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# Recovery Effect of Intramammary Ozone Therapy for Acute Clinical Mastitis in Dairy Cows *

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This research was carried out to determine the recovery effect of ozone gas which is thought to be an alternative method for antibiotic treatment in acute clinical mastitis cases. The material of the study consisted of 23 acute mastitis diagnosed mammary lobes belonging to 22 lactating cows breed. Aseptic milk samples were taken before the treatment. The cows in group I received antibiotic by intramuscular route for 5 days, also an antimastitis drug was injected to affected lobes with 24 hours interval for 3 days. 50 mL ozone/oxygen gase was administered via teat duct with an interval of 24 hours for 3 days to group II. Milk samples were taken and bacteriological examination was applied from the cows after 7 days post treatment. The entire bacteria (100%) isolated form the samples of group I was, coagulase negative staphylococci (CNS). Bacterial and clinical recovery rates were 90.90% (10/11), 90.90% (10/11), respectively. The isolated bacteria in group II were 8 CNS (66.66%), 2 CNS+ Candida spp. (16.66%), 2 Streptococci spp. (16.66%). Bacteriologic recovery rates were obtained as 87.50% (7/8) ve 0% (0/2), 0% (0/2), respectively. Clinical recovery rates were determined as 87.50% (7/8), 50% (1/2) ve 100% (2/2), respectively. As a result, it was concluded that, ozone gas was effective on bacteriological and clinical recovery and can be an alternative to antibiotic treatment in acute clinical mastitis cases caused by CNS, but also it was found that it does not have any effect on recovery of Streptococci and Candida spp. caused mastitis cases.

***Key Words:*** *Acute mastitis, cow, antibiotic, ozone*

### Sütçü İneklerde Akut Klinik Mastitislerde Meme İçi Ozon Tedavisinin İyileştirici Etkisi

Çalışma, ineklerde akut klinik mastitis vakalarında antibiyotik tedavisine alternatif bir yöntem olarak düşünülen ozon gazının iyileştirici etkisinin belirlenmesi amacıyla düzenlendi. Çalışmanın materyalini klinik olarak hasta olduğu tespit edilen laktasyondaki 22 baş ineğe ait 23 adet akut mastitisli meme lobu oluşturdu. Tedaviden önce bakteriyolojik ekim için süt örnekleri alındı. Birinci gruba 5 gün süreyle ve 24 saat aralıklarla 5ml/100 kg dozunda kas içi yolla antibiyotik enjekte edildi, hasta meme loblarına ise 3 gün süreyle ve 24 saat aralıklarla meme içi mastit preparatı uygulandı. İkinci gruba ise meme başı kanalından 50 ml ozon/oksijen gazı 24 saat ara ile 3 gün süreyle uygulandı. Tedavi bitiminden 7 gün sonra tüm ineklerden süt örnekleri alındı ve bakteriyolojik ekim yapıldı. Grup I'de süt örneklerinde üreyen bakterilerin tamamının (% 100) KNS olduğu görüldü. Bakteriyolojik ve klinik iyileşme oranları sırasıyla % 90.90 (10/11), % 90.90 (10/11) olarak saptandı. Grup II'de süt örneklerinde üreyen bakteriler,8 adet (% 66.66) KNS, 2 adet (% 16.66) KNS+maya ve 2 adet (% 16.66) Streptococcus spp. olarak tespit edildi. Bakteriyolojik iyileşme oranları sırasıyla % 87.50 (7/8) ve % 0 (0/2), % 0 (0/2), klinik iyileşme oranları ise sırasıyla % 87.50(7/8), % 50 (1/2) ve % 100(2/2) olarak saptandı. Sonuç olarak, meme içi ozon gazı uygulamasının KNS'dan kaynaklanan akut klinik mastitislerin tedavisinde bakteriyolojik ve klinik iyileşme üzerine oldukça etkin olduğu ve antibiyotiklere alternatif olabileceği, Streptecoccus spp. ve maya kaynaklı akut klinik mastitlerde ise iyileştirici etkisinin olmadığı kanısına varıldı.

***Anahtar Kelimeler****: Akut mastitis, inek, antibiyotik, ozon*

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## Introduction

Due to the economic losses it causes, mastitis is a costly disease in the dairy industry. Mastitis may lead to reduced milk yield, poor quality of milk and milk products resulting from altered milk composition, decrease in the sales value of both milk and dairy cows, milk disposal, and the culling of dairy cows. Mastitis not only leads to treatment costs, but may also cause mortality. Mastitic milk is a risk to public health as it contains a high level of microorganisms as well as antibiotic residues (1-3).

