FULL PAPER *Theriogenology*

Intramammary Application of Ozone Therapy to Acute Clinical Mastitis in Dairy Cows

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ABSTRACT. The infusion of ozone into the inflamed quarter of cows with clinical mastitis was performed and the efficacy of ozone therapy was evaluated. Ozone was infused into the inflamed quarter via a teat canal using ozone gas generating equipment. Nineteen Holstein cows with acute clinical mastitis were divided into two groups: 15 cows treated with ozone therapy, and 4 cows treated with antibiotic therapy. Systemic and local clinical signs, California Mastitis Test scores, the mastitis causing pathogens, electronic conductivity of milk, and somatic cell counts in milk from ozone- and antibiotic-treated quarters, were compared between the groups. Sixty percent (9/15) of cows with acute clinical mastitis treated with ozone therapy, did not require any antibiotics for recovery. This newly developed ozone therapy method was proven to be effective, safe, and cost effective, and carries no risk of drug residues in milk.

KEY WORDS: cattle, intramammary application, mastitis, ozone, therapy.

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Bovine mastitis is the most common and costly disease in the dairy industry [3, 4, 7, 9]. Cost components associated with clinical bovine mastitis are decreased production, discarded milk, culling, medications, and delayed genetic progress [9]. Dairy producers and veterinarians are often presented with the challenge of treating cows affected by clinical mastitis. The intramammary administration of antibiotics is the most common method of treatment of bovine mastitis. However, it is likely that antibiotic treatment during lactation has a low cure rate for many mastitis pathogens [1, 10, 14]. Loss of milk due to drug residues results in a poor cost-benefit ratio for most antibiotic therapy (AT) [9].

Ozone is polymerized oxygen (O_3) created by the passage of air or oxygen over high energy electrodes within an ozone generator or by ultraviolet light [19]. Ozone is currently used for the reduction of microbial populations in rooms for curing cheeses and storing fruits and vegetables, and for the disinfection of water and wastewater. The bactericidal and fungicidal properties of ozone, which have been used in the decontamination of uninhabited bioclean hospital areas has been well documented [5, 17, 18]. Its application in the sterilization of bacteria, fungi and viruses is by strong oxidation [11]. In the veterinary field, however, the application of ozone has not been used for the treatment of inflammatory diseases. The use of ozone therapy (OT) seems to have many advantages in treatment of bovine clinical mastitis. The present trial demonstrates that ozone can be used successfully for the treatment of the bovine mastitis. The aim of this study was to describe the feasibility and effectiveness of OT in bovine acute clinical mastitis.

MATERIALS AND METHODS

Cows and allocation of treatment: Nineteen lactating Holstein cows with acute clinical mastitis were used. All cows were raised in dairy farms in northern Hokkaido, Japan. All dairy cows were diagnosed as having acute mastitis by the following criteria; clinical signs of mastitis, California Mastitis Test (CMT) scores, electronic conductivity (EC), somatic cell counts (SCC) and the presence of pathogens causing mastitis. Four quarters from 4 cows out of 24 quarters from 19 cows with acute clinical mastitis were treated with AT (AT group), as control. For the systemic treatment of clinical mastitis, ampicillin (4 g) or kanamycin (5 g) was given intramuscularly for 1 to 4 days. Then, kanamycin (300 mg) and benzylpenicillin procain (300,000 IU) or cefazolin (150 mg) infusion were used for the treatment of each inflamed quarter for 5 to 9 days. The remaining 20 quarters from 15 cows out of 24 quarters from 19 cows with acute clinical mastitis, were treated with OT (OT group). Seven quarters from 6 cows out of the OT group at initial treatment required AT because the inflammation was not fully recovered on the 3rd day after OT.

Ozone generating system: Ozone generating equipment (OT-31ST-M system, Nippon Ozone Co., Tokyo, Japan), which has the capability of making ozone at a rate of 500 mg/hr was used. Ozone was made from pure oxygen (>99.5 v/v%, Japan Pharmacopoeia, Sapporo, Japan) in a portable oxygen tank, which is required for optimum pressure for intramammary infusion. The equipment consisted of a portable oxygen gas cylinder (capacity 0.15 m^3, Oxygen-6, Sin-Ei Industry Co., Ltd., Saitama, Japan) and double valves attached with flow meter (W-604, Sin-Ei Industry Co., Ltd., Saitama, Japan). The oxygen was introduced through the intake tube of the ozone generator, and the ozone/oxygen mixed gas was blown out from it. The concentration of ozone was regulated by oxygen flow and dial scale on the ozone generator (Fig. 1).

