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Effect of treatment of immunomodulators on recovery and conception rate in endometritic buffaloes

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ABSTRACT

A study was undertaken to evaluate the effect of administration of immunomodulators as an alternative to antibiotics on the recovery of endometritis in buffaloes. Buffaloes (181) were diagnosed for endometritis were randomly grouped to receive one of the following treatments; the animals of group 1 (n = 41) were treated with neem oil *(Azadirachta indica,* 40 ml iu infusion twice at 24 h interval) and the buffaloes (40) in group 2 were administered with 100µg *Escherichia coli* lipopolysaccharide intra uterine at similar times as in group 1. Blood plasma (40 ml) was infused via iu route in animals (40) of group 3, whereas group 4 animals (40) received 20 ml of garlic extract at 2 occasions similar to other groups. The control group 5 animals (20) were given only normal saline twice at 24 h interval. Efficacy of these immunomodulators was assessed by negative reaction to whiteside test and a drastic reduction of bacterial load in cervico-vaginal mucus (CVM) at subsequent estrus. The results indicated that administration of neem oil and *E.coli* LPS retrieved cows from endometritis as majority of them showed negative colour reaction to whiteside test and a significant decline of bacterial load in CVM. A satisfactory recovery rate (75%) in animals of group 3 and 4 was also observed as compared to control (20%). Further, the maximum number of buffaloes were found pregnant (70.73%) following neem oil-treatment. This study indicated therapeutic values of immunomodulators like neem oil, *E.coli* LPS, blood plasma and garlic extract in endometritic buffaloes.

Key words: Buffaloes, Conception rate, Endometritis, Immunomodulators, Recovery rate

Buffaloes are difficult breeders because of their tendency of seasonal breeding and poor conception rate either due to problem of silent ovulations or uterine infections. Intrauterine infusion of different compounds is the mainstay for treatment of bovine endometritis (Gilbert 1992). The increased public health concern about the medicinal residues in edible animal products makes it hard to justify efficacy of dubious intrauterine antimicrobial treatments. Moreover, antibacterial drugs can also markedly inhibit or destroy the phagocytic activity of polymorphonuclear leucocytes, which are responsible for maintaining uterine defense (Massera *et al.* 1980). Hormone therapy is expensive and may lead to several side effects. Further, cost of available treatments, frequency of administration and emergence of bacterial resistance to antibiotics make their use uneconomical. Hence there, is an urgent need to develop an effective, non-antibiotic, ecofriendly and safer drug for treatment of endometritis.

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An alternate approach could be the use of natural substances for activation of natural defense mechanism in uterus. Bacterial endotoxin (Deori *et al.* 2004), serum or plasma (Kumar and Bhooshan 2011), polymorphonuclear leucocytes (PMN) extracts (Sarkar *et al.* 2006) and neem oil (Barman *et al.* 2009, Kumar *et al.* 2009) etc. which stimulate natural uterine defense mechanism (UDM) were suggested as alternative therapies for bovine endometritis. Neem seeds reportedly have antibacterial activity comparable to enrofloxacin, gentamicin, oxytetracycline and streptomycin (Barman *et al.* 2009). Garlic extract also has inhibitory effect on growth of bacteria (Esparza-Borges *et al.* 1996). *E.coli* lipopolysaccharides (LPS), an endotoxins, when placed in uterine lumen, acted as chemoattractants to PMNs (Asbury and Hansen 1987).

The therapeutic efficacy of immunomodulators, namely, neem oil, colostral whey, methanolic fraction of neem seed powder and neem oil, *E.coli* LPS, blood plasma, garlic extract, etc. was tested for bacterial endometritis (Bindrawan *et al.* 2002, Deori *et al.* 2004, Sarkar *et al.* 2006, Kumar *et al.* 2009, Barman *et al.* 2009 and Singh *et al.* 2010 and Kumar and Bhooshan 2011). These substances stimulate the natural

UDM in cows with endometritis and thereby help in exclusion of bacteria from the uterus and indicated a significant success rate in terms of recovery and conception rate under farm conditions. However, there exists a paucity of information in buffaloes at a larger scale. Therefore, before this therapy could go for field use the present study was designed to assess the effect of immunomodulators on recovery and conception rate in endometritic buffaloes under rural conditions.

MATERIALS AND METHODS

Experimental animals and procedure: The present study was conducted in 6 villages adopted by IVRI, Izatnagar, Uttar Pradesh. The animals at estrus showing mucopurulent and hazy discharge as well as positive reaction to whiteside test were included in the experiment and randomly allocated into 1 of the 5 groups: group 1, animals (41) received iu 40 ml of neem oil for 2 occasions at 24 h interval; gr 2 animals (40) received 100µg *E.coli* LPS after dissolving in 20 ml of PBS, two times as similar to gr 1 animals; animals (40) of gr 3 received blood plasma (40 ml) by iu route; animals (40) of gr 4 received garlic extract (20 ml) diluted in normal saline (1:1) two times. Another 20 endometritic buffaloes (gr 5) were treated with normal saline and considered as control.

