International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(3): 285-288

www.veterinarypaper.com
Received: 14-02-2024
Accepted: 29-04-2024

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Intrauterine proteolytic enzyme therapy abates PMN cell count in buffaloes suffering clinical endometritis

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Abstract
The present study was carried out to evaluate the efficacy of proteolytic enzymes therapy in postpartum endometritic buffaloes on recovery in terms of PMN cell percentage and color reaction developed by cervical mucus discharge to white side test. A total of 30 pluriparous buffaloes with signs of clinical endometritis were randomly divided into three groups of ten animals each. The buffaloes of Group I were administered intra uterine with a single dose of proteolytic enzymes *viz*. trypsin, chymotrypsin and papain. Group II buffaloes were treated with ceftiofur sodium intramuscularly and Group III animals served as controls. The cervical mucus was collected from the buffaloes of all groups before and after treatment, and subjected to white side test and endometrial cytology. Before treatment all experimental animals showed positive color reaction to white side test. Whereas, after treatment, 90, 70 and 40 percent of buffaloes were shown negative color reaction to white side test in Group I, Group II and Group III buffaloes, respectively showing highest recovery in Group I treated with proteolytic enzymes. The mean PMN cells percentage before the treatment was 59.3±1.85, 58.9±1.67 and 57.6±2.04, and after the treatment was 6.7±1.13, 21.2±1.35 and 53.8±1.59 in Group I, II and III buffaloes, respectively. The mean PMN percentage revealed significant difference (p<0.05) in Group I and II from pre to post treatment and also revealed significant difference (p<0.05) among groups after treatment. The first service conception rate was highest in Group I (44.44%) followed by Group II (42.85%) and Group III (0%). It can be concluded that intrauterine infusion of proteolytic enzymes declined PMN cells percent in buffaloes with clinical endometritis and might reduce severity of infection.

Keywords: Clinical endometritis, PMN cell percentage, white side test, proteolytic enzymes, endometrial cytology

Introduction
Endometritis, one of the major causes of infertility in bovines is characterised by the presence of purulent or foul discharge, or cervical diameter >7.5 cm between 20 and 33 DIM, or mucopurulent discharge after 26 DIM, when cows are examined between 20 and 33 DIM, and vaginoscopy is performed (LeBlanc *et al*., 2002) [5]. Different diagnostic approaches currently in use for diagnosis of endometritis in cattle and buffaloes include vaginoscopy, bacteriology, white side test, endometrial cytology, ultrasonography and endometrial biopsy. Recent research has shown that, endometrial cytology can quickly diagnose inflammation, as immune cells mainly polymorphonuclear cells attracted to the infection/injury site (Shivhare *et al*., 2018) [9]. In the context of anti-microbial resistance (AMR), finding novel therapeutic approaches have generated some attention, such as use of proteolytic enzymes in endometritis treatment. The proteolytic enzymes *viz* trypsin, chymotrypsin and papain act as biological scalpels, not only have fibrinolytic and proteolytic activity but also supports cellular defence mechanisms (Singh *et al*., 2017) [10]. These enzymes also have hydrolytic properties and cause breakdown of necrotized tissue and debris (Drillich *et al*., 2005) [3]. Additionally, it has been reported that proteases can cause peripheral neutrophil cytotoxicity (Wald *et al*., 2001) [13]. The present investigation was carried out to study the efficacy of proteolytic enzymes in terms of colour reaction of cervical mucus to white side test and PMN cells percentage in buffaloes with clinical endometritis.

Materials and Methods

The present investigation was conducted in buffaloes suffering from clinical endometritis presented to the Department of Veterinary Clinical Complex, College of Veterinary Science, Proddatur and selected nearby Veterinary Dispensaries.

A total of thirty buffaloes, calved at least once and between 90-180 days in postpartum examined by palpation of genital tract per rectum during oestrum and were selected based on the presence of purulent or mucopurulent discharges from vagina. The cervical mucus was collected from all the buffaloes and examined for suppuration and colour reaction to white side test before selection.

