Original Article

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FERTILITY RESPONSE IN SUBCLINICAL ENDOMETRITIC MURRAH BUFFALOES TREATED WITH DIFFERENT THERAPEUTIC REGIMENS

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ABSTRACT

The present study was planned to study the therapeutic efficacy of different therapeutic regimes for enhancing uterine immunity and fertility response in Murrah buffaloes suffering from sub-clinical endometritis. Thirty postpartum buffaloes (28 to 45 days postpartum) found positive for sub-clinical endometritis by endometrial cytology in various dairy farms of Jabalpur (Madhya Pradesh) were included in the study. These animals were randomly divided into five treatment (n=06/group) and were subjected to different treatment regimen. Group 1 animals were intramuscularly injected with 500 mcg cloprostenol, Group 2 animals were given 500 mcg Cloprostenol intramuscularly + 500 mg single intrauterine infusion of cephapirin benzathin, Group 3 animals were given single intrauterine infusion of 100 mcg *E. coli* LPS in 30 ml sterile PBS solution, Group 3 animals were given single intrauterine infusion of 500 mg Oyster glycogen in 30 ml sterile PBS solution and Group IV animals were given single intrauterine infusion of 0.25% Lugol's iodine (20 ml). All the 30 buffaloes were tracked for next successive oestrus and were again subjected to endometrial cytology and were bred by natural service and confirmed for pregnancy between 35 to 60 days post-breeding by trans-rectal ultrasonography.

The pre-treatment PMN percent varied significantly ($P<0.05$) between treatment groups. Significant decline ($P<0.05$) in post-treatment PMN percent was observed in all the treatment groups. The first service pregnancy rate was found to be highest (66.67%) in Group 3 and 4. Whereas overall pregnancy rate was higher (83.33%) in treatment Groups 2, 3 and 4.

Keywords: *Bubalus bubalis*, buffaloes, subclinical endometritis, endometrial cytology, fertility response, cytological endometritis, polymorphonuclear cells

INTRODUCTION

Postpartum period in buffaloes is associated with uterine tissue remodeling, restoration of immunological homeostasis and resumption of ovarian cyclicity necessary for

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subsequent fertility. Invasion of bovine uterus with pathogenic bacteria during first 2 weeks of calving and elimination of this contamination during postpartum period has decisive effect on uterine functions and future fertility (Sheldon and Dobson, 2004). Unlike clinical endometritis, subclinical endometritis (SCE), also known as cytological endometritis (Boby *et al*., 2017), is the inflammation of endometrium without clinical manifestations or systemic illness (Kasimanickam *et al*., 2004). Whilst the effects of clinical uterine diseases are often self-evident, subclinical uterine disease can also cause sub-fertility.

Studies on clinical and subclinical endometritis reported the prevalence of these diseases ranging from 18 to 37% (Drillich *et al*., 2005) and 12 to 94% (Barlund *et al*., 2008), respectively. Since subclinical endometritis remains undiagnosed, it causes serious economic losses. Studies had demonstrated the presence of SCE to be a key factor in decreased pregnancy rate, with an increase in days open ranging from 16 days (Dubuc *et al*., 2011) to 50 days (Brodzki *et al*., 2014) and an increase in number of services per conception (Brodzki *et al*., 2014) in subclinical endometritic animals. Several invasive; histopathology and non-invasive diagnostic tests such as ultrasonography, leucocyte esterase strips (LES), endometrial cytology, optical density of uterine lavage have been developed with varying degree of success for diagnosis of SCE in bovines (de Boer *et al*., 2014; Lee *et al*., 2018).

During the last decade, endometrial cytology by cytobrush method has established its superior efficacy and acceptability for diagnosis of SCE as it is non-invasive, easy, and a practical technique generating good picture of various representative cells (Ghasemi *et al*., 2012). The lack of a standard test necessitates the use of reproductive performance as a reference for evaluating diagnostic methods for SCE (Singh *et al*., 2019). Following diagnostic accuracy, one has to use appropriate therapy for management of this condition. Use of antibiotics have also been tried to combat uterine infections with variable success but this often requires frequent administration and milk disposal for fear of antibiotic residues in milk. Apart from high cost of treatment, there are also chances of development of antibiotic resistance and decrease in phagocytic activity of neutrophils. As an alternate or additional therapeutic measure, modulation of uterine immunity has been proposed as a potential therapy which involves intrauterine administration of immunomodulators in endometritic buffaloes. Keeping this in view, the present study was planned to study the therapeutic efficacy of different therapeutic regimes for enhancing uterine immunity and fertility in Murrah buffaloes suffering from sub-clinical endometritis.

