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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 field trial was conducted to evaluate immunomodulatory prophylactic modalities against persistent uterine infection in 24 Murrah buffaloes and 21 cross-bred Holstein Frisian cows. All animals were treated with intramuscular ceftiofur sodium for 5 days post-calving. On or after 7th day animals were randomly assigned to either; intrauterine normal saline (controls = 15), intrauterine *E. coli* LPS (LPS = 15) or intramuscular Cloprostenol (cloprostenol = 15). Endometritis was Determined at 35 days in Milk (DIM) and estrus, breeding and conception were monitored until 120 DIM. Clinical Endometritis (CE) occurred in 6 (60%), 3 (30%) and 2 (20%) assisted calving animals assigned to control, cloprostenol and LPS groups, respectively ($p = 0.196$). All but one assisted calving animals had cytological uterine inflammation at 35 DIM. Among normal calving animals 1 (20%), 3 (60%) and 4 (80%) of those in control, LPS and cloprostenol groups, respectively showed cytological uterine inflammation ($p = 0.415$). Meanwhile, 93.3, 86.7 and 66.7% of the LPS, Cloprostenol and Control group animals were observed in heat within 120 DIM ($p = 0.229$). Rate of conception within 120 DIM was similar 3 (37.5) among treatment groups in buffaloes but varied in cows from a high of 3 (42.9) in control group to a low of 0 in Cloprostenol group. The application of early puerperal immunostimulatory treatment with cloprotenol or *E. coli* LPS for prevention of endometritis and sub-fertility appears limited requires further validation.

Key words: Buffaloes, cows, cloprostenol, *E. coli* LPS, 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 lead to substantial economic loss. Obstetrical assistance predisposes animals to extensive tissue damage, Retention of the Fetal Membranes (RFM) and heavy bacterial contamination which increase likelihood of uterine infections and sub-fertility (Noakes *et al*., 2009; Azawi, 2013). Prevention of postpartum uterine infections in predisposed dairy animals holds significant economic implications. However, owing to complex and incompletely understood underlying etiopathological mechanisms, effective prophylaxis of persistent uterine infections has remained difficult (LeBlanc, 2008).

Compromised transition period immune competence is an important determinant of persistent uterine infections (Singh *et al*., 2008). Consequently, immunomodulatory approaches have received considerable attention as alternative to antimicrobials for management of uterine infections. Prostaglandin F (PGF)-2α and analogues (Salasel and Mokhtari, 2011) and bacterial lipopolysaccharide (Singh *et al*., 2001; Prasad *et al*., 2009) induce brief rise in trans-uterine leukocyte and innate mediator/effector molecule trafficking which improve clearance of bacteria. It follows from logic that enhancing uterine immune responses particularly in freshly calved high risk dams could help prevent establishment and persistence of uterine bacterial infections.

The present study tested the hypothesis that early puerperal uterine immune stimulation by systemic cloprostenol or intrauterine *E. coli* LPS can reduce risk of endometritis and sub-fertility in dairy cows and buffaloes with variable degrees of predisposition to the condition.

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MATERIALS AND METHODS

Animals and management: A total of 24 Murrah buffaloes and 21 cross-bred Holestain Frasian cows (1st to 6th parity) were purposefully selected (30 assisted and 15 normal, calving) from 16 farms in Ludhiana district-Punjab, India. Animals were kept in tie stall barns, fed on mixed ration (chafed forage, hay/age and concentrates (2-3 kg/animal/day)) and given free access to water and mineral blocks. Milk yield in the first 35 Days In Milk (DIM) ranged from, 2-17 L day^{-1} in cows and 0-11 L day^{-1} in buffaloes.

Calving assistance and treatments: Animals were monitored at calving and obstetrical assistance given to those facing dystocia. The interval to shedding of fetal membranes (before or after 12 h post-calving) was noted. Cows and buffaloes were randomly assigned to three prophylactic treatment modules commencing on day of calving (Table 1). Uniform use of Ceftiofur sodium post-calving was selected with consideration to recommended benefits for preventing metritis (Risco and Hernandez, 2003).

Clinical examination: For the first 35 DIM, animals were clinically monitored on weekly basis for uterine affections according to the criteria outlined by Sheldon *et al*. (2006). Diagnosis of Clinical Endometritis (CE) was established at 35 DIM. Estrus and Artificial Insemination (AI) records 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 and laboratory investigation: Low volume (5 mL) uterine flushing was collected at 35 DIM according to the method outlined by Galvao *et al*. (2011). Half the sample was deposited in screw cup tubes containing Carry Blair Transport Media (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.