In clinical mastitis cases, the symptoms are clearly visible and generally there is no need to perform laboratory tests to confirm the presence of inflammation. As the clinical signs are self-evident, diagnosis can be made by clinical examination methods such as inspection, palpation and examination of the nature of secretions. The general appearance of mastitic milk differs from that of normal milk, such that its colour ranges from a light yellow to a bloody brown, and as a result of the passage of coagulation factors from blood into milk, the presence of flakes, clots and clumps is observed (2, 3).

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In clinical mastitis cases, in order to prevent severe tissue damage, the infectious agent needs to be rapidly eliminated from the body. Therefore, antibiotic therapy is reported as the most effective treatment method for these cases. Antibiotics used for the treatment of mastitis can be administered by intramammary, intramuscular or intravenous route. Due to the presence of the blood-udder barrier, excluding peracute and acute cases, in general antibiotics are preferred to be administered locally, whilst in peracute and acute mastitis cases associated with local oedema, the blockage of the mammary ducts and the impairment of the blood-udder barrier, it is suggested to combine intramammary and systemic antibiotic treatment (4, 5).

The effects of ozone gas administration on the organism include the activation of cell metabolism, the increase of the oxygen transport capacity of haemoglobin, and thus, the increase of tissue oxygen levels through the induction of erythrocyte metabolism, the strengthening of the immune system and the decrease of free radical levels (6, 7). Li et al. (8) reported that the phagocytic activity of neutrophil leukocytes and macrophages increase within 3 to 24 h after ozone gas administration. Medical ozone is a mixture of 5% ozone and 95% oxygen. Ozone gas is produced from pure oxygen using medical ozone generators. The production of ozone is aimed at the supply of the oxygen required by tissues and cells. Ozone gas shows its therapeutic effect by activating the production of cytokines in leukocytes, increasing the production of antibodies, and stimulating phagocytosis. Furthermore, ozone gas has bactericidal effect and disrupts the lipoprotein structure in the cell membrane of bacteria (9-11).

The main problem encountered with mastitis is the obligation to discard milk due to residues resulting from antibiotic treatment. Thus, the use of ozone gas is considered as a feasible alternative method for the treatment of mastitis given that ozone is cheaper and does not cause any harmful residue in milk (12).

This study was aimed at the isolation and identification of the causative agent(s) of clinical mastitis cases encountered in the study region and at the investigation of the therapeutic effect of ozone gas, used as an alternative to antibiotics.

## Materials and Methods

Twenty-three udder lobes belonging to 22 lactating cows, displaying signs of acute clinical mastitis were confirmed to be infected by clinical examination constituted the material of the study. The animals included in the study were housed in tie-stall barns.

The animals were clinically examined for mastitis and their milk was physically examined with the strip-cup test. The teats of the inflamed quarters were cleaned with 70% alcohol solution before milking into a cup, and the milk samples were transferred into 50 mL-sterile tubes for bacteriological analysis. Milk samples were transferred under cold-chain conditions. After the inflamed quarters were emptied, in order to clear the ducts that were blocked by inflammation products, a proteolytic enzyme (Masti Veyxym®, Ekomed) was administered into the udder and this procedure was repeated throughout the treatment period.

Animals displaying a deterioration of the general medical condition were first given 5-10 L of isotonic electrolyte solutions and 500 mL of 30% dextrose solution by IV route, and also flunixin meglumine (Fulimed®, Alke) at a dose of 22 mg/kg to eliminate the effects of inflammation, by IV route within the first 1-2 h. Next, the cows were assigned to two groups. The first group (Group I, n=11) was administered with a parenteral antibiotic containing 200.000 IU/mL of benzylpenicillin procaine and 200 mg/mL of dihydrostreptomycin sulphate (Combiotic S®, Pfizer) at a dose of 5 mL/100 kg by intramuscular route for a period of 5 days at 24 h intervals. An anti-mastitis preparation (Tetra delta LC®, Pfizer) containing 100 mg/mL of novobiocin sodium, 150 mg/ml of neomycin sulphate, 100 mg/mL of procaine penicillin, 125 mg/mL of dihydrostreptomycin sulphate, and 10 mg/mL of prednisolone was administered into the inflamed quarters for a period of 3 days at 24 h intervals. In the second group (Group II, n=11), 50 mL of an ozone/oxygen gas mixture, containing ozone at a concentration of 60 µg/ml, was aspirated from an ozone generator (Genozon®) into a plastic syringe and infused into the teat canal for a period of 3 days at 24 h intervals.