Procedure for intramammary infusion of ozone: The ozone generating equipment was connected by two short plastic tubes (50 cm long, 5 mm in diameter) and a long silicone tube (3 m long, 9 mm in diameter). A short plastic tube was

Fig. 1. Ozone generating system.

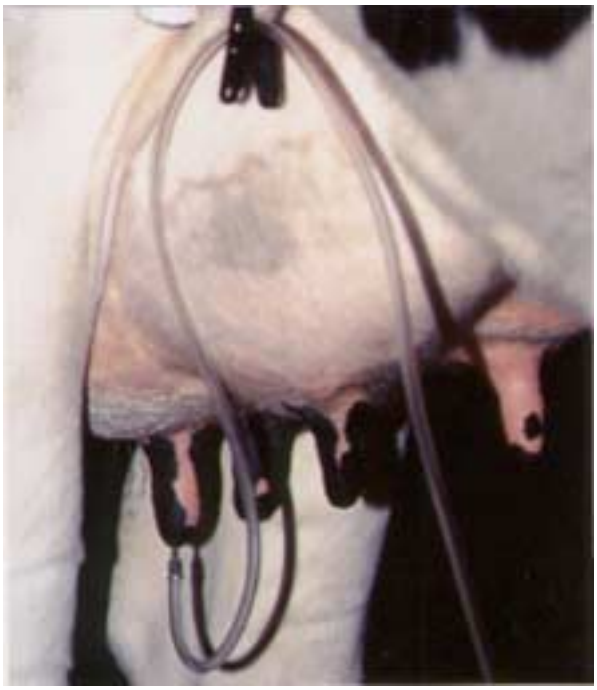

Fig. 2. Intramammary infusion of ozone.

used to connect the outlet of the flow meter (W-604) to the intake of the ozone generator. The long silicone tube was used to connect the outlet of the ozone generator to the infusion tube. The infusion tube consisted of a short plastic tube and the teat cannula (55 mm long, 2.4 mm in diameter) (FH-70, Fujihira Industry Co., Ltd., Tokyo, Japan), sterilized by ethylene oxide gas. Ozone gas produced by the system was infused into the inflamed quarter via the teat canal, after wiping with 70% alcohol, by using a tubing connected system. The teat cannula was inserted into the teat canal and was fixed during ozone infusion (Fig. 2). Then, the oxygen cylinder's valve was opened, and the ozone generator was switched on. Total infusion volume was adjusted to the levels of 1 to 5 liters/quarter depending on the capacity of the quarter. The concentration of ozone was determined by an ozone detector (Nippon Ozone Co., Tokyo, Japan).

Clinical examination: Clinical signs of the acute clinical mastitis were evaluated. Each symptom observed was classified on a score of 0 to 2 as follows: 0 = no clinical signs were observed. 1 = clinical signs indicating: slight improvement, although not fully recovered. 2 = signs indicating abnormal. Other clinical signs of the udder and the quarter milk were evaluated by the following 10 parameters: rectal temperature, appetite, swelling, hardness, heat, redness, pain, milk color, clots in milk and CMT score [13].

Collection of milk samples: One to 2 m*l* of milk samples were collected from each quarter under aseptic condition into sterilized plastic tubes for identification of pathogens and SCCs. Another 10 m*l* of milk samples were collected in other plastic tubes for the measurement of EC.

Bacteriological identification of mastitis causing pathogens: The identification of bacteria in all inflamed quarter milk was carried out according to the procedure recommended by the National Mastitis Council [8].

EC in milk: Five to 10 m*l* of sampled milk were used to determine the EC [2, 15] in milk by the EC detector (Milk Checker, Eisai Inc., Tokyo, Japan).

SCC in milk: The count of SCC in milk from the inflamed quarter was performed according to the routine method described previously [13].

