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Management of Subclinical Endometritis by Immunomodulators and Proteolytic Enzymes

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ABSTRACT

Five hundred repeat breeder cows (2-4 parity) were randomly selected from livestock farm, Kuthuliya and villages of Rewa (M.P.). On the basis of endometrial cytology by cytobrush technique 140 repeat breeder cows (28%) were diagnosed to be suffering from subclinical endometritis. Out of the animals found positive 30 cows were selected and were divided into 5 groups (n=06 per group). The group wise treatment allotted were Group I- Levamisole, 2.5 mg/kg body weight, once for three weeks, S/c; Group II- 100 µgm *E. coli* LPS in 30 ml PBS, single I/U infusion; Group III- 500 mg Benzathine cephapirin, single I/U infusion; Group IV- Proteolytic enzymes in 10 ml distilled water, single I/U infusion and Group V (Control)-no treatment. All the animals were subjected to endometrial cytology at 12 hrs., 24 hrs. and 48 hrs. post-treatment. The animals were bred at next successive oestrus and pregnancy was confirmed 60 days post insemination. Endometrial cytology in treatment group I revealed that the PMN per cent increased significantly ($p<0.05$) from 0 hrs. to 24 hrs. In groups II, III and IV, the increase in PMN per cent was significant ($p<0.05$) from 0 hrs. to 48 hrs. The overall pregnancy rate was higher (83.33%) in treatment groups II and IV as compared to treatment group III (50.00%), group I (33.33%) and group V (0.00%), respectively. It was concluded that immunomodulators like *E. coli* lipopolysaccharide and proteolytic enzymes proved to be better for enhancing uterine immunity and pregnancy rates in subclinical endometritic repeat breeder cows.

HIGHLIGHTS

- Significant increase in PMN percentage was observed in all treatment groups from post-treatment 0 hrs. to 24 hrs.
- First service pregnancy rates were higher in group IV (proteolytic enzymes group).

Keywords: Subclinical endometritis, endometrial cytology, immunomodulators, proteolytic enzymes

High reproductive performance is mandatory for successful management of dairy farms. Reproductive diseases are one of the main hindrances in success of dairy husbandry and causes considerable magnitude of economic losses in livestock sector in India. Repeat breeding has been recognized as one of the worrisome infertility problem in dairy industry.

Repeat breeding syndrome results in lowered dairy profit via wastage of semen and increased insemination cost, increasing intervals to conception, increasing culling and replacement costs, losing genetic gain through increased generation intervals and reducing fertility. Many factors are considered to intervene in repeat breeding, such as impaired embryonic development, peri-ovulatory abnormalities, damage endometrium, stress, and inadequate post-ovulatory progesterone concentrations.

Subclinical endometritis is one of the major contributor to the repeat breeder syndrome (RBS) of bovine subfertility (Ahmadi, 2023). Dairy cattle with 75 per cent exotic blood

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has lower uterine immunity leading to higher incidence (18-25%) of endometritis (subclinical 29.4%, clinical 21.7%), a major cause of repeat breeding syndrome (Singh *et al.,* 2016a).

Subclinical endometritis is defined as an endometrial inflammation occurring 21 days or more after parturition without any clinical signs whereas clinical endometritis is indicated by the presence of purulent/ mucopurulent discharge. Subclinical endometritis causes an abnormal uterine environment and results in repeat breeding. Pathogenic bacteria in the uterus cause inflammation, histological lesions of the endometrium, and a disrupted embryo survival. Therefore, an improvement of the intrauterine environment to enhance embryo survival represents a different therapeutic method for repeat breeding.

Diagnosis of endometritis at field level has been challenging task owing to lack of universally accepted definition of disease as well as the reliability and accuracy of diagnostic tests and treatments (Parikh *et al*., 2022). Routine methods for diagnosing endometritis involve uterine biopsies, lavage, swabs but these may cause irritation and distortion of cells. Recent studies have been focused on sophisticated diagnosis of endometrial alterations beyond clinical signs of endometritis. A novel approach for uterine cytological examination is cytobrush technique which is considered as a reliable method in dairy animals (Bajaj *et al.,* 2016).

Following diagnostic accuracy, one has to use either appropriate antibiotic therapy or any other alternative therapy. The systemic and local (intrauterine) antibiotic therapies have been tried to combat with uterine infections in dairy animals but that after often requires compulsory milk disposed and frequent administration. Apart from high cost of the antibiotic therapy, it also resulted into development of microbial resistance, and decrease in phagocytic activity of polymorphonuclear cells.

