Effect of leukotriene B₄ and oyster glycogen in resolving subclinical endometritis in repeat breeding crossbred cows


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ABSTRACT

In the present study an innovative attempt was made using Leukotriene B₄ (LTB₄) and Oyster glycogen (OG), non-specific immunomodulators, for the treatment of subclinical endometritis (SCE) in repeat breeding crossbred cows. Thirty six subclinical endometritis repeat breeding cows were randomly divided into 3 equal groups (G-I, G-II and G-III). The cows in treatment groups G-I and G-II received single intrauterine (iu) infusion with 50 ml of 30 nmol/L LTB₄ and 10 mg/ml OG, respectively, whereas the cows in G-III served as control. Blood samples collected just before treatment (0 hr), 24 hr post-treatment and at subsequent estrus were assayed for total leukocyte count (TLC), lipid peroxide (LPO) and nitric oxide (NO). Following treatment the recovery rate was assessed at subsequent estrus by determining the pH of cervico-vaginal mucus (CVM), white side test and percentage of polymorphonuclear (PMN) cells in uterine cytology. The recovery rate in G-I (83.3%) and G-II (75.0%) was greater (P< 0.05) than in control (16.7%). All the animals were inseminated with good quality of frozen-thawed semen and pregnancy was confirmed after 60 days. A non significant increase in conception rate in G-I and G-II compared to the control was observed following the treatments. Compared to control, the cows in treated groups had greater (P<0.01) TLC at 24 hr following treatment. The infusion of LTB₄ and OG caused significant (P< 0.05) decline in the plasma levels of inflammatory mediators (LPO and NO) from the day of treatment (667.15±42.85, 753.73±32.78 nmol/L and 71.58±2.57, 76.67±4.59 µmol/L) to subsequent estrus (555.56±42.53, 630.88±31.16 nmol/L and 60.00±2.06, 60.08±2.17 µmol/L), respectively. It can be concluded that administration of LTB₄ and OG resolves SCE in repeat breeding crossbred cows and reduces oxidative stress as evident from the lower levels of plasma LPO and NO following immunomodulatory treatments.

Key words: Conception rate, Leukotriene B₄, Oyster glycogen, Recovery rate, Repeat breeding cows, Subclinical endometritis.

INTRODUCTION

Antibiotics were the previous considerations for the treatment of endometritis and varying degree of success rates have been reported. Due to some major disadvantages of using antibiotics like development of bacterial resistance, diminishing uterine defence mechanism (UDM), high cost of treatment and milk residual effect; clinicians are in quest for alternative approach of treatment. In the recent past, immunomodulatory treatment to combat uterine infections has shown encouraging results (Dhaliwal et al., 2001; Deori et al., 2004; Prasad et al., 2009). Treatment of the condition with various immunomodulators like neem oil (Singh et al., 2010), E. coli LPS (Deori et al., 2004), OG (Prasad et al., 2009) have been studied with varying degrees of success rate. Oyster Glycogen, a branched polymer of glucose synthesized by animal cells for energy storage and release, is a biologically active immunomodulator. Intrauterine (iu) administration of OG in healthy cows, stimulated large number of PMN cells migration into the uterine lumen and upto 90% of the cells identified in uterine secretion were neutrophils (Anderson et al., 1985). The use of OG in repeat breeding crossbred cows with subclinical endometritis (SCE) has not been studied yet. LTB₄ is an arachidonic acid metabolite known to be physiologically produced by stimulated granulocytes, macrophages and mast cells. It is an effective chemoattractant, stimulating preferential migration of PMNs into the lumen of the bovine uterus (Zerbe et al., 1996). However, perusal of literature revealed no information on the use of LTB₄ in the treatment of uterine infection in cattle.

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Inflammatory conditions of the uterus have been shown to produce oxidative stress in animals. Increased concentration of NO in plasma was reported in cows suffering from subclinical and clinical endometritis and the level was related with the degree of endometritis (DeJun et al., 2010). Similarly, elevated LPO levels in plasma were found to be associated with uterine infection (Kizil et al., 2010). However, the work on these inflammatory mediators in relation to treatment with immunomodulators and at subsequent recovery in dairy cows has not been attempted so far. The present study was designed to observe the immunomodulatory effect of LTB₄ and OG in recovery of repeat breeding crossbred cows from SCE and to assess the inflammatory mediators in the peripheral blood following treatments.