Statistical analysis: Data were analyzed statistically by repeated measure ANOVA and Scheffe's F analysis; values of $p<0.05$ were regarded as significant.

RESULTS

Condition of OT applied to inflamed quarter: The conditions such as ozone/oxygen gas flow rate, gas pressure, and the time of treatment, for ozone therapy were determined. The total amount of infused ozone into each inflamed quarter was estimated at 6 to 30 mg, which was equivalent to ozone concentration of 6 mg/*l*, determined by the ozone detector, when 1 to 5 liters of ozone/oxygen gas were infused into a quarter. The rate of oxygen flow was 1 *l*/min, when dial scale 5.5 on the ozone generator was used. The intramammary OT was applied to a quarter at the onset of mastitis.

Changes in clinical signs: The changes in systemic and local clinical signs are shown in Fig. 3. The initial score of clinical signs such as appetite, rectal temperature, swelling, hardness, heat, redness and pain of inflamed quarters, milk color, clots in milk and CMT score was 10 ± 0.97, and the

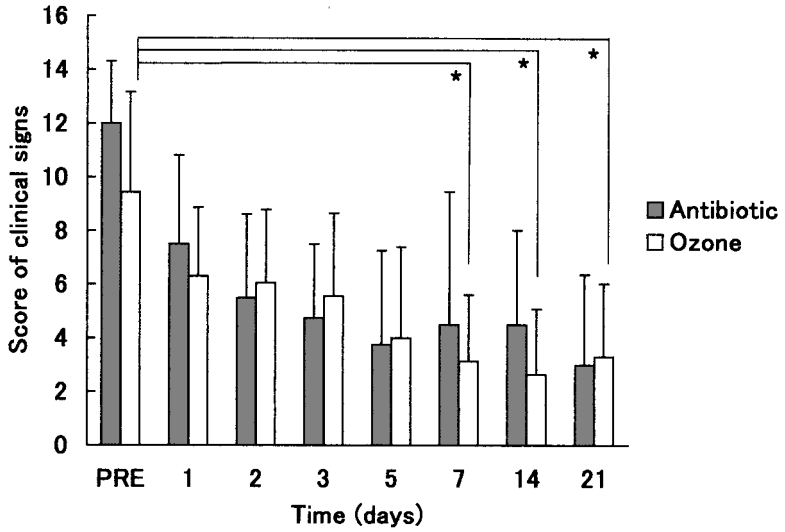

Fig. 3. Changes of scores of clinical signs of mastitic cows after each treatment. The number of samples in the each group was identical to the total number of treated quarters. Data are expressed as the mean ± S.D. of the treated cows. PRE: Before treatment. *: $P<0.05$.

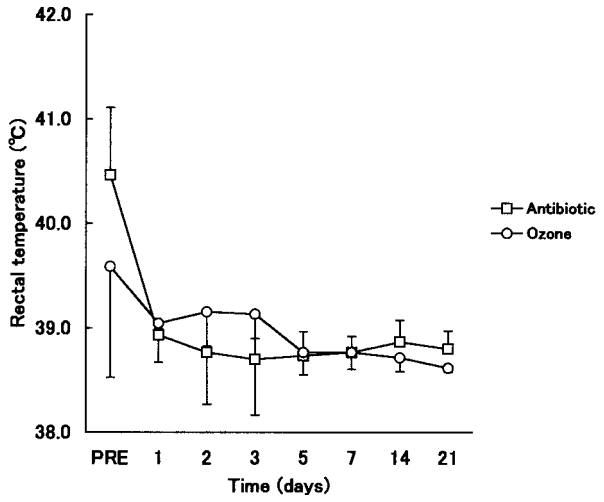

Fig. 4. Changes in rectal temperature of mastitic cows after each therapy. The number of samples in each group indicates the total number of mastitic cows. Data are expressed as the mean ± S.D. of each determination. PRE: Before treatment.

value decreased rapidly to 4.55 ± 0.83 on the 3rd day after OT. The scores of clinical signs of OT-treated cows at 7, 14 and 21 days after OT were significantly ($P<0.05$) decreased, compared to those of before OT (PRE-OT). No significant difference in changes of clinical signs was found between the OT and AT groups.

