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Comparative therapeutic efficacy of levofloxacin, ornidazole and alpha tocopherol combination with prostaglandin F2α on *IL-6* and *IL-10* transcript level in longstanding cases of endometritis in crossbreed Jersey cows

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Summary

This study compared the therapeutic efficacy of levofloxacin, ornidazole and alpha tocopherol combination and prostaglandin F2α (PGF2α) in longstanding cases of endometritis and evaluated their impact on *Interleukin-6* (*IL-6*) and *Interleukin-10* (*IL-10*) transcript level in peripheral blood leukocytes. Eighteen endometritic crossbred Jersey cows were randomly allotted to three groups (six in each) *viz.* Group I (levofloxacin combo treatment I/U), group II (PGF2α treatment I/M), group III (no treatment, control), and group IV (six non-endometritic healthy cyclic) was taken for comparison study. The clinical efficacy was assessed by haematological study (TLC: Total leukocyte count; DC: Differential count), polymorphonuclear leukocytes (PMN) count in uterine cytology and relative mRNA expression of *IL-6* and *IL-10* in peripheral blood leukocytes before and after treatment with respect to conception rate following single and second inseminations. Group I and II registered significant increase in TLC and neutrophil count. PMN cytology was increased two and three fold in group I and II, respectively. The *IL-6* transcript level was increased by 2.5 and 4.6 fold while that of *IL-10* increased by 3.7 and 5.2 fold in group I and II, respectively. Conception rate across group I to IV following single insemination was found to be 66.67%, 50%, 16.67%, and 83.33% and their corresponding values following second insemination were 66.67%, 83.33%, 16.67%, and 83.33%, respectively. Thus, the administration of levofloxacin combo and PGF2α might have better conception rate following first and second insemination, respectively. Our study also reveals that PGF2α could register better clearance of bacteria through stronger PMN cell and cytokine activity in post-treatment period.

Key words: Endometritis, *IL-6*, *IL-10*, Levofloxacin, Prostaglandin F2α

Introduction

Shrinkage of cultivable land and population swell have edged the current scenario of dairy industry into perplex. Odisha constitutes 6.09% of total cattle population of India and witnessed 5.59% decline in total cattle population (19th livestock census 2012). This drastic reduction in cattle population is mostly attributed to "endometritis" contributes around 20% of reproductive disorders in dairy cattle. Endometritis is the inflammation of glandular layer of uterus without any clinical signs and is thought to have a negative impact on reproductive performance, as it increases the services per conception, calving to first service interval, calving to conception interval and reduces risk of pregnancy as well as the conception rate (Kasimanickam *et al*., 2004).

Diagnosis of endometritis can be done based on case history, gynaeco-clinical findings, nature of uterine discharge, uterine palpation per-rectum, vaginal speculum examination, ultrasonography and endometrial cytology however, none of these methods was found to be effective due to lack of standardization and reliability (Mateus *et al*., 2002; Foldi *et al*., 2006). Modified cytobrush has been thought to be advantageous over lavage technique as it is easier, quick and consistent with less distortion of cells (Barlund *et al*., 2008). Uterine biopsy and bacteriological culture have been considered as the gold standard diagnostic methods but seems to be of limited use in field conditions (Gilbert *et al*., 2005; Noakes *et al*., 2001). Apart from these techniques, the chemical mediators of inflammation render scope of diagnosis of endometritis with accuracy (Fischer *et al*.,

2010; Islam *et al*., 2013). Treatment through antibiotics and chemotherapeutic agents has traditionally been used to resolve the endometritis (LeBlanc, 2008; Galvao *et al*., 2009).

An array of treatment regimens has been experimented so far but none could resolve the problem of endometritis in dairy cows. An accurate treatment protocol may attenuate the deleterious effects of endometritis which could enhance the reproductive efficiency of dairy cows of Odisha. Therefore, the present study was aimed to evaluate the efficacy of levofloxacin + ornidazole + α-tocopherol combination and prostaglandin F2α ($PGF_{2\alpha}$) in bovine endometritis with the underlying objectives as follows:

I: To estimate certain hematological parameters like total leukocyte count (TLC), and differential count (DC) before and after treatment (succeeding estrus)

II: To evaluate endometrial cells by modified cytobrush for polymorphonuclear leukocytes (PMN) cell count in 0th and succeeding estrus

