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Evaluation of Prophylactic Cloprostenol and *E. coli* LPS Treatment Against Endometritis in Dairy Cows and Buffaloes

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Abstract: A study was conducted on 24 Murrah buffaloes (9 normal and 15 assisted calving) and 21 cross bred Holstein Frisian cows (6 normal and 15 assisted calving) to evaluate two immunomodulatory prophylactic treatments against endometritis. Animals were randomly assigned to three treatment groups i.e. single intrauterine normal saline on 7 days in milk (DIM) (Controls, n=15), double intramuscular cloprostenol on 8 and 9 DIM (Cloprostenol, n=15) and single intrauterine *E. coli* LPS on 7 DIM (*E.coli*-LPS, n=15). Diagnosis of endometritis was determined at 35 DI and return to estrus, breeding and conception was monitored until 120 DIM. The incidence of clinical endometritis (CE) was higher in control (40%) compared to clorpostenol (20%) and *E. coli* LPS (13.3%) groups (P = 0.415). The latter treatment group also exhibited lower bacterial contamination not involving recognized uterine pathogens. Immunostimulatory treatment related protection from CE was better for animals subjected to obstetrical intervention (P = 0.196) and in those without retention of the fetal membranes (P = 0.222). In contrast, more immunostimulated animals (70%) were diagnosed with cytological evidence of inflammation at 35 DIM as compared to controls (40%) (P = 0.415). Majority of study animals had extended open periods attributed to; prolonged anestrous (14.3% cows and 20.8% buffaloes), extended voluntary waiting periods (33.3% cows and 15.8% buffaloes) as well as low fertility on AI (66.7%% cows and 43.75% buffaloes). Frequency of detection in estrus prior to 120 DIM was higher in immunstimulatory treatment groups (86.7% - cloprostenol and 93.3% - *E. coli* LPS) compared to 66.7% in the control group (P = 0.229). Meanwhile, immunstimulatory treatments did not improve chances of conception by 120 DIM. In conclusion, the application of early puerperal treatment with Cloprotenol or *E. coli* LPS for prevention of endometritis and sub-fertility was rather limited.

Key words: Postpartum · Immunostimulation · Endometritis · Reproductive Performance

INTRODUCTION

Bacterial contamination of bovine uterus is ubiquitous following calving. Establishment and persistence of non-specific uterine infections impairs normal reproductive functions and cause substantial economic loss. Obstetrical assistance predisposes animals to extensive tissue damage, retention of the fetal membranes and heavy bacterial contamination which increase likelihood of persistent uterine infections and sub-fertility [1-3]. Prevention of postpartum uterine infections in dairy animals holds significant economic implications. However, owing to their complex and incompletely understood etiopathological mechanisms, effective prophylaxis of persistent uterine affections remains elusive [4, 5].

Compromised transition immune competence is an important determinant of persistent uterine infection and inflammation [6]. Consequently, immunomodulatory approaches have received considerable attention as

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suitable alternatives to antimicrobials for management of postpartum uterine diseases. Prostaglandin F (PGF)-2α and analogues [7] and bacterial lipopolysaccharide [8, 9] induce brief rise in trans-uterine leukocyte trafficking and production of innate immune mediator/effector molecules which improve clearance of bacteria. It logically follows that, stimulating uterine immune responses in freshly calved high risk dams could help prevent establishment and persistence of bacterial infections.

The present study tested a hypothesis that early puerperal uterine immune stimulation, by systemic cloprostenol or intrauterine *Escherichia coli* LPS (*E. coli* LPS), can help reduce risk of endometritis and sub-fertility in dairy cows and buffaloes subjected to obstetrical assistance at calving.

