RESEARCH ARTICLE

Study on Uterine Environment of Buffaloes during Different Reproductive Phases

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Abstract

This study was undertaken to examine the changes in composition of hormonal and biochemical constituents of uterine secretions of buffaloes (n = 35) during different phases of reproduction, *i.e.,* proestrus, estrus, metestrus and diestrus and anestrus condition. The protein fractioning of blood and uterine secretions was carried out by Gel electrophoresis. Most of the hormonal and biochemical parameters studied revealed higher levels at the estrus phase, indicating peak metabolic activity at the time of estrus, ovulation and fertilization period. The role of enzymes and macro-micro-minerals levels are also reported and discussed. Of the 35 different protein fractions (18 to 192 kDa) observed, the numbers of protein bands present in blood sera were 15 and in uterine fluid secretions 28, of which 8 were common in both the fluids. The findings between normal cyclic and anestrus conditions may be useful to find out the cause-effect relationship of infertility in buffaloes.

Keywords: Buffalo, Hormonal and biochemical constituents, Protein fractions, Reproductive phases, Uterine environment.

Ind J Vet Sci and Biotech (2021): 10.21887/ijvsbt.17.2.17

Introduction

The importance of buffalo as dairy animal in India is well recognized as it contributes 51% of the total milk production (DADF, 2015). Despite having several merits, such as the utilization of poor-quality roughage and resistance to several bovine tropical diseases, it has been blamed for its poor reproductive performance, mainly delayed age at first calving and prolonged inter-calving interval. Anestrus and repeat breeding are the main infertility problems in this species (Gehlot *et al.,* 2018). Earlier, Shoustari *et al.* (2008, 2014) reported the biochemical constituents in blood serum and uterine secretions during different phases of the estrous cycle in dairy cows. However, little information is available on these aspects in buffaloes. Therefore, the present investigation was an attempt to know the various biochemical and hormonal constituents and protein fractions present in uterine secretions during different reproductive phases of buffalo.

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How to cite this article: Sarvaiya, N.P., Parmar, A.P., Pathan, M.M., & Kumar, S. (2021). Study on Uterine Environment of Buffaloes during Different Reproductive Phases. Ind J Vet Sci and Biotech, 17(2): 87-91.

Source of support: Nil

Conflict of interest: None.

Submitted: 12/12/2020 **Accepted:** 21/04/2021 **Published:** 25/06/2021

Materials and Methods

Collection of Uterine Fluid

Reproductive tracts of non-pregnant/cyclic buffaloes (n = 35) having bilateral symmetrical uterine horn with no gross ovarian or uterine pathology were collected from Ahmedabad Municipal Slaughterhouse. Organs were collected immediately after slaughter and brought to the laboratory in ice. The tracts were classified as of (a) Estrus (D1), (b) Metestrus (D2-4), (C) Diestrus (D5-16), (d) Proestrus (D17-20/21), and (e) Anestrus conditions, each comprised of seven numbers.

Uterine lumen of each tract of different categories was washed with pre-cooled (4°C) 20 ml normal saline. The solution was introduced using a blunt 18G needle and 20 mL syringe through the cut tip of one uterine horn and collected from tip of the other horn. Washed fluid was centrifuged at 2500-3000 rpm, and the supernatant was stored frozen at -20°C until analyzed.

Biochemical, Enzymatic and Hormonal Assay:

The biochemical constituents analyzed in the uterine secretions (washings) by respective standard methods were macro (Ca, P) and micro (Cu, Zn, Mn) minerals, enzymes

(AST, ALT, AKP), total protein and total cholesterol, and other metabolites (uric acid, creatinine etc). The hormones progesterone and estradiol were estimated using the coat-a-count RIA method, and gel electrophoresis was used to separate protein bands of different molecular weight in both blood sera and uterine samples.

Poly-Acrylamide Gel Electrophoresis (PAGE):

For PAGE, the **s**ample (10 µl) was mixed with equal volume of gel loading buffer (2x) followed by denaturation for 5 min at 65°C. Electrophoresis was conducted on vertical slab gel PAGE unit (claver) at 20-25 mA till dye reached to running gel, and then the electrical current was increased to 70 mA till complete run. The concentration of resolving gel was 15%, and that of stacking gel was 4%. Each lane was loaded with 20 µl of protein along with 5 µL protein molecular weight marker (10 to 315 kDa). After completion of first electrophoresis, it was stained with 0.1% Commassie Brilliant Blue-R250 in a mixture of methanol: acetic acid: distilled water in the ratio 40:10:50. The gels were destained by using a plain mixture of methanol: acetic acid: distilled water in the ratio 40:10:50 without dye (Sadasivam and Manickam, 1992). The separated bands were visualized under visible light and photographed using Biorad Gel documentation system.