III: To study the differential mRNA expression of *Interleukin-6* (*IL-6*) and *Interleukin-10* (*IL-10*) in peripheral blood leukocytes

IV: To evaluate conception rate following first and second inseminations in endometritic cows

Materials and Methods

Source and selection of animals

The present study was undertaken in the Department of Gynaecology and Obstetrics, C.V.Sc & A.H., O.U.A.T, Bhubaneswar in collaboration with ICAR-PD-FMD, Mukteswar, India from August 2015 to June 2016. The experimental animals were crossbred Jersey cows at oestrus presented to TVCC, C.V.Sc & A.H., Bhubaneswar. Apparently healthy breedable cows of 3rd and 4th parity with 85 to 120 days in milk were considered for the present study.

Gynaecological examination and detection of endometritis

Gynaeco-clinical examination was carried out in all experimental cows by per-rectal examination (Zemjanis, 1970). The cows with history of conception failure for more than three inseminations and having mucopurulent discharge were subjected to uterine cytology examination as described earlier (Sheldon and Dobson, 2004). Arbitrarily >5% PMN threshold was used to diagnose cows having endometritis of clinical or subclinical type (Fischer *et al*., 2010).

Experimental group and treatment protocol

Eighteen endometritic crossbred Jersey cows were selected after screening. Group I cows were treated with 30 ml infusion of levofloxacin + ornidazole + α-tocopherol (I/U; Lenovo AP, Intas Pharmaceuticals Limited, India; n=6), group II cows were treated with 2 ml of injection of PGF2α (I/M; Pragma, Intas Pharmaceuticals Limited, India; n=6), and group III cows were taken as positive control without any treatment (n=6). Additionally, six non-endometritic healthy cyclic cows (group IV, n=6) were used for comparison study.

Collection of blood sample and haematological study

Five ml blood was collected aseptically by jugular venipuncture. Three ml of blood was collected in EDTA treated vial for haematological examination and 2 ml blood was kept in heparinised vial for relative mRNA expression study of target genes. Total leukocyte count and DC were estimated as described earlier (Schalm, 1965).

Endometrial cytology

Collection by cytobrush technique

The endometrial samples for cytology examination were collected by using sterile human cervical cytology brush (Neha Polymers, Mumbai, India) with slight modification suitable for use in large animals (Figs. 1A-B). The normal cytobrush handle was cut to approximately 3 cm in length, threaded onto a solid stainless steel rod with 65 cm length and 4 mm diameter by a sterilized connecting plastic and placed in a stainless steel tube 50 cm in length and 6 mm in diameter for passage through the cervix. The plastic sleeve was then perforated and the stainless steel sheath with cytobrush

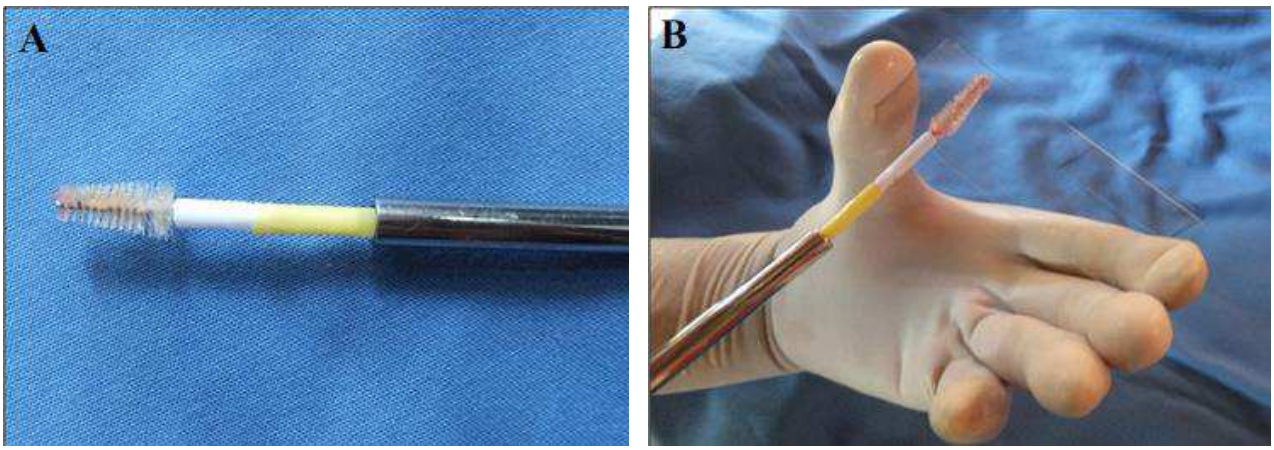

Fig. 1: Endometrial cytology. Modified cytobrush (**A**), and Cytology sample collection and smearing (**B**)

