Effect of Vitamin B\textsubscript{2} on Somatic Cell Counts in Milk of Clinical Staphylococcus aureus Mastitis

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ABSTRACT. Effects of intravenous injection of Vitamin B\textsubscript{2} (VB\textsubscript{2}) on the nitroblue tetrazolium (NBT) reductivity of peripheral blood neutrophils and the somatic cell counts (SCC) in quarter milk of Staphylococcus aureus mastitis were investigated. The NBT reductivities of neutrophils were enhanced at 2 days after single injection of VB\textsubscript{2} (5.0 and 2.5 mg/kg), and were also enhanced at 4 days after initial injection of continuous 3 days of VB\textsubscript{2} (2.5 mg/kg). The SCC in quarter milk were significantly decreased at 3, 7 and 14 days after initial injection of continuous 3 days of VB\textsubscript{2} (2.5 mg/kg), however, S. aureus in the infected quarter was not cured bacteriologically by VB\textsubscript{2} injection. KEY words: somatic cell count, Staphylococcus aureus mastitis, vitamin B\textsubscript{2}.


_**Staphylococcus aureus** is the most common contagious pathogen causing clinical mastitis in dairy cows [4]. A serious problem has been posed the considerable economic loss from reduced milk production and increased somatic cell counts (SCC) in the _S. aureus_ mastitis [8]. Bacteriologic cure rates after antibiotic treatment seldom exceed 50% because of the poor penetration of antibiotics into areas of scarring and inflammation [14]. Therefore, non-antibiotic treatments including the bactericidal enzyme lysostaphin [12], cytokines such as interleukin (IL)-1 and IL-2 [2, 6, 15], and probiotics [3, 5] are the current interest in the treatment of _S. aureus_ mastitis. Vitamin B\textsubscript{2} (VB\textsubscript{2}) has been found to enhance the non-specific host defense mechanisms against bacterial infections such as _Escherichia coli_ and _S. aureus_ in mice by stimulating the generation of neutrophils and enhancing macrophage function [1, 7]. In cows, an intramuscular injection of VB\textsubscript{2} has been shown to stimulate neutrophil function such as nitroblue tetrazolium (NBT) reductivity and phagocytic bactericidal activity of _S. aureus_ [13]. Neutrophils play an important role in the host defense against bovine intramammary infection [11], however, the effect of VB\textsubscript{2} on the SCC in mastitic milk has not been shown. The purpose of the present study is to determine the effects of intravenous injection of VB\textsubscript{2} on the NBT reductivity of peripheral blood neutrophils in the healthy heifers, and on the SCC in quarter milk of the clinical _S. aureus_ mastitis.

VB\textsubscript{2} (Riboflavin sodium phosphate, Wako Pure Chemical Co., Tokyo, Japan) was prepared as a 5.0% w/v solution in injectable sterile water (Otsuka Pharmaceutical Co., Tokushima, Japan), and then passed through a 0.45 µm membrane filter (Millipore Bedford, MA, U.S.A.) before use. Thirteen healthy Holstein heifers aged 20–24 months were used. The heifers were divided into three groups, received single intravenous injection of VB\textsubscript{2} (5.0 mg/kg; n=4, and 2.5 mg/kg; n=4), and continuous 3 days intravenous injection of VB\textsubscript{2} (2.5 mg/kg; n=5). Blood samples were obtained at the before injection, and 1, 2, 3, 4, 5, and 7 days after initial injection. The peripheral blood leukocyte count was determined with an automatic hemocytometer and the neutrophil count was estimated from the differential count determined with a Giemsa-stained blood smear. Isolation of neutrophils from peripheral blood and the NBT reduction assay were performed by the method described previously [9]. The optical density (OD) measured at 565 nm was corrected by subtracting the OD of control specimens which added RPMI-1640 instead of zymosan solution.

In some experiments to examine the effect of VB\textsubscript{2} on the SCC in quarter milk of clinical mastitis, 45 quarters of 40 Holstein cows were used. The mean parity and days after parturition at injection were 1.8 and 52, respectively. The cows were housed in dairy farms, which were confirmed intramammary infection of _S. aureus_ by bacteriologic culture, and showed positive results in the modified California Mastitis Test (PL tester, Nippon Zenyaku Kogyou, Fukushima, Japan) and clinical findings such as decreased appetite and inflammatory symptoms of mammary gland. The cows were divided into two groups, a group received intravenous injection of VB\textsubscript{2} (2.5 mg/kg; 30 quarters of 25 cows) for 3 successive days, and a group of no-treatment (control cows; 15 quarters of 15 cows). Quarter milk samples for the assessment of bacterial culture and SCC were collected at 4–6 hr after morning milking of the before injection, and 3, 7, and 14 days after initial injection. Bacterial culture was on 5% sheep blood agar, and the plates were incubated for 24 hr at 37°C. Tentative identification was made on the basis of Gram-staining, colony morphology and hemolytic pattern. Isolates presumptively identified as _S.aureus_ were tested for tube coagulase reaction. Coagulase-negative isolates were identified as _S. aureus_. The SCC were determined by a Fossomatic milk cell counter (Type 360; Foss Electric, Hillerod, Denmark). Statistical analyses were carried out using the Student’s t-test.

