The present work was carried out to study the variation in biochemical properties of cervico-vaginal mucus of pregnant and non pregnant animals which could help to design suitable tool in future for early pregnancy diagnosis in buffaloes. Cervico-vaginal mucus samples were obtained from 30 buffaloes (12 pregnant and 18 non pregnant) and some biochemical parameters were compared. Means of pH, sodium, potassium and chloride had no significant difference between the estrous cycle and pregnancy. Total sugar content showed significant differences in pregnant and non pregnant animals from 6th to 21st day (P<0.05). In pregnant animals, continuously increasing trend was observed from day 0 to day 21 (25.22±0.5 mg/100 mL on day 0 to 130.5±1.1 mg/100 mL on day 6 and later 156.4±1.8 mg/100 mL on day 21) whereas in non pregnant animals the concentration of total sugar increased from day 0 to day 12 and then started decreasing (24.33±0.6 mg/100 mL on day 0, 118.1±1.7 mg/100 mL on day 6, 127.9±1.5 mg/100 mL on day 12 and then decreased to 90.8±1.2 mg/100 mL on day 21). Protein content of vaginal mucus was different in pregnant and non pregnant animals from 12 to 21 day of experiment. A continuously decreasing trend of protein content was observed in non pregnant group after 12th day (14.3±0.43 mg/mL on day 12 and 9.9±0.55 mg/mL on day 21) whereas in pregnant group, the concentration remained higher up to 21st day without any significant change (26.8±0.33 mg/mL on day 12 to 29.6±0.12 mg/mL on day 21). However, protein content initially increased in both pregnant (42.4±0.22 mg/mL) and non pregnant (40.2±0.03 mg/mL) group and showed highest values on third day. Consequently, significant variation in total sugar and protein in cervico-vaginal mucus could be helpful in designing future biochemical tool for early pregnancy diagnosis in buffalo.

**KEY WORDS** biochemical studies, cervico-vaginal mucus, early pregnancy diagnosis.
MATERIALS AND METHODS

Cervico-vaginal mucus samples were obtained from 30 buffaloes (n=12 pregnant and n=18 non-pregnant) and a comparison was made between values of different parameters of the cervico-vaginal mucus obtained from pregnant and non pregnant animals during about 8 months of experiment (Table 1).

Experimental animal
Selection of animal
The experimental animals were normal buffaloes of 3-6 years age and 2-3 parity, which were free from any genital tract infection.

Induction of heat
2.5 mL of a GnRH analogue (receptal, 0.004 mg/mL buserelin, intervet India private limited) was administered to 100 buffaloes irrespective of the stage of their estrous cycle (day of GnRH treatment and day 0), followed by 2 mL of PGF2α (cyclix, 263 µg/mL cloprostenol sodium, intervet India private limited) on day 7 and a second-GnRH treatment (2.5 mL) 48 h after PGF2α (day 9). Out of 100, those 30 animals which exhibited heat within 24-48 hours of treatment were selected for the experiment.

Heat detection
Estrus was detected twice daily, in morning and evening. The buffaloes were also monitored both for behavioral symptoms (frequent micturition, bellowing, raised tail, restlessness and licking of external genitalia by other animals) and physical changes (vulvular edema and mucus discharge) of the reproductive tract. On the fifth day after estrus, the corpus luteum (CL) was detected by per rectal palpation to confirm the estrus phase.

Breeding of animal
Buffaloes showing signs of true estrus were bred 12 hours after the onset of estrus by natural breeding with high quality bull. Pregnancy was diagnosed after 60 days by rectal palpation.

Collection of cervico-vaginal mucus
Collection procedure
Vulva was washed with a warm dilute solution of savlon. Sterilized vaginal speculum was inserted into vagina and fixed. Sterilized long handle forceps holding cervical sponge was gently inserted through vaginal speculum via vagina up to the cervix. Extreme care being used and line of least resistance were taken. Sponge was left there for few minutes and then taken out. Absorbed cervical mucus was collected in Eppendorf tubes.

Mucus samples were taken from experimental animals at a gap of every 2 day, starting from day of breeding to the completion of one estrous cycle i.e. 21 days, considering day 0 (day of breeding), 3, 6, 9, 12, 15, 18 and 21. Pregnancy was diagnosed after 60 days by per rectal palpation.

Storage of sample
The samples were collected in Eppendorf tubes and immediately transferred to laboratory on ice where they were marked with date, sample number and time of collection. Color, consistency, elasticity and pH of samples were taken immediately. Subsequently they were stored at -20 °C till further processing without added preservative. Pregnancy was confirmed after 60 days on the basis of per rectal palpation. Samples from pregnant and non pregnant animals were separated and samples taken on different days were pooled separately.

Biochemical analysis of cervico-vaginal mucus
Vaginal mucus pH was measured immediately after collection of sample with a pH paper strip. Sodium determination was done by tinder’s method, end point assay (colorimetric) method and potassium estimation was done by tetraphenyl boron method, end point assay (turbid metric). Chloride estimation was done by thiocyanate end point assay method. Results obtained for sodium, potassium and chloride were converted into mg/100 mL for the sake of convenience.

