Report of the Work Done Under UGC project

Project Title : U**terine immunomodulation: a swap of antibiotherapy in endometritic cattle"**

Principal Investigator : **Dr. Mrigank Honparkhe** Principal Scientist cum Head, Department of Veterinary Gynaecology and Obstetrics,GADVASU

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Objectives of the project

To investigate the efficacy of cytobrush technique in diagnosing clinical and subclinical endometritis in cattle.

B. To study the comparative efficacy of certain immunomodulators in enhancing uterine immunity thereby treating endometritis in cow.

C. Based on above, to develop cost effective and convenient therapeutic strategies for the treatment of endometritis in cows.

Methodology

Field surveys were conducted in different villages of Punjab. A total of 200 repeat breeding crossbred cattle at spontaneous estrus were examined for the status of genitalia (through rectal palpation) and cervico-vaginal mucus (CVM) i.e. clear/cloudy/thick/turbid. The cytobrush technique was applied in all the cattle to detect endometrial cytology for confirmation of type of endometritis. The subclinical endometritis was declared on the basis of clear CVM and $\geq$ 4% polymorphnuclear cells (PMN) whereas clinical endometritis diagnosed on the basis of turbid/mucopurulent/cloudy CVM and presence of fibroblast cells in uterine cytology.

Procedure of cytobrush technique-

After proper restraining, the cattle were subjected to evacuation of rectum through back raking. The perineal area and vulva were washed with savlon and water and later on disinfected with sprit swab. The vulvar lips were pulled apart by an assistant and the cytobrush assembly (especially fabricated for bovine contains intrauterine cathter and a stylette attached with cytobrush, Picture 1) was introduced into vagina and then through the cervix to body of the uterus. After assuring its place, the stylette was pushed to expose cytobrush and then screwed gently in both directions (clockwise and anticlockwise). Gentle pressure was applied on its tip against the

uterine body per rectum for proper contact of brush with endometrium. The inner stylette was then withdrawn into the outer cathter to its normal position and then the whole catheter was withdrawn from the reproductive tract.

Picture 1. Showing cytobrush assembly contains outer catheter (A), inner stylette (B) and cytobrush (C).

Staining method for endometrial cytology

Immediatly after removal from reproductive tract, the cytobrush was screwed on clean glass slide. The smear was exposed to Geimsa stain. The stain was prepared adding 1g Geimsa powder in 50 ml glycerol, mixed thoroughly and kept at 4°C for over night at room temperature then 50 ml methanol was added, mixed and homogenised. It was kept for over night again, mixed and filtered with whatman filter paper no.1. The working solution was prepared by diluting it as 3ml Geimsa+2ml PBS +35 ml distilled water) put for 3min. in Concentrated or 30 min. in diluted. After drying the slide, 100 cells were counted under microscope on 40X and oil immersion (endometrial cells + PMN cells) and % PMN cells were calculated.

Microbial assay

The cattle declared on the spot for subclinical endometritis (i.e. presence of ≥ 4% PMN) were subjected to second cytobrush sample for microbial assay.

A. Isolation and identification of bacteria-

Immediately after removal from reproductive tract, the cytobrush were kept in clean sterile glass test tube and were opened in front of a flame and the collected material was put in BHI broth

with the help of sterilized forceps. These were incubated for 6-8 hrs. using nichrome loops. It was then gently streaked on BHI, MLA and EMB agars medium in petri dishes. The plates were incubated in incubator at 37°C and were examined 24 hrs later for growth of bacterial colonies. If no growth was present, the plates were further incubated for 48 hrs at 37°C, before declaring a sample bacteriologically negative (Quinn *et al* 1999a). Isolation and identification of bacteria based on the morphology, cultural characters and biochemical tests as described by Quinn *et al* (1999b).

B. Bacterial morphology

All the isolates obtained were characterized morphologically by using Gram staining, (Quinn *et al* 1999b)

C. **Examination of microbial load**

The bacterial load was calculated using pour plate technique (Apas *et al* 2010). Broth (sample) was thoroughly mixed using a sterile glass rod to make uniform suspension. The above suspension was diluted serially by transferring 1ml from the initial tube to 2nd tube containing 9 ml of PBS and this was followed to make rest of the dilutions in serial order. From each serially diluted tube, 1ml suspension was poured into 20 ml of the molten media, which was pre-maintained at 45°C to 50°C in a water bath. It was mixed thoroughly and was later poured into petri-plate. The petri-plate was swirled thoroughly for uniform mixing of suspension with media. The plate was cooled to solidify the content and then incubated for 12-18 hrs to develop bacterial colonies. These bacterial colonies were counted manually and the total number of bacterial load was calculated on the bases of serial dilution.

