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The effect of liposomal cephalexin on the dry period treatment of bovine *Staphylococcus aureus* mastitis

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젖소의 *Staphylococcus aureus*성 유방염에 대한 liposomal cephalexin의 건유기 치료효과

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초록 : Stable plurilamellar vesicle(SPLV) 제작법을 이용한 liposomal cephalexin으로 젖소의 *Staphylococcus aureus*성 유방염에 대해 건유기 치료를 시도하였다. SPLV의 모양과 크기를 transmission electron microscopy로 관찰하였으며 SPLV의 포획율 및 안정성은 high performance liquid chromatography 및 liquid scintillation counting으로 조사한 바 SPLV의 크기는 대개 직경 0.1~4.0μm였고, cephalexin 포획율은 25.8±3.1%(mean±SE)였으며 4℃ 보관시 0, 24, 48, 72 및 96 시간후의 유리농도백분율은 각각 1.1, 3.9, 6.5, 7.5 및 8.4%였다.

*S aureus*에 감염된 45분방에 대하여 15분방씩 3군으로 나누어 각 분방당 250mg의 liposomal cephalexin(LC군), 250mg free cephalexin(FC)군 및 blank liposome을 첨가한 250mg cephalexin(BLC)군을 건유시 분방내 주입한 후 분만당일에 균분리 및 동정을 실시한 바 LC군, FC군 및 BLC군에서 각각 1, 8 및 7분방에서 상기와 동일한 균이 분리되어 LC군이 타군에 비해 유의한 치료효과를 나타내었다.

Key words : Bovine mastitis, *Staphylococcus aureus*, liposome, stable plurilamellar vesicle.

Introduction

Staphylococcus aureus can survive after being phagocytosed by leukocytes, and the survival seems to be related to the virulence of the organism.[1] Intramammary antimicrobial treatment of chronic bovine mastitis due to *S aureus* during lactation rarely succeeds in a bacteriological cure.[2] The essential feature of chronic mastitis is the survival of the organism in neutrophils and macrophages in the mammary glands.[3,4] The intracellular location of *S aureus* within mammary phagocytes provides protection from many antimicrobial agents effective *in vitro*, particularly the water soluble antibiotics.[5] It was therefore presumed that the administration of an antibiotic in liposomes may promote killing of *S aureus* by delivering the antibiotic to the intracellular site.

Liposomes are closed, unilamellar or multilamellar phospholipid vesicles, having in recent years been investigated for their potential as a drug carrier.[6] Numerous methods for preparation of various types of liposomes have been described[7] since the standard me-

thod for producing multilamellar vesicle(MLV) was developed by Bangham et al.[8] Various chemotherapeutic agents have been incorporated into liposomes, which have been used in *in vitro* and *in vivo* studies to deliver the bioactive agents.[9~13] Because liposomes are readily phagocytosed by macrophages and neutrophils, administration of liposomally-entrapped drug provides a means of direct delivery of antimicrobial drugs which penetrate into cells poorly.[14]

Recent studies have shown that intracellular killing of *S aureus* previously engulfed by mouse peritoneal macrophage,[15] *in vitro* cultured canine monocytes,[9] and bovine phagocytic cells[16] was enhanced by treating with liposomally-entrapped aminoglycoside antibiotics, but free antibiotics had little or no effect on intracellular *S aureus*. No studies have been reported about the dry period treatment of bovine mastitis with liposomally-entrapped drugs. MacLeod and Prescott[17] reported that liposomally-entrapped gentamicin did not significantly enhance the intracellular killing of *S aureus* by *in vitro* cultured bovine mammary macrophages compared with free gentamicin. They also reported that treatment of experimentally induced *S aureus* mastitis in five lactating Holstein cows(20 quarters) failed to show a significant difference in bacterial counts when treatment with liposomally-entrapped gentamicin was compared to that with free gentamicin or blank liposomes plus free gentamicin. Anderson and Kirby[18] found that liposomally-entrapped cloxacillin had not enhanced intracellular killing of *S aureus* in an experimental mouse mastitis model. They suggested that both low metabolic activity of intracellular *S aureus* and low intraphagolysosomal pH made it inappropriate to use liposomes for intracellular delivery of cloxacillin.

