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Effects of Biodegradable Cephalexin Microspheres in Dry Cow Mastitis Therapy

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Abstract : Mastitis is the most costly disease results in lost milk production, decreased milk quality, milk discard, early culling of cows, drug costs and labor costs in dairy cow. Until now, a antibiotic administration at the end of lactation, dry cow therapy has been known the most effective and widely used mastitis control method. However, dry cow therapy do not control a new infection in the late dry and prepartum period because dry cow products have only persistent activity in the early dry period. Therefore, this study was conducted to evaluate clinical effect of sustained released biodegradable cephalexin microsphere using PLGA in bovine mastitis control during dry period. PLGA has been approved as controlled drug release system because of non-toxic, non-tissue reactive and bioerodible characteristics. This study revealed that cephalexin microsphere had a spherical shape with characteristic porous structure on the surface. Also, in vitro drug release studies are clearly observed that the release rate of cephalexin from PLGA microsphere decrease during the first 21 days after initial burst and then increase again between 3 and 4 weeks showing pulsatile releasing pattern. On the other hand, as tried in field the new infection rate, cure rate and mean SCC after parturition in cephalexin microsphere infused group were significantly differenced as compared to the control group. Accordingly, a sustained release of cephalexin from a biodegradable microsphere could make dry cow therapy more efficiently by preventing a new infection and decreasing the number of existing infection of mammary gland during dry period.

Key words **:** mastitis, dry cow therapy, PLGA, cephalexin microsphere

Introduction

Mastitis is defined as an important inflammation of the mammary gland tissue and it is the most important infectious disease affecting dairy cattle[9]. These mastitis is known also the most costly disease result in lost milk production, decreased milk quality, milk discard, early culling of cows, drug costs, veterinary expenses and increased labor costs in dairy cow[13,19]. These economic impacts of bovine mastitis have led to the development of various therapeutic strategies to control intramammary infections (IMIs) and their sequelae.

In many mastitis control methods and management practices, dry cow therapy is the use of intramammary antimicrobial therapy immediately after the last milking of lactation1 and this is reported the most effective and widely used mastitis control method[10,25,30,31,35]. Neave *et al*[26] have also suggested that the new infection rate of mastitis is higher over the entire dry period than during lactation and emphasized the importance of mastitis control during dry period. Especially during the first 3 weeks of the dry period it is more six times higher than during preceding lactation[12,29,36].

But dry cow therapy do not control a new infection in the late dry and prepartum period36 because dry cow products have only persistent activity in the early dry period[38]. In this reason, Bodmeier *et al*[3] have studied advanced drug delivery system of long acting antibiotic product by preparation of ceftiotur microparticles for the purpose of administration to cows at drying-off. Considerable attention has been paid to polyesters such as poly(lactide-*co*-glycolide) since 1973 as the carriers of biodegradable and biocompatible drug delivery system[40].

Poly(d,l-lactide-co-glycolide) (PLGA) copolymer among these synthetic polymer have been approved by the FDA as controlled drug release microsphere because of its non-toxic, non-tissue reactive and bioerodible characteristics[14,21,28]. Besides PLGAs have shown to be biocompatible and they degrade to toxicologically acceptable lactic and glycolic acids that are eventually eliminated from the body[15]. In this experiment, biodegradable PLGA microsphere as a antibiotic delivery device was designed to complement disadvantages of dry cow products.

Consequently, the purpose of this study is to evaluate the clinical effect of sustained released biodegradable PLGA microsphere in bovine mastitis control during dry period. Cephalexin was encapsulated within PLGA microsphere because of its good distributional property throughout udder. The release rate of cephalexin from the prepared microspheres were investigated in vitro and field trial was performed for dry cows.