carrying rod was advanced through the cervix into the uterus. At suitable position, stainless steel tube was retracted to expose the cytobrush and the endometrial cells were collected by rotating the cytobrush in a clockwise direction in contact with the uterine wall. Then the cytobrush was retracted into the stainless steel tube prior to removal from the uterus. The stainless steel instrument was steam sterilized for 4 min between uses.

Cytosmear preparation

The slides for cytological examination were prepared by rolling the cytobrush onto a clean glass microscope slide and sample was fixed with 70% ethyl alcohol. Then the slides were brought immediately to the laboratory and subjected with giemsa stain. Cytological assessment was performed by counting a minimum of 100 cells (endometrial, PMNs and squamous cells) at ×1000 and ×400 magnification to determine the percentage of neutrophils or PMNs (Kasimanickam *et al*., 2005).

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from whole blood using RNA isolation kit (NucleoSpin® RNA Blood, USA) according to the manufacturer's instruction. The extracted RNA was further purified using RNeasy kit (QIAGEN, USA) and treated with DNase-I followed by quantification in nanodrop spectrophotometer. The OD 260/OD280 ratio of all RNA samples were found to be 1.8-2.0 and stored at -80°C till further use. The qRT-PCR was performed using one step SYBR-PrimeScriptTM RT-PCR Kit II (TaKaRa, USA) as per manufacturers' instructions (Applied Biosystems 7500 Real Time PCR System). Briefly, 2 µL (1000 ng) of total RNA was added to 0.8 µL forward primer (0.4 µM), 0.8 µL reverse primer (0.4 µM), 0.8µL of PrimeScript 1 step Enzyme Mix II, 10 µL 2X one step SYBR® RT-PCR Buffer IV, 0.4 µL ROX reference dye along with 5.2 µL RNase free water to a final volume of 20 µL, which was subjected to real time PCR protocol. The qRT-PCR was performed under the following conditions:

Stage 1: Reverse transcription; 42°C for 5 min and 95°C for 10 s

Stage 2: PCR reaction, 40 cycles, denaturation -95°C for 5 s, annealing/extension -60°C for 30 s

Stage 3: Melting step by slow heating from 60°C-95°C with a heating rate of 0.5°C/s with a continuous fluorescence measurement and final cooling down at 4°C.

To calculate relative quantification, 18S was used as endogenous control and the average of ΔCt for samples of healthy non-endometritic cows collected on day 0 before treatment was used as calibrator for each sample. The relative mRNA expression in fold change was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The details of the primers of *IL-6*, *IL-10* and 18S genes (Table 1) along with the melting curve of 18S, *IL-6* and *IL-10* in pre- and post-treatment are presented in Figs. 2A-F.

Pregnancy diagnosis and conception rate

Pregnancy diagnosis was conducted in each experimental cow between 45-60 days post insemination by per rectal examination. Conception rate was assessed by considering pregnant cows against number of insemination and calculated by number of cows diagnosed pregnant to the number of cows inseminated multiplied by 100. All the data were statistically analyzed by paired t-test and one way ANOVA using SPSS 16.0 wherever necessary (Snedecor and Chochran, 1994).

Results

Total leukocyte count and differential count during pre- and post-treatment period

The TLC (thousands/mm^3) values before and after treatment in group I, II, III, and IV were presented in Table 2. There was no significant difference in TLC values amongst various groups before treatment. After treatment, the TLC values were significantly ($P<0.01$) increased in group I and II cows and were found to be highest in group II cows while no significant difference was observed in group III and IV cows. The data on differential count (%) before and following treatment in various groups were presented in Table 3. The neutrophil, lymphocyte, eosinophil and monocyte count did not reveal any significant difference amongst different groups before treatment while the monocyte count did not change before as well as after treatment within and between different groups. The neutrophil count was increased ($P<0.01$) and lymphocyte count was decreased ($P<0.01$) in group I and II while group III and IV did not register any change other than their corresponding values before treatment. The eosinophil count showed a similar pattern as lymphocyte count.

