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Original Article

Intrauterine proteolytic enzymes therapy hastens expulsion of fetal membranes in dystocia affected buffaloes

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Abstract

Background: Difficult calving (dystocia) in buffalo cows is a major obstetrical problem which further leads to metritis complex, encompassing the retention of fetal membranes (RFM), puerperal metritis, endometritis and pyometra with impaired future fertility. **Aims:** The current study aimed to evaluate the effect of the administration of intrauterine proteolytic enzymes on the expulsion of fetal membranes and postpartum fertility in dystociac buffaloes. **Methods:** Proteolytic enzymes consisting of Trypsin (16 mg), Chymotrypsin (16 mg), and Papain (8 mg) were dissolved in 500 ml normal saline were administered after 1 h of assisted delivery in dystociac buffaloes along with the conventional therapy. **Results:** The treated animals (n=15) expelled fetal membranes within a shorter period of time (P=0.043) compared to the control group (n=15) with none in the treatment group retaining it for more than 24 hours. Fewer (26.67 *vs* 73.33%; P=0.027) postpartum uterine infections developed in the treated animals compared to the control group. The interval between first postpartum estrus (P=0.067), service period (P=0.554), and open days (P=0.557) was shorter in the treatment group compared to the control group where postpartum anestrus developed less frequently (26.67 *vs* 66.67%; P=0.066) in the animals treated with enzymatic therapy. Systemic illness (neutrophillia) was reduced in the treatment group compared to the control on day 20 (64.55 ± 1.14% *vs* 70.23 ± 0.99%; P=0.001) and 45 (55.05 ± 1.63% *vs* 64.92 ± 1.45%; P<0.001) postpartum. **Conclusion:** It is concluded that proteolytic enzymes therapy after assisted delivery in dystociac buffalo cows could help in the early expulsion of fetal membranes and reduce uterine infections with decreased neutrophils count.

Key words: Buffalo, Dystocia, Placenta, Proteolytic enzymes

Introduction

Buffaloes are major dairy animal species reared in India in small groups by small and marginal farmers, and their contribution (49%) in the total milk production of the country, despite being almost half in number than cows (20th Livestock Census, 2019; DAHD Annual Report 2019-20), is significant. The profitability of dairy enterprises depends on the number of calf crops produced by a female, which in turn is affected by inter calving intervals. To achieve the target of one calf per 13-14 months in buffaloes, it is a prerequisite that open days not be greater than 3-4 months (El-Wishy, 2007a).

Difficulty in calving (dystocia) is a major problem in buffalos, affecting the puerperium period and thereby prolonging the open days interval and reducing the ultimate number of calf crops from a female animal (Sheldon *et al*., 2006, 2009; El-Wishy, 2007a, b). Retention of fetal membranes (RFM) is the foremost complication of dystocia, making the animal more predisposed to metritis complex. The incidence of RFM in buffaloes varies from 6-14% (Gautam, 2000; Indurkar *et al*., 2019), with an increased incidence of up to 35% in relation to premature calvings, abortions and dystocia (Indurkar *et al*., 2019). The complications of RFM which include postpartum uterine infections such as metritis, clinical and subclinical endometritis and pyometra, lead to adverse effects on uterine involution and ovarian rebound phenomenon, thus causing impaired postpartum fertility (Sheldon *et al*., 2006, 2009; El-Wishy, 2007a, b).

Handling animals in dystocia leads to inflammatory and edematous changes in the birth canal and uterus and predisposes the animals to RFM (Rajala and Grohn, 1998). In addition, the inhibition of collagenase (Sheldon *et al*., 2018) and proteases activities (Eiler and Hopkins, 1992; Grohn and Rajala, 2000) in dystocia affected cases caused by inflammatory changes is a prime factor leading to the failure of the expulsion of fetal membranes. The use of corticosteroids such as dexamethasone in dystocia affected animals also causes

inhibition of the collagenase enzyme activity, thereby, leading to RFM (Sheldon *et al*., 2018).

Hormonal strategies consisting of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and oxytocin have been used in the past to hasten the expulsion of fetal membranes in normal and dystocia affected buffaloes with varying success (Shalaby *et al*., 1994; Wani *et al*., 2019; Singh *et al*., 2020b). Proteolytic enzymes (Trypsin, Chymotrypsin, and Papain) have been used in combination to treat mastitis (Zander *et al*., 1997; Kruger *et al*., 1999) and subclinical endometritis in cattle (Drilich *et al*., 2005; Honparkhe *et al*., 2005; Singh *et al*., 2017) as well as buffaloes (Honparkhe *et al*., 2014; Bhavna *et al*., 2019; Singh *et al*., 2020a). These proteolytic enzymes have hydrolytic properties and cause the breakdown of the necrotized tissue and debris and thus act as biological scalpels (Kruger *et al*., 1999).