MATERIAL AND METHODS

Animals: A random sample of 15 Murrah buffaloes and 15 cross-bred Holstein cows requiring obstetrical assistance at calving were included in the trial. Study animals (Between 1st to 6th parity) belonged to 17 dairy holdings (Small to large scale) in Ludhiana district-Punjab, India. Animals were kept under tie-stall barns and lose housing and fed on; chafed forage and hay/age and concentrates (2-3 kg/animal/day), supplemented with a salt - mineral block. Daily milk yield in the first 35 days in milk (DIM) ranged from, 2 - 17 L/day in cows and 0 - 11 L/day in buffaloes. The interval to shedding of fetal membranes (Before or after 12 hrs, post-calving) after delivery was noted.

Treatment: Uniform treatment with Ceftiofur sodium (BOVICEF, Indian Immunologicals Ltd., India) at 1.5mg/Kg, intramuscular (IM) daily for 5 days post-calving was instituted on account of recommended benefits in preventing metritis [10]. Five animals of each species were subsequently assigned, by random lottery method, to three treatment modalities i.e. Control, Systemic Cloprostenol and intrauterine *E. coli* LPS (Table 1).

Clinical Examination: For the first 35 DIM, animals were monitored weekly for uterine and/or other clinical health problems. Examination and classification of metritis and clinical endometritis (CE) at 35 DIM was done as outlined by Sheldon *et al*. [11]. Estrus and artificial insemination (AI) records of study animals were monitored up to 120 DIM. Pregnancy status of animals subjected to AI within 120 DIM was determined by rectal examination at 60 days post-breeding.

Sampling: Low volume (5 ml) uterine flushing was collected at 35 DIM [12]. Half the flushing sample was deposited in to screw cup tubes containing Carry Blair Transport Media (CBTM) (HiMedia Laboratories Pvt. Ltd. India) and the remaining half placed in a plain tube. Samples were properly labeled and transported to laboratories at Guru Angad Dev Veterinary and Animal Science University (GADVASU) in a thermo cool box.

Cytological Examination: Duplicate thin smears were prepared from each homogenized uterine flushing sample. Smears were stained with Leishman's stain solution (s.d. fine-chem limited, - India) for 2 minutes, stain was subsequently diluted with normal saline for 8 minutes and smears were washed under running water and finally air dried. Microscopic examination of smears (x 100) and differential cell count (Total of 200 cells excluding erythrocytes) was performed to calculate the proportion of polymorphonuclear leukocytes/neutrophils (PMNLs %). Average PMNLs ratio of > 10 % was considered indicative of active uterine inflammation or sub-clinical inflammation (SCE) [11].

Bacteriological Examination: Bacteriological analysis of uterine flushing was conducted for 14 randomly selected animals (5 cows and 9 buffaloes). Samples were inoculated on to Blood agar and Wilkins Chalgreen agar media and incubated at 37°C for 24 to 72 hrs under aerobic and anaerobic conditions, respectively. Identification of bacteria was done on the basis of cultural, morphological and biochemical characteristics [13] using; differential staining (Gram stain and Acid Fast stain), differential/selective media (Manitol Salt Agar (MSA), MacConkey Lactose Agar (MLA), Nutrient Agar (NA) and Eosin Methylene Blue Agar (EMB)) and biochemical identification kit (HiMedia Laboratories Pvt. Ltd, India). Distinct primary colonies were counted to calculate semi-quantitative bacterial load (Score 0-5 scale) [14] and bacterial isolation rate was determined as the number of distinct isolates/sample.

Statistical Analysis: Data was analyzed employing the statistical software SPSS 16 (SPSS Inc., Munich-Germany). Numerical and categorical data were summarized by giving Mean ± SE and %, respectively. Association between categorical determinants and outcome parameters was performed by Fischer's exact test. Group comparison of numeric cytological, bacteriological and reproductive parameters was done by one way analysis of variance (ANOVA). Statistical significance was attributed at $P < 0.05$.