Table 1: Target genes, primer sequence and amplicon length for qRT-PCR

Target gene	Primer sequence	Amplicon length (bp)	Reference
IL-6	For: TCCAGAACGAGTATGAGG Rev: CATCCGAATAGCTCTCAG	236	NM_173923.2
IL-10	For: TGCTGGATGACTTTAAGGG Rev: AGGGCAGAAAGCGATGACA	186	NM_174088.1
18 S	For: CTGAGAAGACGGTCGAACTTGACT Rev: TCCGTTAATGATCCTTCCGCAGGT	90	Ranjan *et al*. (2016)

Table 2: Pre- and post-treatment mean±SE values of total leucocyte count in various experimental groups

Parameters	Day	Groups				
		Group I	Group II	Group III	Group IV	F-value
TLC (10^3/Cu mm)	Pre-treatment	6.86 ± 0.20	7.16 ± 0.14	7.19 ± 0.18	6.81 ± 0.11	1.578^{NS}
	Post-treatment	7.89 ± 0.26^{a}	9.14 ± 0.25^{b}	7.11 ± 0.22^{c}	6.85 ± 0.08^{c}	23.033^{**}
	t-value	2.951^{*}	10.201^{**}	0.379^{NS}	0.043^{NS}	-

Mean±SEM bearing different superscripts in row differs significantly. * P<0.05, and ** P<0.01. NS: Not significant

Table 3: Pre- and post-treatment mean±SE values of neutrophil, eosinophil, lymphocyte and monocyte count in various experimental groups

Parameters	Day	Groups				
		Group I	Group II	Group III	Group IV	F-value
Neutrophil (%)	Pre-treatment	29.83 ± 1.22	30.17 ± 1.53	29.00 ± 1.18	29.67 ± 0.67	0.169^{NS}
	Post-treatment	37.67 ± 0.76^{a}	41.17 ± 1.38^{a}	29.33 ± 0.99^{b}	30.17 ± 0.70^{b}	39.834^{**}
	t-value	6.269^{**}	10.651^{**}	0.326^{NS}	0.406^{NS}	-
Lymphocyte (%)	Pre-treatment	61.00 ± 0.89	61.17 ± 1.78	62.33 ± 1.38	63.50 ± 0.62	0.864^{NS}
	Post-treatment	56.17 ± 0.95^{a}	51.83 ± 1.35^{a}	62.17 ± 1.01^{b}	62.17 ± 0.87^{b}	22.488^{**}
	t-value	3.512^{*}	7.135^{**}	0.255^{NS}	1.512^{NS}	-
Eosinphil (%)	Pre-treatment	8.00 ± 0.36	7.17 ± 0.60	7.00 ± 0.73	5.67 ± 0.42	3.095^{NS}
	Post-treatment	4.83 ± 0.48^{a}	5.83 ± 0.60^{bc}	7.17 ± 0.31^{c}	6.17 ± 0.31^{bc}	4.762^{*}
	t-value	5.836^{**}	1.512^{NS}	0.191^{NS}	1.168^{NS}	-
Monocyte (%)	Pre-treatment	1.33 ± 0.21	1.50 ± 0.22	1.50 ± 0.22	0.83 ± 0.31	1.667^{NS}
	Post-treatment	1.67 ± 0.31	1.00 ± 0.26	1.17 ± 0.17	1.50 ± 0.43	0.473^{NS}
	t-value	1.00^{NS}	1.464^{NS}	1.581^{NS}	1.0^{NS}	-

Mean±SEM bearing different superscripts in row differs significantly. * P<0.05, ** P<0.01. NS: Not significant