In second experiment, a total of 50 repeat breeding cross bred cattle suffering from subclinical endometritis (selected from experiment 1) were divided in to five groups at random (Fig 1). The grouping of the animals was as follows.

Group 1 (Mastivexym ointment; n=10)

These cattle were subjected to intrauterine treatment with Mastivexym ointment (Veyxpharma Gmbh, Germany; contains Trypsin 8 mg, Chymotrypsin 8 mg, papain 4 mg, α-tocopherol acetate 120 mg and retinyl palmitate 58.83 mg) once at day 0 (spontaneous estrus).

Group 2 (Crude formulation of enzymes; n=10)

This group was subjected to intrauterine administration of combination of enzymes viz. Trypsin 8 mg, chymotrypsin 8 mg, papain 4 mg, α-tocopherol acetate 120 mg and retinyl palmitate 58.83 mg (Sigma-Aldrich, USA) dissolved in 10 ml distilled water as a crude formulation not in the form of ointment once at day 0 (spontaneous estrus).

Group 3 (*Escherichia coli* lipopolysacchrides; n=10):

These cattle were treated with intrauterine infusion of 100 µg *Escherichia coli* lipopolysacchrides (LPS) dissolved in 20-30 ml normal saline once at day 0 (Spontaneous estrus).

Group 4 (Inj. Levamisol; n=10):

This group was subjected to inj. Levamisol, 10 ml subcutaneosly once daily for three consecutive days starting from day 0 (Spontaneous estrus) to day 2 of estrous cycle.

Group 5 (Control; n=10)- These animals were not given any kind of treatment at spontaneous estrus.

The cattle belonging to each group were administered intramuscularly with prostaglandin F2 alpha analogue (Inj Estrumate, 500µg cloprostenol, MSD Animal Health, Germany) on day 12 after spontaneous estrus. The estrus detection was done manually by visual signs viz. CVM discharge, kajoling and mounting behavoiur. On the day of start of estrus sign, the cattle received GnRH analogue (20 µg of Buserelin acetate, Inj Receptal) intramuscularly in order to ensure ovulation. Following 12-14 hrs of GnRH injection, all the animals were artificially inseminated twice at 12 hrs interval.

All the cattle from each group were subjected to cytobrush technique and microbial assay (as described in previous experiment; 3.2.1.2 & 3.2.1.3) at spontaneous and induced estrus twice.

The cattle from each group were also subjected to blood sampling on both occasions (pre-treatment spontaneous and post-treatment induced estrus). Blood samples were collected separately in heparinized stoppered centrifuge tubes by jugular venipucture using 18 G needle. All the blood samples were kept in the ice box and were brought to the laboratory. Thereafter, the samples were centrifuged for 15 min at 3000 rpm followed by separation of plasma. The plasma was collected in 2 ml plastic storage vial and stored at -20°C till further analysis.

The milk samples were also collected from each cow at spontaneous and induced estrus by stripping of each teat subsequent to proper cleaning with spirit swab. The samples were analysed for somatic cell count on same day.

Observations recorded:

a) The endometrial samples collected by cytobrush were analysed for percentage of PMN cells, microbial assay (bacterial isolation, bacterial load) in all the groups at spontaneous and induced estrus.
b) Efficacy of each treatment in terms of reduction in PMN cells and bacterial load at induced estrus (subsequent to the treatment) was adjudged.
c) Blood samples analysed for TLC, Hb (SIEMENS-ADVIA 2120 HEMATOLOGY SYSTEM), DLC (Bongh *et al* 1991), total protein (by Lowry *et al* 1951) and Igs (by turbidity method: Mcewan *et al* 1970).
d) Milk samples collected from cattle of each group observed for somatic cell count (by somascope count control pilot) before and after treatment.
e) Pregnancy diagnosis in each cow of different groups was done between days 45-50 by ultrasonography.