This experiment was carried out to determine the effect of liposomal cephalexin on the dry period treatment of bovine mastitis due to *S aureus* using a new liposome carrier called a stable plurilamellar vesicle SPLV[19].

Materials and Methods

Animals : Twenty seven Holstein cow were used on the farm. Intramammary infusions were given into forty five quarters infected naturally by *S. aureus*.

Reagents : Egg phosphatidylcholine, HEPES buffer, Triton X-100, [^{14}C] sucrose, and Arsenazo II dye were purchased from Sigma Chemical Co.(St. Louis). Diethyl ether was purchased from Merck & Co., Inc. and cephalexin from Young-Jin Pharma., Co.(Seoul).

Preparation and characterization of stable plurilamellar vesicles(SPLVs)

1) **Preparation of SPLVs** : SPLVs were composed of egg phosphatidylcholine(EPC). EPC(266μmol in chloroform) was added to a 250ml pear-shaped flask and evaporated to dryness with the aid of a rotary evaporator at 40℃. The lipid was redissolved in 10ml diethyl ether. One ml(10mg) cephalexin solution dissolved in aqueous phase(typically HEPES buffer consisting of 72.5mM KCl, 72.5mM NaCl, and 10mM HEPES, pH 7.4) was added into the lipid-ether mixture. The biphasic solutions were swirled for 30 seconds and then placed in a bath sonicator(model Branson 2200 : Branson Ultrasonics Corp.). A stream of nitrogen was passed into the flask to facilitate evaporation of ether during sonication. Once a paste of SPLVs was formed at the bottom of the flask and no ether could be detected by smell, the SPLVs were dispersed into 20ml of HEPES buffer with the aid of a mechanical vortex mixer. The SPLV suspension was centrifuged at 10,000g for 10min, and the unentrapped cephalexin was decanted. The pellet was resuspended in 20ml of HEPES buffer, and the centrifugation step was repeated. This process of washing was done four times. The final pellet of SPLVs was resuspended in HEPES buffer.

2) **Determination of percentage entrappment** : Cephalexin concentration within SPLVs was determined by high performance liquid chromatography (HPLC) after rupturing the SPLVs with Triton X-100.

3) **Examination of liposome stability** : SPLV-entrapped cephalexin suspension was stored at 4℃ and the supernatants were taken after the suspension was centrifuged at 10,000g at intervals of 0, 24, 48, 72, and 96 hours. The concentrations of cephalexin leaked into the supernatants were examined by HPLC and the percentage release was calculated.

Arsenazo III dye(3 mM) incorporated into SPLVs was suspended in HEPES buffer with 500mM $CaCl_2$ in screw-cap tube and stored at 4℃. The change of

color was examined with the naked eye for 1 month as compared with the color of 3 mM Arsenazo III dye or the dye bound to calcium.

SPLVs containing [^{14}C]-labeled sucrose in iso-osmotic sucrose solution were prepared and stored in 200, 320, and 500 mOsM sucrose solutions at 4℃, respectively. The supernatants were taken after centrifuging at intervals of 0, 24, 48, 72, and 96 hours. Percentage release of radio-labeled sucrose was determined by liquid scintillation counting(model Peckard, Tricard 3000C, United Technologies).

4) Electron microscopy : Shape and size distribution of SPLVs were determined from transmission electron micrographs. SPLVs were negatively stained with 2% phosphotungstic acid(PTA) on 200-mesh carbon coated grids(JEOL Inc., Japan) and examined in a Hitachi 800(Japan) transmission electron microscope (TEM) operated at 100kV. The diameters of SPLVs on the negatively stained electron micrographs were measured by image analyzer(model Q520, Cambridge).