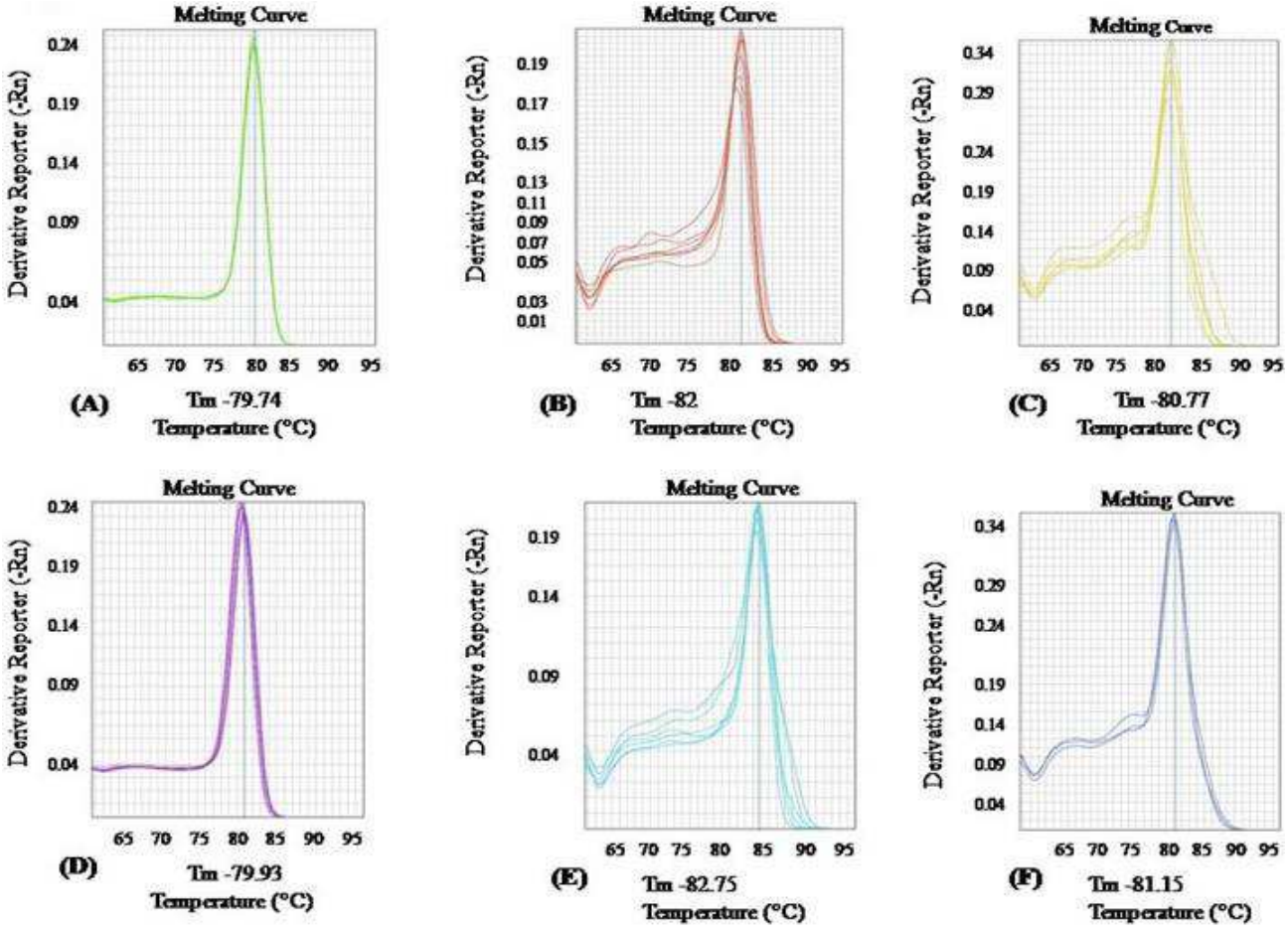

Fig. 2: Melting curve of *IL-6* and *IL-10*. Melting curve of 18 S pre-treatment (**A**), *IL-6* pre-treatment (**B**), *IL-10* pre-treatment (**C**), 18 S post-treatment (**D**), *IL-6* post-treatment (**E**), and *IL-10* post-treatment (**F**) during thermocycling in real time RT-PCR

