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Assessment of reactive oxygen species and phagocytosis of milk leukocytes by alfa-tocopherol and enrofloxacin in bovine mastitis

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ABSTRACT

The effect of enrofloxacin and alfa tocopherol on the capacity of bovine milk leukocytes to generate reactive oxygen species and phagocytic activity after stimulation with phorbol 12-myristate 13-acetate (PMA) for superoxide were studied in bovine clinical mastitis. Group I, consisting of 9 healthy cows, served as the control, whereas groups II and III each contained 9 cows with clinical mastitis on the basis of California Mastitis Test (CMT) positive reaction. Group II cows received 1500 mg of enrofloxacin for 5 days, while group III received 1500 mg of enrofloxacin for 3 days together with 2 injections of alfa-tocopherol and selenium on alternate days by parenteral route. The somatic cell count (SCC) reduced significantly ($P<0.05$) in group III cows, where recovery was 89%, whereas the SCC remained higher in group II cows, with a recovery rate of 78%. Superoxide radical generation in the isolated leukocytes of milk in group II and group III cows was significantly enhanced on day 3 post-treatment (PT). The phagocytic activity of the milk leukocyte was measured by acridine orange dye technique. Phagocytic activity increased significantly in group III cows to an extent of 44.13%, whereas such increase was 10% in group II cows. The phagocytic index (PI) also increased significantly in group III animals. However, a non-significant rise was observed in group II cows. In conclusion, the results of this study show that enrofloxacin and vitamin E have a beneficial effect on the functioning of the immune cells in bovine udders affected by intramammary infection.

Key words: alfa-tocopherol, enrofloxacin, leukocyte, phagocytosis, somatic cell count

Introduction

Mastitis is one of the most costly diseases in dairy cattle and eventually damages the udder tissues (YAGI et al., 2002). Bacterial infection and growth in the udder is the main

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cause of bovine mastitis (BURVENICH et al., 1994). The incidence of clinical mastitis is highest during the periparturient phase in high yielding animals. During this phase negative energy balance is observed in dairy cows, which leads to metabolic disorders and infectious diseases (HOEBEN et al., 1997). Neutrophils are the primary cellular defences of the mammary gland; its main function being phagocytosis and intracellular elimination by generating reactive oxygen species. However, the leukocyte defences are depressed during the periparturient period (CAI et al., 1994). Appropriate clearance of the pathogens from the bovine udder requires both the effectiveness of the drug and optimum functioning of the immune cells (SORDILLO et al., 1997). Antibiotics are the only proven method for treatment of mastitis. However, antibiotic therapy of established mammary infection is only moderately efficacious, while most antibiotics used for the treatment of mastitis further depress the activity of leukocyte defence (HOEBEN et al., 1997). Strategies designed to improve the immune cells of the diseased udder during immunosuppressive stages would greatly impact the ability of the animal to resist the pathogenic infection. A low plasma level of Vitamin E has been recorded in bovine mastitis (HOGAN et al., 1992). However, dietary supplementation of high levels of Vitamin E during the periparturient period substantially benefits udder health (WEISS et al., 1997). EICHER et al. (1994) observed enhanced chemotactic responsiveness of blood neutrophils using Vitamin E therapy.

The purpose of this study was to determine the effects of enrofloxacin, together with Vitamin E treatment, on the SCC, reactive oxygen species and phagocytic activity of the milk leukocytes isolated from milk in bovine clinical mastitis.

Materials and methods

Selection of animals and experimental protocol. Twenty-seven, crossbred lactating cows were randomly selected from an organized dairy farm (Cattle & Buffalo), IVRI, Izatnagar. The cows were maintained in the animal shed of the institute under identical environmental conditions and were divided in 3 equal groups. Group I, consisting of 9 healthy cows served as control. Nine cows in group II and 9 cows in group III (18 cows) positive for clinical mastitis, screened on the basis of California mastitis test (CMT) positive reaction, 3 and 4 point score (SCHALM et al., 1971) were taken for the drug trial. Group II cows were given 1500 mg of enrofloxacin (Enrox-Alembic Chemical works India LTD, 10% injection) by intramuscular route for 5 consecutive days. In group III cows enrofloxacin was given for 3 days and injection of alfa-tocopherol 10 mL per cow (E care Se., Vitamin E-55 IU/ mL as alfa tocopherol acetate and Selenium 1.5 mg. Health Line Pvt. Ltd, Bangalore, India) was also given by subcutaneous route. Second injections was given on day 3 .