Materials and Methods

Materials

Poly(d,l-lactide-co-glycolide) (PLGA) with lactide/glycolide ratio of 65/35 was purchased from Sigma chemical company (St. Louis, MO, USA). The manufacturer reported molecular weight range of the polymer was 40,000-75,000 (weight average molecular weight). Cephalexin (Sigma®) was used in

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this experiment. Dichloromethane (CH_2Cl_2) was supplied by Junsei chemical Co., Ltd. and polyvinyl alcohol (PVA) (MW 30,000-70,000) was purchased from Sigma chemical Co.(USA).

Methods

Preparation of microspheres. PLGA microspheres were prepared using a water-in-oil-in water (W/O/W) solvent evaporation technique essentially according to Sah *et al*[32]. Briefly, 2.5 g of PLGA was dissolved in dichloromethane (DCM, 50 ml) and 200 mg of cephalexin was dissolved in DW (2 ml). The cephalexin solution was added to the PLGA solution and the mixture was emulsified at 15,000 rpm for 5 min with an homogenizer (IKA-T-25 BASIC, IKA LABORTECHNIK D2M-M). Subsequently, the resulting primary emulsion was added to 500 ml of an aqueous PVA solution (1% PVA w/v) and emulsified as the same method of the first emulsion step. The second emulsion was poured in 2 L of DW and stirred for 8hr at room temperature. Non-encapsulated cephalexin was removed by washing the microspheres three times with DW. After 8 hr, the microspheres were collected and freeze-dried (SAMWON, SFDSM12).

Size and morphology of microspheres. The surface morphology, shape and size of cephalexin microspheres were examined by SEM (scanning electron microscopy, JSM-5410LV, JEOL, Japan). Samples for SEM were mounted on metal stubs and coated with gold.

Determination of encapsulation efficiency (EEF). The amount of cephalexin in the microspheres was determined by dispersing 10 mg of the microspheres in 1.0 ml of 0.1 M NaCl/ 5% SDS which dissolved the polymer but not the drug. The samples were centrifuged at 3,000 rpm for 10 min and the cephalexin in the supernatant was quantified using an U-1100 spectrophotometer (HITACHI, Japan) at 260 nm. The EEF was determined as the ratio of the amount analyzed to the initial amount of cephalexin added during preparation.

In vitro drug-release studies. Thirty milligram of microspheres were dispersed in 5 ml phosphate buffered saline (PBS, pH 7.4) in closed plastic tube in an incubator at 37°C and agitated at 75 rpm. Samples of dissolved cephalexin were removed at specified time intervals (1, 2, 4, 7days, once a week for 4 weeks) for analysis and replaced with a fresh buffer. Absorbance measurement was conducted by using an U-1100 spectrophotometer at 260 nm. In vitro release study was performed independently in triplicate.

Degradable appearances of microspheres. Cephalexin microspheres incubated in phosphate buffered saline at pH 7.4 and 37°C for each different time were dried in incubator (VS-1203P3N, VISION SCIENTIFIC Co., Ltd). Dried microspheres were observed using SEM periodically.

Field trial. Dry cows were assigned to control and experimental group consisting of 10 cows per group. Experimental group was received a intramammary infusion of dry cow antibiotic product (Cepha DC ointment, HANDONG Co., Ltd) containing cephalexin microspheres and control group was infused only dry cow antibiotic product through teat end. All teat was cleaned and sanitized with cotton soaked in 70% alcohol before infusing the quarter to avoid introduction of infection and dipped after drug administration with an antiseptic formulation. All cow's milk was collected and examined at 3 wk, 1 wk before dry period and dry-off day. After that, no treatments were performed until parturition and all evaluation was conducted for each quarters of cows.

