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IMPROVING FERTILITY OUTCOMES THROUGH NOVEL INTERVENTIONS IN DAIRY COWS BY FOCUSSING ON PROTEOLYTIC ENZYMES AND ANTIOXIDANTS

Shashank Vishvakarma1, Nitin Kumar Bajaj1*, Vishnu Gupta5, Bhavana Gupta3, Renuka Mishra1, Pankaj Umar2, Aditya Pratap4 and Jyoti Dagar2

1. Department of Veterinary Gynaecology & Obstetrics, College of Veterinary Science & Animal Husbandry, Jabalpur (M.P.), Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.), INDIA-482001
2. Department of Veterinary Pharmacology & Toxicology, College of Veterinary Science & Animal Husbandry, Jabalpur (M.P.), Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.), INDIA-482001
3. Department of Veterinary Public Health, College of Veterinary Science & Animal Husbandry, Jabalpur (M.P.), Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.), INDIA-482001
4. Department of Veterinary Medicine, College of Veterinary Science & Animal Husbandry, Jabalpur (M.P.), Nanaji Deshmukh Veterinary Science University, Jabalpur (M.P.), INDIA-482001
5. Veterinary Assistant Surgeon, Barela (Jabalpur), Department of Animal Husbandry, Government of M.P.INDIA-482001

ABSTRACT

The current investigation aimed to assess the therapeutic effectiveness and fertility outcomes of proteolytic enzymes, namely lysozyme and N-acetyl cysteine, in the management of subclinical endometritis in repeat breeder buffaloes within the framework of Edwin Incorporation. Eighteen repeat breeder buffaloes exhibiting subclinical endometritis were randomly assigned to three treatment groups, each comprising six animals. These buffaloes underwent distinct treatment protocols.

In Group I, animals were administered a 4 mg lysozyme solution in 40 ml normal saline through intrauterine infusion once daily for two consecutive days. In Group II, animals received a single intrauterine infusion of 5% N-acetyl cysteine dissolved in 60 ml normal saline. Group III animals were treated with a crude enzyme formulation, including 8 mg trypsin, 8 mg chymotrypsin, 4 mg papain, 120 mg α-tocopherol acetate, and 58.83 mg retinyl palmitate dissolved in 10 ml distilled water, as a single intrauterine infusion.

Endometrial cytology was conducted at 0 hrs., 24 hrs., and 48 hrs. post-treatment for all 18 animals. Microbial assays were performed at day 0, successive estrus, and post-treatment, followed by breeding after sampling. Pregnancy diagnosis was carried out through per rectal examination at day 60 post-insemination.

A statistically significant increase ($p<0.05$) in PMN percentage was noted between 0 and 24 hrs. post-treatment and 0 and 48 hrs. post-treatment across all treatment groups. Among the 18 post-

treatment uterine samples, seven yielded bacterial isolates, with Staphylococcus species (57.14%) being predominant. The overall conception rate (%) was higher in Group I (100.00%), followed by Group II (83.33%) and Group III (50.00%).Intrauterine administration of lysozyme demonstrated superior efficacy in enhancing uterine immunity and improving the conception rate for the management of subclinical endometritis in repeat breeder buffaloes within the scope of Edwin Incorporation.

Keywords: Subclinical endometritis, endometrial cytology, lysozyme, proteolytic enzymes, N-acetyl cysteine.

INTRODUCTION

Good fertility and optimum reproductive efficiency of dairy animals is the key to economically successful dairy farming. After the postpartum period, repeat breeding is considered one of the most important reproductive disorders in bovines (Yusuf et al., 2010). A repeat breeder affect the production of farm severely as it may be associated with subclinical endometritis, delayed ovulation and corpus luteum deficiency, which cause failure of fertilization or embryonic mortality (Parkinson, 2009). Subclinical endometritis (SE) is an asymptomatic reproductive disorder characterized by presenting an infiltrate of PMN with or without exudate in uterus lumen (Escandon et al. 2020). Prevalence of subclinical endometritis in postpartum lactating dairy cows has varied between 7 and 53% (Lopdell et al., 2011), and its presence was associated with repeat breeder cows after artificial insemination (Salasel et al. 2010; Janowski et al., 2013). Since subclinical endometritis is a non-symptomatic disease, it must be diagnosed and treated promptly to avoid their injurious effects on reproduction (Rinaudo et al., 2017).