Milk sampling. Fifty mL of milk from each cow were collected in sterile vials after cleaning the teat orifice with 70% ethyl alcohol and after discarding the first few streams

of milk. The milk was collected on days 0, 3, 7 and 15 PT. The SCC of the milk samples were carried out as per the method of SCHALM et al. (1971). Milk samples were plated on 5% bovine blood agar plates, and the organisms were identified on the basis of colony morphology, characteristic haemolytic pattern, and Gram's staining (HARMON et al., 1990).

Isolation of milk leukocytes, polymorph nuclear cells (PMNs). The isolation of PMNs from milk samples was carried out as per the method of DALEY et al. (1991). In brief, 50 mL of milk were passed through cheese cloth. The milk was then poured into 50 mL conical tubes and centrifuged at 200 g for 30 minutes at 4 ^{0}C (SORVALL RT 6000, DUPONT). The fat was removed and the skim milk was poured off and discarded. PMN cell pellets were washed twice, re-suspended in sterile PBS. After final wash cells were resuspended in 1 mL of PBS. Differential leukocyte count was performed to ascertain PMN cells. Viability of cells was checked by trypan blue (SRL, India) exclusion technique (COLLIGAN et al., 1994). The cell suspension was adjusted to 1×10^7 and 1×10^6 cells/mL in sterile PBS for superoxide anion (O_2^-) assay and phagocytic activity, respectively.

Measurement of superoxide anion (O_2^-) production. Superoxide anion (O_2^-) of isolated PMNs was carried out as per the method described by NAGAHATA et al. (1986) after stimulation by PMA (Sigma, St Louis, MO, USA). The superoxide production was measured on day 0 and on day 3 PT.

Phagocytic activity and phagocytic index of isolated milk PMNs. Phagocytic activity of isolated milk PMNs was conducted as per the method described by FOX et al. (1987). Briefly: standard strain of *Staphylococcus aureus* was procured from the Division of Standardization (IVRI). Eighteen hours culture was opsonised with pooled bovine serum in an incubator for one hour. An equal volume (500 µl) of PMNs and 500 µl opsonised bacterial suspension was incubated at 37 ^{o}C for half an hour, maintaining PMNs and bacteria at a 1:5 ratio. Thereafter, it was stained with 500 µl of Acridine orange stain (0. 015 %, Sigma, St Louis, Mo, USA), vortexed and centrifuged at 4 ^{o}C, 13000 rpm to obtain cell pellet. Finally, 500 µl crystal violet (0. 05%, SISCO Research Lab, Mumbai, India) was added and centrifuged as above. The pellet was resuspended in cold sterile PBS (500 µl) and wet mount seen under ultraviolet source with excitation filter of 530 nm. Phagocytic activity, expressed by the percentage of phagocytosed neutrophil in 100 cells and phagocytic index, determined on the unit of Staphylococci ingested by single PMNs, was counted in 100 cells. Phagocytic activity and phagocytic index were measured on day 0 and on day 3 PT.

Statistical analysis. Data were analyzed using the one-way analysis of variance. The mean ± SE of the same group of treatment was analyzed using Duncan's Multiple Range Test as per the standard method (SNEDECOR and COCHRAN, 1994).

Results

Effect of enrofloxacin and vitamin E on SCC. Results pertaining to the effect of the drug are presented in Table 1. There were no differences in SCC in the milk sample isolated from healthy cows. SCC in group II cows significantly ($P<0.05$) reduced to an extent of 62.10% on day 3 PT, 72% on day 7 PT and 72.4% on day 15 PT, respectively. The SCC in group II remained more than 5.70×10^5 cells per mL of milk. SCC in group III reduced to 81.14% on day 3, 88.5% on day 7 PT and 88.7% on day 15 PT, respectively. SCC reduced to 3×10^5 cells per mL of milk. Organisms isolated from milk samples were *Staphylococcus aureus* (11%), *Streptococcus agalactiae* (21.5%), *Corynebacterium* spp. (4%), *Micrococci* (41.6%), Coliform bacilli (18%), not isolated (4%).

Table 1. Response of enrofloxacin treatment (group II), enrofloxacin and alfa-tocopherol treatment (group III) on Somatic Cell Count (SCC) ($\times10^5$ cells/mL) in clinical mastitis infected cows as compared to healthy cows (group I) Mean ± SE.

