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Therapeutic efficacy of non-antibiotic protocols for endometritis in crossbred dairy cows

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Abstract
Thirty endometritic crossbred dairy cows diagnosed were randomly allotted to three groups with 10 each to evaluate the therapeutic efficacy of some non-antibiotic protocols. Cows in Group I received 30 ml ozonated oil intrauterine (i.u), Group II 500mg of oyster glycogen in 30 ml PBS i.u and Group III 30ml PBS i.u as control. Uterine flushings were collected on the day of estrus before and after treatment (next immediate estrus) and tested for pH, whiteside test, endometrial cytology and determination of common aerobic bacteria. The mean pH of uterine flushings in all the groups before treatment was 7.95. A significant reduction in mean pH was observed following treatment with ozonated oil (7.46) and oyster glycogen (7.31) whereas no significant change in the untreated cows (7.77) was recorded. Aerobic bacteria isolated in the uterus of endometritic cows were *E. coli*, Staphylococcus sp. Enterobacter sp. and Proteus sp. Following treatment, 60 per cent (6/10) in Group I, 80 per cent (8/10) in Group II and 30 per cent (3/10) in Groups III cows were recovered based on whiteside test and endometrial cytology results. The conception rate was higher (87.50%) in Group II followed by 66.66 per cent in Group I and 33.33 per cent in Group III.

Keywords: endometritis, ozonated oil, oyster glycogen, whiteside test, recovery

Introduction
Endometritis is one of the postpartum uterine diseases that causes reduced fertility with significant economic losses to the dairy farmers. Prevention and treatment of endometritis is necessary to improve reproductive efficiency of dairy cows. Conditions such as decreased immunity, dystocia, unhygienic condition, abnormal gestation length, stillbirth, twins, retained fetal membranes, metabolic disease, etc. may increase the risk of reproductive diseases (Kim and Kang, 2003) [19]. Most commonly isolated bacterial pathogens in postpartum uterus of cows are *Escherichia coli*, Streptococci sp., *Arcanobacterium pyogenes, Bacillus lichiniformis*, Prevotella sp., Staphylococcus sp. and *Fusobacterium necrophorum*. However, *E. coli, A. pyogenes, F. necrophorum* and Prevotella sp. are the most common potential pathogens of the uterus, of which former three bacteria act synergistically to suppress uterine health/defense (Foldi *et al*., 2006) [12].

Various methods to diagnose endometritis in cows including vaginoscopy (Drillich *et al*., 2004) [10], metricheck (Pleticha *et al*., 2009) [32], pH (Sahadev 2005) [38] whiteside test (Pateria and Rawal, 1990)[31], uterine ultrasonography (Barlund *et al*., 2008) [2], uterine biopsy (Kasimanickam *et al*., 2004) [18], bacterial culture (Sahadev, 2017) [39] and endometrial cytology (Sheldon *et al*., 2006) [43] have been used. Several approaches have been employed to treat crossbred cow showing endometritis including antibiotics, antiseptic and hormonal therapy (Hussain and Daniel, 1991) [16]. However, there are certain limitations in the use of antibiotic and hormonal therapy such as development of antimicrobial resistance due to inadvertent use (Durrani *et al*., 2017; Kozat and Okman, 2019) [11, 21], residual effect of antibiotics and hormones in the milk and meat causing health hazards in human (Roberts, 1986) [37], milk disposal, high cost and long duration of treatments and suppression of uterine defense mechanism (Sarkar *et al*., 2016) [40]. In the scenario of increased public health consciousness, scrutinizing for some alternative yet effective non-antibacterial therapeutics like immunomodulators, ozonated oil, herbs and others have become need of the day (Sahadev, 2005; Rajeshkumar, 2016; Nikolaev and Konopeltsev, 2020) [38, 35, 28].

Although spontaneous recovery in some endometritic cows has been reported (Gilbert *et al*., 2005) [14], the negative impact on the reproductive performance compels to initiate treatment for the endometritis.

Materials and Methods

Out of 174 cows presented for artificial insemination were screened for endometritis from October 2019 to September 2020, 34 cows were found positive for endometritis as revealed by nature of vaginal discharge, clinico-gynaecological examination, pH and confirmed by whiteside test. Further, the samples were subjected for endometrial cytology and determination of common aerobic bacteria in the uterine flushings of endometritic cows on the day of diagnosis (before treatment) and in next immediate estrus (after treatment). The cows in Group I received 30 ml ozonated oil, cows in Group II received 500 mg of oyster glycogen in 30 ml PBS and cows in Group III received 30 ml PBS intrauterine on the day of diagnosis. In the study, comparative therapeutic efficacy of ozonated oil and oyster glycogen was evaluated in randomly assigned 10 endometritic cows in each group and also conception rate was recorded following artificial insemination to recovered cows. The data generated were analyzed using student's t test. Consent from the college level Institutional Animal Ethics Committee (IAEC) was obtained to conduct the study (IAEC No. VCH/IAEC/2019/117; dated 03.12.2019).