Sampling and laboratory exam. All individual quarter milk samples from cephalexin microspheres infused cows and control cows were collected once every 1 week for 1 month after parturition and once after 2 month of parturition. About 0.05 ml of individual quarter milk samples were streaked on 5% bovine blood agar plate for bacteriological culture. Plates were incubated aerobically at 37°C and examined after 24 hr incubation. Somatic cell count (SCC) of individual quarter milk was also examined by Fossomatic 90 (A/S N. Foss Electric, Denmark). Colony morphology and hemolytic pattern were observed and bacterial isolation over 3 species had regard as a contamination. Clinical mastitis was identified on the basis of clinical sign, milk appearances, degree of udder's swelling, isolation of Staphyllococcus aureus and Streptococcus spp. as a primary pathogen, SCC ($>2.5\times10^5$ cell/ml).

Statistical analysis. The differences of mean SCC according to change time between experimental and control group were compared by student's *t*-test. The incidence rate of new infection and the cure rate between cephalexin microspheres infused group and control group were also analyzed.

Results

Size and morphology of microspheres

In this study of the microsphere size, a particle size distribution was ranged variably. The particle size of cephalexin microsphere is summarized in Table 1. The study revealed a maximum size distribution ranging from 50 to 62.5 μm. On the other hand, as shown in Fig. 1, the cephalexin-loaded PLGA microspheres had a spherical shape and the surface of the microsphere appeared to be smooth. But on close exam-

Table 1. Size distribution of cephalexin-loaded microspheres using PLGA (65:35)

Size of microsphere	Percent	Distribution*
< 12.5 μm	0%	0
12.5~25.0 μm	0%	0
25.0~37.5 μm	16%	16
37.5~50 μm	24%	24
50~62.5 μm	44%	44
> 62.5 μm	16%	16

* : The numbers of when counted 100 microspheres

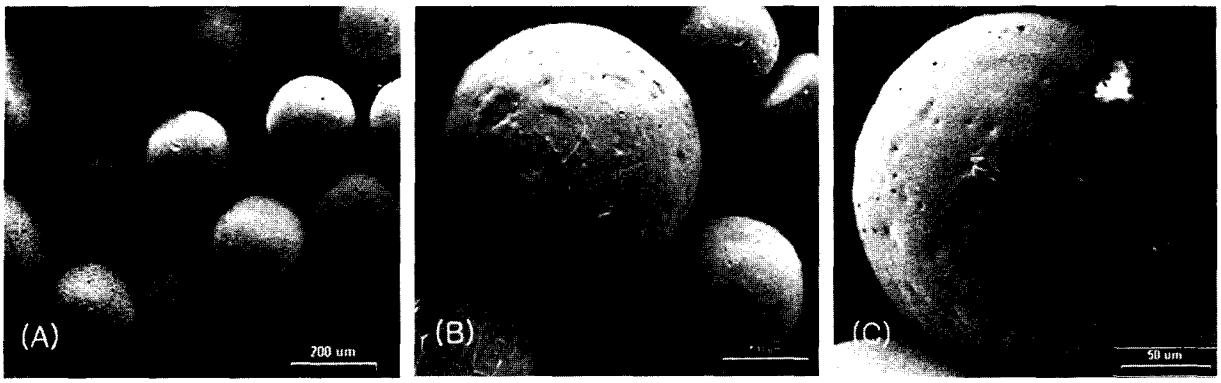

Fig 1. Scanning electron micrographs of cephalexin microsphere using PLGA (lactide/glycolide = 65 : 35)

ination, SEM photographs revealed that microspheres had a characteristic porous structure on the surface.

The productive rate of microsphere and drug encapsulation efficiency

The productive rate of microsphere was 85% (2.3 g) using 200 mg of cephalexin and 2.5 g of PLGA (65 : 35). Also, the encapsulation efficiency and drug content of microsphere were determined and the results are presented in Table 2 for microspheres produced with PLGA. The encapsulation efficiency for PLGA of molecular weight of 40,000 to 75,000 was 68.7%. Drug encapsulation study was performed independently in triplicate.