The change of rectal temperature in cows after therapy is shown in Fig. 4. It showed similar patterns after OT and AT. There were no significant differences in rectal temperature changes between the two groups.

Table 1. The isolation of pathogens in milk from 24 inflamed quarters from 19 lactating cows before ozone and antibiotic treatments

	Ozone (15 Cows-20 Qua.)	Antibiotics (4 Cows-4 Qua.)
S. aureus	6	1
S. uberis	5	
Coliforms	3	2
A. pyogenes	3	1
Strep. spp.	1	2
S. dysgalactiae	2	
CNS	2	
Bacillus spp.	1	

Isolation of bacteria from mastitic milk: Isolated pathogens from the inflamed quarters were *Staphylococcus aureus (S. aureus), Streptococcus uberis (S. uberis)*, Coliforms, *Actinomyces pyogenes (A. pyogenes), Streptococcus* spp., *Streptococcus dysgalactiae*, Coagulase negative Staphylococci and *Bacillus* spp. (Table 1).

Changes in EC and SCC: Data are expressed as the relative percentages of the pretreated values (100%) in Figs. 5 and 6. The differences in changes of EC in milk from quarters treated with OT and AT were not statistically significant (Fig. 5). The number of SCC in milk from quarters treated with OT and AT

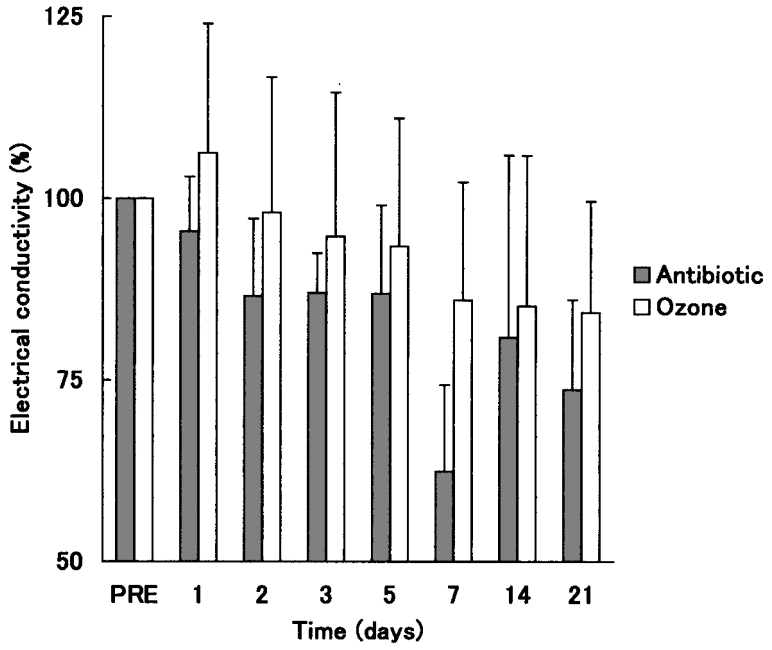

Fig. 5. Changes in the electric conductivity in each quarter milk from ozone- and antibiotics-treated cows. The number of samples in each group was identical to the total number of treated quarters. Data are expressed as the mean ± S.D. of determinations. PRE: Before treatment.

