

## Studies on Biochemical Properties of Cervico-Vaginal Mucus during Early Pregnancy in Buffaloes (*Bubalus bubalis*)

### Research Article

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### ABSTRACT

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 6<sup>th</sup> to 21<sup>st</sup> day ( $P < 0.05$ ). In pregnant animals, continuously increasing trend was observed from day 0 to day 21 ( $25.22 \pm 0.5$  mg/100 mL on day 0 to  $130.5 \pm 1.1$  mg/100 mL on day 6 and later  $156.4 \pm 1.8$  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 ( $24.33 \pm 0.6$  mg/100 mL on day 0,  $118.1 \pm 1.7$  mg/100 mL on day 6,  $127.9 \pm 1.5$  mg/100 mL on day 12 and then decreased to  $90.8 \pm 1.2$  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 12<sup>th</sup> day ( $14.3 \pm 0.43$  mg/mL on day 12 and  $9.9 \pm 0.55$  mg/mL on day 21) whereas in pregnant group, the concentration remained higher up to 21<sup>st</sup> day without any significant change ( $26.8 \pm 0.33$  mg/mL on day 12 to  $29.6 \pm 0.12$  mg/mL on day 21). However, protein content initially increased in both pregnant ( $42.4 \pm 0.22$  mg/mL) and non pregnant ( $40.2 \pm 0.03$  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.

### INTRODUCTION

Pregnancy diagnosis in bovine has been the subject of numerous investigations aimed at developing more practical and reliable diagnostic methods. Manual palpation via rectum generally has been recognized as the standard and common method but it may not be reliable before two months of gestation.

Other methods like radio immune assay (RIA) for progesterone in milk or plasma, early pregnancy factor (EPF)

determinations have limited applicability under field conditions due to high cost of equipments, sophistication as well as non availability of highly trained technical staff. Considering the lack of an ideal method of early pregnancy diagnosis, the present work was planned to study the variation in biochemical properties (pH, sodium, potassium, chloride, total sugar and total protein) 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.

## 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 PGF2 $\alpha$  (cyclix, 263  $\mu$ g/mL cloprostenol sodium, intervet India private limited) on day 7 and a second-GnRH treatment (2.5 mL) 48 h after PGF2 $\alpha$  (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 (vulvar 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.

**Table 1** Details of samples studied

Area	Species	NO. of animal	Samples / animal	Total samples
IDF pantnagar	Buffalo	30	day 0, 3, 6, 9, 12, 15, 18 and 21	240 (8 $\times$ 30)

#### 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  $\pm$  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  $6.66 \pm 0.01$  to  $7.88 \pm 0.14$  in non pregnant group and from  $7.40 \pm 0.01$  to  $8.1 \pm 0.05$  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  $225.5 \pm 0.22$  to  $325.6 \pm 0.45$  mg/100 mL, whereas in non pregnant group it ranged from  $221.3 \pm 0.34$  to  $324.1 \pm 0.33$  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 proestrus phase and lowest around 12<sup>th</sup> 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 in pregnant animals ranged from  $44.6 \pm 0.12$  to  $70.1 \pm 0.08$  mg/100 mL whereas in non pregnant group ranged from  $45.7 \pm 0.55$  to  $72.1 \pm 0.3$  mg/100 mL during different experimental days. The variations in potassium values between pregnancy and the estrous cycle were also non significant. The highest concentration of potassium was seen around 3<sup>rd</sup> day i.e. during early luteal phase and lowest during estrus phase in both pregnant and non pregnant group. Hence, potassium concentration of cervico-vaginal mucus also remained same during both pregnancy and non pregnancy state (Table 2).

The chloride content in cervico-vaginal mucus ranged from  $290.3 \pm 0.11$  to  $652.1 \pm 0.32$  mg/100 mL in non pregnant group and from  $291.1 \pm 0.12$  to  $650.3 \pm 0.08$  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  $25.22 \pm 0.5$  to  $156.4 \pm 1.8$  mg/100 mL in pregnant group and from  $24.33 \pm 0.6$  to  $127.06 \pm 1.2$  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 6<sup>th</sup> day onward which continued up to the 21<sup>st</sup> day ( $P < 0.05$ ). A lower level of total sugar was recorded during estrus in both pregnant ( $25.22 \pm 0.5$  mg/100 mL) and non pregnant ( $24.33 \pm 0.6$  mg/100 mL) group. In pregnant group, a continuously increasing trend was observed from day 0 to day 21 (values ranged from  $25.22 \pm 0.5$  mg/100 mL on day 0 to  $130.5 \pm 1.1$  mg/100 mL on day 6 and later  $156.4 \pm 1.8$  mg/100 mL on day 21) whereas in non pregnant group the sugar concentration in the mucus increased up to 12<sup>th</sup> day of the cycle ( $127.9 \pm 1.5$  mg/100 mL on day 12) and then decreased up to day 21 reaching a value of  $90.8 \pm 1.2$  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  $108.1 \pm 1.3$  mg/100 mL in pregnant group and  $105.1 \pm 1.6$  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 PGF2 $\alpha$  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  $6.2 \pm 0.23$  to  $40.2 \pm 0.03$  mg/mL in non pregnant group and from  $7.1 \pm 0.5$  to  $42.4 \pm 0.22$  mg/mL in pregnant group (Table 2). In both groups, the level of total protein was lowest at estrus ( $6.2 \pm 0.23$  mg/mL and  $7.1 \pm 0.5$  mg/mL in non pregnant and pregnant group respectively) which reached to highest at day 3 ( $40.2 \pm 0.03$  mg/mL and  $42.4 \pm 0.22$  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 21<sup>st</sup> 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 PGF2 $\alpha$  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 PGF2 $\alpha$  induced estrus, while no difference was found among the induced estrus groups.