Modulation of uterine immunity has been proposed as an alternate therapy which involves single intrauterine infusion of *E. coli* Lipopolysaccharide (*E. coli* LPS) or of oyster glycogen in subclinical endometritic cows (Bajaj, 2015). These treatments have cleared the uterine infection in about 75 to 85 per cent cows probably through enhanced phagocytosis and yielded good (45-83 per cent) conception (Bhuyan *et al*., 2014).

The use of proteolytic enzymes for the intramammary treatment of mastitis has been described as non-antibiotic therapy (Macro L.G. 2016). These enzymes not only have fibrinolytic and proteolytic activity but also support the cellular defense mechanisms and inhibit the growth of microorganisms. It has been observed that certain enzymes *viz.* chymotrypsin, trypsin and papain have fibrinolytic and proteolytic activity and supposed to support cellular defense mechanism and inhibit growth of microorganism (Singh *et al*., 2016b) but the extensive studies are lacking on these aspects.

Keeping above the fact in mind, the present study was conducted to study the comparative efficacy of certain immunomodulators and proteolytic enzymes in enhancing uterine immunity and pregnancy rate in repeat breeder cows suffering from subclinical endometritis.

MATERIALS AND METHODS

A total of 500 repeat breeder cows (2-4 parity) reared under similar managemental conditions were randomly selected from college livestock farm, Kuthuliya and different villages of Rewa (M.P.) for the present study. After recording reproductive history all the animals were subjected to gynaeco-clinical examination and endometrial cytology by cytobrush technique. In the present study, a total of 140 repeat breeder cows (28%) were found positive for subclinical endometritis by endometrial cytology, out of these, 30 repeat breeder cows were selected for the various treatments. These animals were randomly divided into 5 groups (4 treatment groups and 1 control group) of 6 cows each and were subjected to different treatment regimen as follows:

Treatment group I: All animals of this group were treated with subcutaneous injection of levamisole (2.5 mg/kg body weight) once at weekly interval for three weeks.

Treatment group II: All animals of this group were treated with single intrauterine infusion of *E. coli* Lipopolysaccharide (LPS) at the dose of 100 µgm (in 30 ml PBS).

Treatment group III: All animal of this group were treated with 500 mg single intrauterine infusion of Benzathine cephapirin.

Treatment group IV : Animals of this group were treated with crude formulation of enzymes, *viz*. Trypsin 8 mg,

chymotrypsin 8 mg, papain 4 mg, α-tocopherol acetate 120 mg and retinyl palmitate 58.83 mg (Sigma-Aldrich, USA) dissolved in 10 ml distilled water as single intrauterine infusion.

Group V / Untreated control group: All animals of this group were not given any treatment.

All the 30 animals were subjected to endometrial cytology at 12 hrs., 24 hrs. and 48 hrs. post-treatment. These animals were bred on next successive oestrus and pregnancy was confirmed 60 days post insemination by per-rectal examination.

Sample collection

After proper restraining, evacuation of rectum of animals was done through back racking. The perineal region and vulva were washed with antiseptic solution and water and later on disinfected with spirit swab. The vulvar lips were pulled apart by an assistant and the modified cytobrush assembly (Madoz *et. al*., 2014) was inserted in the vaginal orifice. This assembly was fabricated stainless steel device and was attached with a sterile endocervical brush used for Pap smear test in humans. The device was covered with sterile plastic sheath to prevent contamination from vaginal discharges. At the external Os of the cervix the outer plastic sheath was perforated and stainless steel guard and cytobrush was passed into and through the cervix. Upon reaching the uterine body, the cytobrush was advanced beyond the stainless steel guard into the lumen of the uterine body where it was rotated clockwise (360 degrees) to obtain cellular material from the endometrium. The cytobrush and rod was retracted inside the guard and carefully removed from the reproductive tract

Staining method for endometrial cytology

Immediately after removal from reproductive tract, the sample taken for cytological analysis was rolled over a glass microscopic slide, air dried and transported to the lab for examination. The cells were fixed and stained with modified Wright-Giemsa stain and examined under microscope at 400x magnification to identify individual cell types including endometrial cells and polymorphonuclear cells (PMN). A total of 300 cells were counted and PMN cell count was expressed as the proportion of PMN cells counted out of combined PMNs plus endometrial cells. The threshold cut off values for diagnosis of subclinical endometritis by endometrial cytology were more than 5 per cent PMNs as described by Gilbert *et al.* (2005).