5) Analytical conditions of high performance liquid chromatography : The chromatograph used was Waters Associates consisted of a Model 510 pump, U6K injector and Model 441 detector at 254 nm with 0.02 absorbance units full scale(AUFS). Micro-Bondapak C_{18} chromatographic column was used and the mobile phase was acetonitrile/water/acetic acid(15+85+0.1, v/v/v, LC grade), which pumped at a flow rate of 1.0ml/min at room temperature. Standard cephalexin was USP Reference Standard (U.S. Pharmacopeial convention, Inc., Rockville, MD) and cephalexin which had been used to incorporate into SPLVs was obtained from Young-Jin Pharma., Co.

Accurately 20mg of cephalexin standard was weighed into a 10ml volumetric flask. Acetonitrile was added to dissolve the substance and then the flask was diluted with distilled water. SPLV-entrapped cephalexin suspension was filtered by use of syringe filter(Acrodisc, LC 13 PVDF, Prod. No. 4455, Gelman Science) after the vesicle membrane was lysed with Triton X-100.

The amount of cephalexin intrapped within SPLVs was calculated as follows :

$$\text{Cephalexin potency(mg/g)} = \text{standard amount(mg/g)} \times \frac{\text{sample peak height or area}}{\text{standard peak height or area}} \times \text{dilution factor} \times \frac{1}{\text{sample weight(g)}}$$

The effect of liposomal cephalexin on the dry period treatment of bovine *Staphylococcus aureus* mastitis : Foremilk quarter samples of the first 15~20ml were asceptically collected twice daily for three days before cow were dried off. The milk samples were examined for infection due to *S aureus*. Somatic cell counts(SCC) of each quarter were determined using a model Fossomatic 90, Somatic Cell Counter. Quarters were classified as infected by *S aureus* when *S aureus* isolated from foremilk for three days was over 100 CFU/ml. Forty five *S aureus*-infected quarters were selected and divided equally into 3 groups at random and each group was composed of fifteen quarters. Infected quarters were infused intramammarily with either 30ml liposomal cephalexin(SPLV-entrapped cephalexin plus free cephalexin, total cephalexin concentration 250mg), 250mg cephalexin with 30mℓ blank SPLV suspesion, or 250mg cephalexin in 30mℓ HEPES buffer as the dry cow treatment.

A treatment was considered effective when no *S. aureus* was isolated from the foremilk on the first day of parturition and when SCC returned to that of noninfected quarters.

Statistical analysis : Chi-square distribution test was used to evaluate a difference among treatment groups on the number of *S aureus*-infected quarters at the 0.05 level of significance.[20] The effect of each treatment group on SCC level was determined by analysis of variance(ANOVA) and significant diferences among groups were examined by Tukey's test.[21]

Results

Preparation and characterization of SPLVs : The mean entrapping efficiency of three different SPLV preparations was 25.8± 3.1% (mean± SE). The percentage release of SPLV-entrapped cephalexin with the passage of time three different SPLV preparations stored at 4℃ in HEPES buffer is shown Fig 1.

The mean percentage release of cephalexin was 1.1,

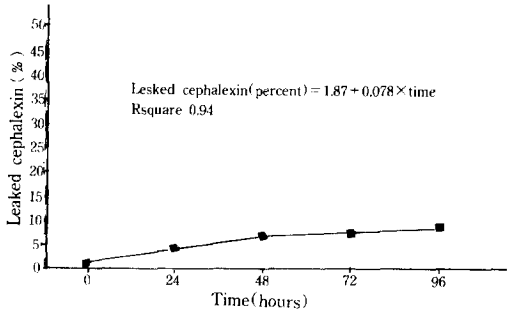

Fig 1. Percentage release of cephalexin from SPLVs stored under 4℃ in HEPES buffer, pH 7.4.

Fig 2. Negatively-stained electron micrograph of SPLV showing 'multilamellar' structures in clumps. Bar = 1μm.

3.9, 6.5, 7.5 and 8.4% at intervals of 0, 24, 48, 72 and 96 hours, respectively. The regression equation was percentage release of cephalexin = 1.87 + 0.076×time with square 0.94.