Collection of cervico-vaginal mucus: The animals were restrained properly and cervico-vaginal mucus (CVM) was sampled at pre-treatment and subsequent estrus. The vulvar and perineum regions were washed with antiseptic solution and wiped properly with absorbable sterile cotton napkins. The internal genitalia were massaged (per rectum) and mucus discharge was collected in sterilized disposable petridish. The CVM was examined for the presence of white flakes, abnormal color, haziness and subjected to whiteside test (Popov 1969) and the pH of the CVM was assessed using pH meter.

Bacteriological examination of CVM: Prior to bacteriological examination, the CVM was mucolysed by mixing with sterile distilled water in a vortex shaker for 10–15 min. Bacterial load was determined by standard plate count method using 10–fold dilution of CVM in sterile normal saline solution. Diluted sample (100 ml) was inoculated in triplicate on nutrient agar plates. The inoculum was spread uniformly with the help of a bent Pasteur pipette on the surface of the agar. The plates were incubated at 37°C for 24 h and individual colonies counted using a colony counter. The bacterial load per ml of CVM was calculated by multiplying with the dilution factor.

$$\text{Bacterial load (per ml)} = (\text{Av. no. of colony} \times \text{dilution factor} \times 0.1 \text{ ml}) \times 2$$

Preparation of blood plasma: Blood (100 ml) was collected from endometritic buffaloes in sterile conical glass flask by jugular vein puncture, with heparin (5 units/ml) as an anticoagulant. Plasma was separated by keeping blood at 4°C in refrigerator and allowing the RBCs to settle down under gravity for 2 h. The separated plasma was stored in sterile vials at –20°C until use.

Alcoholic extract of garlic: The garlic extract was prepared as tincture, using pestle and mortar. The garlic cloves were crushed and mixed with 70% alcohol. The mixture was placed in a water bath for 4–6 h at 65–70°C and was left for overnight incubation. It was filtered and filtrate was used as tincture. The tincture was mixed with saline at equal (1:1) dilution for treatment (Esparza-Borges *et al*. 1996).

Assessment of recovery and conception rate: The clinical recovery was assessed by change in pH, nature of whiteside test of estrual mucus and total bacterial count at subsequent estrus. The buffaloes during subsequent estrus were inseminated twice, 12 h apart using 0.50 ml of frozen/thawed semen from fertile bulls. Pregnancy was confirmed by rectal palpation at 60 days following insemination.

Statistical analysis: Data were analyzed as per Snedecor and Cochran (1994). Pre-and post-treatment means of different parameters were compared by ANOVA, whereas the percentage data were analysed by using Normal Deviate test for proportions.

RESULTS AND DISCUSSION

In the present study before the administration of immunostimulators all endometritic buffaloes had either mucopurulent or hazy discharge with moderate to dark yellow colour reaction to whiteside test. The CVM of endometritic animals revealed large number of leucocytes, which was responsible for change in appearance (hazy or cloudy) and development of colour reaction to whiteside test. Since the pH of CVM turns alkaline in infected cow, the pH of CVM is also an indicator of endometritis as reported earlier by others (Singh and Sastry 1981, Deori *et al.* 2004, Sarkar *et al.* 2006, Kumar *et al.* 2009). Before treatment the pH of CVM varied from 7.75±0.16 to 8.20±0.12 and the mean values did not differ significantly among groups. At subsequent estrus the pH declined significantly ($P < 0.05$) in all treated animals and varies from 6.50 to 6.80. However, in control animals no significant change was observed at subsequent estrus and pH of CVM remained towards alkaline side. Although the effect of pH of cervical mucus on fertility rate is not clearly understood, a higher pH more than 8.5 is reported to be associated with infertility. Alkaline CVM in endometritic animals is caused by metabolites of bacteria and inflammatory exudates (Salphale *et al.* 1993). It appears that the pH drops towards the neutral side, once the infection from the uterus is eliminated (Markusfeld 1984).

The overall mean bacterial load was 93.57±25.20 $\times 10^6$/ml in the estrual CVM of endometritic animals. A significant decline ($P<0.01$) in bacterial load was observed from pre-to post-treatment estrus in all treated animals. The per cent reduction in bacterial load varied from 91 to 96 in animals treated with immunomodulators (Table 1). However, in control animals the decline in bacterial load ($\times 10^6$) was found to be 33.32 ± 15.16% at subsequent estrus. Bacterial load

Table 1. Effect of immunomodulators on recovery and conception rate in endometritic buffaloes

Treatment groups	Pre-Treatment Bacterial load ($\times10^{6}$)	Post-Treatment Bacterial load ($\times10^{6}$)	Reduction in Bacterial Load (%)	Recovery rate	Conception rate
Group I (n=41) (Neem oil)	43.61±13.45	1.01±0.38	96.02±2.02[a]	41 (100%)[a]	29 (70.73%)[a]
Group 2 (n=40) *(E coli* LPS)	45.20±11.55	0.95±0.74	94.0±6.18[a]	32 (80%)[a]	24 (75%)[a]
Group 3 (n=40) (Blood plasma)	48.20±18.52	1.28±0.68	93.10±2.80[a]	30 (75%)[a]	21 (70%)[a]
Group4 (n=40) (Garlic extract)	56.80±20.63	1.55±0.12	91.80±5.30[a]	30 (75%)[a]	20 (66.7%)[a]
Group V (n=20) (Control)	52.68±28.80	26.20±21.87	33.32±15.16[b]	4 (20%)[b]	1 (25%)[b]

