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Application of Silver Ion for Clinical Mastitis in Holstein Cows

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Abstract : Mastitis is an inflammation of the mammary gland that develops as a response to pathogenic microorganisms. Somatic cell count (SCC) and bacteriological analysis are the most accepted tools to determine udder health. This study evaluated the effects of intra-mammary gland infusion of silver ion against clinical mastitis in Holstein cows. Silver ion (20 μg/ml) was infused through the intra-mammary gland in quarters having clinical mastitis and milk was collected to determine SCC, and levels of lactoferrin and bovine serum albumin (BSA). Silver ion infusion decreased udder inflammation, firmness and swelling, and reduced clots, BSA, lactoferrin and SCC in milk. However, milk yield and circulating blood cells remained unchanged. The silver ion-mediated reductions of BSA and SCC indicate reduced inflammation and bacterial activity in silver ion-treated mammary glands in Holsteins with mastitis, which may be exploited in a curative strategy.

Key words **:** Clinical mastitis, somatic cell count, lactoferrin, silver ion, bovine serum albumin.

Introduction

Mastitis is an inflammation of the mammary gland that often develops in response to intramammary bacterial infection (17). The classic symptoms of mastitis are inflammation, swelling of the mammary glands, and increased somatic cell count (SCC) in milk. Mastitis decreases milk production and quality, and increases herd management cost (13).

Many researchers have explored therapeutic effects of various agents against bovine mastitis. Immune modulation of mammary gland by cytokines such as interleukin(IL)-2 and granulocyte colony stimulating facter (G-CSF) (28), β-1, 3-glucan (8,16,20) and ginseng (15) has been reported, as has intramammary infusions of lactoferrin (19), lysostaphin (26) and chitosan (18), ozone treatment (24) and homeopathy (26,32). Despite these varied explorations, mastitis remains a problem to dairy farmers and a treatment challenge for veterinarians.

The disinfection capability of silver has been known for over a century, and silver is used to treat clinical diseases including newborn eye prophylaxis, topical burn wounds, and orthopedic infections (5,6,9), as an antimicrobial materials, in food preservation, and in decontamination of surfaces and gas removal (14,22,25). Several studies have reported *in vitro* experiments using mammalian cells. In one study, silver was shown to exhibit potent antibacterial activity against variety of bacteria while exhibiting low toxicity to mammalian cells (6). In our previous study, silver ion did not harm bovine mammary gland epithelial cells, while negating the harmful effect of *Staphylococcus aureus*-derived α-toxin (28). However, the clinical therapeutic effects of silver ion against mastitis are unclear.

Thus, the objectives of this study were to evaluate the effects of intra-mammary infusion and the possible clinical application of silver ion against clinical mastitis in Holstein cows.

Materials and Methods

Cows and management

Ten quarters of six holstein cows (milk yield 20~40 kg/day) with clinical mastitis were selected on the basis of milk SCC (MSCC, > 2,000 × 10^3 cells/ml) and clinical signs (inflammation; swelling, firmness, redness on udder and clots in milk). Experimental cows were kept on experimental dairy barns at National Institute of Animal Science (NIAS), South Korea and were fed according to the Korean Feeding Standard for energy and protein requirements individually. The cows were housed in a cemented stall bedded with rubber mats and hay. Cows were allowed to exercise outside in a dry lot for 2 hrs. Cows were milked twice a daily at 06:00 and 17:00 with milking machine (Haringbone, Delaval, Sweden).

Evaluation of inflammatory signs

Clinical signs of mastitis were scored to determine the degree of inflammation according to the method of Kai *et al* (18). Scores were assigned on the basis of degree of inflammation, as determined for 3 categories; swelling (0, swelling not

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detected; 1, swelling in part of mammary gland; 2, swelling throughout the mammary gland), firmness of the mammary gland (0, firmness not detected; 1, firmness in part of the mammary gland; 2, firmness throughout the mammary gland), and clots in milk (0, clots not detected; 1, < 0.5 clots/ml of milk; 2, 0.5 to 4 clots/ml of milk; and 3, ≥ 5 clots/ml of milk).