In vitro drug release studies

The W/O/W type PLGA microsphere showed a significant burst effect with 30.2% of the drug released within the first 24 hr of the test period. Also, it is clearly observed that the release rate of cephalexin from PLGA microsphere decreased during the first 21 days after initial burst and then increased again between 3 and 4 weeks, showing the maximum value 28 days after the degradation starts. Therefore, the cephalexin release profiles were biphasic characterized by on initial cephalexin burst followed by a slowly decreased release of cephalexin. The values obtained for the initial burst and in vitro release study are presented in Table 3 and Fig 2.

Table 2. Study on the cephalexin encapsulation efficiency with PLGA microsphere

Numbers	Absorbance	initial dose	encapsulation dose	encapsulation efficiency
1	0.479			
2	0.382	200 mg	137.3 mg	68.7%
3	0.626			

Table 3. In vitro release study in cephalexin microsphere using PLGA (65 : 35)

Released time	Mean absorbance	Released dose
1day	0.119	0.45 mg
2days	0.054	0.19 mg
4days	0.033	0.11 mg
7days	0.016	0.07 mg
2weeks	0.021	0.08 mg
3weeks	0.018	0.07 mg
4weeks	0.136	0.52 mg

Degradable appearances of microsphere

Fig 3a-Fig 3e shows scanning electron micrographs of PLGA microspheres under hydrolytic degradation in a pH 7.4 buffer at 37°C.

The shape of the microspheres was changed remarkably between 24 and 32 days after the degradation starts. A number of fine pores were observed on the surface of PLGA microsphere 3 days after the degradation starts. On the other hand, PLGA microspheres were corrugated surface with many relatively large pores 16 days after degradation starts and then the microspheres were changed their shape continuously.

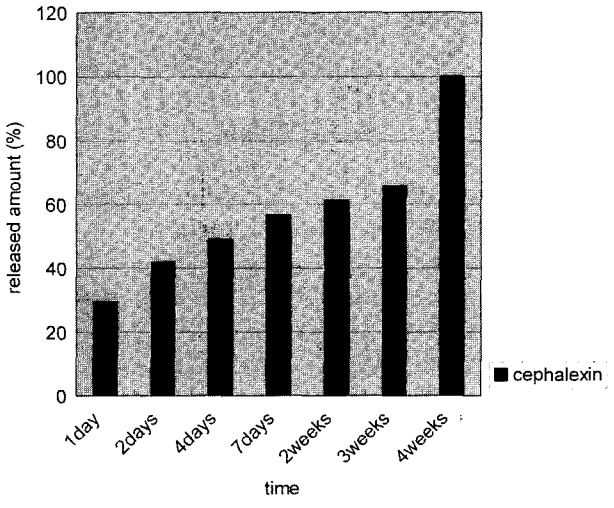

Fig 2. In vitro release of cephalexin microsphere

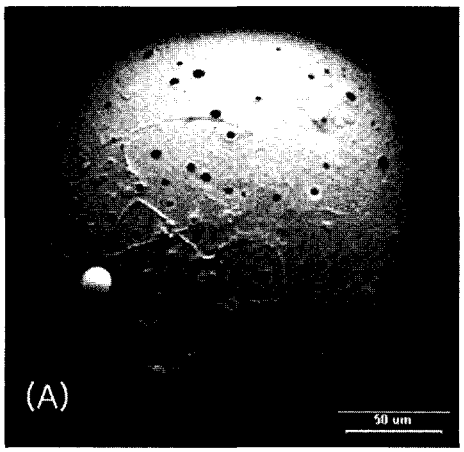

Fig 3a. Scanning electron micrographs of PLGA microsphere after incubation at pH 7.4 and 37°C