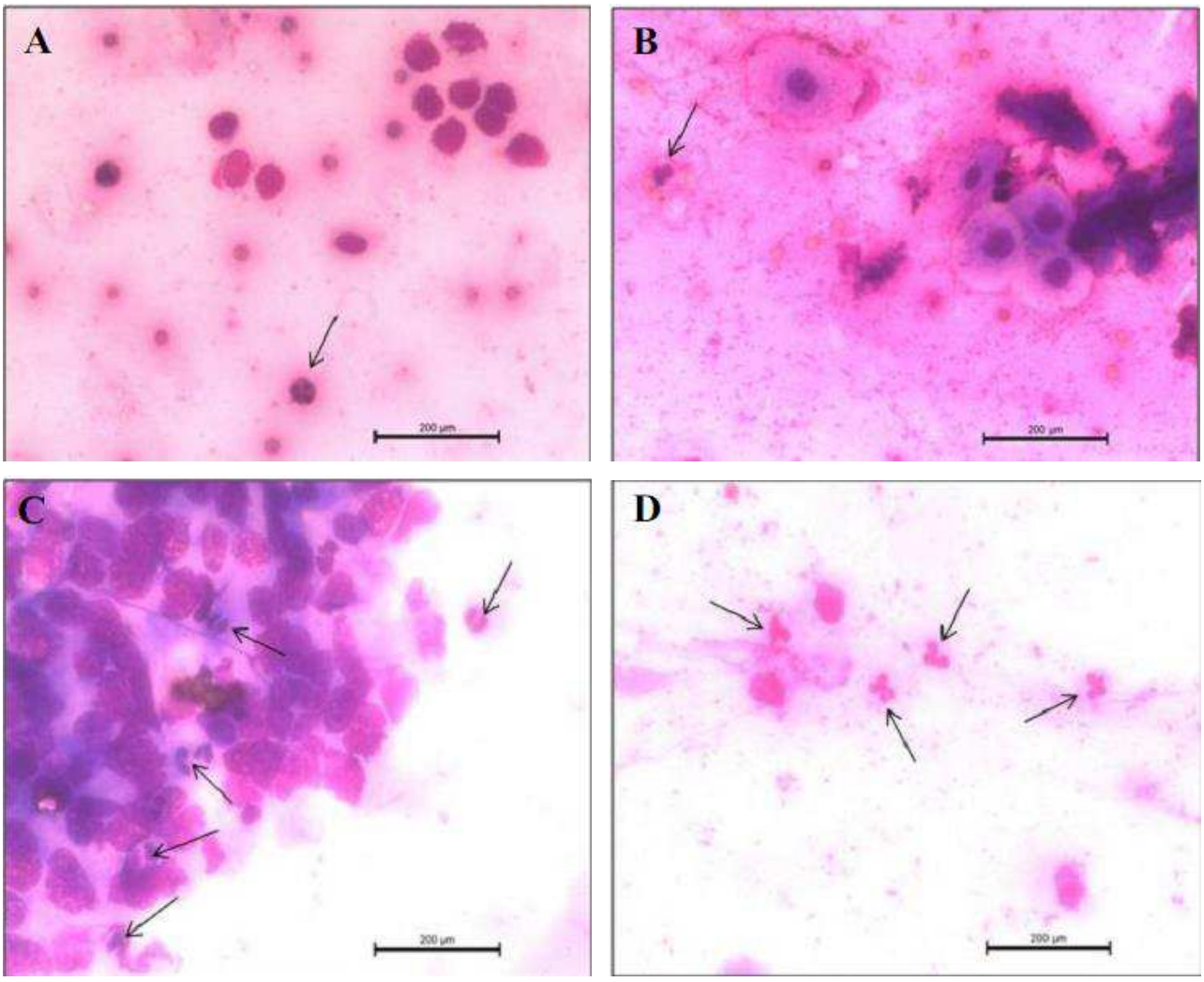

Fig. 3: Polymorphonuclear cells in uterine cytology. PMN cells in uterine cytology sample in pre-treatment period are lower (**A**, **B**), and higher in post-treatment period (**C**, **D**)

Table 4: Pre- and post-treatment mean±SEM values of PMN cells in various experimental groups

Groups	Treatment		
	Pre-treatment	Post-treatment	t-value
Group I	9.67 ± 0.56^{x}	20.83 ± 0.95^{w}	9.815^{**}
Group II	10.17 ± 0.48^{x}	32.00 ± 0.97^{x}	17.860^{**}
Group III	10.33 ± 0.49^{x}	11.33 ± 0.88^{y}	1.464^{NS}
Group IV	3.33 ± 0.33^{y}	3.00 ± 0.36^{z}	0.674^{NS}
F-value	50.880^{**}	227.650^{**}	-