The experimental buffaloes were randomly allocated into three equal groups of ten animals each. The Group I buffaloes with endometritis were administered intrauterine infusion with a single dose of proteolytic enzymes in combination of trypsin 16mg (HIMEDIA® Laboratories Pvt. Ltd. Indra Nagar, Bareilly, Uttar Pradesh), chymotrypsin 16mg (Sisco Research Laboratories Pvt. Ltd. Andheri (E), Mumbai-400 099, Maharashtra, India) and papain 8mg (Loba Chemie Pvt. Ltd. Mumbai, Maharashtra, India) (Drillich *et al*., 2005) [3] (Fig 1). The measured dose of each proteolytic enzyme was taken in a single sterile centrifuge tube and was diluted with 20 ml of phosphate buffer saline (PBS, pH 7.4). The solution was infused into the uterus on the day of estrum with the help of artificial insemination gun barrel and sterile sheath. Whereas, Group II buffaloes were administered with broad-spectrum antibiotic ceftiofur sodium (Xyrofur®, INTAS Pharmaceuticals Ltd., Matoda-382 210, Ahmedabad, Gujarat, India) @2.2mg/kg body weight, SID, intramuscularly for three consecutive days from the day of estrus and Group III buffaloes were recommended for sexual rest for one cycle without any treatment and served as controls.

The cervical mucus of buffaloes was collected by using sterile blue sheath fitted to universal artificial insemination gun (IMV Technologies India Pvt. Ltd. Gurgaon Dist., Haryana). The gun was placed either in the mid cervix or uterus, and mucus was sucked into blue sheath due to negative pressure while withdrawing gun. The aspirated cervical mucus was transferred into a sterile tube aseptically. Thereafter, 2 ml of cervical mucus was transferred to sterile test tube and equal quantity of five percent sodium hydroxide solution was added. The mixture was heated to boiling temperature for 2 to 3 minutes. The cervical mucus samples that developed yellow colour after boiling were interpreted as positive for endometritis and samples that didn't show any colour change were interpreted as negative for endometritis (Pateria and Rawal, 1990) [7]. The severity of endometritis was assessed by the intensity of colour developed as, light yellow, yellow and dark yellow for mild, moderate and intense positive, respectively.

For cytology, a drop of mucus sample was placed on a clean grease free glass side. Another clean grease free glass slide was placed over the sample and was spread it uniformly by keeping the slide in 45º angle on first slide. The slide was air dried completely and fixed the smear in methanol for about 1-2 min. After fixing the slide, smear was stained with Field's stain (A and B) (HIMEDIA® Laboratories Pvt. Ltd. Indra Nagar, Bareilly, Uttar Pradesh). The slide was dipped for 5-10 seconds in stain B and rinsed with distilled water to remove excess stain, later the slide was dipped in stain A for 15-20 seconds and rinsed the slide with distilled water to remove excess stain (Gahlot *et al*., 2017) [4]. The slide was allowed to dry in air. The cytological smears were evaluated by counting hundred cells under high power lens (40x) of a light microscope for percent of PMN cells. The count was made in meandering manner and number of PMN cells were counted in all samples before and after treatment. The data generated was analysed statistically according to the method suggested by Snedecor and Chochran (1989) [12].

The treated buffaloes, which were recovered clinically from endometritis, were artificially inseminated 8- 12 hours after onset of estrus with 0.25 ml of fertile frozen thawed semen, in the next immediate heat subsequent to the treatment. All the inseminated buffaloes were monitored regularly and those animals that did not return to heat were subjected to per-rectal examination to confirm the pregnancy after 60 days of insemination. The buffaloes were restrained properly in trevis and rectal contents were removed using a lubricated, gloved hand. Pregnancy was confirmed by observing the positive signs. The first service conception rate was calculated as number of animals conceived to the number of animals inseminated at first estrus.

Results and Discussion

The recovery in terms of color reaction was varied among animals (Table 1; Fig 2). This might be due to induction of peripheral neutrophil cytotoxicity (Wald *et al*., 2001) [13] by proteolytic enzymes (proteases) triggering immune response to eliminate pathogens and damaged cells. The papain enzyme promotes healing by breaking down cell fragments and necrotic tissue. It lowers the pH, which stimulates the synthesis of cytokines that aid in cell repair and decreasing the growth of microorganisms and inflammatory reactions (Leite *et al*., 2012) [6]. Sharma and Srivastava (2018) [8] recorded 100 percent recovery rate in endometritic buffaloes treated with single dose of intrauterine antibiotic ceftiofur sodium which showed higher results than present study findings. These differences might be due to variation in severity of infection, leucocyte number, cellular debris present in cervical mucus and due to different treatment protocols (Sharma and Srivastava, 2018) [8].