A review of the literature revealed that the use of these proteolytic enzymes in dystocia affected buffaloes has not been studied yet. Dystocia is a prime factor leading to RFM which complicates into subsequent metritis complexes and therefore, results in the animals' impaired postpartum fertility. The current study was carried out with the hypothesis that the intrauterine administration of these enzymes mixed in a normal saline solution after assisted delivery would hasten the expulsion of fetal membranes by disrupting the cotelydeon-curuncle junctions and reducing RFM incidences, thereby improving postpartum fertility in dystociac buffaloes.

Materials and Methods

Selection of animals

The present study was carried out on clinical cases of dystocia affected animals presented at the Teaching Veterinary Clinical Complex, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India. After thorough signalment and anamnesis, the animals were diagnosed with various causes of dystocia. After assisted calf delivery, the animals were assessed for their prognosis depending on the severity of dystocia and only those animals not having any birth canal lacerations, uterine rupture and those with good prognosis were selected for the experiment. All due care was taken to include animals with similar types of dystocia in the present study in both control and treatment groups. A total of 30 dystocia affected buffaloes were included in the study. The prior permission for the experiment was granted from the Committee for the Purpose of Control and Supervision of Experiments on Animals CPCSEA) vide letter number V-11011 (13)/2/2021-CPCSEA-DADF dated 8th February, 2021.

Experimental design

The animals under study (n=30) were equally and randomly divided into the control (n=15) and the treatment group (n=15). The experimental design of the study has been presented in Fig. 1. The animals of both groups were treated with routine therapy consisting of antimicrobials, calcium therapy, analgesics, ecbolic, rumenotrics, liver extracts, antioxidants and fluid therapy. The proteolytic enzymes used in the study were purchased from Sigma-Aldrich, a USA based company (Trypsin product code-048K7021; Chymotrypsin product code-086K7695; Papain product code-76220). In addition to the routine therapy, the animals in the treatment group were administered intrauterine proteolytic enzymes (Trypsin 16 mg, Chymotrypsin 16 mg, and Papin 8 mg) mixed in 500 ml normal saline (NS) solution using a catheter, while the placebo of only 500 ml NS was given to the control group animals an hour after the assisted delivery. Considering the larger size of gravid uterus, the dose of these enzymes was doubled and mixed in more amounts of NS (500 ml) than those used by Singh *et al*. (2020a).

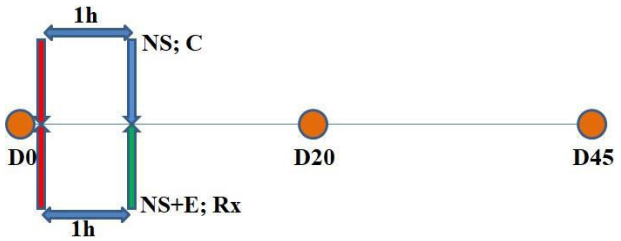

Fig. 1: Schematic representation of the experimental design. D0: Day of dystocia, D20 and D45: Days 20 and 45 postpartum, respectively, NS: Normal saline, C: Control, E: Proteolytic enzymes, and Rx: Treatment. Blood sampling was done on all three days

Blood sampling

The animals under the study were subjected to blood collection via juglar venipuncture after proper restraining. A total of 2 ml blood in EDTA (Ethylenediamine tetra acetate) vials was collected for complete blood count (CBC) estimations on different days i.e day of dystocia; days 0, 20, and 45 postpartum. The blood was assessed for CBC within 30 min after collection.

Haematological analysis

The blood samples collected from the animals were assessed for CBC that included haemoglobin (Hb; g/dL), total erythrocyte count (TEC; million/µL), packed cell volume (PCV; %), total platelets counts (Plate; $\times 10^3$/µL), total leucocyte count (TLC; $\times 10^3$/µL), and differential leucocyte count {DLC; percentage neutrophils (N) and lymphocyte (L) count}. CBC analyses were carried out using an autoanalyzer (ADVIA 2120 Hematology system, Siemens, Germany and Mythic 10 Vet, Orphee, Switzerland).

Observations recorded

After handling dystocia and the treatment with both conventional and proteolytic enzymes therapy (placebo in control), the animals under study were subjected to transrectal examinations on day 20 and 45 postpartum to evaluate uterine involution (Vala *et al*., 2018) and the

presence of vaginal discharge. The following fertility parameters were also evaluated.