MATERIALS AND METHODS

The present study was carried out on 30 postpartum Murrah buffaloes (28 to 45 days postpartum) found positive for sub-clinical endometritis by endometrial cytology in various dairy farms of Jabalpur (Madhya Pradesh). These animals were randomly divided into five treatment groups of 6 animals each and were subjected to different treatment regimen (Table 1).

All the 30 buffaloes were tracked for next successive oestrus and were again subjected to endometrial cytology by cytobrush technique. All the buffaloes were bred by natural service and confirmed for pregnancy between 35 to 60 days post-breeding by per-rectal examination and trans-rectal ultrasonography.

Endometrial cytology by cytobrush technique

After proper restraining, the buffaloes were subjected to evacuation of rectum through back racking. The perineal region and vulva were washed with Chlorhexidine, cetrimide antiseptic liquid 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; Singh, 2014) specially fabricated for buffaloes was introduced in the vagina. The assembly consisted of stainless steel catheter and a stylette attached with cytobrush (Hi-Cytobrush, HI-MEDIA Laboratories Limited, Mumbai, India) and covered with chemie (sterile sanitary over-sheath, 21", IMV Technologies, France). On reaching the external of the cervix, the outer sanitary over-sheath was perforated and the assembly was introduced into the cervix and then to the body of uterus. After assuring its location, the stylette was advanced so as to expose the cytobrush into the lumen of body of uterus where it was gently rotated clockwise and anticlockwise. Gentle pressure was applied on its tip against the uterine body per rectum for proper contact to obtain cellular material from endometrium. The cytobrush and inner stylette were then retracted back into the outer catheter to its normal position and the whole assembly was withdrawn from the reproductive tract. The threshold cut off values for diagnosis of subclinical endometritis by endometrial cytology were >18 % PMNs between day 20 to 33 days postpartum and >10% PMNs between day 34 to 47 days postpartum as described by Kasimanickan *et al*. (2004).

Staining method for endometrial cytology

Immediately after removal from reproductive tract, the cytobrush was rolled over on clean microscopic glass slide, air dried and transported to the laboratory for examination. The slide was exposed to ready to use modified Wright's Giemsa stain solution (Wright's-Giemsa stain, Modified, M/s Sigma-Aldrich Inc., USA) for 2 minutes on staining rack. The stain was then diluted with equal volume of triple glass distilled water and kept for 5 minutes. The slide was then washed with triple glass distilled water and air dried. After drying slides, were screened for the presence of endometrial cells and polymorphonuclear cells (PMN cells) or neutrophils. A total of 300 cells per slide were counted (Melcher *et al*., 2014) counted under the microscope at 400X and 1000X (Oil immersion) and percent PMN cells were calculated.

Statistical analysis

The data was analysed statistically by analysis of variance (ANOVA). The means were compared using Duncan's new multiple range test (DNMRT). Pre-treatment and post-treatment means were compared using Student's t-test as per the standard method described by Snedecor and Cocharn (1994).

RESULTS AND DISCUSSIONS

Endometrial cytology by cytobrush technique

Polymorphonuclear cells (PMN) are the predominant inflammatory cell types found in intrauterine fluid accumulations and determination of the relative proportion of PMN has been shown to be predictors of reproductive performance in the postpartum cows (Kasimanickam *et al*., 2005).

Polymorphonuclear cell (PMN) percent in pre- and post-treatment endometrial cytology samples obtained in different treatment groups of subclinical endometritic buffaloes are presented in Table 2 and Figures 1 to 5.

The percent PMN in pre-treatment

endometrial samples from different treatment groups varied from 18.61±0.96 to 22.89±0.77. The pre-treatment PMN percent varied significantly ($P<0.05$) between Treatment groups. The difference was non-significant ($P>0.05$) between Treatment groups 1 and 3, 1 and 4, 1 and 5 whereas, it was significant ($P<0.05$) between Groups 1 and 2. Percent PMN post-treatment at subsequent oestrus did not varied significantly ($P>0.05$) between different Treatment groups. Significant decline ($P<0.05$) in post-treatment PMN percent was observed in all the Treatment groups.