Preparation of blood smear for DLC-

A drop of blood collected in heparinized vials was put on a clean greas free slide this blood drop was touched with another smooth edged glass slide kept at an angle of 45° with the base slide. The top slide moved in one throw so as to prepare a tongue shaped smear. The smear was then air dried and stained with leishman stain for 2min than diluted by equal amount of buffer an put it for 8 min,washed with tap water and examined under oilimmersion.

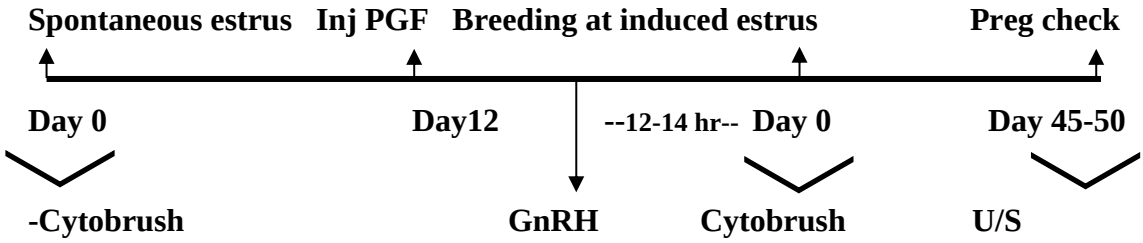
Estimation of total immunoglobulins (Ig) in plasma-

Immunoglobulins were estimated in the blood plasma by turbidity method (Mcewan *et al* 1970). Briefly, 0.5 ml of blood serum or plasma (1:1 diluted with PBS, pH 7.4) was added to 5.0 ml of zinc sulphate (0.025%). Incubated at 25± 1^{o} C for one hour and absorbance was taken at 580 nm. Calculated percentage of total Igs from the standard values of bovine Igs, run along with the sample.

Statistical analysis

The numerical data were presented as Mean ± SEM for all the parameters. A paired t-test was applied to compare the parameters before and after treatment in all groups (Microsoft Excel 2010). The relationship between % PMN cells and microbial assay was calculated by Pearson's correlation coefficient (SPSS 16.0). One way ANOVA was used to find out differences between groups (SPSS 16.0). First service pregnancy rates were compared by chi square test. A P-value of <0.05 was considered statistically significant.

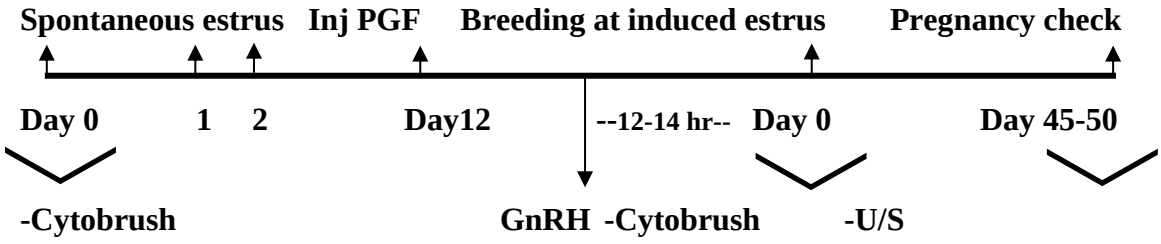
Group 1, 2 & 3



(For PMN cells & Microbial assay) (For PMN cells & Microbial assay)

- Intrauterine treatments
- Blood sample -Blood sample
-Milk sample -Milk sample

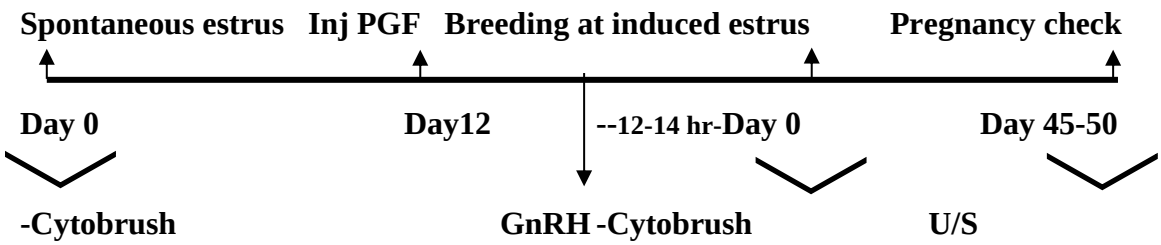
Group 4



(For PMN cells & Microbial assay) (For PMN cells & Microbial assay)