Negatively-stained electron micrographs(Fig 2) showed that SPLVs had 'multilamellar' structure in clumps with evenly distributed aqueous compartments.

Fig 3 shows the results of three different determinations of the mean diameter of SPLVs. SPLVs ranged from 0.1~4.0μm in diameter.

The color of SPLV-entrapped Arsenazo III dye solutions which was suspended in HEPES buffer with 500 mM $CaCl_2$ was unchanged and the supernatants was transparent after 1 month under storage conditions.

When SPLVs containing [^{14}C]-labeled sucrose in iso-osmotic sucrose solution were stored in 200, 320 and 500 mOsM sucrose solutions at 4℃, respectively, the stability of SPLVs stored in 320 mOsM sucrose

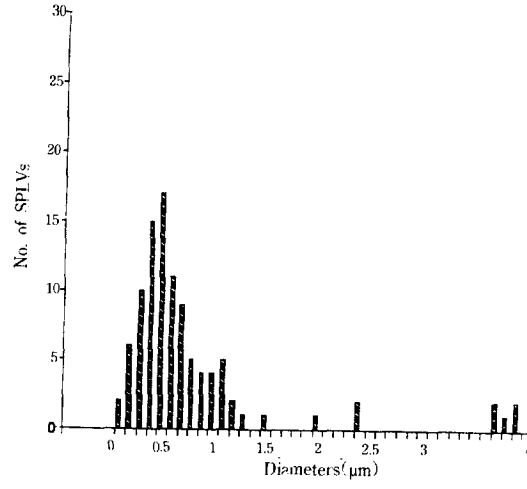

Fig 3. Size distribution of SPLVs obtained by image analysis.

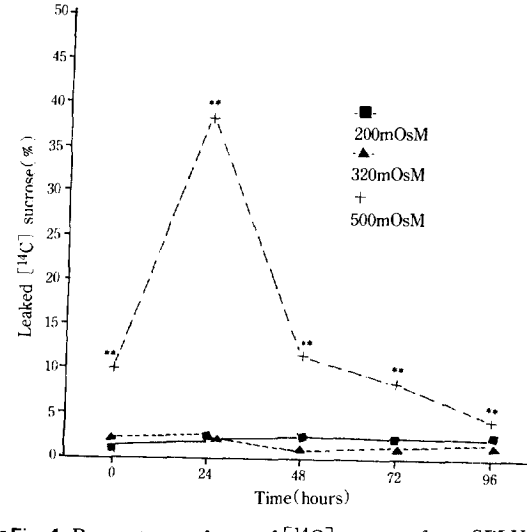

Fig 4. Percentage release of [^{14}C] sucrose from SPLVs stored under 200, 320, or 500 mOsM sucrose solution at 4℃, pH 7.4. ** : Significantly different from the other groups at the same time($p<0.01$).

solution was significantly higher than that of SPLVs stored in 200 and 500 mOsM sucrose solution(Fig 4).

The effect of liposomal cephalexin on the dry period treatment of bovine *Staphylococcus aureus* mastitis : Treatment with 250mg liposomal cephalexin, 250 mg cephalexin with blank SPLV and 250mg cephalexin resulted in 14(93%), 8(53%) and 7(46%) quarters with a bacteriological cure on the first day of parturition in each treatment group, respectively (Fig 5) and