Results and Discussion

Clinico-gynaecological examination:

Diagnosis of endometritis by rectal palpation is subjective and prone for false negative diagnosis as the technique lacks standardization (Foldi *et al*., 2006) [12]. Under field conditions, diagnosis of endometritis is sometimes made based on the pus mixed vaginal mucous on vaginoscopy but LeBlanc *et al*. (2002) [25] and Drillich *et al*. (2004) [10] found vaginoscopy to be 12.3-20 per cent sensitive and 88-90.2 per cent specific in reference to cytobrush cytology results

pH of uterine flushings: The mean pH of uterine flushings of endometritic cows before treatment was 7.95 ± 0.07 whereas 7.46 ± 0.05 in healthy cows. Almost similar pH values (7.7 to 9.0) were reported in endometritis cows (Gupta *et al*., 1981; Kumar *et al*., 2017; Bedewy and Rahawy, 2019) [3, 23, 15] and normal healthy crossbred cows (7.0 to 7.5) (Gupta *et al*., 1981; Tsiligianni *et al*., 2001; Kumar *et al*., 2017) [50, 23, 15]. The inflammation of endometrium due to infection, metabolites of bacteria and inflammatory exudates alters the pH of uterus to alkaline side, which in turn results in failure of conception due to death of spermatozoa and embryo in the uterus (Singla *et al*, 2004 and Sheldon *et al*., 2006) [48, 43]. Following treatment in the next subsequent estrus a significant reduction ($P<0.05$) in pH was noticed in treated Group I (7.46), Group II (7.31) and no significant change in control Group III (7.77) was recorded (Table 1). Comparably substantial decline in pH of uterine flushings after treatment schedules has been reported by several authors (Ravikumar, 2004; Sahadev, 2005) [36, 38].

Table 1: pH of uterine flushings before and after treatment

Treatment group(n=10)	Before treatment	After treatment	Significance
Group I: Ozonated oil	7.97 ± 0.15	7.46 ± 0.11	*
Group II: Oyster glycogen	7.98 ± 0.13	7.31 ± 0.10	*
Group III: Control	7.89 ± 0.07	7.77 ± 0.13	ns

Note: * - significant at $P<0.05$; ns – non significant

Whiteside test

Whiteside test was performed using aseptically collected uterine flushings on the day of diagnosis (day of estrus) and 30 confirmed cows as cases of endometritis. The normal cervical mucus has lesser number of leucocytes which do not cause any change of colour whereas, in clinical or sub clinical endometritis the cervical mucus contains increased number of leucocytes which causes colour reaction with 5% NaOH (Pateria and Rawal, 1990; Sarkar *et al.*, 2006) [31, 41]. Several researchers have stated that the accuracy of WST in detection of endometritis was between 75-92.5 per cent using CVM or uterine flushings of normal cows indicating uterine infection (Ravikumar, 2004; Bhat *et al.*, 2013; Sahadev, 2017) [36, 4, 39]. In response to bacterial infection, number of leucocytes increased in the uterine flushings.

The effect of treatment on number of leucocytes in the uterine flushing of the 30 endometritic cows was evaluated in subsequent estrus. In Group I, 6 out of 10 (60%), 8 out of 10 (80%) in Group II and 3 out of 10 (30%) in Group III were found negative for whiteside test indicative of clearance of endometritis. The present results are in agreement with the findings of Raja *et al*. (2012) [34], Kumar *et al.* (2015) [24] and Sahadev (2017) [39]. The increased number of leucocytes present in the uterine flushings in response to bacterial infection in endometritic cows reduced due to clearance of infection. Therefore, after treatment with ozonated oil and oyster glycogen higher number of uterine flushing samples showed absence of colour reaction. Intrauterine infusion of oyster glycogen increases influx of PMNLs into the uterine lumen with enhanced phagocytic activity and the cellular response persists for 72h thus clears the uterine infection (Singh *et al.*, 2003) [46].