There was significant (p<0.05) decline in the percentage of PMN cells from pre to post-treatment in buffaloes treated with proteolytic enzymes and also highest decline in PMN cells noticed when compared with Group II and controls after treatment (Table 2; Fig 3; Plate 1). The proteolytic enzymes *viz.*, trypsin, chymotrypsin and papain cause the expression of interleukin-1β (IL-β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in mononuclear cells (Desser *et al*., 1997) [2] and that would reduce the uterine epithelial inflammation stimulating various immune responses which might indicated by reduction in neutrophils percentage in uterus after treatment. Similar findings were reported by Singh *et al*. (2017) [10], however low PMN cell count than present study was reported after treatment with enzymes. This difference might be due to subclinical infection of cows in their study and immune status. Singh *et al*. (2020) [11] also recorded significant decline in PMN cell percentage in subclinical endometritis buffaloes. Group II buffaloes treated with antibiotic ceftiofur sodium, showed significant (p<0.05) decrease of PMN cells in pre and post treatment, and also with untreated controls. Azawi *et al*. (2008) [1] recorded less PMN percentage in endometritic buffaloes after treatment with oxytetracycline and tylosine, than present study. These differences might be due to different treatment protocols, bacterial load, type of pathogens associated and route of administration. No significant improvement was seen in untreated controls which was similar to the findings of Azawi

et al. (2008) [1].

The first service conception rates in Group I, II and III were 44.44, 42.85 and 0.0 percent, respectively (Table 3; Plate 2). The first service conception rate was higher in Group I compared to Group II and Group III.

Table 1: Effect of treatment protocols on colour reaction to white side test in buffaloes with clinical endometritis

Groups	Colour reaction to white side test					
	Before treatment (%)			After treatment (%)		
	LY	Y	DY	LY	Y	DY
Group I (n=10)	30 (3)	40 (4)	30 (3)	0 (0)	10 (1)	0 (0)
Group II (n=10)	30 (3)	50 (5)	20 (2)	10 (1)	20 (2)	0 (0)
Group III (n=10)	40 (4)	30 (3)	30 (3)	30 (3)	20 (2)	1 (10)

LY=Light Yellow, Y= Yellow, DY=Dark yellow

Table 2: Mean ± SE of percent of polymorphonuclear cells in cervical mucus of endometritic buffaloes before and after treatment.

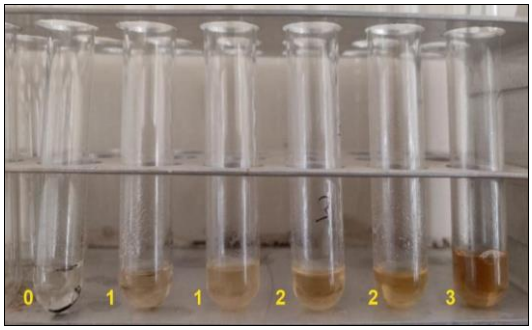
S. No	Groups	No of animals	PMN cells percentage	
			Before treatment	After treatment
1	Group I	10	$59.30\pm1.85^{A,a}$	$6.70\pm1.13^{A,b}$
2	Group II	10	$58.90\pm1.67^{A,a}$	$21.20\pm1.35^{B,b}$
3	Group III	10	$57.60\pm2.04^{A,a}$	$53.80\pm1.59^{C,a}$

Note: Mean ± SE bearing different superscripts (A, B, C column wise and a, b row wise) differ significantly (p<0.05).