Silver ion treatment

Silver ion was obtained from BioPlus Co. Pohang, South Korea. Silver ion was diluted (20 μg/ml) in pyrogen-free distilled water (Jungwei pharma, South Korea). Twenty milliliters silver ion solution (20 μg/ml) was infused in ten quarters of 6 cows once a day for 3 days. Each quarter of cows was examined for following 7 days after the treatment.

Sample preparation

Blood samples were collected from coccygeal vein using K_3 EDTA tube (5 ml, BD Vacutainer®, USA) for hematological analyses at 0, 3 and 7 days after intra-mammary infusion of silver ion. Hematological values were determined within 2 hrs after sampling. Milk samples were aseptically collected from the infused quarters of each cow at 0, 1, 2, 3, 5 and 7 days after intra-mammary infusion of silver ion.

Analysis of somatic cell count (SCC) and hematology

Somatic cell count was performed with somatic cell counter (Foss 300®, Foss Electric Ltd, Denmark). SCC was transformed into somatic cell score (SCS) using method described by Ahn *et al* (1). Hematological values were determined by using multi-species hematology system (HEMA-VET 850®, CDC Tech, USA).

Assays of bovine serum albumin (BSA) and lactoferrin in Milk

Bovine serum albumin (BSA) in milk levels was assayed using a commercially available kit (Bethyl laboratories, Inc., USA) according to methods described by Bannerman *et al* (3). Briefly, 96-well plates were coated overnight at 4°C using 10 μg/ml of sheep anti-bovine BSA diluted with 0.05 *M* sodium carbonate. The plates were washed 4 times with 0.05% Tween 20 and subsequently blocked with TBS for 1 hrs at room temperature. Plates were washed, and 100 μl of diluted curd sample (1:15,000) was added to each well. Plates were incubated for 1 hrs at room temperature. Sheep-anti-BSA conjugated to horseradich peroxidase (HRP) was diluted (1:60,000) in TBS wash buffer and 100 μl of this solution was added to each well. Plates were incubated for 1 hrs at room temperature and washed as described above, and 100 μl of 3,3-5,5-tetramethylbenzidine substrate solution was added to each well. The reaction was stopped by the addition of 100ul of 2 *M* H_2SO_4 and the absorbance was read at 450 nm on a microplate reader (Spectra Rainbow®, Tecan, Austria). Lactoferrin levels in milk were assayed using a commercially available kit (Bethyl laboratories, Inc., USA).

Statistical analysis

The data were analyzed by one-way ANOVA using Statistical Analysis System SAS 9.13 (SAS Institute Inc., Cary, NC, USA). The data presented reveal as mean ± SD and a difference was judged to be significant at $P < 0.05$.

Results

Inflammatory signs of clinical mastitis are diminished by intra-mammary gland infusion of silver ion

Clinical mastitis is characterized by visual signs of inflammation such as clumpy, watery, bloody, or yellowish milk, and intra-mammary pathogens may be isolated. Inflammatory signs were monitored after intra-mammary gland infusion of silver ion in Holstein cows having clinical mastitis. Clinical signs of mastitis were scored according to the aforementioned degree of inflammation. Different responses were observed in the different cows after the intra-mammary introduction of silver ion (Table 1). In general, however, significant reduction were evident in inflammatory signs such as udder swelling (80%), firmness of mammary gland (100%) and clots in milk (86%) after 7 days. SCC in milk is a recognized indicator of mammary health and milk quality, and the risk of clinical mastitis increases with increasing SCC. Presently, mean SCS in milk from quarters having clinical mastitis was reduced from 9.2 ± 0.89 prior to silver ion therapy to 6.7 ± 0.52 7 days after silver ion intra-mammary infusion (Fig 1).