Duplicate thin smears were prepared from each homogenized uterine flushing sample as follows; smear was stained with Leishman's stain solution (s.d. fine-chem limited, Mumbi, India) for 2 min, stain was subsequently diluted with normal saline for 8 min, stain was washed under running water and the smear was finally air dried. Microscopic examination of smears (x100) and differential cell count (total of 200 cells excluding erythrocytes) was performed to calculate the proportion of polymorphonuclear leukocytes/neutrophils (PMNLs %). PMNLs% >10% was considered indicative of Sub-Clinical Endometritis (SCE) at 35 DIM (Sheldon *et al*., 2006).

Bacteriological analysis of uterine flushing was conducted for 25 randomly selected animals (8 cows and 17 buffaloes). Samples in transport media were inoculated on to Blood agar and Wilkins Chalgreen agar media and incubated at 37°C for 24-72 h under aerobic and anaerobic conditions, respectively. Identification of bacteria was done on the basis of cultural, morphological and biochemical characteristics as by Quinn *et al*. (1999) using; differential staining (Gram stain and Acid Fast stain) differential/selective media (Manitol Salt Agar, MacConkey Lactose Agar, Nutrient Agar and Eosin Methylene Blue Agar) and biochemical identification kit (HiMedia Laboratories Pvt. Ltd., Mumbai, India). Primary colonies were counted to calculate semi-quantitative bacterial load (score 0-5 scale) according to Williams *et al*. (2005) 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±Standard Error (SE) and percentage, respectively. Association between categorical determinant and outcome parameters was performed by Fischer's exact

Table 1: Study animals and arrangement of treatment groups

		Experimental treatment groups			
Species	Calving condition	Ceftiofur Sodium* 1.5 mg kg^{-1}, IM, 0-5 DIM +25 mL NS, IU, on 7th DIM (control)	Ceftiofur Sodium* 1.5 mg kg^{-1}, IM, 0-5 DIM+ Cloprostenol natricum**, 2 mL (150 μg (+) IM, 8th) and 9th DIM) (Cloprostenol)	Ceftiofur Sodium* 1.5 mg kg^{-1}, IM, 0-5 DIM+ *E. coli* LPS***, 100μg dilute in 25 mL NS IU, on 7th DIM (*E. coli*-LPS)	Total
Cow	Normal	2	2	2	6
	Assisted	5	5	5	15
Buffalo	Normal	3	3	3	9
	Assisted	5	5	5	15
Treatment group total		15	15	15	45

*BOVICEF, 1 g, 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)

test. Group comparison of numeric cytological, bacteriological and reproductive parameters was done by one way Analysis of Variance (ANOVA). Statistical significance was attributed at $p<0.05$.

RESULTS AND DISCUSSION

Uterine health: Obstetrical assistance was associated with higher incidence of uterine disorders (Table 2). Similarly, 6 (42.9%) animals with and 5 (16.1%) animals without RFM had CE ($p = 0.057$). Similarly, 6 (54.5%), 3 (23.1%) and 2 (9.5%) of the animals having Puerperal Metritis (PM), Clinical Metritis (CM) and normal early purperium (<21 DIM), respectively had CE ($p = 0.005$).

Bacteriological analysis was conducted for 25 animals; 7 (28%) healthy, 11 (44%) SCE and 7 (28%) CE. Only 11 (44%) uterine samples were positive for bacteria including; 1 (14.3%) healthy, 4 (36.4%) with SCE and 6 (85.7%) with CE. Overall bacterial isolation frequency, isolation of recognized pathogens and bacterial load were highest in animals with CE (Table 3).

Reproductive performance: A total of 18 (85.7%) cows and 19 (79.2%) buffaloes came to estrus (at least once) within 120 DIM ($p = 0.567$). The interval to first estrus averaged 68.4±4.5 and 67.2±3.9, days in cows and buffaloes, respectively ($p = 0.831$). 12 (66.7%) and 16 (84.2%) of the estrus cows and buffaloes were submitted to AI within 120 DIM ($p = 0.214$). Average length of the interval to first AI in respective species was 89.7±5.2 and 88.9±3 days ($p = 0.890$). Of the animals subjected to AI at least once on or prior to 120 DIM, 4 (33.3%) cows and 9 (56.2%) buffaloes had successful conception as confirmed by rectal examination at 60 day post breeding ($p = 0.229$). Persistent uterine infection and/or inflammation (endometritis) had a tendency to extend the postpartum anestrus interval as well as reduce chance of conception on AI in cyclic animals (Table 4).

