Effect of Vitamin B_2 on Somatic Cell Counts in Milk of Clinical *Staphylococcus aureus* Mastitis

Shigeru SATO, Hitomi HORI and Keiji OKADA[1)]

Ken-nan Veterinary Clinic Center, Miyagi Prefectural Federation of Agricultural Mutual Aid Association, Shiroishi, Miyagi 989–0731 and [1)]Veterinary Hospital, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–0066, Japan

(Received 5 November 1998/Accepted 12 January 1999)

ABSTRACT. Effects of intravenous injection of Vitamin B_2 (VB_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_2 (5.0 and 2.5 mg/kg), and were also enhanced at 4 days after initial injection of continuous 3 days of VB_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_2 (2.5 mg/kg), however, *S. aureus* in the infected quarter was not cured bacteriologically by VB_2 injection. KEY WORDS: somatic cell count, *Staphylococcus aureus* mastitis, vitamin B_2.

J. Vet. Med. Sci. 61(5): 569–571, 1999

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_2 (VB_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_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_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_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_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_2 (5.0 mg/kg; n=4, and 2.5 mg/kg; n=4), and continuous 3 days intravenous injection of VB_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 subtracing the OD of control specimens which added RPMI-1640 instead of zymosan solution.

In some experiments to examine the effect of VB_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 bacterial 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_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-positive 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_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

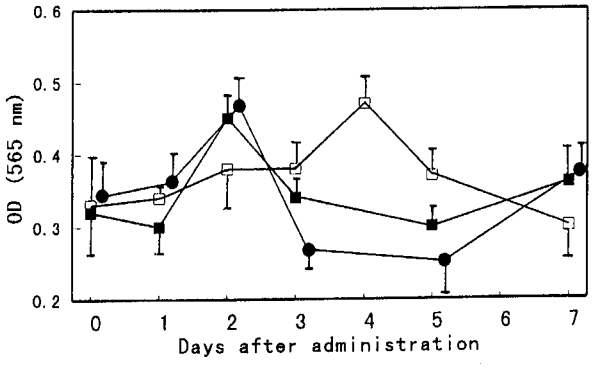

Fig.1. Changes of the nitroblue tetrazolium (NBT) reductivity of peripheral blood neutrophils in the heifers injected intravenously with single (●; 5.0 mg/kg, ■; 2.5 mg/kg) and 3 successive days (□; 2.5 mg/kg) of vitamin B_2. Data are expressed as mean ± S.D.

injection of VB_2 (5.0 mg/kg and 2.5 mg/kg). In the heifers injected intravenously with continuous 3 days of VB_2 (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 VB_2. The mechanism of activation of neutrophil function following VB_2 injection has not been clear, however, VB_2 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 VB_2 (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 also enhanced at 1–6 days after injection [13]. In the present study, the NBT reductivities were enhanced more rapidly and markedly by the single intravenous injection of VB_2 (riboflavin sodium phosphate) in both 5.0 mg/kg and 2.5 mg/kg, compared with the intramuscular injection of VB_2 (riboflavin) [13]. The reason for the difference of peaks in NBT reductivities between the single and continuous 3 days injection of VB_2 was unclear.

Effect of VB_2 injection on the SCC in quarter milk of *S. aureus* mastitis is shown in Table 1. In the VB_2 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 VB_2 injection compared with the cows with low SCC (data not shown). No significant changes was observed in the SCC of control cows. The bacterial 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 VB_2 injection. However, no significant changes between the before and after VB_2 injection, and no significant differences between the VB_2 injected cows and control cows were observed in the bacterial 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 VB_2 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 VB_2 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 VB_2 might be an effective alternative to antibiotic therapy for bovine *S.aureus* mastitis. The mechanism underlying the decreasing of SCC in quarter milk following VB_2 injection has not been clear. The decresing of SCC in the quarter milk of *S. aureus* mastitis following VB_2 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 VB_2 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 B_2 (VB_2) injected cows and control cows

		Days after initial injection			
		0	3	7	14
VB_2 injected cows[a)]	(n=30)[b)]	679 ± 387	451 ± 327*	361 ± 338**	436 ± 329*
Control cows	(n=15)[c)]	733 ± 318	693 ± 315	612 ± 265	655 ± 260

[a)] VB_2 was injected intravenously with 3 successive days (2.5 mg/kg), [b)] 30 infected quarters of 25 cows, [c)] 15 infected quarters of 15 cows. Data (10^4/m*l*) are expressed as mean ± S.D. Remarks (*$p<0.05$,**$p<0.01$) show significant difference against the before injection (day 0) of VB_2 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.

ACKNOWLEDGMENTS. The authors thank the kindly advices of Dr. M. Kimura and Dr. S. Araki in Research and Development Division of Eisai Co., Ltd.

REFFERENCES

1. Araki, S., Suzuki, M., Fujimoto, M. and Kimura, M. 1995. *J. Vet. Med. Sci.* 57: 599–602.
2. Daley, M.J., Coyle, P.A., Williams, T.J., Furda, G., Dougherty, R. and Hayes, P.W. 1991. *J. Dairy Sci.* 74: 4413–4424.
3. Dinsmore, R.P., Cattell, M.B., Stevens, R.D., Gabel, C.S., Salman, M.D. and Collins, J.K. 1995. *J. Dairy Sci.* 78: 1932–1936.
4. Fox, L.K. and Hancock, D.D. 1989. *J. Dairy Sci.* 72: 540–544.
5. Greene, W.A., Gano, A.M., Smith, K.L., Hogan, J.S. and Todhunter, D.A. 1991. *J. Dairy Sci.* 74: 2976–2981.
6. Hogan, J.S., Smith, K.L., Todhunter, D.A., Schoenberger, P.S., and Shuster, D. 1995. *J. Dairy Sci.* 78: 1062–1067.
7. Kimura, M., Suzuki, M. and Araki, S. 1996. *Anim. Sci. Technol.* 67: 368–373.
8. Kirk, J.H., DeGraves, F. and Tyler, J. 1994. *J. Am. Vet. Med. Assoc.* 204: 1152–1158.
9. Nakagawa, J., Osame, S., Ichijo,S., Araki,S. and Kimura, M. 1993. *J. Vet. Med. Sci.* 55: 259–263.
10. Newberne, P.M. and Conner, M.W. 1989. pp.796–834. *In*: Clinical Biochemistry of Domestic Animals, 4th ed. (Kaneko, J.J. ed.), Academic Press Inc., U.S.A.
11. Nickerson, S.C. 1985. *J. Am. Vet. Med. Assoc.* 187: 41–45.
12. Oldham, E.R. and Daley, M.J. 1991. *J. Dairy Sci.* 74: 4175–4182.
13. Osame, S., Araki, S. and Kimura, M. 1995. *J.Vet. Med. Sci.* 57: 493–495.
14. Owens, W.E., Watts, J.J., Boddie, R.L. and Nickerson, S.C. 1988. *J. Dairy Sci.* 71: 3143–3147.
15. Sordillo, L.M., Campos, M, and Babiuk, L.A. 1991. *J. Dairy Sci.* 74: 3370–3375.