**MATERIALS AND METHODS**

**Selection of animals:** A total of 42 crossbred (Haryana and Exotic) dairy cows comprising 36 subclinical endometritic repeat breeders and six normal cows (non-endometritic) were used in the study. All the animals belonged to the Cattle and Buffalo farm of Livestock Production and Management Section, Indian Veterinary Research Institute, Izatnagar, UP, India. These animals were maintained under uniform feeding and managerial conditions during the entire period of the study. All the animals had optimum body condition score (more than three based on 5 point system) and were repeat breeders (3 to 10 times repeaters). These animals were examined per rectum to rule out any anatomical defect of genital tract or of the ovaries. The SCE was confirmed at estrus on the basis of alkaline pH of the cervico-vaginal mucus (CVM) (Tsiligianni et al., 2001), positive colour reaction to white side test (Popov, 1969) and presence of 5% or more neutrophils in uterine cytology (Gilbert et al., 2005). Uterine contents were harvested from the uterine lumen of animals using sterile uterine catheter after flushing the uterus with 20 ml saline. The ratio of PMNs to the epithelial cells was calculated. The cows without subclinical endometritis (normal) were considered on the basis of negative colour reaction to white side test, normal pH of CVM and presence of < 5% PMN cells in uterine cytology smears.

**Experimental design:** The SCE cows were randomly divided into 3 groups (G-I,G-II,G-III) of twelve animals each. G-I animals received single i.u infusion of 50 ml of 30 nmol/L LTB₄ (Sigma, U.S.A) whereas the cows in G-II were administered with single i.u infusion of 50 ml of 10 mg/ml OG in PBS (Sigma, U.S.A). The animals of G-III were not given any treatment and served as positive control and the six normal (non-endometritic) cows served as a negative control (G-IV). LTB₄ was diluted under nitrogen gas with absolute ethanol to a stock concentration of 3mol/L and stored at -100°C in a nitrogen atmosphere (Zerbe et al., 1996). On the day of treatment the stock solution was transported to the farm on ice and protected from light. At the time of treatment, 0.5ml of stock solution was diluted with 50 ml of degassed PBS to make a final concentration of 30nmol/L.

Blood samples (10 ml) from jugular vein were collected in heparinised (20 IU/ml of blood) tubes at pre-treatment estrus (0 hr), 24 hr post-treatment and at subsequent estrus, and divided into two different aliquots viz., one was used for studying the TLC while from the other plasma was separated by centrifugation at 3000 rpm for 10 minutes and stored at -20°C for the estimation of NO and LPO levels. Total leukocyte count in the peripheral blood was determined by the standard procedure using hemocytometer. Plasma nitrate and nitrite concentration were estimated following the method of Sastry et al. (2002). Malonyldialdehyde (MDA) level, an index of lipid peroxidation was measured in plasma by the double heating method of Draper and Hadley (1990). The clinical recovery in cows was assessed at subsequent estrus following treatments by determining the changes in pH of CVM, nature of white side test and reduction of PMN cells in uterine cytology. All cows (G-I, G-II and G-III) were inseminated at subsequent estrus twice, 12 hr apart using 0.5 ml of good quality frozen/thawed semen collected from fertile crossbred bulls. Pregnancy was confirmed by per rectal examination 60 days following A.I.

**Statistical analysis:** The data were analyzed using statistical software SPSS -17 (SPSS Corporation, USA). Paired t test and Duncans multiple range test were used to compare the means of pH and TLC at 0 hr and 24 hr. Whereas, Duncan’s multiple range test was used to compare the means of NO and LPO concentration between and within the groups. The proportional data were analysed by Normal Deviate test.

**RESULTS AND DISCUSSION**

**Colour reaction to whiteside test and pH of CVM at pre and post-treatment estrus:** Before treatment, the CVM of all SCE cows (100 %) showed slight to moderate yellow colour reaction to white side test. Following the administration of LTB₄ and OG, the estrual mucus of 83.3% (10 out of 12) animals in G-I and 75.0% (9 out of 12) in G-II were found negative to whiteside test, however, this test was negative only in 16.7% (2 out of 12) animals of G-III at subsequent estrus. This could be explained on the basis of number of leukocytes present in uterine discharge. The normal uterine discharge contained too low population of leukocytes to cause any change of colour, whereas, the discharge from SCE animals found to possess more number of leukocytes causing colour reaction to whiteside test (Popov, 1969). The absence
of colour development in CVM of cows infused with OG in our study, confirms the earlier reports in cattle (Prasad et al., 2009) and buffaloes (Raju et al., 2009). However, such changes following iu infusion with LTB₄ have not been explored.

