Expression of L selectin molecule on peripheral leukocyte in response to nisin treatment during acute bovine mastitis

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ABSTRACT

Concentration of interleukin 8 (IL 8) in milk serum and blood leukocyte adhesion molecules were studied in healthy cows (group I) and during clinical mastitis with the treatment of Nisin and Vitamin E plus Selenium (group II) and Amoxicillin (group III). The somatic cell count (SCC) was higher in mastitic cows before treatment (P<0.05). However, the count decreased in both the treated groups as compared to pretreatment count (P<0.05). The proinflammatory cytokine i.e. interleukin 8 (IL 8) was higher in milk serum before treatment, but it decreased in both the treated groups on day 7 (P<0.05). The expression of L selectin on peripheral polymorphonuclear cells (PMNs) was lower in mastitic cows both before and after treatment (P<0.05). However, the mean fluorescent intensity (MFI) of L selectin enhanced significantly in group II cows as compared to pretreatment values (P<0.05). The concentration of Selenium and Vitamin E was lower in mastitic cows. Serum Selenium concentration increased significantly in group II cows as compared to pretreatment values (P<0.05). The results indicate that Nisin and Vitamin E plus Selenium therapy enhances the expression of L selectin, which is related to enhancement of the mammary defense. Non antibiotic treatment along with Selenium and Vitamin E was effective in reduction of SCC and IL 8 from the inflamed udder compared to standard antibiotic treatment. Hence combination therapy of nisin and Selenium plus Vitamin E may be recommended for the treatment of mastitis in such farming systems where antibiotics are not allowed. Furthermore development of such combination therapy is important in reducing the antibiotic residue from the human food chain.

Key words: interleukin 8, L selectin, mastitis, nisin, selenium

Introduction

Mastitis is one of the most costly infectious diseases of lactating cattle and buffaloes. Incidence of intramammary infection (IMI) is high during the periparturient period.

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Migration and recruitment of PMNs to the site of infection is a key factor in control of mastitis (HEYNEMAN et al., 1990; BURTON and ERSKINE, 2003). Recruitment of PMNs into the infected udder depends on the coordinated function of selectins adhesion molecules (KEHRLI et al., 1999). It has been observed that there is down-regulation of selectins around the calving period, which affects migration of leukocyte into the udder parenchyma and its functional activities (PAAPE et al., 2002). The clinical efficacy of non antibiotic agents has recently been researched extensively with promising results, including cytokines, micronutrients, vitamins and medicinal herbs (SORDILLO et al., 1997; MUKHERJEE et al., 2005; DE and MUKHERJEE, 2009). Nisin is an antimicrobial peptide extracted from Lactococcus lactis subsp. lactis and is used as an antibacterial food preservative (ANONYM., 1988). CAO et al. (2007) reported that Nisin is effective against intramammary infection caused by gram positive bacteria.

Supplementation of Selenium and Vitamin E in feed helps in reducing the severity and duration of clinical mastitis (HEMINGWAY, 1999; POLITIS et al., 1996). It also helps in alleviating the acute phase response from the site of infection and improves leukocyte migration and intracellular killing respectively (AZIZ and KLESIUS, 1986; SORDILLO et al., 1997; SMITH et al., 1997). Nisin and Nisin based products have been found very effective in the treatment of clinical and sub clinical mastitis. However, the role of Vitamin E plus Selenium along with Nisin therapy has not yet been elucidated.

In this study we determined the effect of Vitamin E plus Selenium and Nisin therapy on L selectin and IL-8 in lactating cows inflicted with clinical mastitis.

**Materials and methods**

**Selection of lactating cows and experimental protocol.** Fifteen lactating crossbred cattle were selected from a dairy farm with more than 200 lactating cattle, aged between 3.6 to 5.6 years, in 1st to 3rd lactation at 5 to 40 days post partum, maintained under identical managemental conditions. The cows were screened for mastitis by the California mastitis test and divided into 3 groups, with 5 cows per group. Group I consisted of 5 healthy cows, where the udder secretions were negative for pathogenic bacteria. Ten mastitic cows (group II, group III), where the udder secretion were positive for pathogenic microorganism and SCC more than 0.5 million cells/mL of milk, formed the drug trial groups. Group II, cows were treated with Nisin 25 lakh IU (A kind gift from Zhejiang Silver Elephant Bio-Engineering Co., Ltd., Zhejiang, China) intramammary after dissolving it in 10 mL of sterile 0.9% saline solution along with Vitamin E plus selenium at the rate of 10 mL per cow intramuscular. Both the drugs were given once a day for 3 days. Group III cows were treated with Amoxicillin intramammary at the dose of 300 mg per infected teat for 3 days.
Collection of milk samples and bacterial isolation. Two hundred mL of milk from each cow was collected, maintaining sterile conditions, after discarding a few streams of milk. The milk was collected on day 0, day 7 and day 15 from diseased and healthy animals and milk was also collected from healthy cows at the same point of time. The SCC of the milk samples was done as per the standard method (SCHALM et al., 1971). The identification of causative organisms in the collected milk samples was carried out by spreading 10 μL of milk over 5% bovine blood agar plate, further the growth of the organisms on selective media. The organisms were identified on the basis of colony morphology, characteristic hemolytic pattern and Gram’s staining and further processed for biochemical tests (BALOWS et al., 1991) bacterial isolation was performed on day 0 and day 14. SCC was performed on day ‘0’ and thereafter on day 7 and 14 of the time period. Similar observations were also taken from the normal healthy cows.