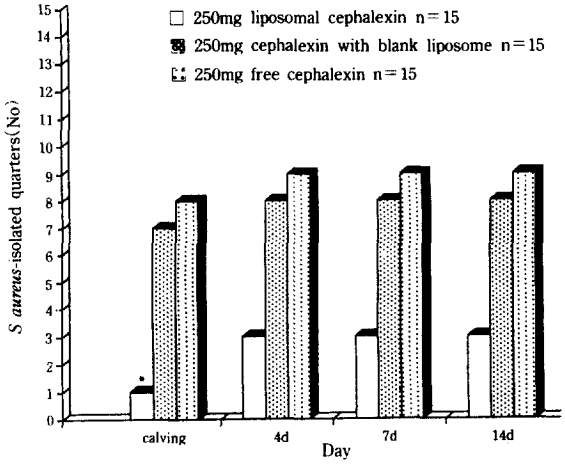

Fig 5. The effect of liposomal cephalexin on the dry period treatment on calving. *S aureus*-infected quarters were dried off with one intramammary infusion of 250mg liposomal cephalexin(15 quarters), 250mg free cephalexin(15 quarters), or 250mg cephalexin with blank liposome(15 quarters). * : Significantly different from the other groups on the same day($p<0.05$).

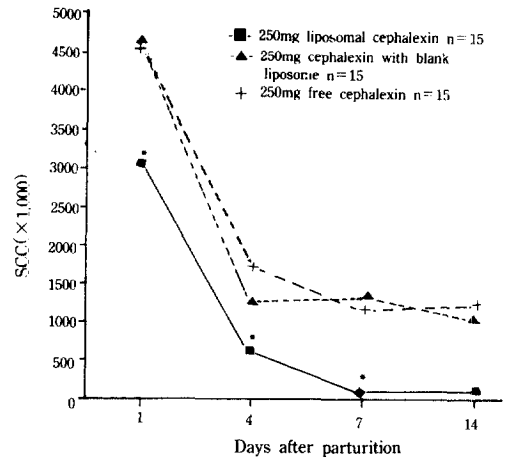

Fig 6. Mean Somatic cell count of each group with the passage of day following parturition after *S aureus*-infected quarters were dried off with one intramammary infusion of 250mg liposomal cephalexin (15 quarters), 250mg free cephalexin(15 quarters), or 250mg cephalexin with blank liposome(15 quarters). * : Significantly different from the other groups on the same day($p<0.05$).

SCC which nearly returned to non-infected quarter levels at fourteen days after parturition(Fig 6). The liposomal cephalexin group was significantly different from the other treatment groups in bacteriological cure rate and SCC($p<0.05$).

Discussion

Bovine mastitis inflicts large economic loss on the dairy industry with a decrease in milk yield, milk loss during and after treatment and increases of culling and veterinary services. Especially intramammary antimicrobial treatment of chronic bovine mastitis due to *S aureus* as an intraalveolar cell invader rarely results in a bacteriological cure.[2]

Generally liposomes have potential as a delivery vehicle of antibiotics because : (1) A specific organ or cell can be a target of drug ; (2) small but effective amounts of drugs can be delivered into a cell[6,22] ; (3) drugs can be existed longer in the body as liposome membranes can be gradually degraded[22~25] ; (4) the toxicity of drugs can be diminished.[16,26,27] Greatest success has been obtained with liposome-entrapped antibiotics against facultative intracellular bacteria such as Brucella[28], Listeria[29], Salmonella[30] and Mycobacteria.[31] These bacteria can survive and multiply within cells of the reticuloendothelial system. Previous studies have shown that free aminoglycosides cannot gain access to the cytosol of macrophages but intracellular killing of *S aureus* can be achieved with aminoglycosides containing liposomes suggesting that liposome delivers the drugs into the cell thus enhancing killing.[9,15,16]

Defense mechanisms of the mammary gland can be divided into three areas : nonspecific resistance, humoral immunity, and cell-mediated immunity. Nonspecific resistance of the mammary gland is characterized by the ability of the gland to resist new intramammary infection without the aid of the immune system. This is conferred by anatomic structures bacteriostatic properties of teat-duct keratin, mucous membrane adherence factors, properties of milk lactoferrin, lactoperoxidase and phagocytic cell.[33] Dees and Schultz[16] reported that enhanced bacterial killing by phagocytes treated with liposomally entrapped aminoglycosides might occur by a number of mechanisms. First, amino-