Statistical Analysis

The data was analysed using SYSTAT software, Version 12, San-Jose California USA. Data from experiment was presented as Mean ±SE. The pair-wise comparison of means was carried out using Fisher's multiple comparison tests as per standard statistical method described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Efficacy of immunomodulators and proteolytic enzymes and PMN percentage

Therapeutic management of subclinical endometritis is a sophisticated term for therapy, but in broader meaning, besides therapy, it involves prevention at individual animals, as well as at herd level. An ideal therapy for uterine infection should involve in eliminating bacteria from the uterus without inhibiting the normal uterine defense mechanism, improve the fertility and reduces the chances of adulteration of milk and meat for human consumption. Endometrial cytology by cytobrush technique and other parameters used earlier were performed in all the repeat breeder cows suffering from subclinical endometritis, pre- and post-treatment 12, 24 and 48 hrs. for evaluation of recovery. Details of polymorphonuclear cell (PMN) per cent in pre- and post-treatment (12, 24 and 48 hrs. post-treatment) endometrial cytology samples obtained in different treatment groups of repeat breeder cows suffering from subclinical endometritis (SCE) are presented in table 1.

In group I, the variation in PMN per cent was significant ($p<0.05$) from 0 hrs. to 24 hrs. while the variation between 0 hrs. and 48 hrs. was non-significant ($p>0.05$). In group II, III and IV, the PMN per cent varied significantly ($p<0.05$) from 0 to 48 hrs. While PMN per cent variation in control group (Group V) was non-significant ($p>0.05$). The difference in PMN cell per cent in pre-treatment (0 hrs.) endometrial cytology samples between treatment groups was significant ($p<0.05$) between I and III and III and V while it was non-significant between group I and

Table 1: PMN percentage in control and different treatment groups of repeat breeder cows suffering from subclinical endometritis

Groups (n=06 / group)	PMN percentage			
	Pre-treatment	Post-treatment		
	0 hrs.	12 hrs.	24 hrs.	48 hrs.
Treatment group I	$16.40^{ap}\pm0.93$	$26.29^{bp}\pm0.79$	$24.05^{cp}\pm0.40$	$18.00^{ap}\pm0.35$
Treatment group II	$15.07^{apq}\pm0.35$	$42.06^{bq}\pm0.37$	$40.11^{cq}\pm0.39$	$33.22^{dq}\pm0.43$
Treatment group III	$14.28^{aq}\pm0.45$	$30.17^{br}\pm0.88$	$26.22^{cr}\pm0.17$	$20.05^{dr}\pm0.61$
Treatment group IV	$15.17^{apq}\pm0.72$	$44.33^{bs}\pm0.75$	$41.29^{cq}\pm0.70$	$37.11^{ds}\pm0.73$
Group V (Control)	$16.44^{ap}\pm0.87$	$15.00^{at}\pm0.73$	$15.00^{as}\pm0.73$	$16.00^{at}\pm0.95$

Mean with different superscripts within group (a,b,c,d) and between groups (p,q,r,s,t) differ significantly ($p<0.05$).

II, I and IV, I and V, II and III, II and IV, II and V and III and IV. These findings were in accordance with the findings of Bajaj (2015) and Bramhanand (2017). The differences in pre-treatment (0 hrs.) PMN cell per cent in endometrial cytology samples may be due to different level of inflammatory response in the animals of different treatment groups and also the level of infection.

The difference in PMN cell per cent in post-treatment (12 hrs.) endometrial samples was significant between all the groups. Since all the animals were subjected to treatment by immunomodulation, antibacterial and proteolytic enzyme therapies there was rise in PMN cell percentage which reflects that the PMN cells infiltration increased in endometrium to clear off infection.