The data generated on uterine constituents were analyzed statistically using a completely randomized design and Duncan's new multiple range test (Snedecor and Cochran, 1994).

Results and Discussion

Biochemical and Hormonal Analysis

The detailed composition of uterine fluid/ secretions observed during different phases of estrous cycle, *viz.,* proestrus, estrus, metestrus, and diestrus and anestrus conditions, is shown in Table 1. The table shows hormonal concentrations (progesterone and estradiol), biochemical constituents (total cholesterol and total protein), macro minerals (calcium and phosphorus), micro minerals (copper, manganese and zinc), enzymes (AKP, AST and ALT) and other metabolites (creatinine and uric acid).

The estradiol and progesterone levels fluctuated as per the estrous cycle phases, but remained at basal level in anestrus condition. These findings were in accordance with the earlier observations in buffalo heifers (Sarvaiya, 1990). Uterine endometrium also synthesise and secrete various biochemical constituents under the influence of these hormones. Total cholesterol and total protein concentrations were also lower than that of blood serum reported earlier by Sarvaiya (1990) and Gehlot *et al*. (2018). Lower levels of these constituents were also reported in buffaloes suffering from retention of placenta (Kumari *et al.,* 2015; Dutta and Dugwekar, 1982) and anetrus condition (Sarvaiya, 1990). Cholesterol plays a vital role as a precursor of steroid hormone synthesis, while protein acts as an enzyme carrier molecule. Hence they fluctuate as per the stage of estrous cycle (Bugalia and Sharma, 1990; Roy *et al.,* 2005). Total protein

Table 1: Compositions of uterine secretions collected from slaughtered buffaloes during different reproductive phases (Mean ± SE)

Parameters	*Proestrus (n = 7)*	*Estrus (n = 7)*	*Metestrus (n = 7)*	*Diestrus (n = 7)*	*Anestrus (n = 7)*
Hormones					
Estradiol (pg/ml)	39.67 ± 3.10^{cd}	48.50 ± 4.42^{d}	20.83 ± 2.60^{b}	10.0 ± 0.00^{a}	11.67 ± 1.80^{a}
Progesterone(ng/ml)	0.71 ± 0.14^{b}	0.25 ± 0.12^{a}	0.58 ± 0.18^{ab}	1.56 ± 0.30^{c}	0.81 ± 0.20^{b}
Biochemical constituents					
Cholesterol (mg/dl)	41.38 ± 3.69^{ab}	49.61 ± 4.62^{b}	37.14 ± 2.33^{a}	47.47 ± 7.01^{ab}	38.50 ± 5.80^{a}
Total Protein (g/dl)	4.00 ± 0.30^{a}	4.30 ± 0.43^{ab}	4.22 ± 0.39^{ab}	4.97 ± 0.63^{b}	3.86 ± 0.58^{a}
Macro-minerals					
Calcium (mg/dl)	6.92 ± 0.47^{a}	7.93 ± 0.28^{b}	7.54 ± 0.47^{ab}	7.33 ± 0.27^{a}	7.44 ± 0.60^{ab}
Phosphorus (mg/dl)	4.13 ± 0.16	4.09 ± 0.23	4.12 ± 0.19	3.93 ± 0.10	4.01 ± 0.15
Micro-minerals					
Copper (ppm)	3.70 ± 0.39^{b}	3.33 ± 0.22^{ab}	3.33 ± 0.63^{ab}	3.58 ± 0.68^{ab}	2.83 ± 0.23^{a}
Zinc (ppm)	20.20 ± 2.00	20.61 ± 3.61	20.37 ± 2.45	24.47 ± 4.12	19.51 ± 0.85
Manganese (ppm)	64.43 ± 8.66	72.83 ± 2.87	73.55 ± 1.60	70.53 ± 1.57	73.78 ± 1.47
Enzymes					
AST (U/L)	39.31 ± 1.31^{ab}	43.10 ± 7.64^{b}	31.60 ± 2.60^{a}	27.76 ± 3.11^{a}	34.03 ± 2.42^{ab}
ALT (U/L)	59.47 ± 2.72	72.44 ± 14.27	52.33 ± 5.87	48.35 ± 8.19	54.65 ± 3.76
AKP (IU/L)	320.0 ± 24.40^{b}	294.0 ± 22.80^{b}	258.6 ± 18.1^{ab}	262.6 ± 20.4^{ab}	240.0 ± 28.0^{a}
Metabolites					
Uric acid (mg/dl)	1.75 ± 0.58^{b}	0.92 ± 0.07^{a}	2.12 ± 0.93^{b}	1.11 ± 0.18^{ab}	1.02 ± 0.01^{a}
Creatinine (mg/dl)	1.33 ± 0.10	1.44 ± 0.28	1.35 ± 0.22	1.42 ± 0.21	1.19 ± 0.08

