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Autogenous toxoid-bacterin treatment for *Staphylococcus aureus* subclinical mastitis in lactating cows

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Abstract : To evaluate clinical effects of autogenous toxoid-bacterin treatment for *Staphylococcus aureus* subclinical mastitis in lactating cows, 22 cows which had at least one *S aureus* infected quarter were selected from one *S aureus* prevalent dairy farm, of which 11 cows were injected their own autogenous toxoid-bacterin and the others were maintain as noninjected control.

In the toxoid-bacterin injected group, 27% of infected quarters were cured during the 12 weeks trials as compared to 5% in the control group. New intramammary infections with *S aureus* were only detected in three-quarters of the control group. Mean IgG antibody titers against *S aureus* somatic antigens and α-toxin in serum and milk were significantly increased in the toxoid-bacterin injected group ($p < 0.05$) and remained higher than those of the control group which showed no significant changes ($p < 0.05$).

From 3 weeks after second injection (at 7 week), mean *S aureus* CFU/ml in milk samples from previously infected quarters with *S aureus* of the toxoid-bacterin injected group was lower than that from preinjection state ($p < 0.05$). In the toxoid-bacterin injected group, significant decrease of mean SCC was detected from milk samples from previously infected quarters with *S aureus* from week 7 to week 10 ($p < 0.05$).

These data suggested that autogenous toxoid-bacterin treatment aganist *S aureus* subclinical mastitis in lactating cows might increase the cure rate of the infections, reduce the severity of the infections and also prevent occurrence of the new infections.

Key words : *Staphylococcus aureus*, mastitis, autogenous toxoid-bacterin, IgG antibody.

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Introduction

Bovine mastitis is one of the most problematic diseases and continues to have major economic impact on the dairy industry throughout the world[1,2]. Although numerous agents can cause mastitis problems in dairy cows, *Staphylococcus aureus* is the most important etiological agent of bovine mastitis[3~5]. Therefore, although various management methods for decreasing the prevalence of *S aureus* mastitis have been improved, many dairies still have some level of infection with *S aureus*[5~7]. To cope with this problem, vaccination to prevent *S aureus* mastitis had been the subject of concern by many researchers[8~10]. These vaccines had been employed as live[11] or killed *S aureus*[12], isolated capsular materials[13], toxoids[14] or combined preparations of killed cells and toxoids[15~18]. These vaccines had been reported to increase specific antibody to *S aureus* antigens both in serum and milk, but only partially prevent new intramammary infections.

Autogenous bacterins are one type of vaccines made from bacteria isolated from infected animals and are inoculated back into those animals. Staphylococcal autogenous bacterins have been used for treatment of staphylococcal infection in humans[19,20] and animals[21,22] for reducing severity of the infection. Based on those results, authors concluded that autogenous bacterin treatment could increase recovery rate in clinical cases.

Therefore, the purpose of this study reported here was to determine the effects of autogenous toxoid-bacterins on their cure rate, changes of antibody level, changes of bacterial counts and somatic cell count (SCC) in milk when applied to *S aureus* subclinical mastitis in lactating cows.

Materials and Methods

Experimental Animals : Twenty two lactating holstein cows which had at least one *S aureus* infected quarter in one dairy farm with mean SCC of bulk tank milk about 450,000/ml were selected. Only detected mastitic pathogen in this farm was *S aureus* by microbiological examination previously. The cows were equally divided into two groups by lactation number and stage of lactation as autogenous toxoid-bacterin injected group or noninjected group as control. All individual quarter milk samples were tested daily to determine bacteriological state and SCC before toxoid-bacterin injection.

Autogenous toxoid-bacterin : To prepare autogenous toxoid-bacterin for each infected cow, *S aureus* was isolated from one of the infected quarters of each cow. Bacteria were cultured in brain-heart infusion broth with 10% (vol/vol) sterile bovine milk whey to enhance pseudocapsule production. After inoculation for 24hr at 37℃, cultures were sterilized by addition of formaldehyde to 1% (vol/vol). Sterility was ensured by inoculation of cultures on brain-heart infusion agar plates for 24hr. If there were no growing bacteria on the plate, cultures were regarded as inactivated. After inactivity was ensured, cells were harvested and washed 3 times with sterile normal saline by centrifugation (4,000rpm, 4℃, 30 min). Finally cells were suspended in sterile normal saline and bacterial concentrations were adjusted to 10^{10} CFU/ml at 450nm with spectrophotometer.