Effect of immunomodulatory treatments: Incidence of CE (Table 5) was relatively lower in intrauterine *E. coli* LPS treatment group both overall ($p = 0.415$) and among animal's receiving obstetrical assistance ($p = 0.196$). A similar relative trend was observed in RFM free animals wherein 36.4, 9.1% and 0 of the control, cloprostenol and intrauterine *E. coli* LPS groups, respectively had CE ($p = 0.222$). On the contrary, SCE was moderately elevated in immunostimulated animals ($p = 0.415$).

In line with clinical disease trends, bacterial isolation rate ($p = 0.578$) and bacteriological load ($p = 0.556$) were relatively lower in LPS group (0.4±0.3 isolates/sample and 0.9±0.6 score) compared to the clorpostenol (1±0.4

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 (21)	8 (46.7)	5 (23.8)	6 (28.6)	6 (28.6)	18 (85.7)
Buffaloes	Normal (6)	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 (21)	6 (25 %)	6 (25)	7 (29.2)	5 (20.8)	20 (83.3)
Total	Normal (15)	0	0	2 (13.3)	0	9 (60)
	Assisted (30)	14 (46.7)**	11 (36.7)**	11 (36.7)**	11 (36.7)**	18 (60)
	Overall (45)	14 (31.1)	11 (24.4)	13 (28.9)	11 (24.4)	27 (60)

Superscript ** and * indicates significant variation between calving condition groups at $p \leq 0.001$ and $p \leq 0.05$)

Table 3: Bacteriological findings (n (%))

	Endometritis at 35 DIM			Species of animal		
Parameters	CE	SCE	Healthy	Cows	Buffaloes	Overall
Bacterial isolates						
E. coli	0	1 (50)	1 (50)	0	2 (100)	2 (10.5)
A. pyogenes.	2 (100)	0	0	0	2 (100)	2 (10.5)
Bacteroides sp.	2 (100)	0	0	0	2 (100)	2 (10.5)
Proteus sp.	1 (100)	0	0	0	1 (100)	1 (5.25)
S. aureus	4 (100)	0	0	2 (50)	2 (50)	4 (21)
P. aeruginosa	0	1 (100)	0	0	1 (100)	1 (5.25)
Staphylococcus sp.	0	2 (100)	0	1 (50)	1 (50)	2 (10.5)
Klebiella sp.	1 (50)	1 (50)	0	2 (100)	0	2 (10.5)
Seratia sp.	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: Effect of endometritis on reproductive parameters

Reproductive parameters	Animal	Uterine health status 35 DIM			p-values
		Healthy	SCE	CE	
Estrus≤120 DIM (%)	Cows (21)	3 (100)	11 (91.7)	4 (66.7)	0.269
	Buffaloes (24)	4 (100)	14 (93.3)	1 (20)*	0.001
	Overall	7 (100)	25 (92.6)	5 (45.5)*	0.001
Interval to first estrus (Mean±SE)	Cows (18)	65.7±10.8	66.5±5.9	75.7±10.8	0.710
	Buffaloes (19)	48.2±6.4	71.2±3.9	86*	0.022
	Overall (37)	55.7±6.4	69.2±3.3	77.8±8.7	0.083
AI≤120 DIM (%)	Cows (18)	2 (66.7)	8 (72.7)	2 (50)	0.711
	Buffaloes (19)	4 (100)	11 (78.6)	1 (100)	0.529
	Overall (37)	6 (85.7)	19 (76)	3 (60)	0.591
Interval to first AI (Mean±SE)	Cows (12)	77±2	92.9±6.4	89.5±21.5	0.588
	Buffaloes (16)	79±5.7	92.7±3.3	86	0.139
	Overall (28)	78.3±3.7	92.8±3.2	88.3±12.5	0.104
Conception on first AI (%)	Cows (12)	0	0	0	-
	Buffaloes (16)	2 (50)	4 (36.4)	0	0.646
	Overall (28)	2 (33.3)	4 (21.1)	0	0.516
Conception on AI ≤120 DIM (%)	Cows (12)	2 (100)	2 (25)	0	0.072
	Buffaloes (16)	4 (100)	5 (45.5)	0	0.086
	Overall (28)	6 (100)*	7 (36.8)	0	0.006

Superscript * indicates significant variation at $p<0.05$

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

Treatment groups	Species	Calving condition	Endometritis	
			CE	SCE
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)	
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)	
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%)

isolates/sample and 2±0.9 score) and control (0.8±0.3 isolates/sample and 2.3±1.1 score) groups. No recognized uterine pathogens were isolated in animals assigned to intrauterine *E. coli* LPS.