The overall mean pH of CVM of SCE cows did not differ significantly between the groups (8.29±0.06, 8.39±0.03 and 8.20±0.09). Several investigators have reported the pH of estrual CVM in the range of 7.40 to 8.00 in repeat breeder cows with SCE (Deori et al., 2004). The alkaline pH of CVM in our study might be associated with degree of infection in experimental cows. Alkaline pH of CVM in endometritic cows is known to be associated with the metabolites of bacteria and the inflammatory exudates (Salphale et al., 1993). However, the treatment of LTB₄ and OG caused a drastic decline (P<0.05) in pH of CVM at subsequent estrus (7.32±0.06 and 7.50±0.08). Similar findings were reported after OG treatment by Singh et al. (2003) and Prasad et al. (2009). However, the CVM from unrecovered cows had the high range of pH (8.08±0.15) as similar to that of untreated control. It is reasonable to suggest that following iu infusion of LTB₄ and OG, once the infection from the uterus is eliminated, the pH drops towards the neutral side. There was no decrease in pH of CVM at subsequent estrus in untreated cows which may be due to inability of the weak uterine defense mechanism in these cows to eliminate the infection.

**Uterine cytology:** Before treatment, all the experimental animals were found to be positive for SCE and the uterine cytology smears indicated the presence of >5-9 % PMN cells. The reduction in PMN cells (<5 %) was observed in 83.3 and 75.0% animals following LTB₄ and OG treatment as against 16.7% in untreated animals at subsequent estrus. It indicated that majority of cows had recovered from SCE following treatments.

Based on reduction in PMN cells (<5 %) a significantly (P<0.05) increased recovery rate was observed in LTB₄ (83.3 %) and OG (75.0 %) treated animals as compared to control (16.7%). LTB₄ is known to be an effective chemoattractant which stimulates migration of PMNs into the lumen of the bovine uterus (Zerbe et al., 1996) and hence causes clearance of uterine bacterial infection. LTB₄ stimulates the phagocytic capacity of PMNs (Ninnemann, 1988). The higher recovery in OG-treated cows is in accordance with the earlier reports in crossbred cows (Singh et al., 2003; Prasad et al., 2009) and buffaloes (Raju et al., 2009). It has been observed that OG treatment induces a large number of PMN cells migration into the uterine lumen and 90% of the cells identified in uterine secretions are neutrophils (Anderson et al., 1985).

**Conception rate:** Further, a total of ten cows, five each in LTB₄ and OG treatment groups were conceived against only one in untreated group. It could be attributed to the higher recovery following these treatments. It is evident from prior studies that the administration of non-specific immunomodulators enhances the UDM and thus clears the bacteria from the uterus and retrieves the cows from subclinical or clinical endometritis (Anderson et al., 1985). The lower conception in present study might be due to the fact that all our experimental cows were repeat breeders (3-10 times repeaters) and the cause of conception failure could be attributed to other factors apart from SCE. Perusal of literature revealed no work on recovery of fertility response in SCE cows following LTB₄ treatment. Though there was a high individual variability probably because of the sensitivity of LTB₄ to light, temperature and oxygen (Zerbe et al., 1996), however by taking appropriate measures to maintain the stability of LTB₄, this molecule could be used to play a major role in ameliorating uterine infection. Further, in this experiment the untreated cows might have failed to resolve the endometritis spontaneously due to weak cellular uterine defense.

**Total Leukocyte Count (TLC):** The LTB₄ and OG infusion caused a significant increase (P<0.01; 40.2 and 30.2%) in TLC observed at 24 hr following treatment (Table 1). The administration of LTB₄ and OG induced leukocytosis as indicated by elevated number of TLC, which may be released from bone marrow stores of neutrophils. A 121-178% increase in TLC within 30 minutes following 0.5-2µg/ml LTB₄ infusion was reported by Griswold et al. (1991) in rabbits. The mean TLC did not differ (P>0.05) between 0 hr (pretreatment estrus) and 24 hr in G-III and G-IV cows.

**Nitric Oxide (NO):** The mean plasma levels of NO were found to be significantly higher in subclinical endometritic than non-endometritic cows (Table 2). The levels of NO (µmol/L), declined significantly (P<0.05) from 71.58±2.57 to 60.00±2.06 and from 76.67±4.59 to 60.08±2.17 in SCE cows following administration of LTB₄ and OG respectively at subsequent estrus. Moreover, following treatments the levels of NO in endometritic cows were found similar to those of normal non-endometritic cows. In contrast, the mean NO concentration in plasma at subsequent estrus was significantly (P<0.05) higher in untreated control animals than their values either at 0 or 24 hr post-treatment. NO is considered as an important mediator in the developmental process of inflammation. The concentration of NO in blood plasma is associated with degree of endometritis (DeJun et al., 2010) who reported a higher levels (µmol/L) of NO in the blood of subclinical (8.15±0.97) and clinical endometritic (8.77±1.35), than normal cows (6.83±0.28).
TABLE 1: Mean(±SE) total leucocyte count (x 10^6 cells/ml) in blood of subclinical endometritic cows treated with LTB_4 and OG and in cows without endometritis