The difference in PMN cell percent in pre-treatment endometrial samples between the Treatment groups was significant ($P<0.05$) in all groups except Group 2. The differences may be due to different level of inflammatory response in the animals of various treatment groups and also the level of infection. However, no significant variation ($P>0.05$) was observed in post-treatment samples between Treatment groups. Since all the animals were subjected to treatment by immunomodulation, hormonal or anti-bacterial therapies, the recovery in all the Treatment groups as reflected by PMN cell percent were below cut off values. This also reflects on the therapeutic efficacy due to various treatments as evidenced by significant variation in the pre- and post-treatment values in PMN cell percent within Treatment groups. The post-treatment reduction in PMN cell percentage in present study in subclinical endometritic buffaloes treated with $PGF_2\alpha$ might be attributed to stimulation of uterine defence mechanism (Paisely *et al*., 1986) and stimulatory effect of $PGF_2\alpha$ on phagocytic activity of uterine PMNs (Paisely *et al*., 1986).

Similarly, in subclinical endometritic buffaloes treated with a combination therapy of $PGF_2\alpha$ and cephapirin (first generation cephalosporin), the reduction in PMN population in uterine lumen during post-treatment subsequent oestrus might be due to action of $PGF_2\alpha$ along with anti-bacterial action of cephapirin on gram positive and gram negative organisms present in uterine lumen thus clearing off the infection.

Immunomodulators like *E. coli* LPS and oyster glycogen 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 and oyster glycogen intrauterine infusion might have caused higher degree of stimulation of uterine defence mechanism (Singh *et al*., 2000).

Treatment with immunomodulators like *E. coli* LPS and oyster glycogen results in significant increase in percent PMN cells from 24 to 72 h of their administration (Singh *et al*., 2000; Prasad *et al*., 2009).

In Lugol's iodine Treatment group, counter-irritant property of iodine increases uterine blood flow thus potentiating uterine defence mechanism and its potent bactericidal action (Ahmed and Elsheikh, 2014) might have resulted in clearing of infection thereby reducing the PMN cell percentage. Comparatively high non-significant PMN cell percent as compared to other Treatment groups was observed which might be due to irritant property of iodine resulting in coagulative necrosis.

Presence of fibroblasts in higher number is indicative of severity of infection. The marginal presence of fibroblasts in post-treatment samples in present study is indicative of recovery as endometrium is regularly sloughed out and the cells are replaced by either endometrial cells

or fibroblasts. In the present study, we could not collect sequential endometrial samples following treatment (at 24 to 72 h post-treatment) due to limitations of field trial. However, the studies related to PMN cell percentage in post-treatment subsequent oestrus are lacking. Subsequent decline in percent PMN cells during post-treatment oestrus is indicative of recovery from infection in all the Treatment groups.

Fertility response of buffaloes in different treatment groups

Fertility response was recorded in the form of pregnancy rate in different Treatment groups of sub-clinical endometritic postpartum buffaloes and has been depicted in Table 3 and Figure 1 to 9.

Figures in paranthesis indicate percentage of animals

The first service pregnancy rate was found to be highest (66.67%) in Group 3 and 4 followed by 1, 2 and 5 (33.33%) groups. Overall pregnancy rate was higher (83.33%) in Treatment groups 2, 3 and 4 as compared to Treatment group 1 (66.67%) and 5 (50.00%).

The higher first service pregnancy rate in immunomodulator (*E. coli* LPS and oyster glycogen) Treatment groups may be attributed 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. Saini *et al.* (1995); Singh *et al.* (2000) reported that a single intrauterine *E. coli* LPS infusion (100 µgm) suppressed the growth of bacterial flora in uterus and up to 80% cattle with sub-clinical endometritis conceived. This supports the findings of the present study. Singla *et al.* (2004) also concluded that intrauterine administration of *E. coli* LPS alone or in combination with autologus serum increased conception rate upto 75 and 80%, respectively.

However, Dadarwal *et al.* (2007) obtained a conception rate of 45 to 66.67% using intrauterine infusion of *E. coli* LPS as compared to untreated controls. Prasad *et al.* (2009) also observed higher conception rate with *E. coli* LPS and oyster glycogen (75%, 9/12) when compared with that obtained by the use of enrofloxacin (58.3%, 7/12) Treatment and Control (16.7%, 2/12).

In another study, Biswal *et al.* (2014) studied the effect of immunomodulators on acute phase proteins and conception rates in endometritic cows and reported an overall conception rate of 60% in oyster glycogen treated group followed by 50% conception rate in levamisol and $PGF_2\alpha$ treated groups. On the other hand, Rathod *et al.* (2014) compared efficacy of levamisol and oyster glycogen on fertility of cross-bred cows suffering from subclinical endometritis and reported higher conception rate (62.50%) in cows treated with oyster glycogen and levamisol (37.50%), however it was 12.50% in Control group.