Antibiotic therapy has various side effects and also needs regular and frequent administration and besides that compulsory milk disposal in dairy animal is recommended; hence it is the need of the hour to opt for alternative therapies (Singh et al., 2009).

As an alternative to therapy immunomodulators and proteolytic enzymes (chymotrypsin, trypsin and papain) were described as non-antibiotic therapy. Intrauterine immunomodulator infusion stimulates uterine defence mechanism (Bajaj, 2015) while proteolytic enzymes have fibrinolytic and proteolytic activity, along with that it also supports the cellular defence mechanisms and inhibit the growth of microorganisms (Singh et al., 2016).

Lysozyme also act as immunomodulator and causes lysis of the cell wall of bacteria which is comprised of mucopolysaccharide, joins by 1,4-glycosidic linkages between N acetyl muramic acid (NAMA) and N-acetyl glucosamine (NAG), lysozyme catalyses the hydrolysis of 1,4-glycosidic bond between NAMA and NAG in cell wall peptidoglycan.

N-acetyl cysteine (NAC) in human medicine widely used in the treatment of chronic obstructive pulmonary diseases (COPD) and tuberculosis. Beside mucolytic effect, NAC, also has antioxidant (AHFS Drug Information, 2010), cytoprotective and anti-inflammatory properties (Melkus et al., 2013). NAC facilitates entry of antimicrobials into mucus and its mucolytic effect is route-independent (Boothe, 2001). Various author states that intrauterine NAC administration to mares with endometritis may help improve pregnancy and cure rates, and that NAC does not adversely

affect endometrial function (Witte et al., 2012). Keeping above facts in mind the present study was designed to evaluate the therapeutic efficacy and fertility response of proteolytic enzymes, lysozyme and N-acetyl cysteine for management of subclinical endometritis in repeat breeder buffaloes.

MATERIALS AND METHODS

The present study was carried out on 18 repeat breeder buffaloes suffering from subclinical endometritis (diagnosed to be suffering from subclinical endometritis by endometrial cytology using cytobrush technique) which were randomly selected from 28 buffaloes suffering from subclinical endometritis in various dairy farms of Jabalpur (Madhya Pradesh). The buffaloes (3rd to 4th parity) selected for study were having body condition score (BCS) between 3-5 in 5-point scale. The buffaloes were stall fed and kept in cemented shed with brick floor under intensive housing system supplemented with mineral mixture. Seasonally available green fodder with wheat straw and concentrate mixture were fed to all animals. During morning and evening, clean drinking water was provided ad lib. The feeding system of private farms in Jabalpur area was almost similar. These animals were randomly divided into three treatment groups of 6 animals each and were subjected to different treatment regimen as follows:

1. Treatment Group I (Group I; n=06): Repeat breeder buffaloes diagnosed as suffering from subclinical endometritis were subjected to treatment with 4 mg lysozyme dissolved in 40 ml normal saline having a pH of 7.4 was infused intrauterine with a sterile AI sheath once for two consecutive days.
2. Treatment Group II (Group II; n=06): Repeat breeder buffaloes diagnosed as suffering from subclinical endometritis were subjected to treatment with 5% N-acetyl cystiene (dissolved in 60 ml normal saline) as single intrauterine infusion.