Mean±SEM bearing same superscript in column does not differ significantly. * P<0.05, and ** P<0.01. NS: Not significant

Table 5: Pre- and post-treatment transcript level of *IL-6* gene in various experimental groups

Groups	Fold change in *IL-6*		Change in transcript level
	Pre-treatment	Post-treatment	
Group I	0.14 (-7.14 fold)	2.51	Increase
Group II	0.23 (-4.35 fold)	4.59	Increase
Group III	0.16 (-6.25 fold)	0.12 (-8.33 fold)	Non significant
Group IV	1	1.44	Non significant

PMN cells count during pre- and post-treatment period

PMN cells (%) in uterine cytology sample in different groups before and after treatment was presented in Table 4 and Figs. 3A-D. PMN cells were significantly higher in group I (2 fold) and II (3 fold), respectively while the group III and IV did not reveal any difference other than their corresponding values before treatment.

Relative *IL-6* transcript level during pre- and post-treatment period

The relative transcript level of *IL-6* in various groups before and following treatment was presented in Table 5. The *IL-6* transcript level was decreased (P<0.01) by 7.14, 4.35, and 6.25 fold in group I, II and III, respectively as compared to group IV cows before treatment. After treatment, the *IL-6* transcript level was increased (P<0.01) by 2.51 and 4.59 fold in group I and II, respectively than their corresponding values before treatment.

Table 6: Conception rate of post-treatment cows with various drug regimens following first and second inseminations

Groups	Total No. of animals inseminated	No. of animals conceived after first insemination	Conception rate after first insemination (%)	No. of animals conceived after second insemination	Conception rate after second insemination (%)
Group I (n=6)	6	4	66.67	4	66.67
Group II (n=6)	6	3	50.00	5	83.33
Group III (n=6)	6	1	16.67	1	16.67
Group IV (n=6)	6	5	83.33	5	83.33

Post-treatment conception rate

The conception rate in group I, II, III, and IV following first insemination was 66.67%, 50%, 16.67%, and 83.33% whereas the corresponding values following second insemination were 66.67%, 83.33%, 16.67%, and 83.33%, respectively (Table 6).

Relative *IL-10* transcript level during pre- and post-treatment period

The relative transcript level of *IL-10* in various groups before and following treatment was presented in Table 7. The *IL-10* transcript level was decreased ($P<0.01$) by 9.09, 5.56, and 2.86 fold in group I, II and III, respectively before treatment than group IV cows. Following treatment, *IL-10* transcript level was increased ($P<0.01$) by 3.68 and 5.20 fold in group I and II, respectively than their corresponding values before treatment. The *IL-10* gene was further down-regulated by 5.56 fold in group III whereas the normal cyclic animals did not show any significant change.

Table 7: Pre- and post-treatment transcript level of *IL-10* in various experimental groups

Groups	Fold change in *IL-10*		Change in transcript level
	Pre-treatment	Post-treatment	
Group I	0.11 (-9.09 fold)	3.68	Increase
Group II	0.18 (-5.56 fold)	5.20	Increase
Group III	0.35 (-2.86 fold)	0.18 (-5.56 fold)	Decrease
Group IV	1	1.04	Non significant

Discussion

Our data on TLC values were within normal physiological range as depicted by Benjamin (1979) and Radostits *et al*. (2007). In contrast, the TLC values before and after treatment deviated from Reddy *et al*. (2012) and Sahoo *et al*. (2014) who did not find any significant difference. Our TLC values also differed from Sarma *et al*. (2012) and Heidarpour *et al*. (2014) who found lower TLC values after treatment. The significant increase in TLC values in group I and II cows after treatment can be attributed to immunomodulation through chemotactic action of cytokines which might result in elevation of leukocytes in systemic circulation (Sheldon *et al*., 2009).