Endometrial cytology:

In the present study, >5 per cent neutrophils after 50 days postpartum was used as the threshold value indicating endometritis in cows as per Sheldon *et al*. (2006) [43] and Gabler *et al*. (2009) [13]. The endometritis cows confirmed based on whiteside test were further subjected to endometrial cytology and it also reconfirmed the whiteside test results. Uterine cytology has been the gold standard for the diagnosis of endometritis in cows (Barlund *et al.*, 2008; Polat *et al*., 2015) [2, 33]. The findings suggest that whiteside and endometrial cytology have more accuracy in identifying the endometritis. Mohammad *et al*. (2017) conducted a similar study in cows and have also reported poor to moderate agreement among PMNLs per cent, bacteriological findings and vaginal discharges.

Bacterial culture:

Uterine flushing samples from all the confirmed endometritic cows based on Whiteside test and endometrial cytology were

subjected for aerobic bacteriological studies. Single isolates were obtained in 14 out of 34 (41.18%) uterine flushings of cows affected with endometritis *viz*., Enterobacter sp. (14.70%) followed by *E. coli* (11.76%), Staphylococcus sp. (8.82%) and Klebsiella sp. (5.88%). Mixed isolates were obtained from 20 out of 34 (58.82%) which include *E. coli* and Staphylococcus sp. (20.59%), *E. coli* and Proteus sp. (8.82%), *E. coli* and Enterobacter sp. (20.59%), and Proteus sp. and Staphylococcus sp. (8.82%). *E. coli* and Staphylococcus sp. and *E. coli* and Enterobacter sp. were most frequently found aerobic organisms in the mixed isolates (Table 2).

The results of the study are in accordance with the findings of several workers (Constantin and Birtoiu, 2016; Durrani *et al*., 2017; Kumar *et al*., 2018) [11, 7, 22]. The most common pathogenic species were *E. coli*, *Archenobacter pyogenes*, *Fusobacterium necrophorum* and Prevotella sp. (Foldi *et al*., 2006) [12]. However, Bhatt *et al.* (2013) [4] recorded single isolates in 76.67 and mixed isolates in 23.33 per cent in cows with endometritis.

The present investigation found that there was a high degree of association between diagnoses made from the studies on WST, EC with bacteriology of the uterus. In the present study after intrauterine treatment with ozonated oil (Group I) and OG (Group II), infection was cleared in 60 and 80 per cent cows, respectively. However, it could not eliminate infection in remaining 40 and 20 per cent cows, respectively in Group I and Group II. Similarly, Jena *et al*. (2018) [17] also reported that OG could not eliminate 10 per cent bacterial isolates in their study. The failure of elimination of infection in some cows might be due to chronic infections where endometrium gets destroyed and there by resulting in poor stimulatory response (Singh *et al*., 2000) [47].

Table 2: Frequency of single and mixed aerobic bacterial isolates in endometritic cows (N=34)

Type of bacterial isolates	Bacteria	No. of cows from which the bacteria was isolated	Percentage
Single isolate	*E. coli*	4	11.76
	Staphylococcus sp.	3	8.82
	Enterobacter sp.	5	14.70
	Klebsiella sp.	2	5.88
Mixed isolate	*E. coli* + Proteus sp.	3	8.82
	E. coli + Staphylococcus sp.	7	20.59
	E. coli + Enterobacter sp.	7	20.59
	Proteus sp. + Staphylococcus sp.	3	8.82
Total		34	100

Assessment of recovery and conception rate

The relative efficacy of treatment by ozonated oils and oyster glycogen was evaluated by comparing with the untreated control group based on the recovery rate and conception following inseminations. Following the treatment, the highest recovery and conception rate (80.00 and 87.5%) was obtained in oyster glycogen treated group followed by ozonated oil treated group (60.00 and 66.66%) and the lowest (30.00 and 33.33%) in untreated control group (Table 3).