To prepare crude toxoid components, same bacteria used for bacterins were grown for 48 hr at 37℃ in brain heart infusion broth. Bacteria were removed by centrifugation (4,000rpm, 4℃, 30 min) and supernatant were concentrated by lyophilization to 12% of the original volume. Formaldehyde was added to 1% (vol/vol) and remained 48hr at 4℃.

Each dose of the toxoid-bacterin consisted of 1 ml of bacterin, 1 ml (40~80 hemolytic unit) of toxoid, and 50mg dextran sulfate (M.W. 500,000) which was emulsified with 2ml of Freund's incomplete adjuvant (Sigma Co, ST. Louis, MO, U.S.A) for a total amount of 4ml.

Safety test of toxoid-bacterin : Safety of each toxoid-bacterin was tested by injecting 1 ml of toxoid-bacterin to 8 weeks old ICR mouse subcutaneously. If mouse remained healthy for 1 week, toxoid-bacterin was regarded as safe.

Injection of autogenous toxoid-baerin : Eleven selected lactating holstein cows were injected with their own toxoid-bacterin subcutaneously in the area of supramammary lymph node and were boosted after 4 weeks. Another 11 cows were maintained as control.

Sampling and laboratory analysis : Individual quarter

milk samples from toxoid-bacterin injected cows and control cows were obtained weekly for bacteriological culture and the determination of SCC. SCC of individual quarter milk was also examined by Fossomatic 90 (Foss Electric, Denmark). About 0.1ml of individual quarter milk samples were streaked on bovine blood agar plate for bacteriological cultures. Plates were incubated aerobically at 37℃ and examined after 24hr incubation. Staphylococcal colonies were identified on the basis of colony morphology, hemolytic patterns and microscopic appearance and the colonies were counted. Remained individual milk samples were centrifuged (3,000rpm, 4℃, 15 min) and skim milk were separated. The skim milk were stored at -20℃ for detecting antibody titers. Blood was also collected two weeks intervals and sera were stored at -20℃ until antibody detecting.

Antibody detection by indirect ELISA : The sera and milk samples were analyzed for specific IgG antibodies to *S aureus* somatic antigens and to *α*-toxin in an indirect ELISA.

To prepare staphylococcal somatic antigens for each toxoid-bacterin injected cows, same bacteria used for each bacterin preparation were grown and harvested as described in the preparation of the toxoid-bacterin. To prepare staphylococcal somatic antigens for each control cow, one of *S aureus* infected quarters was selected from each cow and *S aureus* was isolated by the method as described in the preparation of the toxoid-bacterin. Somatic antigen preparation for control cows was same as that of toxoid-bacterin injected cows. All staphylococcal somatic antigens were suspended (10^{10}CFU/ml) in coating buffer (0.05M carbonate buffer, pH 9.6) immediately before the plates were coated on. The *α*-toxin antigens (Sigma) also were suspended (20μg/ml) in coating buffer immediately before the plates were coated on.

The wells of microtiter plates were coated by incubation with 100μl antigen in coating buffer overnight at 4℃. The coated wells were washed 4 times with phosphate buffered saline with 0.05% Tween 20 (PBS-T). The serum samples were diluted 1 : 500 in phosphate buffered saline (PBS) for somatic cell ELISA and 1 : 200 for *α*-toxin ELISA. The skim milk samples were diluted 1 : 3 in PBS for two ELISAs.

The plates were incubated with 100μl/well of diluted serum and skim milk at 37℃ for 2hr. After incubation the plates were washed 4 times with PBS-T. Subsequently, peroxidase-conjugated rabbit anti-bovine IgG (Sigma) diluted 1 : 1000 in PBS was added and incubated at 37℃ for 1 hr. The plates were washed again 4 times with PBS-T, and 100μl of 0.4mg/ml O-phenylenediamine in H_2O_2 inserted (0.04%) citrate buffer (pH 5.0) was added. The enzyme reaction was allowed to continue for 30 min and stopped by addition of 50μl H_2SO_4. The absorbance was read at 405nm with automatic ELISA reader (Titertek Multiscan Mcc/340, Flow labratories, Inc, Mclean, VA, USA).

Statistical analysis : The mean absorbance between toxoid-bacterin injected group and control group was compared by Student's *t*-test. The differences of weekly mean SCC, CFU/ml in individual quarter milk samples and mean absorbance for each group were also analyzed by Student's *t*-tests. A value of $p < 0.05$ was considered statistically significant.