The neutrophil count before and following treatment irrespective of drug treatment was found to be within normal physiological range between 15 and 45% (Chauhan, 1995). In our study, the higher neutrophil count in group I and II cows following treatment was consistent with earlier findings (Ahmad *et al*., 2003; Biswal *et al*., 2014). The increase in neutrophil count before and following treatment might be due to higher concentration of cytokines which could result in elevation of neutrophils in systemic circulation (Hogarth, 1982). Our finding on neutrophil count following treatment was not in line with the agreement of Sarma *et al*. (2012) and Heidarpour *et al*. (2014) who found a lower value in endometritic cows following treatment. Our result also deviated from Reddy *et al*. (2012) who did not notice any change before and following treatment. Our data on lymphocyte count was in accordance with Heidarpour *et al*. (2014) who reported a significant decrease in lymphocyte count in both clinical and subclinical endometritis. The significant variation of lymphocyte count can be attributed to immuno-modulatory action of treated drugs in uterine environment causing migration of lymphocytes to the site of action (Hogarth, 1982). In our study, the eosinophil count showed a significant decline in group I following treatment while changes in other groups were non-significant which agrees with Heidarpour *et al*. (2014) and Sahoo *et al*. (2014). The higher value in the beginning and subsequent dip at day 21 following treatment might be due to release of histamine and relief of infection by different drugs during post-treatment period (Sahoo *et al*., 2014). The monocyte count corroborates with Pathan *et al*. (2011) who recorded 1.62 ± 0.12 and 1.53 ± 0.05 in cyclic and non-cyclic cows, respectively. However, the alteration in monocyte count might be due to migration of monocytes to uterine lumen due to opsonisation of uterine microbes. Higher PMN count in group I and II after treatment was identical to Biswal *et al*. (2014) and Sahoo *et al*. (2014). However, our PMN count differs from Kasimanickam *et al*. (2004) who obtained lower PMN counts following treatment. This might be due to chemotactic action of cytokines released from damaged bacteria which could have triggered the neutrophil count in uterus (Sharma and Dhaliwal, 2009).

IL-6 and *IL-10* transcript level was higher in endometritic cows than healthy cows in early post parturient period (Islam *et al*., 2013; Patra *et al*., 2014; Brodzki *et al*., 2015). However, no significant difference was observed in *IL-10* transcript level in peripheral blood of endometritic and healthy animals (Duvel *et al*., 2014). In our findings, the *IL-6* and *IL-10* mRNA expression were down-regulated before treatment compared to healthy control group. Further, Galvao *et al*. (2011) revealed that, lower *IL-6* expression might lead to poor chemotaxis and activation of neutrophils and monocytes which could impair bacterial clearance. Lower *IL-6* mRNA expression in peripheral blood could be due to an

intrinsic defect in endometrial cell function (Nino-Soto *et al*., 2008). In the present study, neutrophil count did not differ before treatment in various experimental groups which substantiate the lower transcript level of *IL-6* and *IL-10* (Butterfield *et al*., 2006; Chapwanya *et al*., 2009). In the present study, the *IL-6* and *IL-10* transcript level was up-regulated in group I and II following treatment rather than non-endometritic healthy control which can be attributed to local clearance of bacteria by the immunomodulatory action of different drugs.

Our findings on conception rate in group I cows were consistent with earlier reports (Singh *et al*., 2011; Kumar *et al*., 2014) and group II cows (Biswal *et al*., 2014) following treatment. In our study, group II cows registered better bacterial clearance with stronger PMN cell activity and cytokine expression compared to group I cows following treatment. However, group I cows witnessed greater conception rate than group II cows following first insemination. This might be due to powerful antioxidant property of α-tocopherol coupled with bactericidal action of levofloxacin. It has been suggested that, higher PMN influx coupled with higher *IL-6* expression (4.59 fold in group II against 2.51 fold in group I) in uterine lumen tends to lower pregnancy rate in cows (Kasimanickam *et al*., 2004) which further validates the lower conception rate in group II than group I cows. But the conception rate following second insemination indicates that group II cows registered better conception rate (83.33%) than group I cows (66.67%).

Our study indicates that, the administration levofloxacin + ornidazole + α-tocopherol combination may provide better conception rate following single insemination while administration of PGF2α could register better efficacy in clearing bacterial infection through stronger PMN cell along with cytokine expression and better conception rate after second insemination in post-treatment period. Elevation in PMN cell influx in uterine lumen with up-regulation in cytokines causing bacterial clearance justifies the efficiency of different drugs.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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