Total sugar content of mucus samples were determined colorimetrically by phenol sulphuric acid method of Roughan and Batt (1968). The absorption maxima was recorded in a Boasch and Lomb colorimeter (spectronic 20) at 495 nm using pure D-glucose (sigma) as a standard and the sugar content expressed as mg glucose / 100 mL of mucus. Total protein concentration in cervico-vaginal mucus was determined by the method of Lowry et al. (1951) as modified by Peterson (1977). This method is based on the reaction of aromatic groups of tryptophan and tyrosine of proteins with folin’s reagent to form a blue color. Results obtained were converted to mg/mL.

Statistical analysis
The results were analyzed by one-way ANOVA, followed by the least significant difference (LSD) post hoc test and were expressed as mean ± SEM. The level of statistical significance was set at P<0.05 for all analyses.
RESULTS AND DISCUSSION

The cervico-vaginal mucus of buffaloes was predominantly alkaline during different phases of the estrous cycle. The results were in conformity with Sood et al. (1950). Alkalinity was highest during estrus phase in both pregnant and non pregnant group.

The values of pH ranged from 6.66 ± 0.01 to 7.88 ± 0.14 in non pregnant group and from 7.40 ± 0.01 to 8.1 ± 0.05 in pregnant group which were non significant. The mean of pH was not different between pregnant and non pregnant animals (Table 2).

The mean values of sodium concentration in cervico-vaginal mucus in pregnant animals ranged from 225.5 ± 0.22 to 325.6 ± 0.45 mg/100 mL, whereas in non pregnant group it ranged from 221.3 ± 0.34 to 324.1 ± 0.33 mg/100 mL during the days of experiment. These variations in values between pregnancy and the estrous cycle were not significant. The highest concentration of sodium was seen from day 18 to day 21 i.e. during prooestrous phase and lowest around 12th day in both pregnant and non pregnant group. Hence, sodium concentration of cervico-vaginal mucus remained same in both pregnancy and non pregnancy state (Table 2).

The concentration of potassium in cervico-vaginal mucus of buffaloes was predominantly alkaline during different phases of the estrous cycle. The results were in conformity with Sood et al. (1950). Alkalinity was highest during estrus phase in both pregnant and non pregnant group.

The values of pH ranged from 6.66 ± 0.01 to 7.88 ± 0.14 in non pregnant group and from 7.40 ± 0.01 to 8.1 ± 0.05 in pregnant group which were non significant. The mean of pH was not different between pregnant and non pregnant animals (Table 2).

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The chloride content in cervico-vaginal mucus ranged from 290.3 ± 0.11 to 652.1 ± 0.32 mg/100 mL in non pregnant group and from 291.1 ± 0.12 to 650.3 ± 0.08 mg/100 mL in pregnant group which were non significant. Highest chloride content was seen at the time of estrus and lowest on days 9 in both experimental groups. However, there was no significant difference in chloride concentration in cervico-vaginal mucus of pregnant and non pregnant group during examined days (Table 2).

The data for sodium, potassium and chloride content in cervico-vaginal mucus are in general agreement with Bhatia and Kohli’s (2007) and El-Naggar (1975) results. The total sugar content of cervico-vaginal mucus of experimental animals ranged from 25.22 ± 0.5 to 156.4 ± 1.8 mg/100 mL in pregnant group and from 24.33 ± 0.6 to 127.06 ± 1.2 mg/100 mL in non pregnant group (Table 2). A significant difference in values of total sugar in cervico-vaginal mucus between pregnant and non pregnant group was observed from 6th day onward which continued up to the 21st day (P<0.05). A lower level of total sugar was recorded during estrus in both pregnant (25.22±0.5 mg/100 mL) and non pregnant (24.33±0.6 mg/100 mL) group. In pregnant group, a continuously increasing trend was observed from day 0 to day 21 (values ranged from 25.22±0.5 mg/100 mL on day 0 to 130.5±1.1 mg/100 mL on day 6 and later 156.4±1.8 mg/100 mL on day 21) whereas in non pregnant group the sugar concentration in the mucus increased up to 12th day of the cycle (127.9±1.5 mg/100 mL on day 12) and then decreased up to day 21 reaching a value of 90.8 ± 1.2 mg/100 mL on day 21. However on third day the total sugar content increased by a noticeable amount in both pregnant and non pregnant group and reached to 108.1 ± 1.3 mg/100 mL in pregnant group and 105.1 ± 1.6 mg/100 mL in non pregnant group. Tsiligianni et al. (2001) observed that glucose and fructose concentrations of cervical mucus do not differ significantly in cows with normal and PGF2α induced estrus whereas El-Naggar (1975) found higher fructose concentration in cervical mucus of cows with normal estrus. Bane and Rajakoski (1961) merely traced glucose in the estrous cervical mucus of cow, using colorimetric tips and they also observed that lowest levels of total sugar concentration on the day of estrus which increased later. Total protein concentration of the mucus ranged from 6.2 ± 0.23 to 40.2 ± 0.03 mg/mL in non pregnant group and from 7.1 ± 0.5 to 42.4 ± 0.22 mg/mL in pregnant group (Table 2). In both groups, the level of total protein was lowest at estrus (6.2±0.23 mg/mL and 7.1±0.5 mg/mL in non pregnant and pregnant group respectively) which reached to highest at day 3 (40.2±0.03 mg/mL and 42.4±0.22 mg/mL in non pregnant and pregnant animals, respectively). Then, a continuously decreasing trend of protein concentration was observed in non pregnant group, whereas in pregnant group, the concentration slightly decreased (day 6) and remained higher up to 21st day without any significant change. In our study, total protein concentration in the cervico-vaginal mucus collected from buffaloes was similar to that observed by Prasad et al. (1981). They found that total protein concentration is higher (almost double) in cervico-vaginal mucus collected the day before estrus manifestation than that collected during the day of estrus in the PGF2α induced estrus cows. They observed lower total protein concentration on the day of estrus. They also observed increased proteins in cervical mucus collected from cows that did not exhibit estrus signs. Moreover in a study by Tsiligianni et al. (2001), it was shown that concentration of total protein was significantly lower (P<0.001) in normal than in PGF2α induced estrus, while no difference was found among the induced estrus groups.
They also observed that pregnancy rates of the animals did not differ significantly among the normal and the induced-estrus groups.