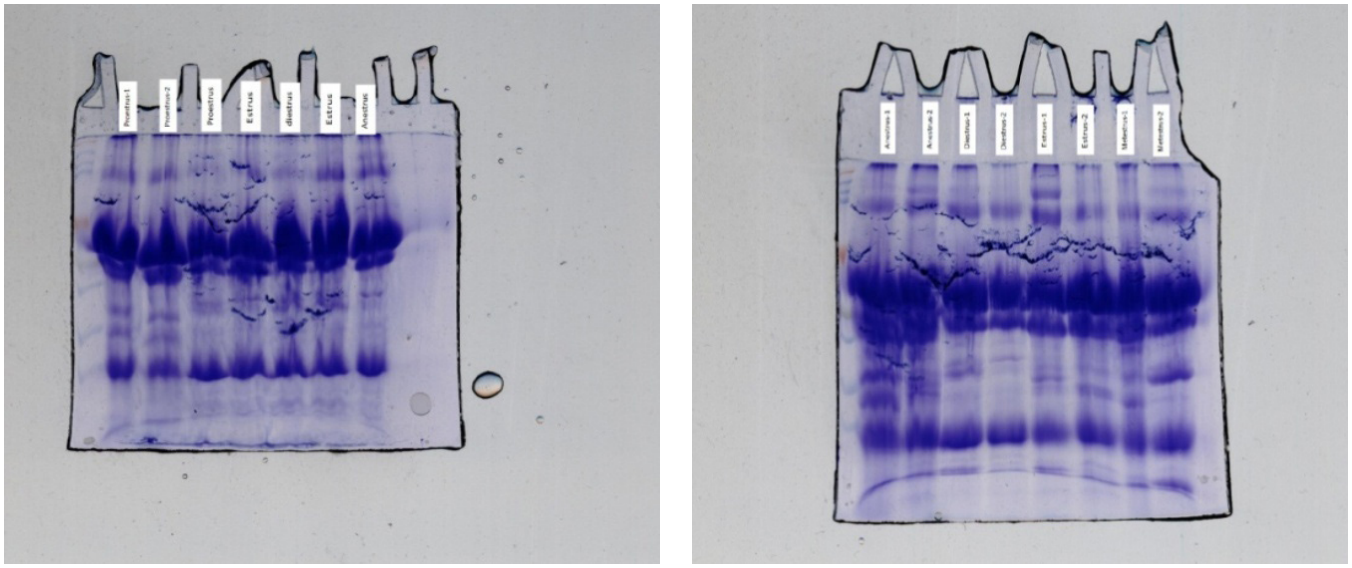
n = number of samples, Means with common superscripts within the row do not differ significantly between the phases at 5% level .

Table 2: Molecular weight of protein (kDa) in blood serum and uterine fluid of buffaloes during different reproductive phases

Stage	*Proestrus*		*Estrus*		*Metestrus*		*Diestrus*		*Anestrus*	
Protein M.W. kDa/ Sample	*Serum*	*UF*	*Serum*	*UF*	*Serum*	*UF*	*Serum*	*UF*	*Serum*	*UF*
18	P	P	P	P	P	P	P	P	P	P
20	-	P	-	-	-	-	-	-	-	-
27	-	-	-	P	-	P	-	-	-	P
29	-	P	-	P	-	-	-	-	-	-
30	-	P	-	-	-	-	-	-	-	P
33	-	-	-	P	-	P	-	P	-	P
36	-	P	-	P	-	-	-	-	-	-
37	P	P	-	P	-	-	P	P	-	-
39	-	-	-	P	P	-	-	P	-	P
40	P	-	P	-	-	-	P	-	-	-
43	-	-	-	-	P	P	-	P	-	-
47	-	-	-	-	-	P	-	-	P	P
52	-	-	-	P	-	P	-	-	-	-
53	-	-	-	P	-	-	-	P	-	-
54	-	P	-	-	-	-	-	P	-	P
57	-	-	-	-	-	-	P	-	-	-
59	P	-	P	-	P	-	-	-	-	-
62	-	P	-	-	-	-	-	-	P	-
64	-	P	-	-	-	-	-	-	-	-
68	-	-	-	P	-	P	-	-	-	-
69	-	-	-	P	-	-	-	P	-	-
70	-	-	-	-	-	-	-	P	-	P
73	P	P	P	-	P	-	P	-	-	-
76	-	-	-	-	-	-	-	-	P	-
141	-	-	-	-	-	P	-	-	-	-
143	-	-	-	P	-	-	-	-	-	-
144	-	-	-	P	-	-	-	-	-	-
148	-	-	-	-	-	P	-	P	-	-
149	-	-	-	-	-	-	-	P	-	-
155	-	P	-	-	P	-	-	-	-	P
158	-	-	-	-	-	-	-	-	P	-
161	P	-	P	-	-	-	P	-	-	-
178	-	-	-	P	-	P	-	-	-	-
182	-	-	-	-	-	-	-	P	-	P
192	-	-	-	-	-	-	-	-	P	
Total = 35	6	11	5	14	6	10	6	12	6	10