Prophylactic immunomodulatory treatments were not associated with significant improovment of reproductive outlooks (Table 6). Nevertheless, relatively more immunostimulated than controls animals came to estrus within 120 DIM ($p = 0.229$) whereas the reverse trend was apparent with regards to probability of successful conception to AI within 120 DIM ($p = 0.601$) in case of cows.

Present findings indicate a higher risk of CE in animals having obstetrical assistance, RFM and metritis. These conditions are recognized risk factors for metritis and endometritis (Bell and Roberts, 2007; Noakes *et al*., 2009). Animals with CE had higher bacterial contamination involving *A. pyogenes*, *Bacteroides* sp., *Proteus* sp. and *S. aureus*. These uterine pathogens are frequently associated with infertility in cattle (Dohmen *et al*.,1995; Williams *et al*., 2005) as well as buffaloes (Azawi, 2010, 2013).

Optimum financial return from milk production entails that dairy cows and buffaloes conceive within 95-100 days and 120 DIM, respectively (Prasad and Neeraj, 2010). Majority of animals in the current study had extended open periods attributed to prolonged anestrous and voluntary waiting periods as well as low fertility on AI both of which were pronounced in case of endometritis (CE and SCE). Uterine infection and inflammation can prolong postpartum acyclicity (Sheldon *et al*., 2002; Williams *et al*., 2007) as well as reducing fertility on service (Azawi, 2010).

Information on prophylactic applications of immunomodulators against persistent uterine infection and sub-fertility are scarce and often contradicting. Some indicated that single or double early puerperal systemic prostaglandin treatment enhanced uterine involution and reduced persistent inflammation in metritis affected buffaloes (Nak *et al*., 2011; Prabhakar *et al*., 2011) and cows (Pecsi, 2007). In contrast, Hendricks *et al*. (2006) reported that repeated administration of PGF-2α between 7 and 14 days postpartum did not reduce prevalence of CE on days 22 or 58. On the other hand, Prabhakar *et al*. (2007) found that intrauterine application of *E. coli* LPS after dystocia treatment improved subsequent PMNLs infiltration and tissue repair discouraging establishment of persistent uterine infections.

Despite statistical limitations, current findings tend to suggest more favorable prospect for early puerperal intrauterine *E. coli* LPS treatment. The modality was associated with persistent uterine inflammatory cellular

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

Treatment groups	Species	Estrus (%)	Interval to 1st estrus (Mean±SE)	AI (%)	Interval to 1st AI (Mean±SE)	Conception (%)
Control (15)	Cows (7)	5(71.4)	65.2±11.5	4 (57.1)	81.25±10.9	3 (42.9)
	Buffaloes (8)	5 (62.5)	65±8.6	4 (50)	93.75±4.5	3 (37.5)
Cloprostenol (15)	Cows (7)	6 (85.7)	66±9	4 (57.1)	86.25±6.8	0
	Buffaloes (8)	7 (87.5)	62±3.4	7 (87.5)	86±3.4	3 (37.5)
E. coli LPS (15)	Cows (7)	7 (100)	72.9±4.5	4 (57.1)	101.5±7.9	1 (14.3)
	Buffaloes (8)	7 (87.5)	73.9±8.2	5 (62.5)	89±7.9	3 (37.5)

response, higher clearance of uterine bacterial pathogens, lower incidence of CE and higher return to estrus by 120 DIM. The observed relative difference could reflect superiority of intrauterine application route which delivers higher concentrations of drug to the uterine cavity and endometrium (Masera *et al*., 1980). In the case of cloprostenol, systemic route of application and rapid metabolism could limit the magnitude, duration and efficacy of immunostimulatory benefits at uterine level. Farms variations with regards to hygienic management could facilitate bacterial recontamination of uterus from the environment further obscuring prophylactic benefits of immunomodulatory treatments (Lewis, 1997; Szenci, 2010).

CONCLUSION

Early puerperal uterine immune stimulation by systemic cloprostenol or intrauterine *E. coli* LPS did not result in significant reduction of endometritis or improvement of the open period. Meanwhile, there exists indication of potential prophylactic benefits particularly for puerperal intrauterine *E. coli* LPS treatment. Strictly controlled investigations involving substantial sample sizes and more comprehensive health and fertility parameters are mandated to validate practical values of immunomodulators in the prevention of postpartum uterine diseases

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