Protein content was different in pregnant and non pregnant animals from 12th to 21 day. A continuously decreasing trend of protein concentration was observed in non pregnant group after 12th day (14.3±0.43 mg/mL on day 12 and 9.9±0.55 mg/mL on day 21) whereas in pregnant group, the concentration remained higher up to 21st day without any significant change (values ranging from 26.4±0.33 on day 12 to 29.6±0.12 mg/mL on day 21).

However, the level of protein initially increased in both pregnant (42.4±0.22 mg/mL) and non pregnant (40.2±0.03 mg/mL) group and showed highest values on third day.

The increased amount of cervical mucus during estrus might cause a dilution effect and therefore show the lowest protein concentration. This might indicate that protein is mainly of endometrial origin and is diluted by the increased mucus volume at estrus.

In a study by Zaaijer et al. (1992), highest protein concentration was observed at day 6 of the cycle but in that experiment protein analysis was done by cellulose acetate electrophoresis. They also observed the lowest protein concentration at the day of estrus.

The mucus present in estrus appears to contain very little protein and is readily differentiated from the mucus of days 9 and pregnancy. In general, protein concentration is significantly higher in pregnant animals than in non pregnant. Proteins have been shown to mediate between the conceptus and maternal endometrium and may be indicative of fertility status at estrus.

CONCLUSION

Biocohemical studies like pH, sodium, potassium and chloride content of cervico-vaginal mucus did not show any remarkable differences between the estrous cycle and pregnancy, but significant differences were seen in total protein and total sugar contents. Total sugar content was different in pregnant and non pregnant animals from 6th to 21th day of estrous cycle in pregnant buffalos, a continuously increasing trend was observed during subsequent days of pregnancy (values ranged from 130.5±1.1 mg/100 mL on day 6 to 156.4±1.8 mg/100 mL on day 21) whereas in non pregnant group the sugar concentration in the mucus increased up to 15th day of the cycle and then decreased (values ranged from 118.1±1.7 mg/100 mL on day 6 to 90.8±1.2 mg/100 mL on day 21). Total protein levels increased in cervico-vaginal mucus in both pregnant and non pregnant animal at day 3 after which a continuously decreasing trend of protein concentration was observed in non pregnant group, whereas in pregnant group, the concentration remained higher up to 21th day without any significant change (values ranging from 26.8±0.33 on day 12 to 29.6±0.12 mg/mL on day 21).

<table>
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<th>Days of estrous Cycle</th>
<th>pH</th>
<th>Sodium (mg/100 mL)</th>
<th>Potassium (mg/100 mL)</th>
<th>Chloride (mg/100 mL)</th>
<th>Total sugar (mg/100 mL)</th>
<th>Total protein (mg/mL)</th>
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<tr>
<td>0</td>
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<td>320.4±0.22</td>
<td>105.1±1.6</td>
<td>40.2±0.32</td>
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<td>245.2±0.34</td>
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<td>408.1±0.02</td>
<td>90.8±1.8</td>
<td>9.9±0.09</td>
<td>29.6±0.12</td>
</tr>
</tbody>
</table>

* (P<0.05).

1 Day 0, day of estrus and day 3-21, days of the estrous cycle or pregnancy.

1 (P<0.05).
vaginal mucus may pave the way for development of biochemical kit which could be used for early pregnancy diagnosis in buffaloes.

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