**Microbiological Examination:** Before starting treatment, the milk samples taken from the teats of the inflamed quarters were inoculated at the Microbiology Laboratory for isolation and identification. At the laboratory, the milk samples were first applied Gram staining for bacterioscopy, and the findings obtained by microscopic examination were recorded. The milk samples were inoculated onto blood agar and MacConkey's agar and incubated at 37 $^{o}$C for 2-3 days under aerobic conditions. The colonies that grew upon cultivation were applied bacteriological biochemical tests for species identification. Furthermore, all of the samples were also inoculated onto Sabouraud's dextrose agar and incubated at 25 $^{o}$C for 7-10 days for species identification at genus level for yeast and fungi. Seven days after the end of treatment, again milk samples were taken from both groups for bacteriological inoculation. The clinical cure rate was determined on the basis of the detection of the normalisation of the udder tissue and milk upon examination performed 7 days after the end of treatment.

**Ethical Considerations:** All the experimental procedures followed in this study were approved by the Animal Experiments Local Ethics Committee of Hatay Mustafa Kemal University with the number of Decision 2014-8 / 6.

**Statistical Analysis:** The data obtained in the present study were analysed with the chi-square test using the Statistical Software Package for the Social Sciences (SPSS 21.0).

## Results

At the clinical examination performed before the starting the treatment, 13 (59%) of the cows were determined to have a body temperature above 39.5°C. The general medical condition of the animals was determined to have deteriorated more or less, and inflammation signs of pain, swelling, heat and redness were observed in the inflamed quarters. Furthermore, none of the milk samples taken from the inflamed quarters displayed a normal appearance.

In the study, 23 quarters belonging to 22 cows were determined to be inflamed due to acute mastitis. In one of the animals included in the group that received ozone treatment, 2 quarters were determined to be inflamed. The microbiological inoculation of the 23 milk samples taken from the 22 cows, which were confirmed to be clinically ill, resulted in the isolation of coagulase-negative staphylococci (CNS) from 19, *Streptococcus* spp. from 2, and both CNS and *Candida* spp. from 2 of the samples (Table 1).

It was determined that all of the bacteria (100%) that grew in the milk samples belonging to Group I were CNS (Table 1). In Group I, the bacteriological and clinical cure rates were both determined to be 90.90% (10/11) (Table 2).

In Group II, the bacteria that grew in the inoculated milk samples were determined to be CNS in 8 (66.66%), CNS + *Candida* spp. in 2 (16.66%) and *Streptococcus* spp. in 2 (16.66%) of the samples (Table 1). The bacteriological cure rates for these samples were determined as 87.50% (7/8), 0% (0/2), and 0% (0/2), respectively (Table 3), whilst the clinical cure rates were ascertained to be 87.50% (7/8), 50% (1/2), and 100% (2/2), respectively (Table 3). In Group II, the total bacteriological and clinical cure rates were determined as 58.33% (7/12) and 83.33% (10/12), respectively (Table 3).

**Table 1.** The microorganisms isolated from the milk samples.

| Microorganisms | Group I | | Group II | |
|---|---|---|---|---|
| | n | % | n | % |
| CNS | 11 | 100 | 8 | 66.66 |
| CNS + *Candida* spp. | - | - | 2 | 16.66 |
| *Streptecoccus* spp | - | - | 2 | 16.66 |
| Total | 11 | | 12 | |

**Table 2.** Signs of microbiological and clinical cure observed for the microorganisms isolated from the milk samples belonging to Group I

| Microorganism | n | Bacteriological cure rates (%) | Clinical cure rates (%) |
|---|---|---|---|
| CNS | 11 | 90.90 (10/11) | 90.90 (10/11) |
| Total | 11 | 90.90 (10/11) | 90.90 (10/11) |

**Table 3.** Signs of microbiological and clinical cure observed for the microorganisms isolated from the milk samples belonging to Group II

| Microorganism | n | Bacteriological cure rates (%) | Clinical cure rates (%) |
|---|---|---|---|
| CNS | 8 | 87.50 (7/8) | 87.50 (7/8) |
| CNS + *Candida* spp. | 2 | 0 (0/2) | 50 (1/2) |
| *Streptecoccus* spp. | 2 | 0 (0/2) | 100(2/2) |
| Total | 12 | 58.33 (7/12) | 83.33(10/12) |

P=0.178

## Discussion

The present study was designed to isolate and identify the causative agent(s) of clinical mastitis cases encountered in the study region and to determine the therapeutic effect of ozone gas treatment, employed as an alternative to antibiotic therapy, in these cases.