Results

Changes of intramammary infection state : The changes of intramammary infection state of toxoid-bacterin injected group and control group were summarized in Table 1. In toxoid-bacterin injected group, 27% of infected quarters was cured during experimental period as compared to 5% in the control group.

Table 1. Changes of Intramammary infection state

Treatment (n)	No. of intramammary infections	No. of persisting infections	No. of cured quaters (%)	No. of new infections
Toxoid-bacterin (11)	33	24	9(27)	0
Control (11)	18	17	1(5)	3

New intramammary infections with *S aureus* were detected in 3 quarters in control group but no new intramammary infection was detected in toxoid-bacterin injected group.

IgG levels against *S aureus* somatic antigens in serum and milk : Mean IgG antibody titers against *S aureus* somatic antigens in serum was shown in Fig 1. Mean IgG antibody titers was significantly increased in toxoid-bacterin injected group and remained higher than those of control group after toxoid-bacterin injection ($p < 0.05$), but the control group showed no significant changes throughout trials ($p < 0.05$).

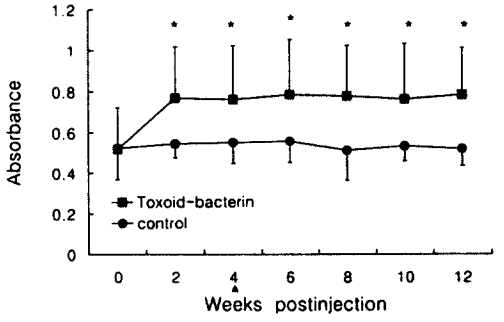

Fig 1. Changes of mean IgG antibody titers against *S aureus* somatic antigens in serum.
*$p < 0.05$, ▲ : boost injection, bar represents±2 standard deviation.

Mean IgG antibody titers in milk also significantly increased in toxoid-bacterin injected group after toxoid-bacterin injection and maintained higher titers than that of control group ($p < 0.05$) which had no significant changes (Fig 2 ; $p < 0.05$).

IgG levels aganist *α*-toxin in serum and milk : In serum samples, toxoid-bacterin injected group showed significant responses in IgG antibodies against *α*-toxin after first toxoid-bacterin injection and mean IgG antibody titers remained higher than that of preinjection state ($p < 0.05$) and that of control group ($p < 0.05$). In control group, there were no significant changes of mean IgG antibody titers against *α*-toxin throughout trials (Fig 3 ; $p < 0.05$).

In milk samples, mean IgG antibody titers in toxoid-bacterin injected group was increaed after first injection, but statistical significant changes were detected from 2 weeks after second injection (week 6) (Fig 4 ; $p < 0.05$). In control group, mean IgG antibody titers against *α*-toxin in milk was not significantly changed throughout trials ($p < 0.05$). Significant differences of mean IgG antibody titers against *α*-toxin in milk between baterin-toxoid injected group and control group were detected from 6 weeks (week 10) after second injection ($p < 0.05$).

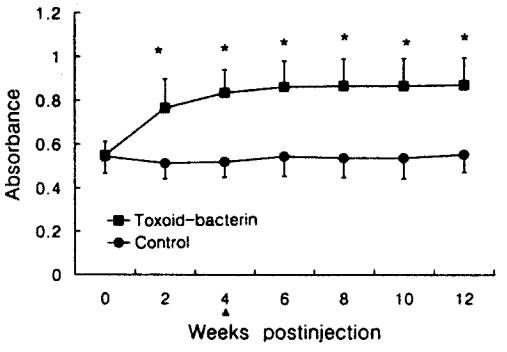

Fig 2. Changes of mean IgG antibody titers against *S aureus* somatic antigens in milk.
*$p < 0.05$, ▲ : boost injection, bar represents±2 standard deviation.

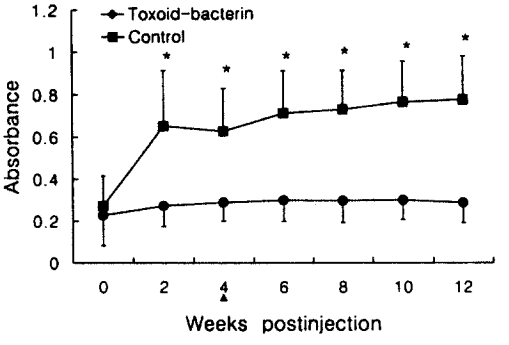

Fig 3. Changes of mean IgG antibody titers against *S aureus α*-toxin in serum.
*$p < 0.05$, ▲ : boost injection, bar represents±2 standard deviation.