1) Uterine infections (%) on day 20 and 45 postpartum
2) Spontaneous estrus rate (%)
3) Interval from calving to first postpartum estrus (days)
4) Service period (days)
5) Non-return to estrus after first AI (%)
6) Open days/calving to conception interval (days)
7) Number of inseminations for pregnancy
8) Overall pregnancy rate (%)
9) Animals became anestrus (%)

Statistical analysis

The data obtained in the study was assessed for normal distribution, and extreme values were not included for the statistical analyses. The obtained data is expressed as mean±standard error of mean (SEM) and percentage. Chi-square tests were used to compare pregnancy rates between the treated and non-treated groups. Student's t-test compared the timing of expulsion of fetal membranes and other postpartum fertility parameters in both groups. One way analysis of variance (ANOVA) test was used for the comparative analysis of haematological parameters on different days in the control and treatment groups. Pearson correlation analysis was carried out between the period of expulsion of fetal membranes, blood neutrophils counts on various days and calving to the first estrus interval. Binomial logistic regression was carried out to assess the predicted probability of pregnancy (irrespective of the treatment) based on various factors including the period of expulsion of fetal membranes, neutrophils counts (%) on the day of dystocia, day 20 and 45 postpartum, the presence of uterine infections (%) on day 20 and 45 postpartum and the interval to the first postpartum estrus (days). All the statistical analyses were performed using Graphpad Prism statistical software version 9.0.0 (121) except for the logistic regression analysis which was assessed via computer based SPSS software. The level of significance for the parameters was predicted at 95% confidence interval ($P<0.05$) while, the tendency to be significant was evaluated at a P-value of less than 0.07.

Results

The mean time period taken for expulsion of fetal membranes (h), RFM (%) in both control and treatment groups is presented in Table 1. The animals subjected to the proteolytic enzymes treatment took a mean shorter duration ($P<0.05$) for expulsion of fetal membranes than those not treated. None of the animals in the treatment group took more than 24 h for the expulsion of fetal membranes, whereas 73.33% (11/15) of those in the control developed RFM after assisted delivery.

Uterine involution scores were based on transrectal examinations and are presented in Table 2. Uterine involution was faster in those animals treated with proteolytic enzymes than those of the control.

Haematological parameters of both groups on days 0, 20, and 45 are presented in Table 3. The N count (%) reduced significantly ($P<0.05$) on day 20 and 45 postpartum in the treatment group compared to those not treated with proteolytic enzymes.

Pearson correlation coefficient was calculated between the timing of expulsion of fetal membranes (h), neutrophils counts on day 0, 20, and 45 and the calving

Table 1: Time period of the expulsion of fetal membranes after assisted delivery, and retention of fetal membranes in dystocia affected buffaloes

S. No.	Parameter		Control (n=15)	Treatment (n=15)
1	Period of expulsion of fetal membranes (h)		27.40 ± 6.33^a	8.80 ± 1.50^b
2	Retention of fetal membranes (%)		73.33^a	26.67^b
3	Perentage of animals expelling fetal membranes in varying time duration (h)	≤6 h	13.33	40.00
		6-12 h	13.33	33.34
		12-24 h	40.00	26.66
		>24 h	33.34^a	00.00^b

Mean±SEM with superscripts [a] and [b] differ significantly within rows ($P<0.05$)

Table 2: Per rectal findings scorecard on uterine involution in dystocia affected buffaloes

S. No.	Parameter		Control (n=15)				Treatment (n=15)			
			Day 20		Day 45		Day 20		Day 45	
			No. of animals	%	No. of animals	%	No. of animals	%	No. of animals	%
1	Uterine location	3	00	-	06	40.00	09	60.00	15	100.00
		2	15	100.0	09	60.00	06	40.00	00	-
		1	00	-	00	-	00	-	00	-
2	Gravid uterine horn size	3	07	46.67	03	20.00	00	-	13	86.67
		2	07	46.67	10	66.67	06	40.00	02	13.33
		1	01	06.66	02	13.33	09	60.00	00	-
3	Non-gravid uterine horn size	3	15	100	10	66.67	08	53.33	15	100.00
		2	00	-	03	20.00	07	46.67	00	-
		1	00	-	02	13.33	00	-	00	-
4	Uterine tonicity	3	03	20.00	04	26.67	02	13.33	06	40.00
		2	06	40.00	09	60.00	10	66.67	06	40.00
		1	06	40.00	02	13.33	03	20.00	03	20.00

