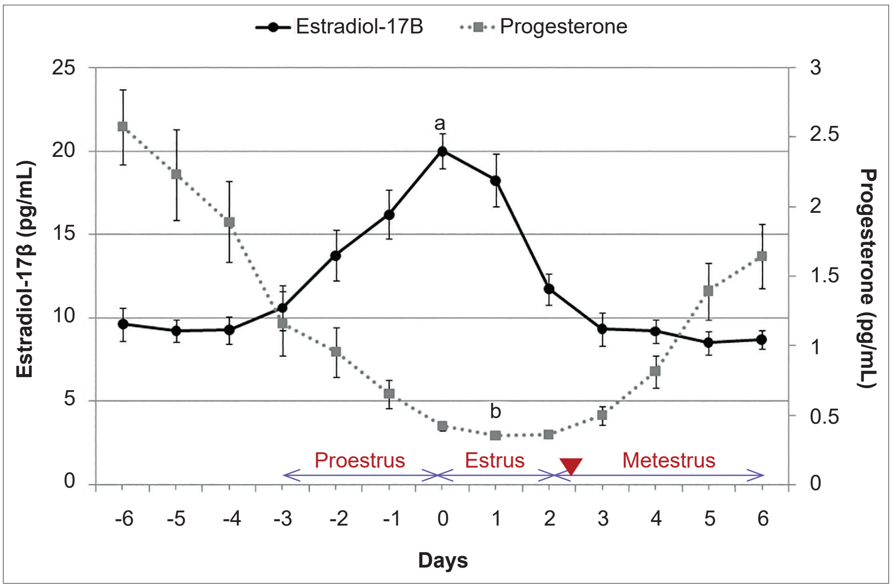


Fig. 3. Mean (±SEM) concentrations of estradiol-17β (E2) and progesterone (P4) in plasma during the estrous cycles (n=18) in Mithun. Day 0 was the day of the onset of estrus. ▼ Indicates ovulation time point, ([a,b]) indicates significant differences (P<0.05) at highest and nadir concentrations of estradiol-17β (pg/mL) and progesterone (ng/mL) within groups, respectively.

patterns as compared to atypical or nil arborization patterns (Alena *et al.* 2008).

Evaluation of cervical mucus smears revealed intermediate cells and a greater number of superficial cells with pyknotic nuclei and degenerated cytoplasm during early estrus, superficial cells with pyknotic nuclei and degenerated cytoplasm during mid estrus and a large number of parabasal cells along with polymorphonuclear neutrophils (PMN) cells during metestrus. The percentages of epithelial cells were significantly (P<0.05) higher in early estrus and mid estrus than in other phases, and percentages of PMN were significantly (P<0.05) higher in metestrus were higher than in other phases (Table 1). The E2 and P4 profiles during proestrus, estrus and metestrus are presented in Fig. 3. The E2 concentrations increased gradually in the proestrus phase and reached its peak concentrations on the day of estrus (day 0) in presence of dominant follicle. The P4 concentrations were lower than 0.5 ng/mL at the onset of estrus, during the estrus phase, and before ovulation and increased sharply after ovulation and achieved ≥1 ng/mL on day 5 or 6 when the corpus luteum started the growth. The conception rate at day 60-70 as confirmed by ultrasound post-insemination was (28.33%, 17/60).

Table 1. Mean (±SEM) epithelial and polymorphonuclear neutrophil cells in the cervical mucus smear during early estrus, mid estrus and metestrus in Mithun

| Estrus phase (n=18) | Epithelial (%) | Polymorphonuclear neutrophil cells (%) |
|---|---|---|
| Early estrus | 98.94±0.26[a] | 1.05±0.26[a] |
| Mid estrus | 99.11±0.19[a] | 0.88±0.19[a] |
| Metestrus | 55.27±2.25[b] | 44.72±2.25[b] |

*a, b means significant (P<0.05) within column.

In the present study, observed caseous or white-coloured thick consistency cervical mucus during metestrus can be used as an ovulation predictor in cycling Mithuns as ovulation occurred during or in and around the appearance of mucus. Ultrasonography may precisely detect ovulation; however, the development of less labor-intensive methods will be of great benefit. Recently, in goats, vaginal cytology and cervical mucus were evaluated to determine ovulation time with high accuracy (Souza-Fabjan *et al.* 2021) and cervical mucus appeared as a caseous mass with evident flocculation nearing ovulation. In cattle, a strong relationship exists between the spinnbarkeit value and arborization pattern with plasma P4 concentration and ovulation time and is used as a tool to decide optimal insemination time (Layek *et al.* 2013). In the present study, the caseous or white cervical mucus showed parabasal cells and PMN cell infiltration, PMN is possibly having a role in clearing the unwanted microflora post-estrus or breeding. Further, the decrease of P4 in proestrus and the probable increase in uterine blood flow by increased E2 caused the migration of PMN cells to the reproductive tract lumen (Azawi 2008, Hafez and Hafez 2000). Similarly in cattle and buffalo, evaluation of cervical mucus revealed a higher % of neutrophils in metestrus and late estrus, respectively than in other phases (Ahmadi *et al.* 2006, Suebkhampet and Chaikhun-Marcou 2019). Apart from the concentrations of steroid hormones, the arachidonic acid metabolites also affect neutrophil infiltration which may arise from a normal inflammatory response (Hoedemaker *et al.* 1992). In cattle, during metestrus, the external os is constricted with scanty, sticky, and pale yellow or brown cervical mucus and leukocytic invasion of the vaginal mucosa is maximal 2-5 days after estrus (Noakes *et al.* 2001). The results indicate that the ideal time to perform AI (artificial insemination) in Mithun is during the midestrus (typical arborization pattern). The AI in late estrus or nearing metestrus may interrupt the spermatozoa progression by phagocytosis by

PMN cells. In Mithun, the present study and earlier study demonstrated that AI during midestrus and the presence of caseous or white cervical mucus did not affect the conception rates (Nadaf *et al.* 2022). The epithelial cells in the cervical mucus smears of Mithun are an indicator of the progression of the estrus phase and a greater number of superficial cells observed during the midestrus represent greater estrogen concentrations (Sharma and Sharma 2016). The cervical mucus characteristics and vaginal cytology are used to determine the ideal time of insemination in cattle and buffalo (Mingoas *et al.* 2009, Duran *et al.* 2015). As Mithun exhibits silent estrus, the cervical mucus arborization pattern and cytology are beneficial in determining the ideal time for insemination.

In conclusion, cervical mucus shows a typical arborization pattern in midestrus which is an ideal time for AI in Mithun and the appearance of characteristic sticky caseous or white cervical mucus during metestrus did not affect the conception rates and can be used as a tool to predict ovulation time.

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