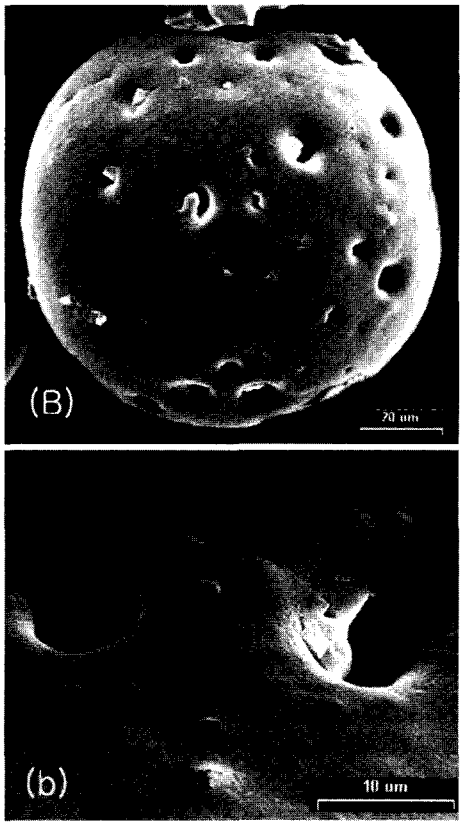

Fig 3b. PLGA (65:35) microsphere surface (B) and pore structure (b) at different degradation stages.

So, after 24 days the PLGA microspheres were observed to develop a highly porous outer appearance. Consequently, 32 days after degradation starts, mass loss from polymer occurred because the particles had lost their structure and degradation products could escape from the matrix. In conclusion, SEM of microspheres after incubation for 1 and 32

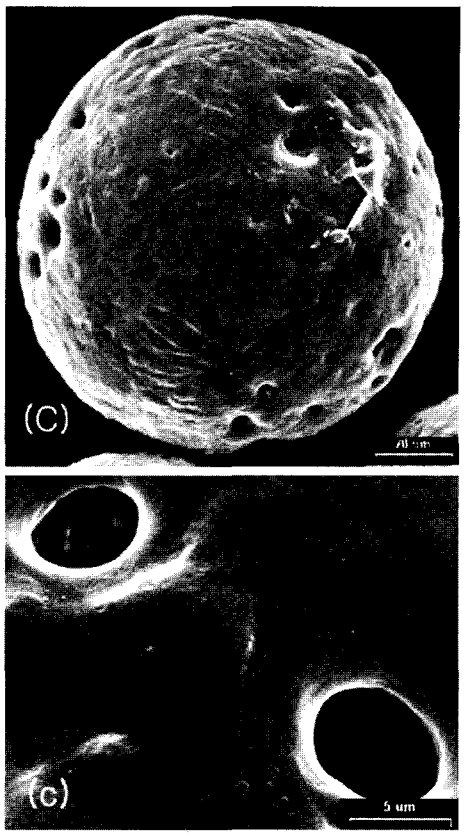

Fig 3c. PLGA (65:35) microsphere surface (C) and pore structure (c) at different degradation stages.

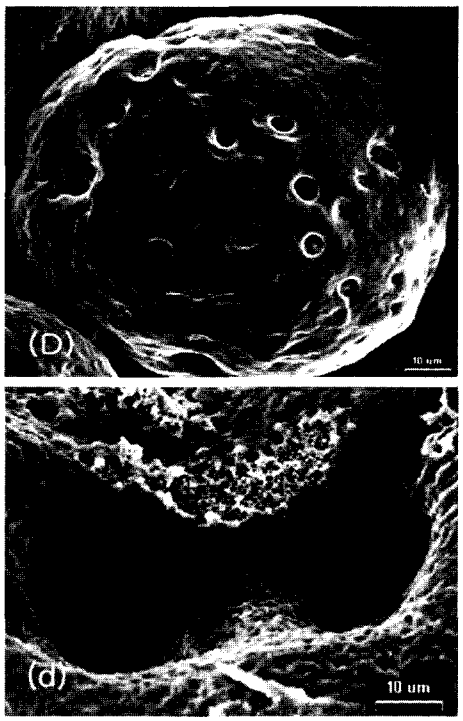

Fig 3d. PLGA (65:35) microsphere surface (D) and pore structure (d) at different degradation stages.