Plate 01: Endometrial cytology sample collection by cytobrush technique

3. Treatment Group III (Group III; n=06): Repeat breeder buffaloes diagnosed as suffering from subclinical endometritis were subjected to treatment 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. Endometrial cytology was done at 0, 24, 48 hours post-treatment in all the treatment group animals. Oestrus detection were be done by visual signs viz. CVM discharge and mounting behaviour etc. Culture isolation was done at day 0 and next successive oestrus in all treatment group buffaloes. All the treatment group buffaloes were bred by artificial insemination on next successive oestrus. Pregnancy diagnosis was done by per rectal examination at day 60 post insemination.

A. Endometrial cytology by cytobrush technique

i. Collection of endometrial sample by cytobrush technique - After proper restraining, the animals were subjected to evacuation of rectum 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) specially fabricated for buffaloes was introduced in the vagina.

The assembly consisted of stainless-steel catheter and a stylette attached with cytobrush and covered with chemie. Endometrial cytology was carried out according to Senosy et al. (2012). Endometrial cytology samples were collected by rotating the brush while in contact with the uterine wall (uterine body). The brush was retracted into the stainless-steel tube prior to removal from the uterus (Plate 01). Cytology slides were prepared by rolling the brush on two clean glass microscope slides for each sample and fixed with cytofixative (Ahmadi et al., 2012).

The threshold cut off value for PMN percentage for diagnosis of subclinical endometritis by cytobrush technique was >5% PMNs (Gilbert et al., 2005).

ii. Staining of endometrial cytology slide

Slide was stained with modified Giemsa stain solution for 2 minute and after 2 minutes slide was washed with running tap water and then air dried. After drying slides, were screened for the presence of polymorphonuclear cells (PMNs) or neutrophils endometrial cells, fibroblasts and RBCs if any. A total of 300 cells were counted under the binocular microscope at X400 and X1000 (oil immersion) and per cent PMN cells were calculated.

B. Bacteriological examination and culture isolation from endometrial tissue:

The endometrial samples collected aseptically by cytobrush technique were also be subjected to culture isolation and identification of bacterial micro-organisms.

Statistical analysis

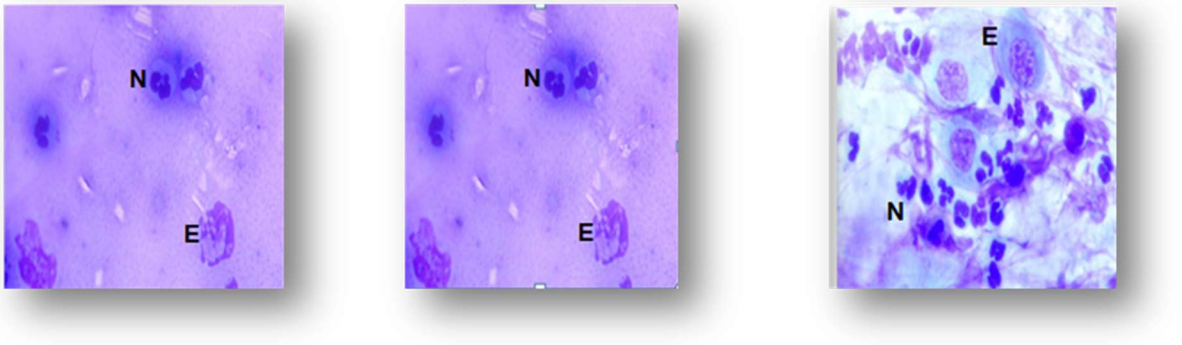
The data ware analysed statistically by analysis of variance (ANOVA). The means were compared using Duncan's multiple range test (DMRT). Pre-treatment and post-treatment means were compared using f-test as per the standard method described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Elimination of pathogen from the genital tract of animal is necessary to restore the fertile life of animal and to improve the production of farm. Hence, the therapeutic management of subclinical endometritis must be aimed towards the same without causing any harm to future fertility. In order to maintain the same, proper diagnosis with high degree of certainty must be applied. Since long, the diagnosis and treatment of repeat breeder buffaloes suffering from subclinical endometritis remains a challenging task for veterinarians. The efficacy of treatment depends on the accuracy of diagnosis, line of treatment chosen and individual response of an animal towards the treatment. The success of treatment must be calculated with regard to healing rate and subsequent reproductive performance in the form of future fertility.