Significant ($p<0.05$) variation in PMN per cent values at 24 hrs. post-treatment was observed between group I and II, I and III, I and IV, I and V, II and III, II and V, III and IV and III and V while non-significant ($p>0.05$) variation was observed in between II and IV. The rise in PMN cells is maximum in post-treatment 24 hrs. endometrial cytology samples reflects their influx/ infiltration to fight infection. Immunomodulators like *E. coli* LPS and levamisole cause PMN influx in uterine lumen. The influx of PMNs may involve either or combination of mechanisms, *viz.,* vasodilation, chemo attraction and increased population of interleukin-1, interleukin-8 and granulocyte macrophage cell stimulating factor (Tizard, 1998). *E. coli* LPS intrauterine infusion might have caused higher degree of stimulation of uterine defense mechanism (Moraes *et al*., 2017). Prasad *et al.* (2009) reported that treatment with immunomodulators result in significant increase in per cent PMN cells from 24-72 hrs. after administration. Singh *et al*. (2017) advocated the use of levamisole to treat uterine infections due to its potent immunomodulatory action. Kswain *et al*. (2011) also reported that administration of levamisole potentiates both systemic and uterine defense mechanism favoring fertility with subsequent conception rate. This variation in PMN per cent values reflects the efficacy of the therapies used to clear off infection which can also be seen in the form of pregnancy rate.

At post-treatment 48 hrs., significant difference ($p<0.05$) in PMN per cent was observed in all the treatment groups. The proteolytic enzymes *i.e.,* chymotrypsin, trypsin and papain are considered as biological scalpels and have fibrinolytic and proteolytic activity in the inflamed tissue resulting in the breakdown of products of infection, damaged cells and tissues (Singh *et al.,* 2016b). The Gram-positive and Gram-negative bacteria, yeasts and toxins contain protein and lipids that are degraded directly by these enzymes leading to stasis in growth or death of bacterium. Subsequent decline in per cent PMN cells during post-treatment 24 and 48 hrs. after initial PMN influx is indicative of recovery from infection in all the treatment groups.

Fertility response in repeat breeder cows suffering from subclinical endometritis

Fertility response was observed in terms of pregnancy rate on the basis of pregnancy diagnosis by rectal palpation after 60 days of insemination in different treatment groups of repeat breeder cows suffering from subclinical endometritis. The result of pregnancy rate in treatment and control groups has been presented in table 2.

The overall pregnancy rate was higher in *E. coli* LPS and proteolytic enzymes treatment groups may be attributed

Table 2: Pregnancy rate in control and different treatment groups of subclinical endometritic repeat breeder cows

Groups (n=06 per group)	I service pregnancy rate	II service pregnancy rate	Overall pregnancy rate
Group I	1 (16.67)	1 (16.67)	2 (33.33)
Group II	3 (50.00)	2 (33.33)	5 (83.33)
Group III	2 (33.33)	1 (16.67)	3 (50.00)
Group IV	4 (66.67)	1 (16.67)	5 (83.33)
Group V	0 (0.00)	0 (0.00)	0 (0.00)

Figures in paranthesis indicate percentage of pregnancy.

to better clinical recovery as compared to other treatment groups. This may be due to their potent immunomodulation action and chemotactic action increasing PMN cell influx eliminating infection from uterus. Moreover, Singh *et al.* (2016b) reported that the proteolytic enzymes (Trypsin, chymotrypsin, papain, α-tocopherol acetate and retinyl palmitate) not only have fibrinolytic and proteolytic activity but also support the cellular defence mechanism and inhibit the growth of microorganism. Venkatesh *et al*. (2022) evaluated efficacy of various immunomodulators for management of endometritis in postpartum Murrah buffaloes and reported overall conception rate of 88.88, 71.42, 66.66, 75.00 and 33.33 per cent in *E.coli* LPS, Lysozyme, cephalexin and lysozyme + cephalexin and control group animals, respectively. As per the above findings, similar findings were also reported by Bajaj (2015) who obtained a conception rate of 83.33 per cent using intrauterine infusion of *E. coli* LPS as compared to other treatment groups in buffaloes and Bhuyan *et al.* (2015) in cows (83.33 %). The pregnancy rate obtained in present study are higher than the findings of Honparkhe *et al.* (2014) and Singh *et al.* (2016b) who reported 60 per cent conception rate after treatment with single intrauterine infusion of proteolytic enzymes. The pregnancy rates in benzathin cephapirin group in present study are higher than that reported by Ahmadi *et al.* (2005) as 37.50 per cent. None of the animals in untreated control group become pregnant that might be due to their no recovery was found from subclinical endometritis during the study period. The above explanations justify the therapeutic effect of *E. coli* LPS and proteolytic enzymes used in the present study yielding overall better pregnancy rate as compared to other treatment groups.

CONCLUSION

Based on the above study, it can be concluded that Immunomodulators like *E.coli* lipopolysaccharide and proteolytic enzymes proved to be better for enhancing uterine immunity and pregnancy rates (83.33%) in subclinical endometritic repeat breeder cows.

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