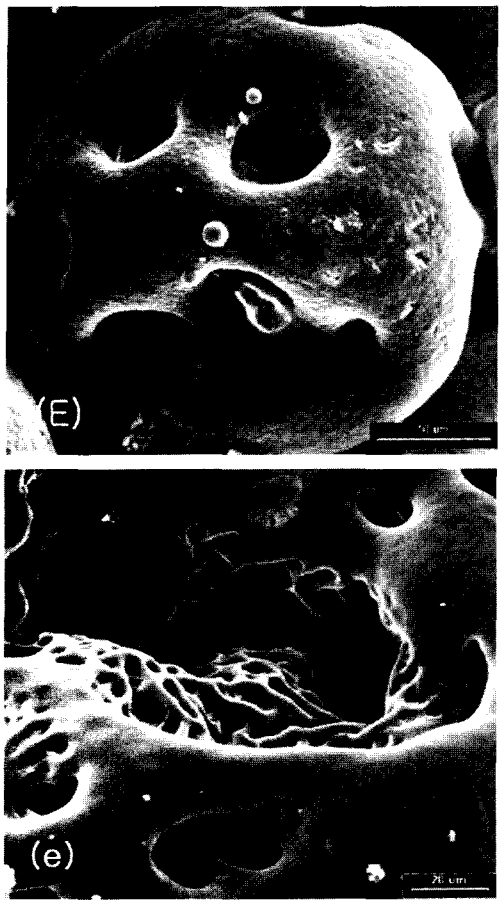

Fig 3e. PLGA (65:35) microsphere surface (E) and pore structure (e) at different degradation stages.

days showed particle coalescence and the deveplopment of a porous matrix.

Analysis of field trial

The changes of intramammary infection states. The changes of intramammary infection state between cephalexin microsphere infused group and control group were presented in Table 4.

In experimental group, 73% of infected quarters was cured during dry period as compared to 50% in the control group. New intramammary infections were detected in 2 (8%) of 25 quarters in the experimental group while it was detected in 4 (13%) of quarters in the control group. Therefore, as shown in cure rate and new infection rate, the cephalexin microsphere infused group had a significant difference as compared to the control group.

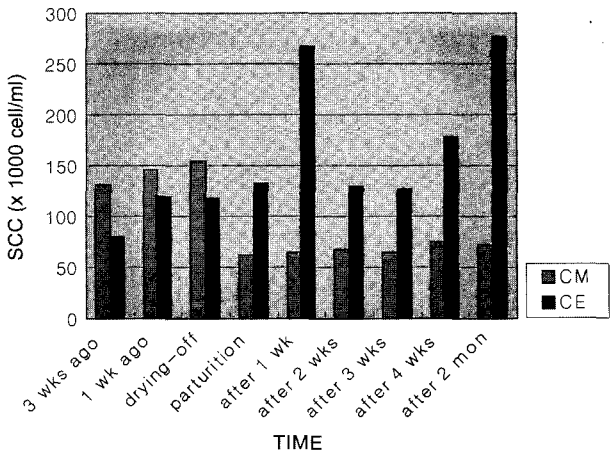

Fig 4. The changes of mean SCC in individual quarter milk
*CM : cephalexin microsphere treatment
CE : dry cow product (cepha DC) treatment

The changes of mean SCC in individual quarters milk. In cephalexin microsphere infused group, mean SCC was decreased significantly after parturition as compared to dry-off day ($P < 0.05$). In contrast, mean SCC was recorded variably and was showed increased pattern after parturition in control group. The changes of mean SCC according to time were presented in Fig 4.