Estimation of interleukin-8 (IL-8) in milk serum. Milk serum was collected by centrifugation of milk, the translucent supernatant collected and stored at -20 °C. IL-8 in milk serum was quantified by using a commercial anti-human IL-8 ELISA kit (Quantikine, R and D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Cytokine level was measured before treatment and on day 7, and similar observations were recorded in healthy cows.

Expression of leukocyte adhesion molecules in peripheral PMNs. Expression of L-Selectin was done using a commercial kit (CD 18, Clone- BAQ30A, Isotype- IgG1, VMRD, Inc. Pullman, WA, USA and L-Selectin, Clone- DU1-29, Isotype- IgG1, VMRD, Inc. Pullman, WA, USA) as per the method described by SOLTY and QUINN (1999). The cellular concentration was determined by flow cytometry (Becton Dickinson Immunocytometry System, Bioscience, USA). A total of 10,000 events were counted for each sample. Dot plots were gated for PMNs. The cells were assayed for size by forward scattering and granularity by side scattering. The expression of leukocyte adhesion molecules in terms of mean fluorescence intensity (MFI) was calculated after plotting the fluorescence of the histograms with CellQuest software (Becton Dickinson, Bioscience, USA). For isotype control, a similar procedure was followed, but instead of adding monoclonal antibodies, isotypes (FITC labeled rabbit antimouse IgG) were added to the sample.

Estimation of selenium and vitamin E in serum. Blood was collected before treatment and on day 10 of the time period for estimation of selenium and Vitamin E. The serum was separated and digestion was performed as per the procedure described by KOLMER et al. (1951). Serum Selenium was estimated before treatment and on day 10 by Atomic Absorption Spectrophotometry (AAS 4141, ECIL, India). Estimation of serum vitamin E was performed before treatment and on day 10 as per the method described by NAIR and MAGAR (1955).
Statistical analysis. Somatic cell count data was analyzed using the repeated measurement model with animals as subjects and the time period as a repeated measurement. The data for L selectin, IL-8 and Vitamin E and Selenium concentration, were analyzed applying one-way analysis of variance (ANOVA) to determine the level of significance between the groups, and Duncan’s Multiple Range Test (DMRT) was applied to determine the significance within the group at different time intervals with a statistical software (SPSS, Version 10, South Asia, Bangalore, India).

Results

Effect of treatment on SCC. The SCC ranged from $2.87 \pm 0.09$ to $3.01 \pm 0.16 \times 10^4$ and $19.17 \pm 1.02$ to $20.02 \pm 0.99 \times 10^4$ cells per mL of milk samples isolated from normal healthy cows and mastitic cows respectively. There were no differences in SCC in the milk sample isolated from healthy cows at different time intervals of the study period. The SCC in group II and group III, decreased (P<0.05) on day 7 compared to pre treatment values. The cell count on day 15 was significantly lower (P<0.05) in group II cows compared to group III (Table 1).

Table 1. Somatic Cell Count (SCC) $1 \times 10^5$ cells/mL of milk in response to treatment with Nisin and Vitamin E plus Selenium (group II), Amoxicillin (group III) mastitic cows and in normal healthy cows (group I) (mean ± SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>$3.91 \pm 0.31^a$</td>
<td>$3.87 \pm 0.39^a$</td>
<td>$3.61 \pm 0.46^a$</td>
</tr>
<tr>
<td>Group II</td>
<td>$15.62 \pm 0.99^{a,y}$</td>
<td>$5.21 \pm 1.09^{b,y}$</td>
<td>$4.72 \pm 1.11^{b,x}$</td>
</tr>
<tr>
<td>Group III</td>
<td>$16.02 \pm 1.23^{a,y}$</td>
<td>$7.05 \pm 1.87^{b,x}$</td>
<td>$6.03 \pm 1.23^{b,y}$</td>
</tr>
</tbody>
</table>

*Values with different superscripts in each rows (a, b) and each column (x, y, z) differ significantly (P<0.05)

From the 12 milk samples collected from mastitic cows, the organisms isolated were *Staphylococcus aureus* (41.66%), *Streptococcus agalactiae* (16.66%), *Streptococcus uberis* (16.66%) and coliforms (25%). No bacterial growth could be observed in the milk samples collected from cows treated with Nisin and Vit. E plus selenium, however growth of *Staphylococcus aureus* was observed in the milk sample collected from one cow of group III on day 14 of the treatment time period.