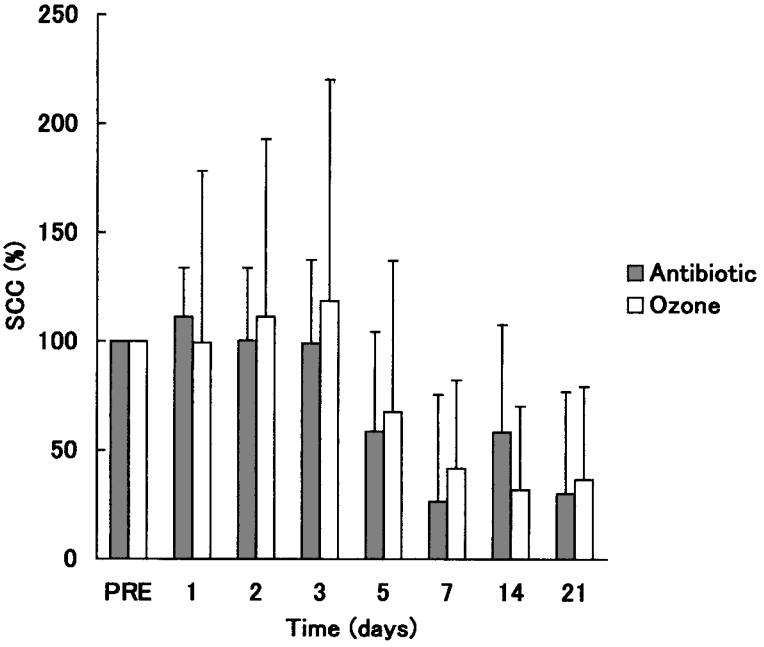

Fig. 6. Changes in the number of SCC in quarter milk from ozone- and antibiotics-treated cows. The number of samples in each group was identical to the total number of treated quarters. Data are expressed as the mean ± S.D. of determinations. PRE: Before treatment.

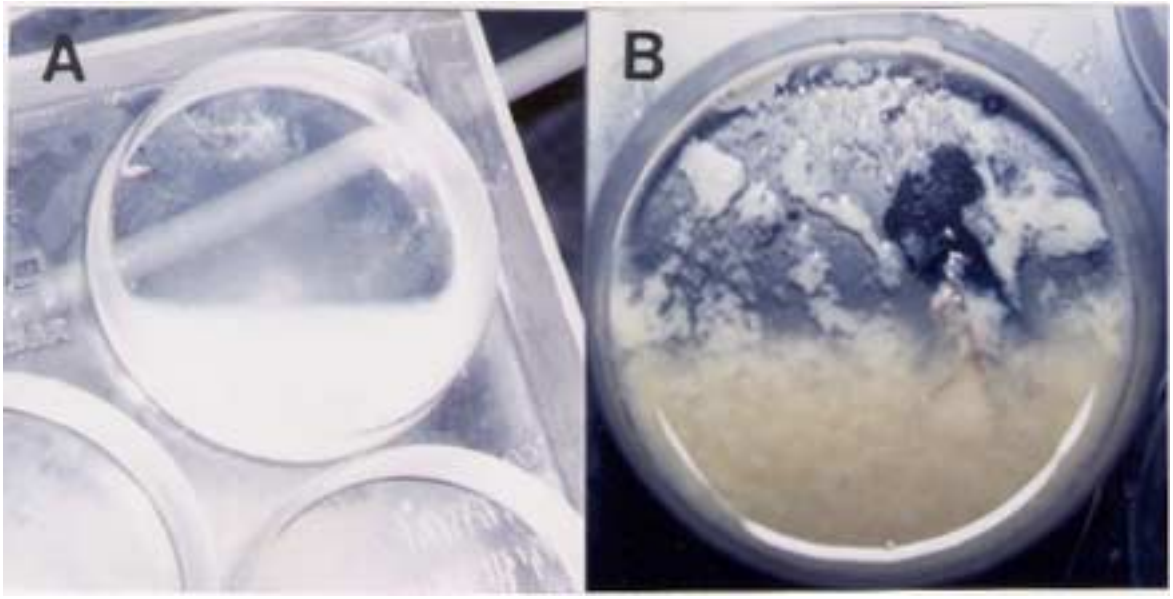

Fig. 7. Changes in the quality of mastitic milk from inflamed quarter. In this case, the infective agent was *Escherichia coli*. A: No clots or discoloration were seen in excreted milk from the inflamed quarter before OT. B: Large quantity of clots was seen in excreted milk from the quarter after OT.

was compared (Fig. 6). There were no significant differences in SCC changes between the two groups. The changes in EC and SCC levels decreased gradually as time elapsed during the observation period (1–21 days) after each treatment as shown in Figs. 5 and 6.

Other findings of OT: A large quantity of clots was seen in excreted milk from the inflamed quarters at 12–24 hr after OT (Fig. 7). Slight hypodermic ozone gas edema and decrease in production of the milk were rarely seen at the next milking. No apparent side effects were observed in the OT-administered area, such as irritative effects to the mammary glands and necrotising effects to the teat.