Effect of intravenous injection of VB\textsubscript{2} on the NBT reductivity of peripheral blood neutrophils is shown in Fig. 1. The NBT reductivities of neutrophils were elevated and reached its maximum level at 2 days after single intravenous
injection of VB2 (5.0 mg/kg and 2.5 mg/kg). In the heifers injected intravenously with continuous 3 days of VB2 (2.5 mg/kg), the NBT reductivities were elevated and reached its maximum level at 4 days after initial injection. Thereafter, the NBT reductivities were diminished and returned to the level of before injection at 7 days after initial injection. No significant changes was observed in the peripheral blood leukocyte and neutrophil counts after single and continuous injection of VB2. The mechanism of activation of neutrophil function following VB2 injection has not been clear, however, VB2 is converted within the body to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are known to act physiologically as coenzymes for redox reactions [10].

By the intramuscular injection of VB2 (riboflavin, 5.0 mg/kg) to cows, the NBT reductivity was enhanced at 3–6 days after injection, and the phagocytic bactericidal activity of S. aureus was enhanced at 1–6 days after injection [13]. In the present study, the VB2 injection involved an intravenous injection of VB2 (riboflavin sodium phosphate) in both 5.0 mg/kg and 2.5 mg/kg, compared with the intramuscular injection of VB2 (riboflavin) [13]. The reason for the difference of peaks in NBT reductivities between the single and continuous 3 days injection of VB2 was unclear.

Effect of VB2 injection on the SCC in quarter milk of S. aureus mastitis is shown in Table 1. In the VB2 injected cows, the SCC of infected quarter milk by PL tester were increased temporarily at 1 day or 2 days after initial injection. Thereafter, the SCC in quarter milk were significantly (p<0.05 and p<0.01) decreased at 3, 7 and 14 days after initial injection compared with the before injection, which were significantly (p<0.05) lower than those of the control cows, respectively. In the cows with high SCC, the SCC were decreased rapidly after VB2 injection compared with the cows with low SCC (data not shown). No significant changes was observed in the SCC of control cows. The bacterical counts of S. aureus in the infected quarter milk were decreased in 12 of 30 quarters to 7 days after initial injection, and S. aureus was not detected temporarily in 7 of 30 quarters after VB2 injection. However, no significant changes between the before and after VB2 injection, and no significant differences between the VB2 injected cows and control cows were observed in the bacterical counts (data not shown). For the controlling S. aureus mastitis, a program of early identification, segregation and culling of the mastitic cows has been proposed the best management approach, because of the low bacteriological cure rate after antibiotic treatment in the S. aureus mastitis. In the present study, the VB2 injection appeared to have little or no effect on the clearance of S. aureus from mastitic quarter. We did not adjust for the differences in stage of lactation, milk production and parity, all of which have major effect on SCC, however, the SCC in quarter milk of S. aureus mastitis were decreased by VB2 injection. The positive results of PL tester and the inflammatory symptoms of mammary gland were also improved by the decreasing of SCC, however, the changes of SCC were not related to the bacterial counts of S. aureus. Therefore, an intravenous injection of VB2 might be an effective alternative to antibiotic therapy for bovine S.aureus mastitis. The mechanism underlying the decreasing of SCC in quarter milk following VB2 injection has not been clear. The decreasing of SCC in the quarter milk of S. aureus mastitis following VB2 injection may be related to the enhancement of the NBT reductivity of peripheral blood neutrophils and the activation of the host defense mechanism against bacterial infection in the mammary gland. It has been suggested that the mechanism of activation of neutrophil function following VB2 injection involved cytokines such as granulocyte colony-stimulating factor (G-CSF) rather than a direct mechanism [13]. It is suggested

Table 1. Somatic cell counts (SCC) in the quarter milk of Staphylococcus aureus mastitis in the vitamin B2 (VB2) injected cows and control cows

<table>
<thead>
<tr>
<th>Days after initial injection</th>
<th>0</th>
<th>3</th>
<th>7</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>VB2 injected cows✩ (n=30)✩</td>
<td>679 ± 387</td>
<td>451 ± 327**</td>
<td>361 ± 338**</td>
<td>436 ± 329*</td>
</tr>
<tr>
<td>Control cows (n=15)✩</td>
<td>733 ± 318</td>
<td>693 ± 315</td>
<td>612 ± 265</td>
<td>655 ± 260</td>
</tr>
</tbody>
</table>

✩VB2 was injected intravenously with 3 successive days (2.5 mg/kg), ♦15 infected quarters of 25 cows, ♠30 infected quarters of 15 cows. Data (10⁴/ml) are expressed as mean ± S.D. Remarks (*p<0.05, **p<0.01) show significant difference against the before injection (day 0) of VB2 injected cows.
that the neutrophil function is activated and the SCC are decreased because of increased and/or regulated cytokine production induced by VB$_2$ injection. Further studies will be required to clarify whether the effect of VB$_2$ injection on decreasing SCC may be due to cytokines including G-CSF.

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**REFERENCES**