Discussion

Antibiotic therapy at drying off is the most effective and widely used mastitis control method during the non-lactating period[11]. The advantages of dry cow therapy are well-known and have been reviewed[10,25,30,31,35]. But despite complete dry cow therapy some cows calve with infected quarters and some with clinical matitis. The dry period lasts for 60 days. One of the important causes of this failure is that most antibiotic formulations do not cover the entire dry period because they only persist during the early dry period[37]. Smith *et al.*[39] reported that dry cow therapy reduced new streptococcal infection rate in the first 25% but not in the remainder of the dry period, and that coliform infection rate was not reduced at all. Todhunter *et al*[42] showed that mammary secretion collected from dry treated quarters at 7 days

Table 4. The changes of intramammary infections (IMIs)

treatment	No. of cows	No. of quarters investigated	No. of preexisting IMI	No. of cured quarters	No. of new infection	No. of persisting infection
CM	10	40	15	11 (73%)	2 (8%)	4 (27%)
CE	10	40	10	5 (50%)	4 (13%)	5 (50%)

* CM : cephalexin microsphere treatment CE : dry cow product (cepha DC) treatment

after drying off gave no evidence of inhibition of streptococcal growth due to antibiotics. This raise concern about the persistence of dry cow products presently available.

In this experiment, cephalexin microspheres using PLGA by solvent evaporation technique showed a pulsatile releasing pattern increased again between 3 and 4 weeks after initial burst showing a persistent effect of drug release over 4 weeks. These findings were similar to those of studies on PLGA microspheres encapsulating recombinant human growth hormone[8]. Also, these effects are thought to make antibiotics therapy for dry cows more efficiently. On the other hand, the initial burst effect is attributed to release of the drug from the surface regions of the microspheres followed by a slower release of drug as water penetrated the polymer matrix[4,27]. The changes of surface morphology of PLGA microspheres observed by SEM support these findings. An expansion in the pore sizes was noticed following the release. Most of all, it could be known in this experiment that the release rate of cephalexin from PLGA microspheres showed a maximum at 28 days after incubation and that the shape of the microspheres changed remarkably between 24 and 32 days after the degradation starts. Therefore, it is thought that drugs are released from PLGA matrices accompanied with the removal of the degraded segments of the matrices.

The drug release from PLGA microspheres depends on various factors including the polymer composition, polymer molecular weight, drug loading particle size, porosity and the microstructure of microspheres[6,16,18]. The parameters which affect the preparation of microspheres are also variable. These factors which affect a drug release and the preparation of microspheres were not investigated in this study. However, in vitro drug release study showed a relationship with the change of porous structure of microspheres and a drug release characteristics indirectly.

The biocompatibility of PLGA used in this experiment has been demonstrated in many biological sites[22]. Except antibiotic encapsulation using in this study various therapeutic agents such as anti-inflammatory drugs, anticancer drug, steroids, peptides and proteins have been incorporated in the lactide/glycolide copolymer system[2,7,17,20,23,27,33,44].

In the present study, antibiotic encapsulated PLGA microspheres were applied to the bovine mastitis control method during dry period. Selection of a suitable dry period treatment should take into account that Gram negative infections are not common at that time because of the high concentration of lactoferrin in the dry secretion[24]. Accordingly, cephalexin was selected because of a potent antibiotic effect against *Streptococcus* spp., β-lactamase producing *Staphylococcus aureus* and a good distributional property throughout udder by intramammary route[13,41].

As tried in field, 73% of the infected quarters was cured during dry period in experimental group while only 50% in control group. Moreover, a new infection rate in cephalexin microsphere infused group was less than in control group. In addition to these studies the persistent infection rate was also only 27% in experimental group as compared to 50% in control group. These results may be attributable to increasing inhibition of bacterial growth by long acting drug effect from cephalexin microspheres. On the other hand, in the cephalexin microsphere infused group, the significant decreased changes of mean SCC in individual quarter milk were detected after parturition as compared to the drying-off day ($P < 0.05$). In contrast, mean SCC was highly increased after 1 week of parturition in the control group ($P < 0.05$). These findings suggest that the intramammary infusion of cephalexin microspheres could reduce the intramammary infection more efficiently.