**Table 2** Concentrations of various biochemical parameters of cervico-vaginal mucus in pregnant and non pregnant animals

Days of estrous Cycle <sup>1</sup>	pH		Sodium (mg/100 mL)		Potassium (mg/100 mL)		Chloride (mg/100 mL)		Total sugar (mg/100 mL)		Total protein (mg/mL)	
	Non pregnant (n=18)	Pregnant (n=12)	Non pregnant (n=18)	Pregnant (n=12)	Non pregnant (n=18)	Pregnant (n=12)	Non Pregnant (n=18)	Pregnant (n=12)	Non Pregnant (n=18)	Pregnant (n=12)	Non Pregnant (n=18)	Pregnant (n=12)
0	7.88±	8.1±	295.1±	290.1±	45.7±	44.6±	652.1±	650.3±	24.33±	25.22±	6.2±	7.1±
	0.14	0.05	0.45	0.5	0.55	0.12	0.32	0.08	0.6	0.5	0.23	0.5
3	7.75±	7.96±	250.4±	251.2±	72.1±	70.1±	320.4±	324.2±	105.1±	108.1±	40.2±	42.4±
	0.1	0.1	0.68	0.12	0.3	0.08	0.22	0.11	1.6	1.3	0.03	0.22
6	7.45±	7.40±	245.2±	246.8±	69.4±	66.2±	294.1±	300.5±	118.1±	130.5±	28.1±	29.6±
	0.11	0.14	0.56	0.22	0.45	0.23	0.21	0.23	1.7	1.1*	0.32	0.3
9	7.09±	7.49±	234.1±	232.1±	65.4±	65.4±	290.3±	291.1±	125 ±	135.2±	28.3±	29.4±
	0.14	0.05	0.55	0.21	0.55	0.44	0.11	0.12	1.6	1.1*	0.12	0.16
12	6.66±	7.40±	221.3±	225.5±	65.4±	65.3±	291.4±	291.4±	127.9±	140.2±	14.3±	26.8±
	0.01	0.01	0.34	0.22	0.34	0.33	0.09	0.32	1.5	1.2*	0.43	0.33*
15	7.00±	7.95±	231.4±	233.8±	69.2±	65.2±	299.1±	295.5±	127.06±	141.8±	14.4±	27.2±
	0.16	0.05	0.65	0.05	0.34	0.45	0.02	0.23	1.2	1.1*	0.22	0.15*
18	7.49±	7.7±	324.1±	325.6±	66.7±	66.1±	410.4±	411±	109.6±	149.4±	16.1±	26.4±
	0.17	0.06	0.33	0.45	0.45	0.65	0.22	0.12	7.6	1.2*	0.09	0.22*
21	7.75±	7.66±	312.5±	315.8±	67±	63.5±	408.1±	410±	90.8±	156.4±	9.9±	29.6±
	0.1	0.08	0.56	0.66	0.08	0.45	0.00	0.03	1.2	1.8*	0.55	0.12*

<sup>1</sup> Day 0, day of estrus and day 3-21, days of the estrous cycle or pregnancy.  
\* (P<0.05).

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 12<sup>th</sup> to 21 day. A continuously decreasing trend of protein concentration was observed in non pregnant group after 12<sup>th</sup> 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 21<sup>st</sup> 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, 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 concentration of protein. 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 mucin present in estrus appears to contain very little protein and is readily differentiated from the mucin 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

Biochemical 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 6<sup>th</sup> to 21<sup>st</sup> 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 15<sup>th</sup> 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 21<sup>st</sup> day without any significant change. Total protein concentration in cervico-vaginal mucus also showed a significant difference between the estrous cycle and pregnancy from 12<sup>th</sup> day onward (P<0.05). A continuously decreasing trend of protein concentration was observed in non pregnant group after 12<sup>th</sup> day (values ranging from 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 21<sup>st</sup> day without any significant change (values ranging from 26.8±0.33 on day 12 to 29.6±0.12 on day 21). Total sugar and protein in cervico-

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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