Effect of treatment on IL-8. The IL-8 concentration was significantly higher (P<0.05) in the milk serum collected from mastitic cows as compared to normal healthy cows. IL-8 levels decreased on day 7 (P<0.05) in both the treated groups compared to pretreatment values. However, a lower level was observed in group II cows on day 7 compared to group III cow (P<0.05) (Table 2).
Table 2. Expression of L selectin on peripheral PMNs and concentration of Interleukin 8 pg/mL milk serum in response to treatment with Nisin and Vitamin E plus Selenium (group II), Amoxicillin (group III) in mastitic cows compared with normal cows (group I) (mean ± SE)

<table>
<thead>
<tr>
<th>Group</th>
<th>L selectin (MFI)</th>
<th>Interleukin 8 (pg/mL milk serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>Group I</td>
<td>4.99 ± 1.09*</td>
<td>5.12 ± 0.61*</td>
</tr>
<tr>
<td>Group II</td>
<td>1.91 ± 1.12××</td>
<td>4.32 ± 1.27××</td>
</tr>
<tr>
<td>Group III</td>
<td>2.03 ± 1.00×</td>
<td>3.02 ± 1.02×</td>
</tr>
</tbody>
</table>

Values with different superscripts in each rows (a, b) and each column (x, y, z) differ significantly (P<0.05)

**Effect of treatment on L selectin.** The expression of L-selectin in peripheral PMNs isolated from normal healthy cows ranged from 4.56 ± 0.17 to 4.66 ± 0.24 mean fluorescent intensity (MFI). Expression of L selectin was lower in mastitic cows (P<0.05) before treatment. However, the expression increased in group II cows on day 7 (P<0.05), as compared to pretreatment values and group III cows, whereas the changes in group III was insignificant in post treated cows (Table 2).

**Effect of treatment on concentration of vitamin E and selenium.** The Vitamin E concentration ranged from 4.66 ± 0.91 to 4.71 ± 0.67 μg/mL and selenium ranged...
from 0.69 ± 0.29 to 0.71 ± 0.16 µg/mL of serum in blood collected from normal healthy cows. The Vitamin E and Selenium concentration was lower (P<0.05) in serum collected from mastitis cows before treatment. The selenium concentration increased significantly in group II cows on day 10 (P<0.05), whereas the vitamin E concentration remained unchanged in response to therapy on day 10 (Fig. 1).

**Discussion**

In large animal practice mastitis is one of the most challenging diseases to manage. Mastitis is generally caused by intramammary infection, attributes to inflammation and induces an acute phase response (HARMON, 1994). In the present study the SCC and IL-8 was higher in mastitic cows before treatment (P<0.05), both the parameters are related to the infiltration of PMNs into the mammary glands in response to bacterial infection (RIOLLET et al., 2000). Many chemotactic factors, such as interleukin-8 (IL-8), leukotriene and complement fragment rise in mastitic milk in response to bacterial stimuli during inflammation (SHUSTER et al., 1997). Antibiotics are used to treat mastitis and to prevent disease in healthy cows, although there is no convincing evidence that antimicrobials are effective in the management of mastitis (ERSKINE, 2000), further they cannot remove the inflammatory metabolites from the site of infection. To end inflammation appropriate exclusion of PMN is essential from the infected site. In this study we recorded, reduction of SCC and IL 8 concentration in milk serum and enhancement of L selectin of the milk PMNs in cows treated with Nisin plus vitamin E and selenium. Nisin is an antibacterial polypeptide, it is highly effective against gram positive microorganisms. It exhibits antibacterial activity through the formation of pores in the cell membrane (BROUGHTON, 2005). Reduction of inflammation in post treated cows could be due to the synergistic effect of Nisin and Vitamin E plus selenium in reducing the infection from the infected udder. The nutritional status of the cow is directly related to overall health and the ability of an animal to fight diseases. It has been observed that supplementation of micronutrients like Selenium and Vitamin E helps in reducing somatic cell count, and the severity and duration of clinical mastitis (HEMINGWAY, 1999; POLITIS et al., 1996). Selenium deficiency has a negative effect on leukocyte migration and phagocytosis (POLITIS et al., 1996), whereas, Vitamin E prevents lipid peroxidation and improves cellular defenses (SORDILLO et al., 1997). Interleukin 8 is an inflammatory mediator released by the resident cells and induces tissue injury. It has been recorded that Vitamin E therapy helps in modulation of prostaglandins and thromboxane pathways and prevents cellular insult by the toxic inflammatory metabolites (SMITH et al., 1997).