Changes of mean *S aureus* colony forming unit (CFU) in individual quarter milk : In toxoid-bacterin injected group, mean *S aureus* CFU/ml in milk samples from previously infected quarters with *S aureus* was slightly increased until 2 weeks after toxoid-bacterin injection (Fig 5). From 3

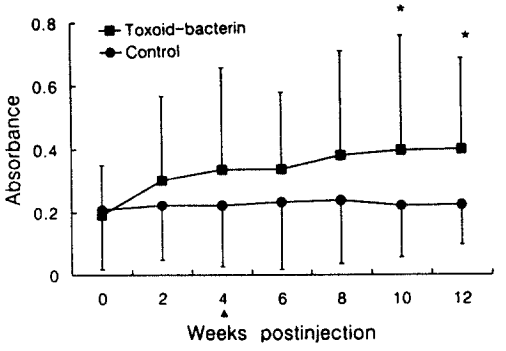

Fig 4. Changes of mean IgG antibody titers against *S aureus* α-toxin in milk.
*p ⟨ 0.05, ▲ : boost injection, bar represents±2 standard deviation.

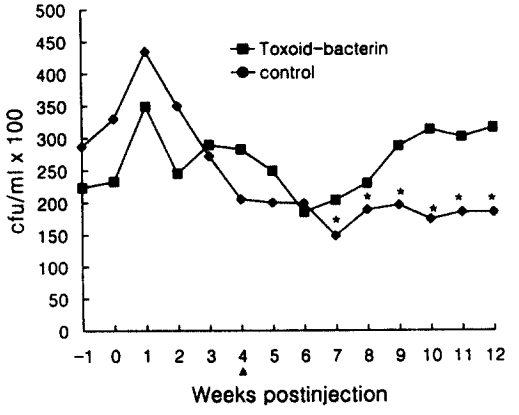

Fig 5. Changes of *S aureus* CFU/ml in individual quarter milk.
*p ⟨ 0.05, ▲ : boost injection.

weeks after first injection, however, that was decreased and finally remained significantly lower than that of preinjection state (p ⟨ 0.05) from 3 weeks after second injection (week 7).

In control group, significant changes of mean *S aureus* CFU/ml milk samples from previously infected quarters with *S aureus* were not detected but remained higher than that of preinjection state from week 8 (p ⟨ 0.05).

Changes of mean SCC in individual quarter milk : In toxoid-bacterin injected group, mean SCC in milk samples from previously infected quarters with *S aureus* was slightly changed at every week (Fig 6) and significant decreased changes were detected from week 7 to week 10 (p ⟨ 0.05). There were no significant changes of mean SCC in individual quarter milk samples from non-infected quarters (p ⟨ 0.05).

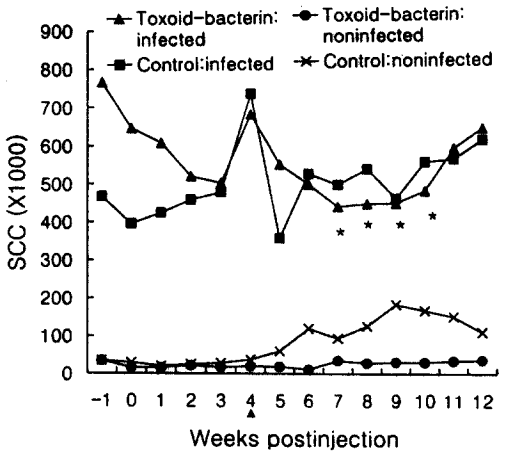

Fig 6. Changes of mean SCC in individual quarter milk.
*p ⟨ 0.05, ▲ : boost injection.

In control group, mean SCC in milk samples from previously infected quarters with *S aureus* was also variable during the experiment (Fig 6), but significant changes were not detected (p ⟨ 0.05). Mean SCC in individual quarter milk samples from non-infected quarters was increased from week 6 and remained higher than that of preinjection state, but statistical significance was not detected (p ⟨ 0.05).