In the present study, cephalexin microspheres were infused through intramammary route with antibiotic ointment for dry cows and after administration no side effect were observed until parturition. Therefore, these results in this studies revealed that cephalexin microspheres enhanced elimination of infections present at drying-off and reduced the new infection rate during the dry period. However, the limitations of the mastitis control scheme based on teat dipping and dry cow therapy still exist. Further study for environment pathogen and proper control of the environment of the cows is needed. Besides, disadvantages of microsphere including relatively complicated manufacturing procedures to produce a sterile, stable and reproducible product and expensive production costs are needed to overcome to practice in large animal clinical medicine.

Conclusion

Cephalexin microsphere in this studies had a spherical shape with a porous surface and showed the maximum size distribution ranging from 50 μm to 62.5 μm. The productive rate of microspheres was 85% (2.3 g) using 200 mg of cephalexin and 2.5 g of PLGA (65 : 35) and this study showed a 68.7% of encapsulation efficiency. Cephalexin microsphere showed a pulsatile releasing pattern increased again between 3 and 4 weeks after initial burst showing persistent effect of drug release over 4 weeks. In cephalexin microsphere infused group, 73% of infected quarters were cured during dry period as compared to 50% in the control group. Also, a new infection rate in experimental group was lower than in the control group. In experimental group, mean SCC in individual quarter milk was significantly reduced after parturition as compared to the drying-off ($P < 0.05$). In contrast, mean SCC was highly increased after 1 week of parturition in the control group ($P < 0.05$). These results in this studies revealed that cephalexin microspheres enhanced elimination of infection present at drying-off and reduced the new infection rate during the dry period.

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젖소의 건유기 유방염 치료에 있어서 생분해 cephalexin microspheres의 효과

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요 약 : 유방염은 젖소에 있어서 유량의 감소, 유질 저하로 인한 우유 폐기문제, 젖소의 도태, 치료비 및 노동력의 부담등을 초래하는 가장 경제적 손실이 큰 질환이다. 현재까지 비유기 최종 착유 후에 항생제를 주입하는 건유기 치료법이 유방염 관리에 가장 효과적이며 폭넓게 쓰이고 있는 방법으로 알려져 있다. 하지만, 건유기 치료는 건유기 주입 항생제 제품들이 건유기 초기에만 지속적인 활성을 지니기 때문에 건유기 말기와 분만 전기에 있어서의 신규 감염은 방어할 수 없다. 따라서 본 연구는 건유기 유방염 관리를 위해 PLGA를 이용한 서방형 생분해 cephalexin microsphere를 제조하여 이의 임상적 효능을 평가하고자 하였다. PLGA는 무독성의 조직 반응을 일으키지 않는 특성으로 인해 약물 방출 조절 체계의 일환으로 인정을 받아왔다. 본 연구에서 cephalexin microsphere는 표면에 특징적인 구멍을 가지고 있는 구형 모양으로 확인이 되었으며 약물 방출 시험에서 초기 과다 방출 이후로 21일간 약물의 방출이 점차 감소한 뒤 3주와 4주 사이에 2차 방출을 보이는 맥동성 방출 양상이 관찰되었다. 현장 적용 시험에서는 cephalexin microsphere를 주입한 실험군에서 대조군에 비해 분만 후 신규 감염율 및 치료율, 평균 체세포 수의 변화의 측면에서 볼 때 유의성 있는 차이가 있음을 확인하였다. 따라서 생분해 microsphere를 이용한 건유기 치료법은 건유기 동안 신규 감염을 예방하고 기감염을 감소시킴으로써 종전의 건유기 치료를 좀 더 효과적으로 증진시킬 수 있을 것으로 사료된다.

주요어 : 유방염, 건유기 치료, PLGA, cephalexin microsphere