Silver ion infusion reduces levels of BSA and lactoferrin in milk

A various factors were used to assess proteolytic activities in mastitic milk and mammary tissue during inflammation. We observed the BSA and lactoferrin concentration in milk be acquired from quarter of clinical mastitis after silver ion intra-mammary gland infusion. Mean BSA and lactoferrin concentrations in milk from quarters having clinical mastitis was reduced from 3.32 ± 1.04 mg/ml and 350.4 ± 180.42 mg/ml,

Table 1. The inflammatory signs of clinical mastitis after silver ion infusion through intra-mammary gland (quarters=10)

Infusion time (Days)	0				3				7			
Score	0*	1	2	3	0	1	2	3	0	1	2	3
Swelling	5	3	2	-	8	2	-	-	9	1	-	-
Firmness	5	-	5	-	10	-	-	-	10		-	-
Clots in milk	3	3	2	2	7	2	1	-	9	1	-	-

*Score of clinical signs for each affected mammary gland were assigned for swelling of the mammary gland (0, swelling not detected; 1, swelling in part of mammary gland; 2, swelling throughout the mammary gland), firmness of the mammary gland (0, firmness not detected; 1, firmness in part of the mammary gland; 2, firmness throughout the mammary gland), and clots in milk (0, clots not detected; 1, < 0.5 clots/ml of milk; 2, 0.5 to 4 clots/ml of milk; and 3, ≥ 5 clots/ml of milk).

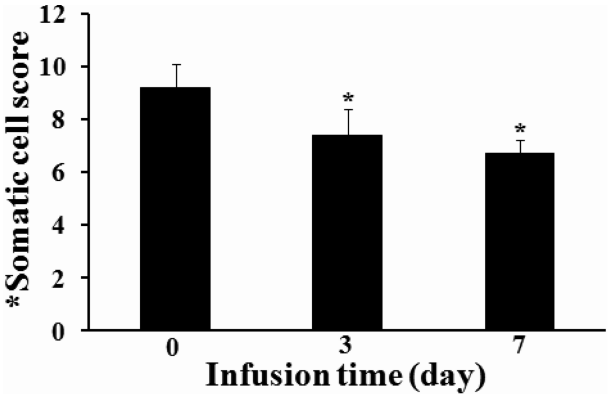

Fig 1. Somatic cell score in milk be acquired from the quarter having clinical mastitis after silver ion (20 μg/ml) infusion. Silver ion was infused through intramammary gland route and milk acquired from cows at 0, 3 and 7 days after silver ion treatment. Somatic cell count was performed with somatic cell counter as described in methods. Bars indicate standard deviation. *P < 0.05. *Somatic cell score = log2(SCC/100,000) + 3.

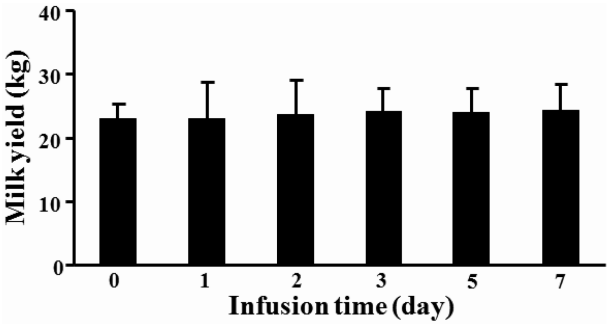

Fig 3. The change of total milk yield be obtained from cows after silver ion infusion through intra-mammary gland. Milk samples were aseptically collected from the infused quarters of each cow at 0, 1, 2, 3, 5 and 7 days after intra-mammary gland infusion of silver ion. Milk yield checked with automatic milking machine as described in methods. Mean (± SD) of milk yields are reported in kilograms. Bars indicate standard deviation.

respectively, prior to silver ion infusion to 0.55 ± 0.56 mg/ml and 142.2 ± 137.25 mg/ml, respectively, at 7 days after silver ion intra-mammary gland infusion (Figs 2A and 2B).

Silver ion infusion does not affect milk yield and circulating blood cells

We also investigated whether milk yield and blood components were changed by the intra-mammary gland infusion of silver ions against clinical mastitis. Daily milk yield of cows under clinical mastitis didn't affected by the intra-mammary gland infusion of silver ions (Fig 3) and circulating blood cell counts also were not affected by the intra-mammary infusion of silver ions (Table 2).