UF = Uterine fluid, P = Present.

concentrations in uterine secretions were higher in cyclic buffaloes than anestrus buffaloes. Macro-minerals (Ca and P) and micro minerals (Cu, Zn, and Mn) play vital role in many enzymes functions, carbohydrate metabolism, and intra- and extra-cellular cations regulatory mechanism (Shoushtari *et al.,* 2015). These constituents are found somewhat higher in uterine fluid and again in the proestrus or metestrus phase. Higher concentration of enzyme AKP in uterine fluid and again during the proestrus phase is in accordance with the findings of Schultz *et al.* (1971) and Sarvaiya (1990), indicating its anabolic activity under the hormone estrogen control. The concentration of uric acid and creatinine in uterine fluid in the present study did not show any specific trend. In earlier studies, nitrogenous substances like urea, uric acid



Blood serum **Uterine fluid**

Fig. 1: Comparison of protein fractions in blood serum and uterine fluid of buffaloes during different phases of estrous cycle and anestrus condition by PAGE

and creatinine were associated with reduced reproductive efficiency (Gehlot *et al*., 2018), and they responsible for innate immunity and create inflammation for clearing pathogenic agents. Overall, results clearly revealed fluctuations in the parameters as per the demands, synthesis, and utilizations; as per the need for ovulation, fertilization, early embryonic survival, and implantation of the early embryos.

Gel Electrophoresis

The uterine luminal fluid proteins are altered during various reproductive phases of many domestic animal species, including pig (Kayser *et al*., 2006) and cattle (Forde *et al*., 2014). Very few reports are available on these aspects in buffalo (Kumar and Purohit, 2018). Hence, the protein fractions were estimated and compared from both blood serum and uterine fluid (Table 2). The electrophoresis pattern suggested that a total of 35 protein bands were present in blood and uterine fluids collected from buffaloes. Analysis of blood samples indicated a total of 15 peptide bands; while in uterine fluid 28 peptide bands were detected (8 bands were common in both the fluids). A band of 18 kDa was present in both samples (blood serum and uterine fluid), irrespective of stages (Table 2, Fig. 1 and 2).

Out of 15 bands in blood sera, 5 bands were detected in the estrus stage, while remaining stages had six bands. Out of 28 bands in uterine fluid, 11, 14, 10, 12, and 10 bands were present during proestrus, estrus, metestrus, diestrus and anestrus phase, respectively (Table 2). Almost double the protein bands were synthesized by uterine tissues than in blood, which means they might not be merely secretions or diffusions from the blood serum, since only about 2 % of proteins in uterine fluid varies according to reproductive stages of cattle (Roberts and Parker, 1974). Progesterone is the main hormone that controls the uterine protein secretion, which is of high molecular weight (Knight *et al.,* 1974; Roberts *et al*., 1987).

A higher number of bands in the estrus phase in uterine fluid indicates the synthesis of required proteins for fertility under estradiol. A band of 20 kDa was only present in a uterine fluid sample in proestrus stage. Earlier, Roy *et al.* (2006) also reported uterine-specific protein of 20 kDa in buffalo; like-wise 27 and 29 kDa uterine specific proteins were also reported by them, which might be associated with fertility. Overall, a combined analysis inferred that peptide of <100 kDa were more dominant. The peptide weight in blood serum ranged from 18 to 192 kDa, while in the uterine fluid it ranged between 18 and 182 kDa. Only 11 peptides were above 100 kDa. A band of 73 kDa in blood was present in all four stages of the estrous cycle but absent in anestrus samples, which may be associated with infertility.

Conclusions

The uterine fluid is a product of active synthesis and secretion of the endometrium, and not of simple diffusion from blood. Further secretion of various biochemical and mineral constituents into the uterine lumen is under hormonal control. A higher number of protein bands during estrus phase in uterine secretion is associated with estradiol; whereas, secretion of the uterine protein of high molecular weight is under the influence of progesterone. These comparative findings are most useful between normal cyclic and anestrus buffaloes to find out the cause-effect relationship of infertility in buffaloes.

Acknowledgement

Authors are grateful to the Dean of Veterinary College and Authorities of Anand Agricultural University, Anand, for the facilities provided for this research work.

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