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Treatment groups</th>
<th>Control groups</th>
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<tbody>
<tr>
<td></td>
<td>G-I (n=12)</td>
<td>G-II (n=12)</td>
</tr>
<tr>
<td></td>
<td>LTB_4</td>
<td>OG</td>
</tr>
<tr>
<td>0 hr (pre-treatment)</td>
<td>6.96±0.21^A</td>
<td>9.17±1.03^A</td>
</tr>
<tr>
<td>24 hr (post-treatment)</td>
<td>9.76±0.54^B</td>
<td>11.94±1.31^B</td>
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LTB_4: Leukotriene B_4; OG: Oyster glycogen; UT: Untreated control; HC: Healthy control
Values with different superscripts in each column (A, B) differ significantly (P<0.01).

TABLE 2: Mean (±SE) Nitric oxide (µmol/L) in blood plasma of subclinical endometritic cows treated with LTB_4 and OG and in cows without endometritis

<table>
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<tbody>
<tr>
<td></td>
<td>G-I (n=12)</td>
<td>G-II (n=12)</td>
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<tr>
<td></td>
<td>LTB_4</td>
<td>OG</td>
</tr>
<tr>
<td>0 hr (pre-treatment)</td>
<td>71.58±2.57^Bb</td>
<td>76.67±4.59^Bb</td>
</tr>
<tr>
<td>24 hr (post-treatment)</td>
<td>70.25±2.50^Bb</td>
<td>77.25±2.92^Bb</td>
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<tr>
<td>Subsequent estrus</td>
<td>60.00±2.06^Aa</td>
<td>60.08±2.17^Aa</td>
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</tbody>
</table>

LTB_4: Leukotriene B_4; OG: Oyster glycogen; UT: Untreated control; HC: Healthy control
Values with different superscripts in each column (A, B) and in each row (a, b) differ significantly (P<0.05).

Present study revealed a significant decrease in NO concentration following treatments, whereas, the levels remained high at subsequent estrus in untreated cows. This observation corroborates the fact that administration of immunostimulatory substances (LTB_4 and OG) retrieves the cows from SCE by eliminating the bacterial infection and reducing uterine inflammation as indicated by lower plasma NO concentration. This contention receives support from the findings of De (2004) who observed a higher concentration of NO in isolated blood PMN cells from mastitic cows and a significant (P<0.05) decline in its level 3 days after the treatment with alcoholic extract of Azadiracta indica.

Lipid Peroxide (LPO): A significantly lower levels of LPO was observed for normal control (G-IV) than the SCE cows (G-I, II and III) at 0 and 24 hr. Subsequently, following the administration of LTB_4 and OG the LPO levels were declined significantly at subsequent estrus in G-I and G-II and became similar to those of non-endometritic cows (Table 3). In contrast, no significant change in LPO levels was observed in untreated endometritic animals either at 0 hr (pre-treatment estrus), 24 hr (post-treatment estrus) or at subsequent estrus. This observation receives support from Kizil et al. (2010) who reported a higher LPO levels in cows with acute puerperal metritis than healthy ones.

The present study reveals a lower plasma MDA concentration in healthy cows than those which recovered from subclinical endometritis, indicating a slightly higher oxidative stress in recovered animals than the healthy ones. The lower LPO levels in RBC haemolysate have been

TABLE 3: Mean(±SE) Lipid peroxide levels (nmol MDA/L plasma) in blood plasma of subclinical endometritic cows treated with LTB_4 and OG and in cows without endometritis

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<th>Treatment groups</th>
<th>Control groups</th>
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<tr>
<td></td>
<td>G-I (n=12)</td>
<td>G-II (n=12)</td>
</tr>
<tr>
<td></td>
<td>LTB_4</td>
<td>OG</td>
</tr>
<tr>
<td>0 hr (pre-treatment)</td>
<td>667.15±42.85^Bb</td>
<td>753.73±32.78^Bb</td>
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<tr>
<td>24 hr (post-treatment)</td>
<td>683.21±42.53^Bb</td>
<td>748.39±40.54^Bb</td>
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<tr>
<td>Subsequent estrus</td>
<td>555.56±42.53^Ab</td>
<td>630.88±31.16^Ab</td>
</tr>
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LTB_4: Leukotriene B_4; OG: Oyster glycogen; UT: Untreated control; HC: Healthy control
Values with different superscripts in each column (A, B) and in each row (a, b, c) differ significantly (P<0.05).
observed by administration of immunomodulators, like methanolic fraction of neem oil and neem seed powder in endometritic cows (Singh et al., 2010).

From this study it can be concluded that Leukotriene B$_4$ and Oyster glycogen stimulated the uterine defence mechanism and reduced oxidative stress in subclinical endometritic cows.

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REFERENCES