Table 3: Recovery and Conception rate after treatment in endometritic buffaloes

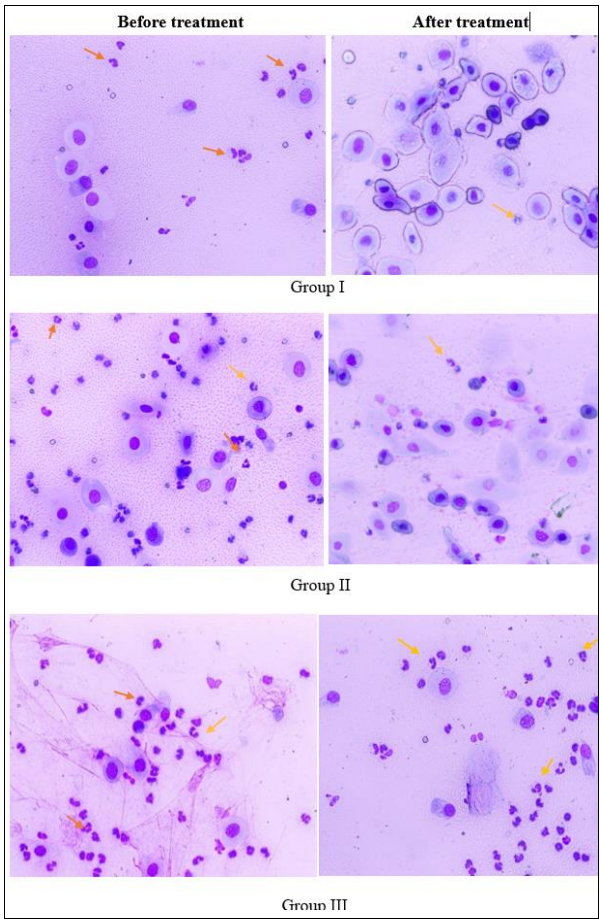
Groups	No of animals	No of animals recovered/inseminated (%)	First service conception rate (%)
Group I	10	9 (90.00)	4 (44.44)
Group II	10	7 (70.00)	3 (42.85)
Group III	10	3 (30.00)	0 (0.0)

Fig 1: Proteolytic enzymes: Papain, Trypsin and Chymotrypsin



0 – no colour, 1- light yellow, 2- yellow, 3 – dark yellow

Fig 2: Colour reaction developed in cervical mucus by white side test



Note: All arrows pointing towards neutrophils (40X)

Fig 3: Effect of treatment protocols on PMN cell percentage in buffaloes with clinical endometritis

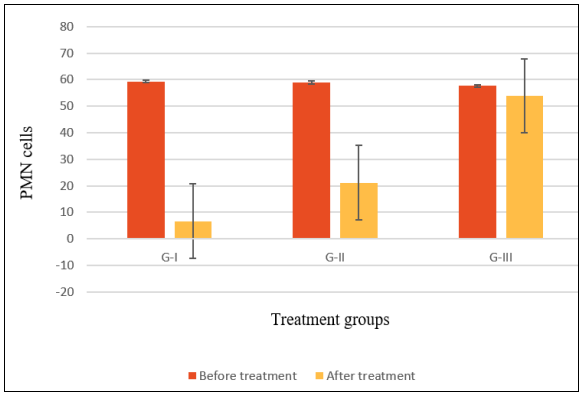


Plate 1: Mean (±SE) PMN cell percentage before and after treatment in different groups of buffaloes with clinical endometritis

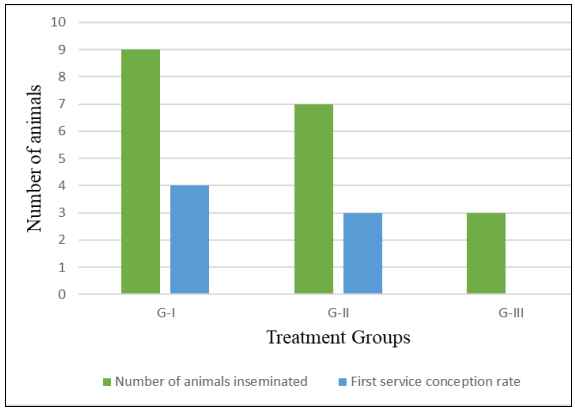


Plate 2: First service conception rates after treatment in different groups of buffaloes with clinical endometritis

Conclusion

The use of hormones and antibiotics in the therapy of uterine infections is under critical public discussion. The alternative therapeutic approaches need to be evaluated. The present study concluded that, the buffaloes with clinical endometritis can be effectively treated with the intrauterine infusion of proteolytic enzymes containing trypsin, chymotrypsin and papain for reducing severity of infection and decreasing PMN cell load and can be considered as an alternative to antibiotic treatment.

Conflict of interest

The authors declare no conflict of interest.

Financial Support

Not available

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How to Cite This Article

Zaben SN, Jyothi K, Anusha K, Raghunath M. Intrauterine proteolytic enzyme therapy abates PMN cell count in buffaloes suffering clinical endometritis. International Journal of Veterinary Sciences and Animal Husbandry. 2024;9(3):285-288.