- S/C, Levamisol (3 inj on Days 0, 1 & 2)
- Blood sample -Blood sample
-Milk sample -Milk sample

Control group



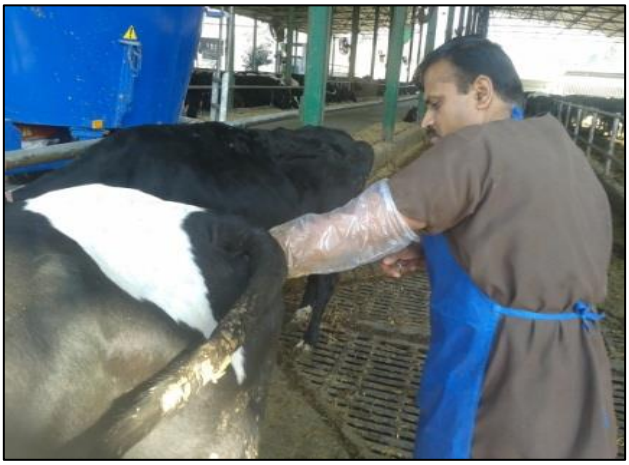
(For PMN cells & Microbial assay) (For PMN cells & Microbial assay)

- Blood sample -Blood sample

-Milk sample -Milk sample

Field survey, screening and treatment

Halwara
University dairy farm
Bhago Majra, Kharar
Kheri khurd, Sangrur
Tharike, Ludhiana
Kanganwal, Ludhiana



Results:

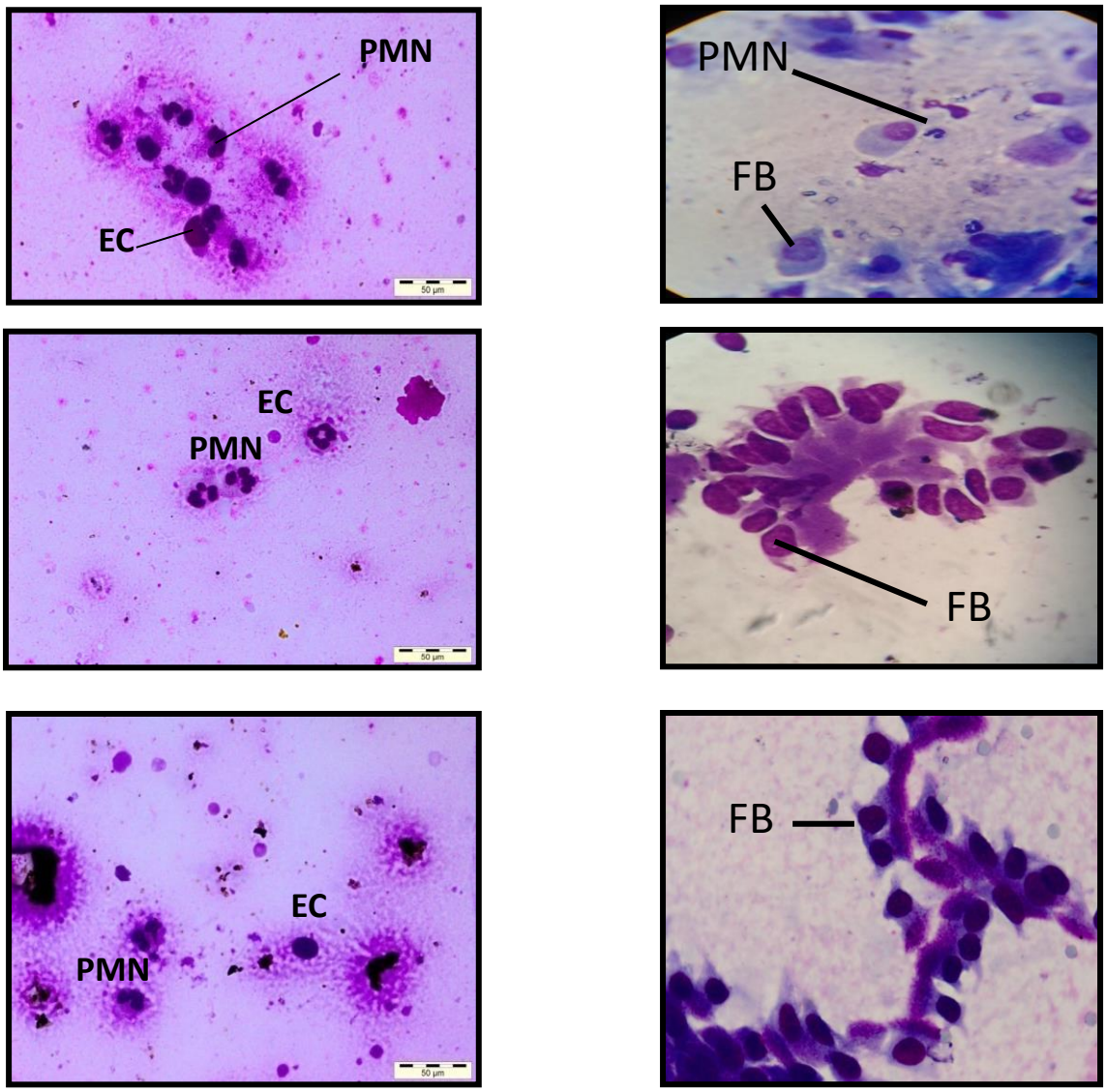
Diagnostic efficacy of uterine cytobrush