Table 3: Efficacy of various treatment regimens for recovery from endometritis and conception rate following AI in crossbred cows (n=10)

Group	No. of cows recovered	Recovery rate	Conception among the recovered cows	Conception rate
I (Ozonated oil)	6	60.00%	4	66.66%
II (OG)	8	80.00%	7	87.50%
III (Control)	3	30.00%	1	33.33%

The recovery rate for cows treated with OG in the present study is in accordance with the findings of Sahadev (2005) [38] and Sarma *et al*. (2012) [42] where they have recorded a recovery rate of 80 per cent. Singh *et al*. (2018) [45] and Solanki *et al.* (2019) [49] recorded a lower recovery rate of 60-70 per cent following intrauterine infusion of oyster glycogen in endometritic cows. Conception rate of 88.70 per cent was reported by Sahadev (2005) [38] is in accordance with the present study. Lower conception rate of 50 -70 per cent has been reported by Sarma *et al*. (2012) [42], Biswal *et al*. (2013) [5], Singh *et al*. (2018) [45] and Solanki *et al.* (2019) [49] after oyster glycogen treatment for endometritic cows. Following infusion of biologically active immunomodulators like OG, the mechanisms involved in the influx of PMNLs into the uterine lumen include the chemoattractants that cause vasodilation, act directly as chemoattractant, stimulates monocyte and/or macrophages that in turn produce interleukin -1α and -8 which are responsible for influx of PMNLs (Dhaliwal *et al*., 2001) [8].

Similar to the present study recovery rate of 60 per cent for endometritic cows treated with ozonated oil has been reported by Mali *et al*. (2020) [26]. However, Nikolaev and Konopeltsev (2020) [28] reported a higher recovery rate of 93.3 per cent. The conception rate recorded in this study is in accordance with the conception rate (50- 63.3%) reported by Mali *et al*. (2020) [26] and Nikolaev and Konopeltsev (2020) [28]. Aguilar *et al.* (2019) [1] have obtained the first AI conception rate of 43.33 per cent following intrauterine infusion of ozone in distilled water for treatment of endometritis in cows.

Ozone is a powerful oxidant with instability and quick transformation into oxygen (Di Paolo *et al*., 2004) [9]. The oxidative processes in peroxidase activity destroy bacteria by disrupting the capsules and cell membranes and then blocking the replication of DNA. Gram-positive bacteria are reported to be more sensitive to ozone than Gram-negative. Immunomodulatory activity of ozone by stimulating activation of lymphocytes or monocytes to release several cytokines, *viz*., IFN α, β, γ, TNFα, IL-1, -2, -4, -6, -8 and -10, GM-CSF and TGFβ has been reported (Ohtsuka *et al*., 2006; Korzun *et al*., 2008) [29, 20]. Ozmen *et al.* (1993) [30] opined that ozone may further activate the Kreb's cycles and stimulate release of enzymes to cause elimination of free oxygen radicals and thus may protect the host cells.

The unsaturated fatty acid contents of vegetable oils including

flaxseed, olive oil and sunflower oil, interacts with ozone to form ozonides which has antimicrobial effects (Nikolaev and Konopeltsev, 2020) [28]. Mali *et al*. (2020) [26] recently reported that the improved conception rate obtained in their study in the normal cyclical and repeat breeder cows with subclinical uterine infection might be due to the beneficial effect of ozone gas in improving the uterine environment, tissue oxygenation and blood flow towards uterus. The reason for reduced recovery and conception rate obtained in the present study may be explained by the facts that though ozone has strong disinfection and immunomodulation activity, it fails to destroy intracellular bacteria and viruses (Bocci and Di Paolo, 2004) [6].

Out of 10 untreated endometritic cows (Control), three cows (30%) found complete recovery from endometritis and one spontaneously recovered cow conceived following insemination (Table 3). Spontaneous recovery in dairy cows have been reported by several authors as 8.33 (Sahadev, 2005) [38], 40.00 (Sheldon *et al*., 2009) [44], 16.66 (Rajeshkumar, 2016) [35], 40.00 (Sahadev, 2017) [39] and 20 per cent (Singh *et al*., 2018) [46].

Conception rate among the spontaneously recovered cows after insemination was reported as 41.66 per cent by Ravikumar (2004) [36] and 10 per cent by Singh *et al*. (2018) [46] and no conception by Rajeshkumar (2016) [35].

Concentration of some metabolic or steroid hormones and production and reproduction factors influenced self-recovery of cows from clinical endometritis. Size and position of reproductive tract influence self-healing from clinical endometritis (Mogheiseh *et al*., 2020) [27].

Conclusion

The findings of this study indicated that oyster glycogen can be effectively used for treatment of endometritis. The non-antibiotic therapy for endometritis with oyster glycogen and ozonated oils could overcome the known disadvantages of antibiotics. Ozonated oil, a non-antibiotic is a new hope for effective treatment of endometritis in cows. However, use of ozonated oil for endometritis treatment needs proper standardization of dose, duration of treatment, frequency of administration and evaluation on a larger number of cows before its recommendation.

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Conflict of interest

Authors have no conflict of interest

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