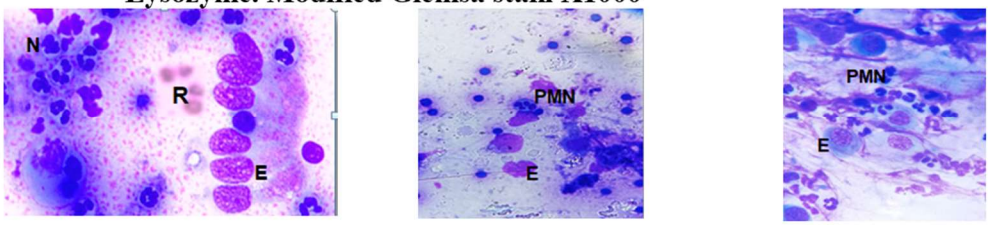
A. Endometrial cytology by cytobrush technique

Endometrial cytology findings and Polymorphonuclear cell (PMN) per cent at 0 hrs. (pre-treatment), 24 hrs. and 48 hrs. (post-treatment) endometrial cytology samples obtained in different treatment groups of repeat breeder buffaloes suffering from subclinical endometritis (SCE) are presented in table 01-02 and plates 02-04.



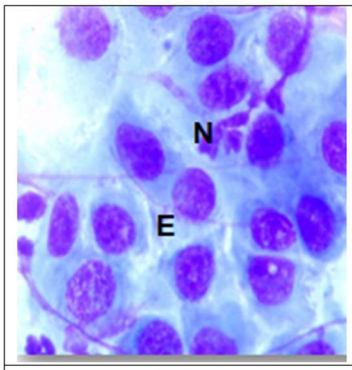
0 Hours **24 Hours** **48 Hours**

Plate 02: Endometrial smear from repeat breeder buffalo suffering from subclinical endometritis at 0 hrs., 24 hrs. and 48 hrs. showing endometrial cells (E) and polymorphonuclear cells (N) after treatment with intrauterine infusion of 4mg Lysozyme. Modified Giemsa stain X1000

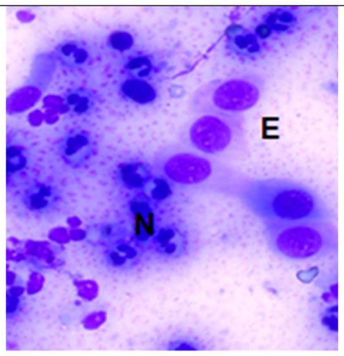


0 Hours **24 Hours** **48 Hours**

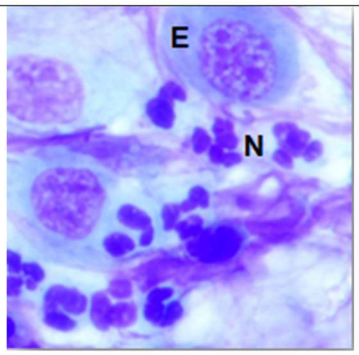
Plate 03: Endometrial smear from repeat breeder buffalo suffering from subclinical endometritis at 0 hrs. 24 hrs. and 48 hrs. showing endometrial cells (E) and polymorphonuclear cells (PMN) after treatment with intrauterine infusion of 5% N- acetyl cysteine. Modified Giemsa stain X1000



0 Hours



24 Hours



48 Hours

Plate 04: Endometrial smear from repeat breeder buffalo suffering from sub-clinical endometritis AT 0 hrs., 24 hrs. and 48 hrs. showing endometrial cells (E) and polymorphonuclear cells (PMN) after treatment with with intrauterine infusion of proteolytic enzymes. Modified Giemsa stain X1000

Table 01: Endometrial cytology findings in different treatment groups of repeat breeder buffaloes suffering from subclinical endometritis