Leukocyte adhesion molecules are sensitive indicators of cell activation. The inability to express adhesion molecules on leukocytes results in poor migration of PMNs at the site of infection, which could lead to acute infection (SHUSTER and HARMON, 1992).
L-selectin works as cytokine as a molecule in priming host defense and is mediated through CD18. L-selectin also regulates the accumulation of leukocytes at the site of infection and control inflammation (DIEZ-FRAILE et al., 2002; SOLTYS and QUINN, 1999). LEE and KEHRLI (1998) recorded poor expression of adhesion molecules on neutrophils during the periparturient period and suggested that this could be due to the release of acute phase cytokines from the fetal-maternal interface breakdown prior to parturition. In our study, we recorded lower expression of L selectin in mastitic cows, although the expression of L selectin increased in group II cows treated with Nisin and Vitamin E plus Selenium (P<0.05). Many researchers have reported down regulation of L selectin on peripheral PMNs in Streptococcal, Staphylococcal and E. coli mastitis (SOLTYS and QUINN, 1999; SHUSTER et al., 1997). In the present study the enhanced expression of L selectin in post treated cows could be due to the synergistic effect of Nisin and Vitamin E plus selenium treatment. STICKEL et al. (1997) observed a reduction of mucosal 6-keto prostaglandin F-1 alpha concentrations and inflammation by Vitamin E treatment in mice. Selenium is a cellular antioxidant and scavenger of free radicals, and a key regulator of lipid peroxidation (SMITH et al., 1997). Others have also recorded release of high levels of prostaglandin in mastitic cows with high SCC and lower levels of serum selenium (PARANTAINER et al., 1987).

**Conclusion**

Nisin and Vitamin E plus Selenium treatment reduced the SCC and concentration of IL 8 but the level of L selectin enhanced in mastitic cows. The therapy indicated a reduction in infection and inflammation. Non antibiotic treatment, along with Selenium and Vitamin E, was effective in the treatment of mastitis compared to standard antibiotic treatment. Therefore combination therapy of Nisin and Selenium plus Vitamin E may be recommended for the treatment of mastitis in order to reduce the harmful antibiotic residue from milk and milk products. Furthermore, this alternative approach is also useful for farming systems where antibiotics are not allowed.

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**References**


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SAŽETAK
Koncentracija interleukina 8 (IL-8) u mlječnom serumu i adhezijske molekule krvnih leukocita istraživane su u zdravim krava (skupina I), zatim u krava s kliničkim mastitismom liječenim nizinom i vitaminom E uz dodatak selena (skupina II) i krava liječenih amoksicilinom (skupina III). Broj somatskih stanica bio je povećan.

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u krava s mastitisom prije liječenja (P<0,05). Njihov broj smanjio se u objema liječenim skupinama u odnosu na broj prije liječenja (P<0,05). Koncentracija proupalnoga citokina, tj. interleukina 8 (IL-8), bila je viša u mliječnom serumu prije liječenja, ali se sedam dana nakon liječenja smanjila u objema liječenim skupinama (P<0,05). Ekspresija L selectina na perifernim polimorfonuklearnim stanicama bila je niža u krava s mastitisom u usporedbi s zdravim kravama i prije i nakon liječenja. Srednja flaorescencija L selectina značajno se povećala sedmoga dana u krava skupine II (P<0,05). Koncentracija selena i vitamina E bila je manja u krava s mastitisom. Koncentracija selena u serumu značajno se povećala u krava skupine II u usporedbi s vrijednostima prije liječenja (P<0,05). Rezultati pokazuju da liječenje nizinom i vitaminom E s dodatnim selenom povećavaju ekspresiju L selectina što upućuje na pojačanu obranu mliječne žlijezde. Liječenje antibiotikom uz dodatak selena i vitamina E nije imalo učinka na smanjenje broja somatskih stanica i na koncentraciju IL-8 u upaljenom vremenu u usporedbi s uobičajenim liječenjem antibioticima. Stoga se kombinacija nizina i selena s dodatkom vitamina E preporučuje za liječenje mastitisia u uvjetima u kojima nije dopuštena upotreba antibiotika. Daljnji razvoj lako kombiniranoga liječenja važan je za uklanjanje ostataka antibiotika iz ljudskoga prehrambenoga lanca.

Ključne riječi: interleukin 8, L selektin, mastitis, nizin, selen