Based on uterine cytobrush cytology and characteristics of CVM, 51% crossbred cattle suffered from endometritis. The incidence of subclinical and clinical endometritis was recorded as 29% and 21%, respectively. The cattle with subclinical endometritis had clear CVM whereas, cattle with clinical endometritis showed turbid/cloudy/mucopurulent discharges at the time of estrus. The uterine cytology through cytobrush revealed PMN cells (≥4 %) and endometrial cells in subclinical endometritis whereas; clinical endometritic cases revealed abundant fibroblast cells (according to the severity of the infection) in addition to PMN cells and endometrial cells or epithelial cells (Picture 2). The bacteria were isolated from all the cattle (100%) those declared with subclinical endometritis on the basis of % PMN and yielded significant positive correlation (r=1). Various bacterial isolates recovered are shown in Table I. There was prevalence of *bacilli* (47.94%) followed by *E coli* (24.65%) and Staphylococcus (12.32%) in this study. The false positive cases for subclinical endometritis were not found as all the animals with ≥ 4 % PMN revealed bacterial isolates. In conclusion, cytobrush technique is an efficient method and can be considered as a cow side test for the diagnosis of subclinical endometritis at field level.

Comparative efficacy of various immunomodulators used in subclinical endometritic cattle

In the present study, a total of 50 repeat breeding cross bred cattle suffering from subclinical endometritis (selected from experiment 1) were divided in to five groups at random according to their treatment at spontaneous estrus as Group 1 (Mastivexym ointment), Group 2 (Crude formulation of enzymes) Group 3 (*Escherichia coli* lipopolysacchrides), Group 4 (Inj. Levamisol) and Group 5 (control). The cattle belonging to each group were administered intramuscularly with prostaglandin F2 alpha analogue (Inj Estrumate, 500µg cloprostenol, MSD Animal Health, Germany) on day 12 after spontaneous estrus followed by GnRH analogue (Inj. Recepetal, 5 ml, i.m.) on the day of induced estrus. After 12-14 hrs.of GnRH, all the cattle were inseminated twice at 12 hrs intrval.

Subclinical Endometritis **Clinical Endometritis**



Picture 2. Showing uterine cytology of subclinical and clinical endometritic cattle. PMN- polymorphonuclear cells, EC- epithelial cells, FB- fibroblast cells

Per cent PMN cells and bacterial load during spontaneous (Pre Rx) and subsequent induced estrus (post Rx)

The % PMN cells and bacterial load at spontaneous and subsequent induced estrus are presented in Table 2. The % PMN cells and bacterial load during post-treatment induced estrus was significantly (P<0.05) reduced in all the treated groups except control. In control group, the % PMN cells and bacterial load were significantly higher (P<0.05) than other treated groups during subsequent induced estrus which was suggestive of no improvement at subsequent estrus in this group. There was significant decline in % PMN cells during subsequent induced estrus indicative of recovery from infection in treatment groups.

Table 2. Per cent PMN cells and bacterial load at spontaneous and induced estrus in cattle of various groups suffering from subclinical endometritis.