Figures in parenthesis indicate the percentage of animals; Values within same column ([ab]) having different superscript differ significantly ($p<0.05$).

was used to assess the level of infection and as a diagnostic indicator to assess health status of organs including uterus (Dhaliwal *et al.* 2001). In our study the bacterial load in CVM of endometritic cows is in accordance with the findings of others (Sarkar *et al.* 2006, Barman *et al.* 2009).

Further, 100% animals treated with neem oil discharged clear CVM with negative colour reaction to whiteside test and reduction of bacterial load was 96.02±2.02% at subsequent estrus (Table 1).A similar decline in bacterial load was recorded with 88% recovery in endometritis cows, which were given 40 ml crude neem oil, 3 times, at 24 h interval (Bindrawan *et al.* 2002). This might have occurred either due to antibacterial activity of neem compounds fractions and increase in phagocytosis or elimination of pathogen due to stimulation of uterine defense mechanism. Extracts of neem were found to contain non-specific immunostimulant, antimicrobial, anti-viral and antifungal properties (Talwar *et al.* 1993). This contention receives support from the findings of (Barman *et al.* 2009), who reported that neem oil showed antibacterial sensitivity against 95% bacterial isolates belonging to CVM of endometritic cows, that was comparable to antibiotics such as enrofloxacin and gentamicin, further it was also observed that 1 mg of nimbidine – a principal compound of neem oil, was equivalent to 800 units of penicillin and 0.5 mg of streptomycin sulphate (Singh and Sastry 1981). Intrauterine infusion of neem oil also known to cause an infiltration of neutrophils in rats (Upadhyay *et al.* 1990). The decline in bacterial load thus, appears to have occurred due to suppressive action of neem preparations on growth of both gram positive and gram negative bacteria (Barman *et al.* 2009, Koul *et al.* 1990). Further, the overall conception rate was higher in animals treated with neem oil (70.73%) than the control animals (Table 1).

The infusion of 100 µg *E.coli* LPS by iu route in endometritic buffaloes caused 80% recovery as indicated by negative colour reaction of CVM to whiteside test and 94.0± 6.18% reduction in bacterial load at subsequent estrus and followed by 75% conception (Table 1). This finding is similar to earlier reports of Singh *et al.* (2000) and Deori *et al.* (2004). Endotoxins such as *E.coli* lipopolysaccharides when placed in the uterine lumen by infusion are thought to act as chemoattractants to polymorphs and thus, provide cellular mediated immunity into the uterus. This increase in the number of PMNs in the endometrium could help to resolve endometritis in both cows and mares (Asbury and Hansen 1987). In addition to PMN infiltration, an influx of serum proteins also occurs following the intrauterine infusion of LPS (Deori *et al.* 2004).

Similarly, the infusion of blood plasma caused a drastic decline in bacterial load (93.10 ± 2.80%) and recovered as much as 75% buffaloes as indicated by negative colour reaction of CVM to whiteside test at subsequent estrus and out of that 70% were conceived (Table1).This observation is in accordance with the reports of Kumar *et al.* (2004) and Kumar and Bhooshan (2011). As plasma contains immunoglobulins which acts as an opsonin and thus enhances phagocytosis and clears the infection from the uterus.

The recovery rate, assessed by negative colour reaction of CVM to whiteside test at subsequent estrus following treatment with garlic extract was also found to be 75%.In this group the decline in bacterial load was 91.80 ± 5.30% and further an 66.67% conception rate was obtained (Table 1). In this study a higher recovery and conception was observed than reported by Sarkar *et al.* (2006). Garlic, possesses remarkable immunopotentiating action and its extract was highly effective against *E.coli* even at 1% concentration. The therapeutic efficacy of garlic lies in its cytotoxic, antitumor, antifungal, antibacterial, antiviral as well as antiprotozoal properties for which it is being widely used traditionally since long time, which in turn could be attributed to an organic sulphur compound 'Ajoene' (4,5,9-trithiadodeca 1,6,11-triene-9-oxide) (Ledezma and Apitz 1998). In a freshly crushed clove, alliin is broken down by alliinase enzyme into allicin. Allicin, along with other constituents of the volatile oil is highly antiseptic and antibiotic against both gram-positive and gram-negative bacteria (Ahsan and Islam 1996).

This study indicated therapeutic values of immunomodulators like neem oil, *E.coli* LPS, blood plasma and garlic extract in endometritic buffaloes. It can be concluded that particularly neem oil appeared superior for endometritis as its intrauterine infusion caused drastic decline in bacterial load and resulted in 100% recovery rate and

allowed higher number of animals to conceive.

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