DISCUSSION

Ozone (O_3) is a kind of active oxygen, but is not radical itself, and is important as a trigger matter for oxidation of lipids. Treatments utilizing ozone have been used in human medicine for over a century in Germany, and a large number of hard-to-cure diseases have been safely remedied by ozone [11]. Little attention has been paid to the clinical application of ozone to animal diseases. The application of ozone to bovine acute clinical mastitis, infusing of ozone into the inflamed quarter, appears to have great possibilities for sterilization of causative agents, and detoxicating of the inflamed quarter through oxidation with ozone.

After OT application to the inflamed quarter, acute clinical mastitis showing systemic and local symptoms was greatly improved. The rectal temperature decreased to a normal range, there was an improvement in appetite, and the general conditions of the inflamed quarters were improved, though an increase in clots were found in the quarters during the first few days after OT. The excretion of a large quantity of clots in milk from the inflamed quarter appeared to be one of the characteristic findings of OT (Fig. 7). It is likely that application of OT to the inflamed quarter has an enhancing effect on the excretion of clots from the quarter. The mechanism for this is still unclear. It may be associated with increased functions of leukocytes, increased respiratory burst which appear to be modulated by the action of active oxygen, or improved circulation in the ozone-infused quarter (unpublished data). The levels of SCC and EC values were not fully recovered 3 weeks after treatment in the OT and AT groups, however, these values decreased gradually towards the normal range (Figs. 5 and 6).

Ozone therapy requires a minimum contact time, adequate volume and concentration of ozone to sterilize the causative agents of mastitis, as the infused ozone needs direct contact with the causative agents. The anatomical structures of the udder was very advantageous when OT was applied using compressed gas, because a degree of compressed gas could be kept in the udder as the teat canal prevented the gas from easily leaking out. If enough compressed ozone gas was infused into the quarter, inflamed milk was discharged from the teat canal by the gas pressure. The excessive infusing gas was easily discharged out of the quarter from the teat canal depending on the pressure of the quarter, i.e., liminal inside pressure, by the mechanism of the sphincter muscle of the teat. However, it was understood that the ozone had resolved into normal oxygen [11] and disappeared completely during the contact time. In fact, no smell of ozone was exuded from the treated quarter at the next milking after OT.

It was evident that there was very little difference in the effectiveness and convalescence of both AT and OT therapies.

However, OT appears to have a great advantage over AT, because dairy farmers are required to discard all the milk treated with antibiotics. No milk withdrawal time is required with OT. In addition, there is no risk of drug residue in the milk. OT would be a very useful method as a first selection for the treatment of acute bovine clinical mastitis. It is not known whether ozone functions only in the killing of pathogens, or also has a role in enhancing the host defense mechanism [11].

Some cases of clinical mastitis, however, were found not to be efficiently cured by OT as a method of first choice, therefore, AT was applied to these cows. The causative pathogens included: *S. aureus, A. pyogenes* and *S. uberis*. In most cases of mastitis with *S. aureus* and *A. pyogenes*, there was no change and/or returning symptom by OT, but these cases appeared to be associated with chronic mastitis. In particular, mastitis with *S. uberis* showed a strong resistance to OT. About 60% (9/15) of cows with acute clinical mastitis recovered by OT alone.

Changes in the EC and SCC showed similar patterns for the cows in the OT and AT groups, in relation to the recovery of the inflamed quarters after each therapy. The change of milk quality concerning EC and SCC in the treated quarter showed few differences between AT and OT. The changes observed in OT and AT were similar, indicating that the efficacy of OT was equivalent to that of AT. However, the exact mechanisms for recovery from clinical mastitis through the use of OT remain to be elucidated.

It is understood that ozone gas toxicity causes irritation to the nose and eyes [6, 16 –18]. However, only slight or no smell of ozone was sensed at treatment or from the treated areas. This indicates that the concentrations of 0.01 to 0.05 ppm by volume were much lower than the reported toxic levels of 1 ppm or more [16].

In conclusion, a newly developed method, OT, has proven to be effective, safe, and cheap, and leaves no drug residues in milk. Costs associated with OT are low, as it involves only the cost of the gas (oxygen). Moreover, no milk was discarded from the treated quarters, because there was no ozone residue in the quarter at next milking. OT is readily available and has more advantages over the widely used AT for the treatment of acute bovine clinical mastitis.

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