Group	% PMNs cells		Bacterial load (log_{10}CFU/ml)	
	Spontaneous estrus	Induced estrus	Spontaneous estrus	Induced Estrus
Group 1 (Mastivexym: n=10)	5.5 ± 0.78^{a}	2.7 ± 0.34^{bA}	5.80 ± 0.24^{a}	4.28 ± 0.52^{bA}
Group 2 (FE; n=10)	4.6 ± 0.22^{a}	2.3 ± 0.26^{bA}	5.76 ± 0.17^{a}	4.55 ± 0.54^{bA}
Group 3 (LPS; n=10)	5.2 ± 0.42^{a}	2.5 ± 0.27^{bA}	5.96 ± 0.18^{a}	2.90 ± 0.80^{bA}
Group 4 (levamisol; n=10)	4.4 ± 0.27^{a}	1.9 ± 0.46^{bA}	5.67 ± 0.12^{a}	4.28 ± 0.50^{bA}
Control (n=10)	5.4 ± 0.52	4.8 ± 0.63^{B}	6.04 ± 0.25^{a}	6.58 ± 0.31^{B}

a & b significant (P<0.05) within group ; A & B significant (P<0.05) between different groups.

Blood parameters during spontaneous and subsequent induced estrus

The TLC range in different groups varied between 9250.9±946.10 to 13614.2 ± 3131.24 cells /ml at spontaneous estrus prior to treatment and between 9891.8 ± 1396.85 to 12496 ± 1452.18 cells/ml at subsequent induced estrus. The TLC recorded at both occasions did not vary significantly (P>0.05) within and between groups. The DLC values did not vary significantly (P>0.05) within and between groups except few occasions. Non-significant differences were observed in haemoglobin levels within and between various groups at spontaneous and induced estrus. The values varied between 7.54 ± 0.25 to 8.82± 0.26 g/dl. This indicated healthy status of animals before and after treatment and there was no adverse effect of any treatment. Total plasma immunoglobulins (Igs) concentration on both occasions (spontaneous and induced estrus) did not differ significantly (P>0.05) in all the groups except in LPS group. Igs values were significantly higher in groups 4 (levamisol) and 2 (formulated proteolytic enzyme) at induced estrus than control

group (3.24 ± 0.51 and 3.43 ± 0.38 vs 1.80 ± 0.34 mg/ml). The values of total plasma protein varied between 7.172 ± 0.38 to 9.248 ± 0.83 g/dl in different groups. Non-significant (P>0.05) differences were observed in total plasma protein levels within and between various groups at spontaneous and subsequent induced estrus. Non-significant differences were observed in haemoglobin levels within and between various groups at spontaneous and induced estrus. The values varied between 7.54 ± 0.25 to 8.82± 0.26 g/dl. This indicated healthy status of animals before and after treatment and there was no adverse effect of any treatment. Total plasma immunoglobulins (Igs) concentration on both occasions (spontaneous and induced estrus) did not differ significantly (P>0.05) in all the groups except in LPS group. Igs values were significantly higher in groups 4 (levamisol) and 2 (formulated proteolytic enzyme) at induced estrus than control group (3.24 ± 0.51 and 3.43 ± 0.38 vs 1.80 ± 0.34 mg/ml). The values of total plasma protein varied between 7.172 ± 0.38 to 9.248 ± 0.83 g/dl in different groups. Non-significant (P>0.05) differences were observed in total plasma protein levels within and between various groups at spontaneous and subsequent induced estrus.

Milk somatic cell count (SCC) during spontaneous and subsequent induced estrus

Average milk SCC changes after immunomodulator treatment in cattle of the various groups and data are (cells/ml) presented in Table 3. Somatic cell count were significantly (P<0.05) decreased following treatment with Mastivexym, LPS, Levamisol. SCC values were also reduced in Group 2 but it was statistically non significant. In control group the SCC was increased during subsequent induced estrus. The result revealed that immunomodulatory treatment used in different groups was efficient.

Table 3. Milk somatic cell count (cell/ml) during spontaneous and subsequent induced estrus

Sr.no.	Treatment group	Somatic cell count (cell/ml)	
		Spontaneous estrus	Induced estrus
1	Group 1 (Mastivexym; n=10)	2402.60 ±857.67[a]	1227.30 ± 452.63[b]
2	Group2 (FE; n=10)	1611.10 ± 493.33	1218.60 ± 463.17

3	Group 3 (LPS; n=10)	600.80 ± 177.98[a]	376.60 ± 111.49[bA]
4	Group 4 (Levamisol; n-=10)	2119.10 ± 844.59[a]	1226.40 ± 605.61[b]
5	Group 5 Control (n=10)	2396.20 ± 857.67	2456.10 ± 178.31[B]

a & b significant (P<0.05) within group; A & B significant (P<0.05) between groups