Table 1: Study animals and arrangement of treatment groups

		Experimental treatment groups			
Species	Calving assistance	Ceftiofur Sodium* 1.5mg/Kg, IM, 0-5 DIM+25 ml NS, IU, on 7 DIM(Control)	Ceftiofur Sodium* 1.5mg/Kg, IM, 0-5 DIM+Cloprostenol natricum **, 2ml (150µg (+) IM, 8 and 9 DIM(Cloprostenol)	Ceftiofur Sodium* 1.5mg/Kg, IM, 0-5 DIM+ *E. coli* LPS***, 100µg diluted in 25 ml NS[7], IU, on 7 DIM (*E. coli*-LPS)	Total
Cow	No	2	2	2	6
	Yes	5	5	5	15
Buffalo	No	3	3	3	9
	Yes	5	5	5	15
Treatment group total		15	15	15	45

*BOVICEF, 1gm, Ceftiofur Sodium Sterile Powder for Injection, Indian Immunologicals Ltd. Mumbi- India)

**ESTROPURR, (+) Cloprostenol Inj. B.P. (Vet), Bioveta, a.s. Check Republic)

*** *E. coli* LPS, *E. coli* serotype 026: B6 containing 10,000 endotoxin units per mg of LPS; Sigma, USA)

RESULTS AND DISCUSSION

Uterine Health: Obstetrical assistance was associated with higher incidence of uterine disorders (Table 2). Meanwhile, 42.9% animals with and 16.1% animals without retention of the fetal membranes (RFM) had CE (P = 0.057). Similarly, 54.5%, 23.1% and 9.5% of the animals having puerperal metritis (PM), clinical metritis (CM) and normal early purperium (< 21 DIM), respectively had CE at 35 DIM (P = 0.005). This agrees with established notion that, calving assistance and RFM are important risk factors for metritis and endometritis [1, 2, 15].

Bacteriological analysis was done for 7(28%), 11 (44%) and 7(28%) of the healthy, SCE and CE animals, respectively. Eleven (44%) samples were positive for bacteria, including; 1(14.3%) healthy, 4(36.4%) SCE and 6 (85.7%) CE. Animals with CE had higher bacterial contamination involving uterine pathogens *A. pyogenes* and Bacteroides spp. (18%), *S. aureus* (36%) and Proteus spp. (9%) (Table 3). These uterine pathogens are frequently associated with infertility in cattle [14, 16, 17] as well as buffaloes [3, 18].

Reproductive Performance: Optimum financial return from milk production entails that dairy cows and buffaloes conceive within 95-100 days and 120 DIM, respectively [19]. In contrast, majority of animals in the current study had extended open periods attributed to; prolonged anestrous (14.3% cows and 20.8% buffaloes), extended voluntary waiting periods (33.3% cows and 15.8% buffaloes) as well as low fertility on AI (66.7%% cows and 43.75% buffaloes). Pregnant animals had earlier return to estrus (55.7±4.1 days) compared to open animals (74.3±3.3 days) (P = 0.001). Clinical endometritis had negative effects on reproductive parameters (Table 4). Chance of conception within 120 DIM was also lower in buffaloes with SCE (25%) compared to healthy buffaloes (100%) (P = 0.012). A similar but relative (P = 0.080) variation was observed in cows (11.1% vs. 66.7%). Uterine infection and inflammation reduce reproductive performance by causing prolonged acyclicity [20, 21] and/or reducing fertility on service [22, 18].

Effect of Immunomodulatory Treatments: Incidence of CE (Table 5) was relatively lower in assisted calving animals receiving intrauterine *E. coli* LPS (P = 0.196). A similar trend was observed in RFM free animals wherein 36.4%, 9.1% and 0 of the control, cloprostenol and *E. coli* LPS groups, respectively had CE at 35 DIM (P = 0.222). Meanwhile, SCE was moderately higher in control compared to immunostimulated animals (P = 0.415). In line with clinical findings, bacterial isolation rate (P =0.578) and bacteriological load (P =0.556) were relatively lower in *E. coli* LPS (0.4 ± 0.3 isolates/sample and 0.9 ± 0.6 score) compared to clorpostenol (1 ± 0.4 isolates/sample and 2 ± 0.9 score) and control (0.8 ± 0.3 isolates/sample and 2.3 ± 1.1 score) groups. Moreover, no recognized uterine pathogens were isolated in animals given intrauterine *E. coli* LPS. Immunostimulated animals came to estrus within 120 DIM more frequently (P = 0.229) than controls but the reverse was true (P = 0.601) for conception rates (Table 6).