glycosides entrapped inside the liposome may be delivered into phagocytic cell where it can kill intracellular bacteria normally resistant to cellular killing mechanisms. Second, blank liposomes may alter cellular membrane permeability so that free antibiotic, normally excluded from cell, can enter. Third, enhanced intraphagocytic killing of bacteria might result from nonspecific stimulation of natural killing mechanisms after exposure to SPLVs with no contribution of the aminoglycosides. Fourth, what appears to be enhanced intraphagocytic killing might really be bactericidal activity of free antibiotic in the tissue culture media as opposed to activity of intracellular aminoglycosides. They concluded from their data that enhanced intracellular killing in phagocytic cells after liposomal delivery is directly related to bacterial killing by the antibiotic. MacLeod and Perscott[17] found it to be important to control extracellular growth of *S. aureus* by the use of free gentamicin in all treatments, since liposomally-incorporated gentamicin did not control extracellular growth either in tissue culture medium or in broth. For these reasons we chose liposomal cephalexin composed of SPLV-entrapped cephalexin and free cephalexin in this experiment.

On the other hand, MacLeod and Prescott[17] reported that liposomally-entrapped gentamicin by reverse-phase evaporation preparations did not significantly enhance the intracellular killing of *S aureus* by *in vitro* cultured bovine mammary macrophage compared with free gentamicin. They also reported that treatment of experimentally induced *S aureus* mastitis in five lactating Holstein cows(20 quarters) failed to show a significant difference in bacterial counts when treatment with liposomally-entrapped gentamicin was compared to that with free gentamicin or blank liposomes plus free gentamicin. Anderson and Kirby[18] also found that liposomally-entrapped cloxacillin by dehydration-rehydration preparations did not enhance intracellular killing of *S aureus* in an experimental mouse mastitis model. They suggested that both low metabolic activity of intracellular *S aureus* and low intraphagolysosomal pH made it inappropriate to use liposomes for intracellular delivery of cloxacillin. Therefore these experiments were carried out by use of SPLV preparations and acid stable cephalexin which was known to exhibit the acitivity against pathogenic gram-positive and gram-negative bacteria isolated from bovine udders and neonatal calf diseases.[34]

SPLVs in this study had a mean encapsulation efficiency of 25.8%. The size of SPLVs were examined from negatively-stained electron micrographs and ranged from 0.1~4.0μm in diameter. These results were similar to those reported by Gruner et al.[19] SPLVs were relatively stable with regard to leakage of cephalexin(Fig 1), Arsenazo III dye and [^{14}C] sucrose(Fig 4) at storage temperatures(4℃) or in isotonic solution, at least over 96 hours.

In this study cephalexin associated with SPLVs were found to significantly enhance intracellular killing of *S. aureus* during the dry period($p<0.05$). No difference between free cephalexin and blank SPLV plus free cephalexin was noticed in killing bacteria. Blank SPLVs showed that SPLVs themselves did not enhence bacterial killing activity and this result was similar to those reports by others.[13,17,32]

The use of SPLVs to enhance the therapeutic activity of antibiotics such as cephalexin may afford the possibilities of more effective use of current antibiotics and enhancing the activity of newly developed antibiotics.

Summary

Liposomal cephalexin was used in the dry period treatment of bovine mastitis due to *Staphylococcus aureus*. Liposomes were prepared by stable plurilamellar vesicle(SPLV) process. The shape and size of SPLVs were examined by transmission electron microscopy. The entrapping efficiency and stability of SPLVs were determined by high performance liquid chromatography or liquid scintillation counting. The size of SPLVs ranged from 0.1 to 4.0μm in diameter, with an entrapping efficiency of cephalexin of 25.8%. The formulation of liposomal cephalexin was used for treatment were SPLV-entrapped cephalexin and free cephalexin with total cephalexin concentration of 250mg per quarters. All quarters were infused intramammarily at the end of lactation period by liposomal cephalexin, free cephalexin, or blank liposome with free cephalexin. The number of quarters cured by liposomal cephalexin(14/15

quarters, 93%) was significantly higher than that by free cephalexin(7/15 quarters, 46%), or by blank liposome with free cephalexin(8/15 quarters, 53%)($p<0.05$).

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