Table 3: Haematological parameters in dystocia affected buffaloes

Parameter		Control (n=15)			Treatment (n=15)		
		Day 0	Day 20	Day 45	Day 0	Day 20	Day 45
Hb (g/dL)		10.55±0.60	10.37±0.71	11.06±0.72	10.55±0.31	10.74±0.35	11.68±0.33
PCV (%)		31.99±1.65	31.71±2.26	32.58±1.97	31.86±0.99	32.77±1.49	34.85±0.94
TLC ($\times10^3$/µL)		11.75±0.92	13.52±0.92	11.04±0.80	11.06±0.76	10.68±0.58	9.45±0.57
TEC ($\times10^6$/µL)		6.78±0.43	6.34±0.44	6.73±0.41	6.90±0.22	6.61±0.15	6.54±0.20
DLC	N (%)	68.38±1.50	70.23±0.99^a	64.92±1.46	68.90±0.84^{ab}	64.55±1.14^b	55.05±1.63^c
	L (%)	31.62±1.49	29.77±0.99^a	35.08±1.46	31.10±0.84^{ab}	35.05±0.96^b	44.85±1.59^c
Platelets ($\times10^3$/µL)		280.8±23.84	303.8±35.68	321.7±30.90	247.4±18.35	287.8±21.66	261.6±14.65

Hb: Haemoglobin, PCV: Packed cell volume, TLC: Total leucocyte count, TEC: Total erythrocyte count, DLC: Differential leucocyte count, N: Neutrophils, and L: Lymphocytes. Mean±SEM with superscripts a and b are statistically significant (P<0.05) within rows

to estrus interval. Neutrophils counts (%) on the day of dystocia had no correlation (r=-0.09; P>0.05) with the period of the expulsion of fetal membranes; however, a positive moderate correlation was found on days 20 (r=0.31; P=0.100) and 45 (r=0.41; P=0.026), indicating that animals with RFM had higher N (%) on subsequent days postpartum. The first postpartum estrus interval (days) also had a weak positive, non-significant correlation (r=0.12; P=0.544) with the period of expulsion of fetal membranes (h), meaning that animals with RFM showed their first postpartum estrus later than those without RFM.

The predicted probability of pregnancy based on logistic regression analysis was analyzed. None of the parameters assessed via logistic regression had a significant (P>0.05) effect on the predicted probability of the animals, irrespective of the treatment; however, the period of expulsion of fetal membranes (h) showed a linear trend (P<0.05; likelihood ration=15.16), which indicates longer periods of reduced probability of animal pregnancy could be a marker of future fertility after dystocia.

Postpartum fertility parameters of animals of both groups were also evaluated. The number of animals showing spontaneous estrus after calving (100% *versus* 86.67%; P=0.483; treatment *versus* control), interval to first AI (74.53 ± 5.95 *versus* 80.64 ± 8.70 days; P=0.554; treatment *versus* control), and open days (91.00 ± 10.05 *versus* 79.90 ± 17.00 days; P=0.557; treatment *versus* control) varied non-significantly (P>0.05) in both groups. The interval between calving to first estrus (44.80 ± 3.06 *versus* 54.15 ± 3.89 days; P=0.067; treatment *versus* control) and non-return rate to first AI (73.33 *versus* 33.33%; P=0.066; treatment *versus* control) tended to vary significantly. The overall pregnancy rate was greater (40% (6/15) *versus* 20% (3/15); P=0.427; treatment *versus* control) in the animals treated with proteolytic enzymes therapy than those of the control, whereas more animals became anestrus in the control group (26.67% (4/15) *versus* 66.67% (10/15); P=0.074; treatment *versus* control).

Discussion

The release of afterbirth (fetal membranes) after calving in buffaloes is a multifactorial association of biochemical, hormonal, metabolic, and immunological changes that start even prior to parturition (Beagly *et al*., 2010). The results obtained in this study supported the hypothesis that intrauterine administration of proteolytic enzymes could hasten the expulsion of fetal membranes, thereby reducing RFM occurrence in the assisted delivery in buffalo cows. The established concept that RFM was associated with reduced protease and collagenase activity (Gunnink, 1984; Musah *et al*., 1987; Eiler and Hopkins, 1992; Bourne *et al*., 2007; Eilder and Fecteau, 2007) could be met with the exogenous supplementation of hydrolytic enzymes which would help in the disruption of caruncle-cotyledon junctions causing ease in the expulsion of fetal membranes.