Pregnancy rates in different groups

The first service pregnancy rates in Groups 1, 2, 3, 4 and 5 were 50 %, 50%, 40%, 40%, 20%, respectively (Table 4). The total number of pregnant cattle belonging to treatment groups was significantly higher (P<0.05) than control. The cattle treated with proteolytic enzymes (Group 1 and 2) yielded better pregnancy rates (14/20; 70.0%) than other groups. Therefore, it was concluded that immunomodulators like proteolytic enzymes, LPS and levamisol can be used to enhance uterine immunity and pregnancy rates in repeat breeding cattle suffering from subclinical endometritis.

Tables 4. Pregnancy rates in different treatment groups.

Groups	**First service pregnancy rate (%)**	**Number of cattle pregnant after 2nd/3rd service**	**Total number of pregnant cattle (%)**
Group 1 (Mastivexym; n=10)	5/10 (50.00)*	1	6 (60.00)*
Group 2 (FE;n=10)	5/10 (50.00)*	3	8 (80.00)*

Group 3(LPS; n=10)	4/10 (40.00)	2	6 (60.00)*
Group 4 (Levamisol; n=10)	4/10 (40.00)	3	7 (70.00)*
Group 5 (Control; n=10)	2/10 (20.00)	0	2 (20.00)

*****- P<0.05 (significant than control group)

Conclusions

- Uterine cytobrush technique is an efficient and reliable method for diagnosing sub-clinical endometritis in dairy cattle.
- Uterine cytology revealed that the increase in severity of endometritis resulted more no of fibroblasts cells.
- Immunomodulation treatment through proteolytic enzymes, LPS, and Levamisol significantly reduced % PMN cells and bacterial load during subsequent estrus.
- The administration of proteolytic enzymes resulted better first service pregnancy rates (50 %), followed by LPS (40%) and Levamisol (40%) treatment in subclinical endometritic cattle.

Dr. Mrigank Honparkhe
Principal Investigator
Principal Scientist cum Head (Department of Veterinary Gynaecology and Obstetrics)
Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana

PUBLICATIONS

Papers Presented and Abstracts Published

Singh J, Honparkhe M, Ghuman SPS, Kumar A and Chandra M. 2015. Intrauterine proteolytic enzyme therapy for the treatment of subclinical endometritis in dairy cattle. *In: XXXI Annual Convention and National Symposium of ISSAR on Current Challenges and Opportunities In Animal Reproduction,* Department of Veterinary Gynaecology and Obstetrics, Veterinary College, Hebbal, Bengaluru from Dec 3-5, 2015 IFF-P10, pp 45.

Full papers presented and published in compendium

Honparkhe M. 2015. Clinical and research techniques for the diagnosis of subclinical endometritis dairy animals. In Compendium "Improving reproduction rate in ruminants by suitable reproductive technologies" . eds SPS Ghuman, M Honparkhe and PS Brar Under CAFT programme held at Department of Vety Gyanecology & Obst, GADVASU Ludhiana, Punjab, Sept 02-22, 2015, pp 45-48.

Honparkhe M. 2016. Evaluation and strategies to improve uterine health in dairy animals. In compendium "The cutting edge technologies to enhance fertility in farm animals" eds. M Honparkhe Ajeet Kumar and PS Brar Under CAFT programme held at Department of Vety Gyanecology & Obst, GADVASU Ludhiana, Punjab, Nov 04-24, 2016, pp 1-6.

Papers Published

Singh J, **Honparkhe M**, Chandra M, Kumar A, Ghuman S P S and Dhindsa S S. 2016. Diagnostic efficacy of uterine cytobrush technique for subclinical endometritis in crossbred dairy cattle Indian *Indian Veterinary Journal* **93** (02): 11 – 13.

Singh J, **Honparkhe M**, Ghuman S.P.S, Kumar A, Dhindsa S and Chandra M. 2017. Intrauterine proteolytic enzyme therapy for subclinical endometritis in dairy cattle. *Indian Journal of Animal Reproduction* **38**

Master's research thesis published:

Immunomodulation therapy as an alternative approach to antibiotic therapy in endometritic dairy cattle (Jasveer Singh L-2012-V-34-M)

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