Group of cows	SCC ($\times10^5$ cells/mL)/days post treatment			
	0 day	3 days	7 days	15 days
I	4.93 ± 0.448	4.58 ± 0.445	4.56 ± 0.445	4.56 ± 0.445
II	20.66 ± 0.741[a]	7.83 ± 0.350[b]	5.78 ± 1.90[c]	5.70 ± 1.122[c]
III	26.73 ± 2.387[a]	5.04 ± 0.224[b]	3.06 ± 0.118[c]	3.00 ± 0.106[c]

*Values with different superscripts in each row (a, b, c) differ significantly ($P<0.05$)

Table 2. Response of enrofloxacin treatment (group II), enrofloxacin and alfa-tocopherol treatment (group III) on phagocytic activity and phagocytic index in clinical mastitis infected cows compared to healthy cows (group I) Mean ± SE.

Group of cows	Days post treatment			
	Phagocytic activity		Phagocytic index	
	0 day	3 days	0 day	3 days
I	30.33 ± 0.879	31.03 ± 0.878	3.20 ± 0.086	3.21 ± 0.086
II	20.82 ± 0.860	22.06 ± 0.940	1.86 ± 0.126	2.21 ± 0.142
III	21.14 ± 0.89[a]	30.47 ± 0.487[b]	1.80 ± 0.145[a]	3.21 ± 0.487[b]

*Values with different superscripts in each row (a, b) differ significantly ($P<0.05$)

Production of superoxide anion. The ability of the neutrophil to produce superoxide (measured by nitro blue tetrazolium reduction) in response to the treatment of enrofloxacin and Vitamin E is presented in Fig. 1. There was a non-significant enhancement (21%)

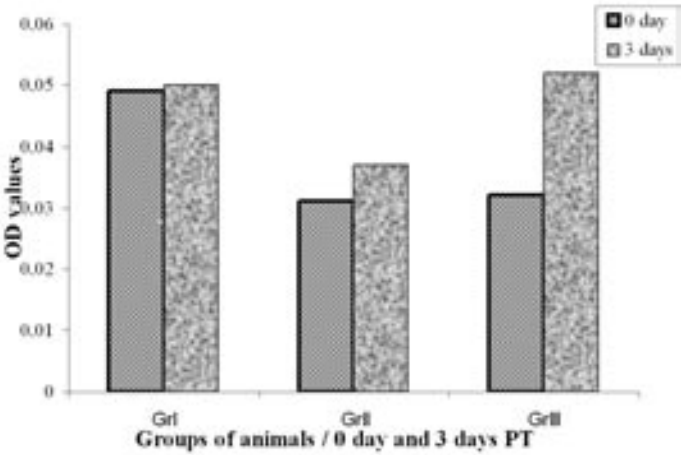

Fig. 1. Production of superoxide anion (Delta OD/ 1×100,000,00 cells/3 mL of DMF layer) by milk leukocytes in response to treatment by enrofloxacin (group II) and enrofloxacin and alfa-tocopherol (group III) in mastitic cows compared to healthy cows (group I)

of superoxide level from the milk leukocytes in group II cows on day 3 PT, whereas the superoxide production was significantly enhanced to an extent of 59.4% in group III cows on day 3 PT. Significant effects of time and treatment also were detected ($P<0.05$).

Phagocytic activity and phagocytic index. There were no differences in phagocytic activity in the milk sample isolated from healthy cows (Table 2). Similarly, phagocytic activity in group II cows did not show any significant difference. However, activity increased significantly ($P<0.05$) in group III cows and was enhanced to 44.13% on day 3 PT. PI increased in group II animals to an extent of 15.8%. However, it increased significantly ($P<0.05$) in group III cows on day 3 PT. PI increased to 43.9% in group III cows with combined treatment of antibiotic and tocopherol.

Discussion

The interaction between invading pathogens and the immune responses of the host greatly determines the outcome of the disease. For appropriate clearance of the infection from the udder, bacteria must be recognized by the phagocytic cells and eliminated by the intracellular mechanism of the respiratory burst. In the present study, cases of clinical mastitis were selected from a lactating dairy herd. The SCC was observed in more than 20×10^5 cells/mL of milk, which was drastically reduced by parenteral administration of enrofloxacin on days 3, 7 and 15 PT. However, the SCC counts remained more than normal

on day 15 PT in group II cows where the recovery rate was 78%. Similarly, SCC reduced drastically to 81.14% on day 3 PT. The SCC count on days 7 and 15 was observed as a less than normal count in group III cows, where the recovery rate was 89%.

The respiratory burst activity of bovine leukocyte isolated from milk was measured by superoxide anion production induced by PMA. PMA is a soluble stimulant of respiratory burst. Decreased phagocytic activity, intracellular elimination and respiratory burst have been observed in leukocytes due to fat and casein globules already ingested by milk leukocyte (MEHZARD et al., 2001). However, this problem can be overcome by processing milk in cold conditions. In the present work O^-_2 production was non-significantly enhanced in group II cows to an extent of 21%, whereas in group III cows it enhanced significantly ($P<0.05$) to an extent of 59.4% on day 3 PT. Similarly, the phagocytic activity and phagocytic index of the milk leukocytes enhanced to only 5.95 % and 15.83 % in group II cows, respectively. Such enhancement was 44.13% and 43.9% on day 3 PT in group III cows.