The more frequent incidence of RFM in association with dystocia could be due to smaller gestation length. Therefore, immature cotyledon-caruncle junctions lead to the failure to separate the fetal membranes (Rajala and Grohn, 1998). Moreover, the use of steroid drugs such as dexamethasone which cause disruption in the activity of collagenase enzyme has been cited as a major contributory factor to the occurrence of RFM (Eilder and Fecteau, 2007; Sheldon *et al*., 2018). The higher levels of cortisol caused by stress, in conjunction with greater serotonin levels in dystociac animals, inhibit the activity of matrix metalloproteinases (MMPs) and could be an attributing factor to the occurrence of RFM (Eiler and Hopkins, 1992; Grohn and Rajala, 2000; Fecteau and Eiler, 2001; Sathya *et al*., 2005; Singh *et al*., 2011).

The endometrial injury created by traction and manipulation at the time of dystocia handling induces the release of heparin from the mast cells leading to the inhibition of collagenase activity (Gross *et al*., 1985; Au *et al*., 1992). In addition, immunosuppression disrupts the release of protease enzymes from the inflamed endometrial epithelium (Fecteau and Eiler, 2001; Eiler and Fecteau, 2007). The external support of proteolytic enzymes, which is due to their hydrolytic activity, could act on the junction of cotyledon and caruncles and therefore, separate the fetal membranes. The increased contractility of the uterine smooth muscles caused by chymotrypsin (Drillich *et al*., 2005) could also be associated with the early expulsion of fetal membranes and the reduction of RFM occurrences.

The buffaloes suffering with difficult calving were found to be more predisposed to uterine infections. This

could be due to the handling of those animals at the time of dystocia, RFM, and inflammation of the endometrium all causing the establishment of pyogenic microbes in the uterus (Usmani *et al*., 2001; Jadon *et al*., 2005; Azawi *et al*., 2008). Also in the present study, we found that more retained fetal membranes in the animals that were not treated with proteolytic enzymes led to delayed involution and development of uterine infections. These proteolytic enzymes act as biological scalpels whereby they hydrolyze the necrosed cells and debris, hence being a prime factor in decreasing the chances of uterine infections postpartum.

The reduced concentration of neutrophils in the treatment group on day 20 and 45 postpartum compared to the control animals could be attributed to the fact that a smaller number of animals had uterine infections, and therefore reduced systemic illness. The presence of higher leucocyte counts on day 20 in the control group could be due to the fact that more animals had uterine infections and showed systemic reactions in response to the illness.

It has been established earlier that the retained fetal membranes have negative impacts on the fertility, production, and economics of dairy enterprise (Dubac *et al*., 2010). The post-partum fertility of the dystocia affected buffaloes in the current study was impaired as compared to that of normal calving. The treatment with proteolytic enzymes helped in the early restoration of the uterine epithelium after the early expulsion of fetal membranes; therefore, more pregnancies were observed in the treatment group compared to the control. The fertility findings of dystocia affected buffaloes with proteolytic enzymatic therapy have not been studied previously. Quershi *et al*. (1997) evaluated the effect of vitamin E and selenium or Bacillie Calmette Guerin (BCG) on post-partum reproductive performance of Nili-Ravi buffaloes and observed that the expulsion of fetal membranes, uterine involution, calving to first postpartum estrus and service were lesser in the treated animals.

Gohar *et al*. (2018) used intrauterine oxytetracycline systemically in buffaloes with retained fetal membranes and found that the interval to the first estrus, number of services per conception and days open were reduced compared to the control animals. Wani *et al*. (2019) supplemented dystocia affected buffaloes with GnRH on various days and found beneficial effects on first postpartum estrus, service period as well as conception rate. Singh *et al*. (2020b) concluded that the administration of $PGF_2\alpha$ and GnRH hastened uterine involution in dystocia affected buffalos.

It could be inferred from the present study that the intrauterine use of proteolytic enzymes i.e Trypsin, Chymotrypsin, and Papain at the 2:2:1 ratio in buffalos suffering from difficult parturition hastened the expulsion of fetal membranes. The reduced occurrence of RFM in treated animals led to decreased uterine infections, and diminished systemic illness in terms of neutrophillic counts. Therefore, an early occurrence of the first postpartum estrus could be observed in these animals as well as those with a well involuted uterus without pregnancy infections compared to animals that have uterine infections. This is the first report on the use of these intrauterine proteolytic enzymes in dystociac buffaloes. A similar study could be carried out on a larger number of animals without using any antimicrobials to test the efficacy of these enzymes as an alternative to antibiotic therapy in dystocia affected animals for the prevention of postpartum uterine infections.

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Conflict of interest

The authors declare no conflict of interest among themselves.

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