The above explanations justify the therapeutic effect of immunomodulators and $PGF_2\alpha$ along with benzathin cephapirin used in the present study yielding overall better conception rate as compared to other Treatment groups.

CONCLUSION

It could be concluded that immunomodulators like *E. coli* lipopolysaccharide and oyster glycogen and combination of $PGF_2\alpha$ and cephapirin benzathin were found to be the most effective treatments followed by $PGF_2\alpha$ and Lugol's iodine. Administration of immunomodulators like

Table 1. Therapeutic regimen followed in different treatment groups of subclinical endometritic postpartum Murrah buffaloes.

Treatment Groups (n=06 animals/group)	Treatment given
Group 1	Single intramuscular injection of 500 mcg cloprostenol after CL palpation
Group 2	Combination therapy of single intramuscular injection of 500 mcg Cloprostenol after CL palpation and 72 h later followed by 500 mg single intrauterine infusion of cephapirin benzathin
Group 3	*E. coli* lipopolysaccharide (M/s Sigma-Aldrich Inc., USA) at the dose rate of 100 mcg in 30 ml sterile phosphate buffer saline solution (PBS, pH=7.0, M/s HI-MEDIA laboratories Pvt. Ltd., Nasik, India), single intrauterine infusion
Group 4	Oyster glycogen (M/s Sigma-Aldrich Inc., USA) at the dose rate of 500 mg in 30 ml sterile phosphate buffer saline solution (PBS, pH=7.0, M/s HI-MEDIA laboratories Pvt. Ltd., Nasik, India), single intrauterine infusion
Group 5	0.25%, 20 ml Lugol's Iodine solution, single intrauterine infusion

Table 2. Endometrial cytology in different treatment groups of subclinical endometritic postpartum Murrah buffaloes.

Sub-clinical endometritis treatment groups (n=06 per group)		Endometrial cytology				
		PMN*	Endometrial cells*	Fibroblasts*	RBC*	PMN %
Group 1	Pre-treatment	66.83^{a}±2.21	236.50^{b}±3.53	0.00±0.00	0.00±0.00	22.28^{a}±0.74
	Post-treatment	13.00±5.94	285.00±7.30	1.33±0.80	0.00±0.00	4.33±1.99
Group 2	Pre-treatment	55.83^{b}±2.87	244.17^{a}±2.87	0.00±0.00	0.00±0.00	18.61^{b}±0.96
	Post-treatment	6.83±1.28	292.17±1.52	0.50±0.34	0.00±0.00	2.28±0.424
Group 3	Pre-treatment	68.33^{a}±2.88	229.17^{a}±2.37	0.50±0.34	0.67±0.42	22.78^{a}±0.96
	Post-treatment	7.67±1.12	291.33±1.20	1.00±0.82	0.00±0.00	2.56±0.37
Group 4	Pre-treatment	68.67^{a}±2.31	228.33^{a}±1.56	0.33±0.21	2.83±1.52	22.89^{a}±0.77
	Post-treatment	6.50±1.60	291.33^{a}±2.60	0.17±0.17	1.83±1.32	2.17±0.54
Group 5	Pre-treatment	66.00^{a}±2.10	229.17^{a}±2.26	0.17±0.17	6.17±1.64	22.00^{a}±0.70
	Post-treatment	20.67±7.35	276.33±6.39	2.50±1.15	1.17±0.83	6.89±2.45

The means with the same superscript within the column did not differ significantly (P>0.05).

*Numbers per 300 cells counted

Table 3. Pregnancy rate in different treatment groups of sub-clinical endometritic postpartum buffaloes.

Sub-clinical endometritis treatment groups (n = 06 per group)	Group I service pregnancy rate	Group II and III service pregnancy rate	Overall pregnancy rate
Group 1	2 (33.33)	2 (33.33)	4 (66.67)
Group 2	2 (33.33)	3 (50.00)	5 (83.33)
Group 3	4 (66.67)	1 (16.67)	5 (83.33)
Group 4	4 (66.67)	1 (16.67)	5 (83.33)
Group 5	2 (33.33)	1 (16.67)	3 (50.00)

E. coli lipopolysaccharide and oyster glycogen resulted in better first service pregnancy rate (66.67%), which might be due to the enhancement of uterine immunity in postpartum sub-clinical endometritic buffaloes.

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