CNS are minor mastitis agents, which are frequently isolated all over the world. They are natural saprophytes found in the skin of the teats, and under favourable conditions they colonize the teat canal. In general, CNS are known to cause subclinical mastitis, but they may also cause clinical mastitis. Varying symptoms are observed in CNS-induced mastitis cases, yet the inflammation observed in these cases is of moderate severity. Random sampling in Finland revealed an isolation rate of 50% for CNS. Again, in Finland, CNS were isolated at the highest rate (18%) from clinical mastitis cases, in particular from cases of moderate severity (13, 14). The isolation rate of CNS from clinical mastitis cases has been reported as 17% in Switzerland (15) and 9% in Israel (16). In the present study, the microbiological inoculation of the 23 milk samples, resulted in the isolation of CNS from 19 (82.60%) of the samples and both CNS and *Candida* spp. from 2 (8.69%) of the samples. The isolation rate of both CNS and *Candida* spp. being much higher than the rates previously reported by other researchers was noteworthy.

In cases of acute clinical mastitis, treatment should firstly focus on signs such as depression, progressive dehydration, fatigue and increased body temperature (17). Treated animals should be given an adequate volume of isotonic electrolyte solutions by IV route within a period of 1-2 hours. Fluid therapy restores the vital fluids of the animals and dilutes the toxins. In order to neutralize the effects of the prostaglandins generated as a result of inflammation, it is required to administer nonsteroidal anti-inflammatory drugs (4, 18). Furthermore, in order to ensure a better distribution of the drugs administered by intramammary route, it is suggested that proteolytic enzyme preparations be administered to lyse the epithelial sloughing, blood clots, flakes and pus, and thereby, to help clear the blocked ducts (18, 19). In the present study, after the inflamed quarters were milked and emptied, a proteolytic enzyme preparation was administered into the udder to help

clear the ducts that were blocked due to inflammation products, and this procedure was repeated throughout the treatment period. For treatment, firstly, the animals showing signs suggestive of a deteriorated general medical condition, such as a body temperature of ≥39ºC, depression, short and rapid respiration, tremor, fatigue and anorexia, were administered 5-10 L of isotonic electrolyte solutions and 30% dextrose solution by IV route within 1-2 h, as well as an antihistaminic and nonsteroidal anti-inflammatory drugs. Non-steroidal anti-inflammatory drugs were administered to neutralize the effects of the prostaglandins generated as a result of inflammation. No mortality was observed in any of the groups throughout the study period.

In clinical mastitis cases, in order to prevent severe tissue damage, it is required to rapidly eliminate the infectious agent from the body. Reports indicate that the most effective method of rapidly eliminating the infectious agent is to administer a combination of parenteral and intramammary antibiotics (5). It has been reported that the bacteriological cure rates of clinical mastitis cases caused by Gram-positive bacteria range from 15.4% to 91.6%, depending on the intervention time, type of antibiotic used for treatment, and administration route and administration period of the antibiotic (20).

Baştan et al. (21) reported the clinical and bacteriological cure rates they achieved with the administration of a combination of intramammary amoxicillin + clavulanic acid + prednisolone and systemic amoxicillin + clavulanic acid for the treatment of acute mastitis as 84% and 88%, respectively. In the present study, the animals included in the antibiotic treatment group were administered with a parenteral antibiotic preparation containing benzylpenicillin procaine and dihydrostreptomycin sulphate and an anti-mastitis preparation containing novobiocin sodium, neomycin sulphate, procaine penicillin, dihydrostreptomycin sulphate and prednisolone into the inflamed quarters. The bacteriological and clinical cure rates achieved with this treatment regimen were both determined as 90.90%. Researchers (22, 23) have reported that a bacteriological cure rate ranging between 70-90% is achieved with the use of beta-lactam antibiotics in the treatment of CNS-induced mastitis cases.

Reports indicate that the administration of ozone gas at a dose range of 10-80 ug/ml does not cause any adverse effect (6, 24). In the present study, 50 mL of an ozone/oxygen gas mixture, containing ozone at a concentration of 60 µg/mL, was aspirated from an ozone generator into a plastic syringe and infused into the teat canal at 24 h intervals for a period of 3 days.