Information on prophylactic applications of, single or repeated, puerperal treatment with PGF-2α against persistent uterine infection and sub-fertility are contradicting. Some observed enhanced uterine involution and reduced persistent inflammation in metritis affected buffaloes [23] and cows [24, 25]. In contrast, [26] reported that repeated administration of PGF-2α between 7 and 14 days postpartum did not reduce prevalence of CE on days 22 or 58 postpartum. Systemic route of application and rapid metabolism could be limiting factors to efficacy of immunostimulatory benefits of PGF-2α at uterine level.

Table 2: Incidence of uterine diseases according to species and calving condition

		Incidence of uterine diseases (N (%))				
Species	Calving condition	RFM	PM	CM	CE	SCE
Cows	Normal (6)	0	0	0	0	3(50)
	Assisted (15)	8(53.3)*	5(33.3)*	6(40)*	6(40)	15(100)*
	Overall	8(46.7)	5(23.8)	6(28.6)	6(28.6)	18(85.7)
Buffaloes	Normal (9)	0	0	2(22.2)	0	6(66.7)
	Assisted (15)	6(40)*	6 (40) *	5(33.3) *	5(33.3)	14(93.3)
	Overall	6(25 %)	6(25)	7(29.2)	5(20.8)	20(83.3)

Superscript * indicates significant variation between calving condition groups ($P < 0.05$)

Table 3: Bacteriological findings (n (%))

	Endometritis at 35 DIM			Species of animal		
Parameters	CE	SCE	Healthy	Cows	Buffaloes	Overall
Escherichia coli	0	1(50)	1(50)	0	2(100)	2(10.5)
Arcanobacterium pyogenes	2 (100)	0	0	0	2(100)	2(10.5)
Bacteroides spp.	2 (100)	0	0	0	2(100)	2(10.5)
Proteus spp.	1(100)	0	0	0	1(100)	1(5.25)
Staphylococcus aureus	4(100)	0	0	2(50)	2(50)	4(21)
Pseudomonas aeruginosa	0	1(100)	0	0	1(100)	1(5.25)
Staphylococcus spp.	0	2(100)	0	1(50)	1(50)	2(10.5)
Klebiella spp.	1(50)	1(50)	0	2(100)	0	2(10.5)
Seratia spp.	0	2(100)	0	0	2(100)	2(10.5)
Anthracoides	1(100)	0	0	1(100)	0	1(5.25)
Total isolates	11(57.9)	7(36.8)	1(5.3)	6(31.6)	13(68.4)	19(100)
Bacterial isolation rate	1.6±0.4*	0.64±0.3	0.14±0.4	0.75±0.3	0.76±0.3	0.76±0.2
Bacteriological load	4.3±1.2*	1.2±0.6	0.3±0.3	1.75±0.7	1.8±0.7	1.8±0.5

Table 4: Reproductive findings according to exposure to clinical endometritis (CE)

		CE	
Reproductive parameters	Species	Negative	Positive
Estrus in 120 DIM (N (%))	Cows	14(93.3)	4(66.7)
	Buffaloes	18(94.7)*	1(20)
	Overall	32 (94.1)*	5(45.5)
Interval to first estrus (Mean ± SE)	Cows	66.4 ± 5	75.7 ±10.9
	Buffaloes	66.1 ± 4	86
	Overall	66.2± 3.1	77.8 ± 8.7
AI in 120 DIM (N (%))	Cows	10(66.7)	2(33.3)
	Buffaloes	15(78.9)*	1(20)
	Overall	25(73.5)*	3(27.3)
Interval to first AI (Mean ± SE)	Cows	89.7 ± 5.5	89.5 ± 21.5
	Buffaloes	89.1 ±3.2	86
	Overall	89.3 ± 2.8	88.3 ± 12..5
DO < 120 DIM (N (%))	Cows	4(26.7)	0
	Buffaloes	9(47.4)	0
	Overall	13(38.2)*	0