Endometrial Cytology (Total cell = 300)	Pre / Post-treatment		Group(n=06/group)		
			I	II	III
PMN (n)	Pre-treatment	00 hrs.	$26.00^{c}\pm3.47$	$36.83^{b}\pm7.63$	$37.50^{b}\pm5.59$
	Post-treatment	24 hrs.	$58.00^{b}\pm4.99$	$51.50^{a}\pm6.56$	$68.00^{a}\pm5.49$
		48 hrs.	$70.00^{a}\pm4.95$	$56.17^{a}\pm8.93$	$66.33^{a}\pm4.57$
Endometrial Cells (n)	Pre-treatment	00 hrs.	$268.83^{a}\pm3.52$	$251.17^{a}\pm8.02$	$255.67^{a}\pm5.58$
	Post-treatment	24 hrs.	$236.50^{b}\pm5.52$	$241.83^{b}\pm6.66$	$225.33^{b}\pm5.53$
		48 hrs.	$224.33^{c}\pm5.22$	$237.67^{b}\pm8.94$	$227.50^{b}\pm5.51$

Erythrocytes (n)	**Pre-treatment**	00 hrs.	1.83^{a}±0.60	2.17^{a}±0.60	2.00^{a}±0.73
	Post-treatment	24 hrs.	2.67^{a}±0.71	2.50^{a}±0.50	2.17^{a}±0.60
		48 hrs.	2.50^{a}±0.43	3.00^{a}±0.93	2.00^{a}±0.52
Fibroblasts (n)	**Pre-treatment**	00 hrs.	3.33^{a}±0.33	3.83^{a}±0.87	4.83^{a}±0.83
	Post-treatment	24 hrs.	2.83^{a}±0.95	4.17^{a}±0.98	4.50^{a}±0.72
		48 hrs.	3.17^{a}±0.60	3.17^{a}±0.48	4.17^{a}±1.19
PMN %	**Pre-treatment**	00 hrs.	8.67^{b}±1.16	12.28^{b}±2.32	12.50^{b}±1.86
	Post-treatment	24 hrs.	19.33^{a}±1.66	17.17^{a}±2.19	22.67^{a}±1.83
		48 hrs.	23.33^{a}±1.65	18.72^{a}±2.98	22.11^{a}±1.52

Mean values bearing different superscripts (a, b) in a column differ significantly ($p<0.05$)
*Average of mean number per 300 cells counted

The per cent PMN in pre-treatment endometrial samples from different treatment groups varied from 8.67±1.16 to 12.50±1.86. The variation in pre-treatment PMN per cent (0 hrs.) was non-significant ($p>0.05$) between different groups treatment groups.

Table 02: Effect of different treatment regimens om PMN% in endometrial cytology at 0hrs., 24hrs. and 48hrs. post-treatment in repeat breeder buffaloes suffering from subclinical endometritis

Groups (n=06/ group)	**PMN percentage**		
	Pre-treatment	**Post-treatment**	
	0 hrs.	**24 hrs.**	**48 hrs.**
I	08.67^{b}±1.16	19.33^{a}±1.66	23.33^{a}±1.65
II	12.28^{b}±2.32	17.17^{a}±2.19	18.72^{a}±2.98
III	12.50^{b}±1.86	18.72^{a}±2.98	22.11^{a}±1.52

Mean values bearing different superscripts (a, b) in a column differ significantly ($p<0.05$)
*Average of mean number per 300 cells counted

The variation in post-treatment PMN per cent at 24 hrs. and 48 hrs. was also non-significant ($p<0.05$) between different groups treatment groups. Significant increase ($p<0.05$) in PMN per cent was observed between 0 and 24 hrs. post-treatment and 0 and 48 hrs. post-treatment in all the treatment groups while this increase was non-significant ($p>0.05$) between 24. and 48 hrs. Post treatment in all the treatment groups. The findings of present study are in corroboration with the findings of Manjhi (2018) who also observed significant ($p<0.05$) increase in PMN per cent in all the treatment groups (Levamisole, E.coli LPS, cephaprrin benzahthin and proteolytic enzymes groups) from 0 hrs. to 24 hrs. post-treatment while the variation was non-significant ($p>0.05$) between 24 and 48 hrs. post treatment.