In their study on 19 cows with clinical mastitis, upon assigning the animals to two groups and infusing ozone gas aspirated from an ozone generator into the inflamed quarters of 15 of the cows and administering systemic and local antibiotics to the remaining 4, Ogata and Nagahata (12), determined that 9 (60%) of the 15 cows, which were administered ozone gas, completely healed. They suggested that ozone gas therapy could be an alternative treatment for clinical mastitis. In another study conducted by Yanliang et al. (25) 30 cows with clinical mastitis were randomly allocated to 2 equal groups, such that Group I was given combined ozone gas and antibiotic therapy and Group II was given antibiotic therapy alone. In result, the researchers determined that in Groups I and II, 93.3% (14/15) and 73.33% (11/15) of the animals were cured, respectively. In the present study, the 11 cows included in Group I were administered with intramammary and systemic antibiotics, and their bacteriological and clinical cure rates were both determined as 90.90%. The 11 animals included in Group II, which were administered with ozone gas, displayed bacteriological and clinical cure rates of 58.33% and 83.33%, respectively. The cure rates achieved in the group, which was administered with ozone gas in the present study, were found to be higher than the cure rate reported by Ogata and Nagahata (12) and lower than the cure rate reported by Yanliang et al. (25) for animals administered with combined antibiotic and ozone gas therapy.

In a research conducted on 79 infected udder lobes belonging to 32 lactating cows with clinical mastitis, Enginler et al. (26) randomly assigned the animals to 5 groups and administered Group I with 100 mL of an ozone/oxygen gas mixture, containing ozone at a concentration of 70 µg/mL, into the teat canal at 24 h intervals for a period of 7 days, Group II with cephalosporin by intramuscular route for a period of 5 days, Group III with an ozone/oxygen gas mixture containing ozone at a concentration of 70 µg/mL + antibiotics, Group IV with an ozone/oxygen gas mixture containing ozone at a concentration of 30 µg/mL, and Group V with an ozone/oxygen gas mixture containing ozone at a concentration of 60 µg/mL. The general medical condition of the animals was determined to normalize in Groups I, III and V, and the best clinical and microbiological cure results were achieved in the group, which was administered with a combination of an antibiotic and a high concentration of ozone gas. In the present study, the infected quarters were infused with 50 ml of an ozone/oxygen gas mixture, containing ozone at a concentration of 60 µg/mL, at 24 h intervals for a period of 3 days. The general medical condition of all of the animals returned to normal and the bacteriological and clinical cure rates were determined as 58.33% and 83.33%, respectively. In the present study, the treatment period was shorter than that of the study conducted by Enginler et al. (26) and the amount of ozone gas used for treatment was less than that given to Groups I and V and equal to that administered to Group IV by Enginler et al. (26). The results obtained in the present study being better than the results achieved by Enginler et al. (26) in Group IV with the administration of an equal amount of ozone gas for a longer time period demonstrated the significance of the restoration of the general medical condition in the first place in animals with acute clinical mastitis.

Streptococci are ubiquitous bacteria and major mastitis agents. The most significant species of this genus are *S. agalactia*, *S .dysgalactia*, and *S. uberis* (27). In their study in cows with acute clinical mastitis, Ogata and Nagahata (12) determined that *S. uberis* was the microorganism resistant to ozone gas administered by intramammary route. In the present study, *Streptococcus* spp. were isolated from 2 milk samples belonging to the group administered with ozone gas and no subspecies was able to be identified. Treatment with ozone gas yielded bacteriological and clinical cure rates of 0% and 100%, respectively. Thus, it was determined that ozone gas did not have any therapeutic effect in mastitis cases caused by *Streptococcus* spp.

*Candida* spp. are classified as environmental mastitis agents. They emerge after the long-term use of antibiotics, and it is reported that antibiotics create a favourable environment for the growth of *Candida* spp.. Furthermore, they are transmitted with contaminated cannulae (28). In the present study, *Candida* spp. grew in 2 milk samples belonging to the group administered with ozone gas, and post-treatment isolation showed that *Candida* spp. grew once more in these two samples after ozone therapy.

As a result, it was concluded that the intramammary administration of ozone gas was very effective in the treatment of CNS-induced acute clinical mastitis cases by enabling both bacteriological and clinical cure, and that ozone could be used as an alternative to antibiotics. On the other hand, ozone gas was found to show no therapeutic effect in mastitis cases caused by *Streptococcus* spp. and *Candida* spp..

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