Discussion

Clinical mastitis signs include depression, anorexia, fever or hypothermia, muscle weakness, and inflammation of the mammary gland with an abnormal mammary secretion (23), and severely affected cows become dehydrated, recumbent, and can go into shock. Especially, the diagnosis of acute clinical mastitis was based on the presence of clinical signs such as redness, swelling, hardness and pain in the udder, or the presence of clots in the milk. In this study, the change of clinical signs after intra-mammary gland infusion of silver ion was monitored in Holstein cows with clinical indications of mastitis. Silver ion infusion through the intra-mammary gland significantly reduced inflammatory symptoms including udder swelling, udder firmness, and clots in milk after 7 days. This suggests that silver ion can relieve the inflammation of mammary gland by virtue of its potent inhibitory and bactericidal effects against mastitis-causing pathogens

Subclinical mastitis is associated with increased (SCC) and has several negative consequences. The risk of clinical mastitis increases with increasing SCC (4,32). Somatic cell count (SCC) in milk is a well known indicator reflecting mammary health and milk quality. Excessive amounts of neutrophils, macrophages, lymphocytes, eosinophils and various epithelial

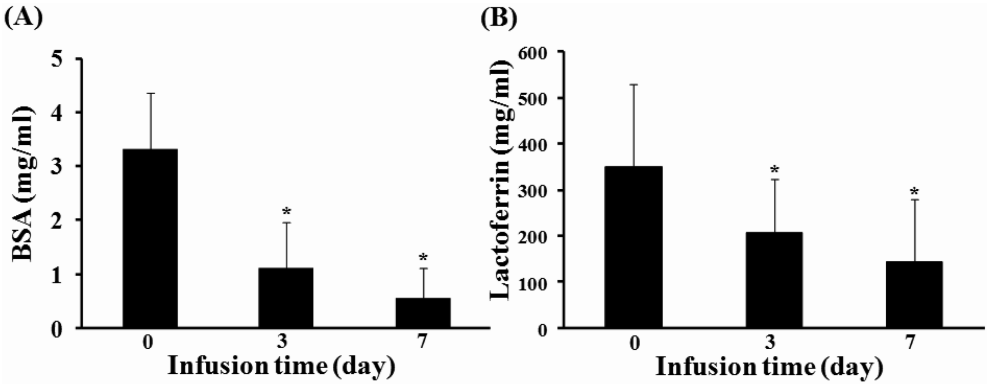

Fig 2. The concentration of bovine serum albumin (BSA) and lactoferrin in milk after silver ion (20 μg/ml) infusion. Silver ion was infused through intra-mammary gland route and milk acquired from cows at 0, 3 and 7 days after silver ion treatment. Bovine serum albumin (A) and lactoferrin (B) concentration in milk were assayed by ELISA. Bars indicate standard deviation. *P < 0.05.

Table 2. The evaluation of blood components after silver ion infusion through intra-mammary gland

Treatment		Infusion time (Days)		
		0	3	7
Silver ion (20 μg/ml) (N=6)	WBC	8.98 ± 1.05	9.61 ± 0.63	9.26 ± 2.39
	NE	3.96 ± 0.91	4.04 ± 0.85	3.37 ± 0.86
	LY	4.64 ± 0.95	4.04 ± 1.02	4.49 ± 0.82
	MO	0.46 ± 0.19	0.36 ± 0.28	0.42 ± 0.44
	EO	0.13 ± 0.14	0.32 ± 0.35	0.11 ± 0.17
	BA	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
	RBC	6.27 ± 0.87	6.86 ± 0.80	6.23 ± 0.97

Blood samples collected from coccygeal veins of six cows at 0, 3 and 7 days after intra-mammary gland infusion of silver ion (20 μg/ml). The circulating hematological counts (mean ± SD) are reported × 1,000/μl. WBC: white blood cells, NE: neutrophil, LY: lymphocyte, MO: monocyte, EO: eosinophil, BA: basophil, RBC: red blood cell × 10^6/μl.

cells of mammary tissue in milk is considered as a response of mammary tissue to microorganisms in part of inflammation of mammary gland (21,30). We investigated SCC in milk be acquired from the quarter having clinical mastitis after silver ion infusion, and SCS was reduced after silver ion intra-mammary infusion. This result suggests that white blood cells (WBC) in milk may be decrease by inflammation reduction in mammary gland after silver ion infusion.