Caissie et al. (2020) studied effect of 3.3% NAC on mares susceptible to persistent breeding induced endometritis (PBIE) and reported that per cent PMN was significantly higher in 12 hours post-AI compared to 60 hrs. post AI. They opined that NAC infusion was associated with a significant and prolonged acute inflammatory reaction of the endometrium, which was suspected due to hyperosmotic nature of NAC.

Choudhary (2022) reported significant decrease in the pre- and post-treatment (subsequent oestrus) PMN per cent within treatment groups suggestive of good efficacy of all the treatment groups. Similar significant decrease in PMN cell per cent in subsequent oestrus were also reported by Bajaj (2015).

Unlike increase in PMN percentage, there was an increase in PMN counts within the group that shows significant difference ($P<0.05$) from 0 hrs. to 24 hrs. and non-significant difference ($P>0.05$) from 24 hrs. to 48 hrs. in all the treatment groups except that of group I.

In group I, the PMN cell counts increase significantly ($P<0.05$) from 0 hrs (pre-treatment) to 48 hrs. post treatment. The repeat breeder buffaloes in lysozyme group (group I) might be due to intra-uterine infusion 4 mg of lysozyme consecutively for 2 days (0 hrs. and 24. hrs.). This significant increase may be because of lysozyme's continued immunomodulatory activity that allows the infiltration of neutrophils within uterine lumen and supports antibacterial property (Manjhi, 2018). Similarly it has been reported that immunomodulators like E. coli LPS and lysozyme are related to significant PMN influx in uterine lumen from 24-72 hours after administration (Manjhi, 2018). The acute increase in PMN count at different time interval in different treatment groups might be due to the immunomodulator property of lysozyme, N-acetyl cysteine and proteolytic enzyme, which marks the beginning of healing mechanism and increased phagocytosis by PMN cells ultimately supporting the uterine defence mechanism in order to clear the uterine infection.

PMN influx may involve single or combined mechanisms of vasodilation, chemo-attraction, and increased populations of interleukin-1, interleukin-8, and granulocyte-macrophage cell-stimulating factor (Tizard, 2017). a proteolytic enzyme, i.e. Chymotrypsin, trypsin, and papain, which are considered biological scalpels, have fibrinolytic and proteolytic activity in inflamed tissue, causing the degradation of infectious products, damaged cells and tissues (Singh et al., 2016). Gram-positive and Gram-negative bacteria, yeasts, and toxins contain proteins and lipids that are directly degraded by these enzymes, resulting in bacterial growth arrest or death (Kruger

et al., 1999). Fibroblasts were also observed in the endometrial cytology in both pre- and post-treatment samples.

According to Bajaj et al. (2018) high involvement of fibroblast is indicative of severe degree of infection while the marginal count post treatment is related to healing.

B. Bacteriological examination and culture isolation from endometrial tissue:

The detailed results of bacterial isolates recorded from uterine samples at day 0 and next successive oestrus in various treatment groups of subclinical endometritic repeat breeder buffaloes are presented in table 03. The post-treatment increase in PMN per cent might have reduced the bacterial load by their phagocytic activity in the uterine lumen. The difference in the lysozyme group, N-acetyl cysteine and proteolytic enzymes group with respect to bacterial isolates obtained may be because of higher efficacy of the therapeutic agents used in lysozyme group.