Infusion of enrofloxacin and Vitamin E in clinical cases of mastitis resulted in a drastic reduction of SCC on day 3 PT. The respiratory burst activity (O_2^-) of milk neutrophils was significantly ($P<0.05$) enhanced in group III cows. Similarly, the phagocytic activity and phagocytic index also increased significantly in group III cows The most important role of phagocytosis is to eliminate foreign cells, which is accomplished by oxygen-dependent and oxygen-independent mechanisms. However, impaired functioning of neutrophil was observed in periparturient disorders (CAI et al., 1994). MAYER (1987) reported low oxygen concentration in mastitic milk. This may reduce oxygen radical production and consequently impair neutrophil oxygen-dependent bactericidal activity. Sudden and marked decrease in reactive oxygen species production have been seen in acute mastitis, the diminished burst activity may be most probably due to immature cell type (HEYNEMAN et al., 1990). Enrofloxacin belongs to the fluoroquinolone group, which does not affect phagocytosis of bovine granulocyte (PAAPE et al., 1991). HOEBEN et al. (1997), reported enrofloxacin increases the chemiluminescence of milk PMNs and release of respiratory burst enzymes. This increased enzymic activity of enrofloxacin could be due to improved penetration into the PMNs and stimulation of H_2O_2. However, the production of O_2^- was unchanged. Previous studies have shown enhanced release of myeloperoxidase enzyme from milk leukocyte by enrofloxacin treatment in bovine sub clinical mastitis (MUKHERJEE and DASH, 2003). In recent years a number of substances have been identified which increase the activity of the immune cells (VOJTIC, 1998; MUKHERJEE et al., 2004). NDIWENI and FINCH (1995; 1996) recorded enhanced production of chemotaxin by blood lymphocytes and milk macrophages by *in vitro* treatment of Vitamin E and Selenium. Similarly, increased phagocytosis and O_2^- of bovine PMN cells were also observed by supplementation of these vitamins and trace minerals.

Conclusion

SCC significantly reduced in group III animals on day 15 PT, whereas SCC remained $>5.7\times10^5$ cells/mL of milk in group II on day 15 PT. The O_2^- was significantly increased in group III cows, such enhancement being only 21% in group II cows. Similarly, phagocytic activity and phagocytic index was significantly higher in group III cows. Hence it is concluded that alfa-tocopherol can be used in bovine mastitis together with enrofloxacin in the treatment of bovine mastitis for better and earlier recovery.

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SAŽETAK

Istraživan je učinak enrofloksacina i alfa tokoferola na sposobnost leukocita mlijeka u tvorbi reaktivnih kisikovih metabolita i na njihovu fagocitnu aktivnost nakon davanja phorbol 12-myristat 13-acetata kod mastitisa krava. Skupinu I sačinjavalo je devet zdravih kontrolnih krava, dok je skupinu II i III sačinjavalo po devet krava s kliničkim mastitisom dokazanim pomoću kalifornijskog mastitis-testa. Krave skupine II dobivale su po 1500 mg enrofloksacina tijekom pet dana, dok su one iz skupine III dobivale 1500 mg enrofloksacina tijekom tri dana, uz dvije injekcije alfa-tokoferola i selena naizmjenično. Broj somatskih stanica značajno se smanjio ($P<0,05$) u krava iz skupine III, od kojih je 89% bilo izliječeno, dok je u krava iz skupine II broj somatskih stanica ostao visok s izliječenjem od 78%. Tvorba slobodnih kisikovih radikala u izdvojenim leukocitima mlijeka krava II. i III. skupine značajno se povećala trećeg dana nakon liječenja. Fagocitna aktivnost leukocita u mlijeku određivana je tehnikom bojanja s akridinskim bojama. Fagocitna aktivnost značajno se povećala u krava skupine III i dosegnula je 44,13%, dok je takav porast u krava iz skupine II iznosio 10%. Fagocitni indeks značajno se povećao u životinja skupine III, dok u krava skupine II nije ustanovljen statistički značajan porast. Rezultati istraživanja su pokazali da enrofloksacin i vitamin E imaju povoljan učinak na funkcioniranje imunosnih stanica u vimenu krava nakon infekcija.

Ključne riječi: alfa-tokoferol, enrofloksacin, leukociti, fagocitoza, broj somatskih stanica