BSA in milk can be a marker of blood-mammary gland barrier function and mammary gland pro-inflammatory responsiveness. The decreased BSA level in milk after silver ion treatment can be indicative of decreased permeability of the blood-milk barrier. The observation that BSA levels declined rapidly after its acute surge is evidence that silver ion had not damaged the blood-milk barrier, and supports the idea that silver ion infusion does not damage mammary tissue. It is appropriate to suggest that silver ion infusion decreases the permeability of the blood-milk barrier for a short period of time, which halts inflammation.

Lactoferrin is an iron-binding glycoprotein synthesized by specific granules in polymorphonuclear leukocytes (2) and mammary epithelial cells (12). It has broad-spectrum antimicrobial properties and is thought to contribute to the host defense system active at mucosal surfaces (7). In this study, decreased lactoferrin concentration in milk after silver ion infusion was probably because of the decreased influx of neutrophils in the mammary system. Neutrophils may partly contribute to increased lactoferrin concentration in mastitis-infected quarter milk, because neutrophils are also a source of lactoferrin apart from mammary epithelial cells (10,11). Neutrophils produce toxic oxygen radicals and metabolites are thought to play a role in chronic inflammation and tissue destruction (31). silver ion may exeet its anti-inflammatory effect by inhibiting superoxide-mediated production of oxygen radicals by neutrophils.

Conclusion

Intra-mammary gland infusion of silver ion in quarters of Holstein cows having clinical mastitis decreased udder inflammatory signs including udder swelling, udder firmness, and clots and SCC in milk. BSA and lactoferrin levels in milk were also reduced by silver ion infusion. The reduction of BSA and SCC level in milk after silver ion infusion indicates a positive effect to reduce the inflammation and bacterial activity in mammary glands. Further, the intra-mammary gland infusion of silver ion did not damage the mammary system or compromise the general hematology and milk yield of the treated cows. These results suggest that intra-mammary infusion of silver ion has potential in treatment of clinical mastitis.

Acknowledgements

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임상형 유방염을 가진 홀스테인(Holstein) 소에 대한 은 이온의 적용 연구

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요 약 : 유방염은 병원성 미생물들에 의한 유선 내에 염증이 생기는 질환으로, 유방 조직과 우유 내 체세포수 (SCC)와 세균학적 분석이 유방염 진단을 위해서 사용된다. 본 연구에서는 임상형 유방염이 걸린 홀스테인 소의 유선 내로 은 이온(20 μg/ml)을 투입하고, 유방염의 임상 증상의 변화와 우유 내 somatic cell count, 소 혈청 알부민과 lactoferrin 등과 같은 유방염 관련 인자들을 조사하였다. 은 이온의 유선 내 투입은 유방의 부종과 견고함 등의 염증 증상을 완화시켰으며, 우유의 응고와 체세포수를 감소시켰다. 또한, 우유 내 소 혈청 알부민과 lactoferrin의 농도가 은 이온의 투입에 의해 감소되었다, 그러나 임상형 유방염이 걸린 소의 유선 내 은 이온의 투입은 착유된 우유의 양과 혈액 내 백혈구와 적혈구 수를 변화시키지는 않았다. 이러한 결과는 유방염이 걸린 소에 은 이온을 유선 내로 투입 함으로서 염증과 세균의 활성을 감소시킨다는 것을 보여주며, 은 이온을 임상형 유방염의 치료에 적용할 수 있는 가능성을 보여주는 것이다.

주요어 : 소 유방염, 체세포수, lactoferrin, 은 이온, 소 혈청 알부민