Table 03: Bacterial isolates obtained from uterine sample of repeat breeder buffaloes suffering from subclinical endometritis

Groups (n=06/ group)	Treatment	Bacterial Isolates		Positive Isolates			Type of bacterial isolates			
		Negative	Positive	Single type	Mixed type	Total	*Streptococcus* spp.	*Staphylococcus* spp.	*E. coli*	*Bacillus* spp.
I	Pre-treatment	0 (00.00)	6 (100.00)	3 (50.00)	3 (50.00)	9	3 (33.33)	5 (55.55)	0 (00.00)	1 (11.11)
	Post-treatment	4 (66.67)	2 (33.33)	2 (100.00)	0 (00.00)	2	0 (00.00)	1 (50.00)	0 (00.00)	1 (50.00)
II	Pre-treatment	0 (00.00)	6 (100.00)	2 (33.33)	4 (66.67)	10	1 (10.00)	6 (60.00)	2 (20.00)	1 (10.00)
	Post-treatment	3 (50.00)	3 (50.00)	3 (100.00)	0 (00.00)	3	1 (33.3)	2 (66.67)	0 (00.00)	0 (00.00)

III	Pre-treatment	0 (00.00)	6 (100.00)	2 (33.33)	4 (66.67)	10	2 (20.00)	4 (40.00)	3 (30.00)	1 (10.00)
	Post-treatment	4 (66.67)	2 (33.33)	2 (100.00)	0 (00.00)	2	1 (50.00)	1 (50.00)	0 (00.00)	0 (00.00)
Overall Pre-treatment (n=18)		0 (00.00)	18 (100.00)	7 (38.89)	11 (61.11)	29	6 (20.69)	15 (51.72)	5 (17.24)	3 (10.34)
Overall Post-treatment (n=18)		11 (61.11)	7 (38.89)	7 (100.00)	0 (00.00)	7	2 (28.57)	4 (57.14)	0 (00.00)	1 (14.28)

Figure in paranthesis indicate percentage

Solomon et al., (2009) also reported extreme decrease in bacterial count and increased recovery rate (83.30%) and conception rate (53.30%) by using lysozyme (2 mg) as intrauterine immune modulators for management of endometritis as compared to phosphate buffer saline (50 ml).
However, many research workers like Palanisamy et al. (2015) observed that LPS treatment was most effective in controlling uterine infections by reducing bacterial count followed by lysozyme and oyster glycan.

C. Fertility response of in subclinical endometritic repeat breeder buffaloes

Fertility response in the present study was observed in terms of conception rate on the basis of pregnancy diagnosis by per-rectal palpation on day 60 of post-insemination in different treatment groups of repeat breeders buffaloes suffering from subclinical endometritic. The result of conception rate in treatment groups has been presented in table 04.

Table 07: Conception rates in different treatment groups of repeat breeder buffaloes suffering from subclinical endometritis

Groups (n=06/group)	Conception rate		
	I service	II service	Overall
I	5 (83.33)	1(16.66)	6 (100.00)
II	3 (50.00)	2 (33.33)	5 (83.33)

III	2 (33.33)	1 (16.33)	3 (50.00)

Figure in parenthesis indicate percentage of animals.

The higher first service conception rates in treatment group I (Lysozyme group) may be because of 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 and also because of potent bactericidal action of lysozyme on gram positive bacteria. In the present study lysozyme was used twice at 24 hrs. interval which might have potentially increased PMN influx to combat infection which was also evident by significant rise in PMN numbers in 24 and 48 hrs. post treatment endometrial cytology samples as compared to day 0 sample and decrease in bacterial isolates of post treatment in subsequent oestrus. Choudhary (2022) reported first service conception rates of 50.00 per cent using 4 mg lysozyme single dose in repeat breeder buffaloes suffering from subclinical endometritis.

Solomon et al, (2009) by using lysozyme (2mg) in endometritis affected bovine as compared to phosphate buffer saline (50 ml) reported increased recovery (83.30%) and conception rate (53.30%). All these works and their results support the finding of present study.

CONCLUSION

Based on the above study, it can be concluded that the intra uterine administration of lysozyme proved to be better for enhancing uterine immunity and conception rate (100.00%) for management of subclinical endometritis in repeat breeder buffaloes.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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