Discussion

Traditionally, treatment of *S aureus* mastitis has been limited to intramammary infusion of antibiotics on dry cows and lactating cows with clinical or subclinical mastitis. The use of intramammary antibiotic therapy for *S aureus* during lactation however has given very poor results.

Since *S aureus* respond poorly to antibiotics, vaccines against this organism have been studied extensively and now focused on enhancing specific antibodies against pseudocapsular antigens[15-18,23,24].

Pseudocapsule allows complement and antibodies reach to cell wall to penetrate but interferes recognition of the complexed antibody by polymorphonuclear neutrophil (PMN), so prevents activation of complement (C_3)[25]. In addition, pseudocapsules are poor immunizing agents because they are

week immunogens and T cell independent[26]. However, when antibodies are produced against capsule, they are effective opsonins for PMN in cattle[27] and can increase phagocytic efficacy of PMN[13,27]. Therefore to enhance pseudocapsule production to prepare autogenous toxoid-bacterin, isolated *S aureus* were cultured in brain-heart infusion broth which was added to 10% (vol/vol) sterile bovine milk whey in the present experiment. The production of pseudocapsule during preparation of toxoid-bacterin was not determinated in this study. However, pseudocapsule might be contained in the preparation of autogenous toxoid-bacterin in this study and this possibility was supported by the results of preventing the occurrence of the new infection and reducing the severity of the infection by increasing antibody titers in the toxoid-bacterin injected group.

In the present experiment, the cure rate of *S aureus* infection was 27% in the toxoid-bacterin injected group. This cure rate was slightly lower than those of antibiotic treatment[28], but in the non-treated control group the cure rate was only 5%. However, antibiotic therapy for *S aureus* mastitis during lactation can cause milk residue problems. In this reason, autogenous toxoid-bacterin treatment for *S aureus* subclinical mastitis during lactation as tested in the present experiment can be substitute for the antibiotic therapy without considering the problem of antibiotic residues in milk.

Mean *S aureus* CFU/ml in individual milk samples of toxoid-bacterin injected group was decreased from 2 weeks after first injection and remained significantly lower than that of preinjection state from week 7 ($p < 0.05$). These results reflected that some *S aureus* infected quarters were cured. In control group, however, mean *S aureus* CFU/ml was remained higher than that of preinjection state from week 8. These results indicated that autogenous toxoid-bacterin injection was effective in elimination of existing *S aureus* infection to some extent. New intramammary infection with *S aureus* were not detected in the toxoid-bacterin injected group but were detected in 3 quarters in control group. These results may be attributable to increasing opsonization and phagocytosis by PMN resulted from increased specific IgG antibodies against *S aureus* in serum and milk[8,29].

In the toxoid-bacterin injected group, significant decreased changes of mean SCC in milk samples from previously infected quarters with *S aureus* was detected from week 7 to week 10 ($p < 0.05$). These results suggested that the toxoid-bacterin treatment could reduce the severity of infection. Anderson[30] reported that risk of higher milk SCC might be occurred due to enhancing immunity after *S aureus* vaccination. But, in the present study, any similar risk was not occurred in the toxoid-bacterin injected group.

α-toxin is a product of *S aureus* and is believed to be responsible for the gangrenous form of *S aureus* mastitis[30]. Specific antibodies against α-toxin is effective on reducing *S aureus* adherence to mammary epithelial cells and epithelial cell damage[31]. In the present experiment, specific IgG antibody titer against α-toxin was increased in serum and milk of the toxoid-bacterin injected group. These increased antibodies against α-toxin can minimize tissue damages by neutralizing α-toxin produced by certain infected *S aureus* and can reduce *S aureus* adherence to mammary epithelial cells.

In the previous study, subcutaneous vaccination in the area of the supramammary lymph node enhanced local production of antibody in the udder[15]. Some swellings at the injection site however were observed in most of injected cows after first and second injection. This result was similar to that of previous study[16]. However, none of toxoid-bacterin injected cows showed signs of pain when the swelling were palpated. Systemic adverse reactions like reduced appetite, short time lethargy after *S aureus* vaccination were also reported in the previous study[16], otherwise these adverse reactions were not occurred in the present experiment.

The results of the present study showed that autogenous toxoid-bacterin treatment for subclinical *S aureus* mastitis in lactating cows could be an alternative management method to increase the cure rate of the infections, reduce the severity of the infections and prevent the occurrence of the new infections in highly *S aureus* prevalent dairy farm without remarkable adverse effects.

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