Superscript * indicates significant difference at $P < 0.05$

On the other hand, intrauterine treatment with *E. coli* LPS after dystocia management was reported to improve PMNLs infiltration and tissue repair thereby discouraging establishment of persistent uterine infections in buffaloes [27]. Despite statistical limitations, current findings also suggest a relatively better prophylactic prospect of intrauterine *E. coli* LPS treatment, which was associated with; persistent inflammatory cellular response, higher clearance of bacterial pathogens, lower incidence of CE and higher return to estrus by 120 DIM. This could reflect superiority of intrauterine route of treatment in delivering higher concentrations of drug to uterine cavity and endometrium [28].

As observed in the current study, factors like obstetrical intervention and RFM could alter response to immunomodulatory prophylactic treatment by virtue of their deleterious effects on local immune competence, tissue integrity and level of bacterial contamination [2, 3]. Farms variations with regards to hygienic management could facilitate bacterial recontamination of uterus from the environment thereby obscuring clearing effects of prophylactic immunomodulatory treatments [29, 30].

Table 5: Incidence of endometritis according to treatment and calving condition (N (%))

			Endometritis	
Treatment group	Species	Calving condition	CE	SCE
Ceftiofur (Control)	Cows (7)	Normal (2)	0	1(50)
		Dystocia (5)	3(60)	2 (40)
	Buffaloes (8)	Normal (3)	0	1(33.3)
		Dystocia (5)	3(60)	2(40)
	Overall (15)		6(40)	6(40)
Ceftiofur + Cloprostenol	Cows (7)	Normal (2)	0	1(50)
		Dystocia (5)	2(40)	3(60)
	Buffaloes (8)	Normal (3)	0	3(100)
		Dystocia (5)	1(20)	3(80)
	Overall (15)		3(20)	10(66.7)
Ceftiofur + *E. coli* LPS	Cows (7)	Normal (2)	0	1(50)
		Dystocia (5)	1(20)	4(80)
	Buffaloes (8)	Normal (3)	0	2(66.7)
		Dystocia (5)	1(20)	4(100)
	Overall (15)		2(13.3)	11(73.3%)

Table 6: Reproductive findings according to prophylactic treatment groups (≤ 120 DIM)

Treatment group	Species	Estrus	Interval to 1st estrus	AI	Conception
Control	Cows (7)	5(71.4)	65.2 ± 11.5	4 (57.1)	3 (42.9)
	Buffaloes (8)	5 (62.5)	65 ± 8.6	4 (50)	3 (37.5)
Systemic Cloprostenol	Cows (7)	6 (85.7)	66 ± 9	4 (57.1)	0
	Buffaloes(8)	7 (87.5)	62 ± 3.4	7 (87.5)	3 (37.5)
Intrauterine *E. coli* LPS	Cows (7)	7 (100)	72.9 ± 4.5	4 (57.1)	1 (14.3)
	Buffaloes (8)	7 (87.5)	73.9 ± 8.2	5 (62.5)	3 (37.5)

CONCLUSION

Early puerperal uterine immune stimulation by systemic cloprostenol or intrauterine *E. coli* LPS did not result in significant protection from endometritis or improvement of open periods. However, there is indication as to prophylactic potentials of early puerperal intrauterine *E. coli* LPS treatment. Small sample size and variable management conditions could be obscuring prophylactic benefit of the immunostimulatory treatments. Strictly controlled investigations employing more substantial sample sizes and comprehensive health and fertility parameters are required to validate practical values of immunomodulators for preventing persistent uterine infection and inflammation.

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