International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2025; 10(1): 226-229

www.veterinarypaper.com
Received: 27-11-2024
Accepted: 30-12-2024

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Evaluation of Intramammary ozone therapy for clinical mastitis in dairy cows

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DOI: https://doi.org/10.22271/veterinary.2025.v10.i1d.2028

Abstract
The effectiveness of ozone therapy was assessed after administering ozone to the inflammatory quarters of cows suffering from clinical mastitis. Using ozone gas generating apparatus, ozone was injected via a teat canal into the inflammatory area. Thirty cattle with clinical mastitis were divided into three groups: Ten cows were treated with ozonated normal saline (NS), ten cows with ozonated NS + antibiotic and Ten cows were treated with antibiotic therapy. Systemic and local clinical signs, California mastitis test scores, mastitis-causing pathogens, pH and somatic cell counts in milk were compared across and within groups. 60% (6/10) of cows with clinical mastitis treated with ozonated NS, 70% (7/10) treated with sensitive antibiotic, and 100% (10/10) of cows treated with ozone + antibiotic therapy recovered. It was demonstrated that this recently developed ozone therapy method is both safe and effective. The combination therapy also sterilized the udder quarters as no bacterial isolate could be detected in post-treatment milk samples.

Keywords: Clinical mastitis, Antibiotic, Intramammary application, Ozone therapy

Introduction
Mastitis is a complex illness that affects livestock all over the world and is strongly linked to the environment and the production system (Mekibib *et al*., 2010) [10]. Mastitis is defined as an increase in somatic cell counts (SCC) in secreted milk or the udder's mammary tissue, local udder inflammation with swelling, heat, pain, redness and abnormal milk production, such as discoloration or clots (described as clinical mastitis, CM) or an increased SCC with normal milk appearance (subclinical mastitis, SCM) (De Vliegher *et al*., 2012) [4]. A number of diagnostic techniques, such as the California mastitis test (CMT), the Modified Whiteside test (MWT), the Somatic cell count (SCC), pH, Chlorine and Catalase tests can be used to indirectly identify changes in mastitis.

Antimicrobial therapy is commonly used to prevent and treat mastitis. Unfortunately, despite the best antimicrobial treatments available, antimicrobial resistance (AMR) is regarded as one of the reasons for low cure rates (Barkema *et al.*, 2006) [1]. Ozone (O_3) is an unstable polymerized oxygen which is created by the passage of air or oxygen over high energy electrodes within an ozone generator system or by ultraviolet light (Yang and Chen, 1979). After a short period of exposure, bacteria, spores and viruses may be inactivated by ozone therapy (Bocci, 1996) [2]. Ozone shows its efficacy with different mechanisms including the activation of erythrocytes and immune cells and it is a disinfectant against the anaerobic bacteria (Zobel *et al.*, 2014) [17]. In cases of mastitis, ozone has been shown to lower the somatic cell count (SCC). Mastitis can be safely and effectively treated with ozone therapy, leaving no trace in milk (Ogata and Nagahata, 2000) [11]. The use of ozone therapy (OT) seems to have many advantages in treatment of bovine clinical mastitis. The aim of this study was to describe the feasibility and effectiveness of OT in bovine acute clinical mastitis.

Materials and Methods

The current study was conducted, following approval by IAEC, at Teaching Veterinary Clinical Complex, Krantisinh Nana Patil College of Veterinary Science, Shirwal, Satara (Maharashtra, India) and nearby villages of Shirwal. Lactating cows presented with clinical signs like reduced milk yield, painful, hot, hard and swollen udders and abnormal milk were suspected for mastitis. Out of these 30 animals which were positive for CMT and somatic cell count more than 200,000 cells/mL were included in the present therapeutic study. These 30 cows were randomly divided into 3 treatment groups consisting 10 cows in each group (Group A, Group B, Group C).

Clinical Examination of Cows under Study

General clinical examination included rectal temperature (°F), pulse (/min), heart rate (beats/min) and respiratory rate (breaths/min). Changes in teat and udder conformation, and physical characteristics of milk like smell, taste, colour, consistency and pH were also recorded.

Diagnostic Tests

The pH of milk samples was recorded by Digital pH meter (Laqua Benchtop). The pH electrode was calibrated to 7.00. The California mastitis test (CMT) was conducted during milking of lactating dairy animals as per the instructions of manufacturer of the reagent. The manual somatic cell count (SCC) in milk samples was done as per the method described by Schalm *et al*. (1971) [13].

Bacteriological Culture of Mastitis Milk Samples and AbST of Isolates

Milk samples from mastitis positive quarters were inoculated on various media and characterization of bacterial isolates was performed based on colony morphology and Gram's staining as per the method described by Cowan (1993) [3]. *In vitro* antibacterial sensitivity pattern of the micro-organisms isolated was carried out by disc diffusion method using nine antibiotics discs.

Treatment Regime

The ozone machine, which already had an integrated system for ozonizing water, was used to prepare the ozonated saline. The gas was added for a predetermined amount of time until it reached the right concentration of ozone (60 µg/mL), which was then used for intramammary administration of antibiotics and anti-inflammatory drugs.

Cattle in group A were treated with ozonated normal saline (@ 60 µg/mL) intramammary 30 mL per quarter for 5 days; cattle in group B were treated with ozonated normal saline (@ 60 µg/mL) intramammary 30 mL along with most sensitive antibiotic, *i.e.* Ceftriaxone + Tazobactum (@ 10 mg/kg b.wt.) / Cefoparazone (250 mg intramammary) for 5 days with supportive NSAID as Tolfenamic acid (2 mg/kg IM); cattle in group C were treated with most sensitive antibiotic with supportive NSAID as in group B without ozonated saline.

Statistical Analysis

The data generated was analysed statistically for two-way repeated measures ANOVA by using online statistical software, SPSS 20.0. The p-value < 0.05 was regarded as significant.

Results and Discussion

In present study, we aimed to compare the treatment efficacy of ozonated NS insufflation via latex free 30 mL syringe alone and with combination of antibiotic and only antibiotic treatment in dairy cows with clinical mastitis. However, prolonged use of antibiotic can leave behind residues in milk that pose a major risk to the general public's health (Gruet *et al.*, 2001) [7]. The application of ozone infusion into the inflamed quarter in clinical mastitis of dairy cattle can result in the sterilization of causative agents as well as detoxification of the inflamed quarter (Ogata and Nagahata 2000) [11]. Shinozuka *et al.* (2009) [14] found that mammary irrigation with ozone water was more effective at treating mastitis in dairy cows than systemic antibiotic administration.

The results of CMT, SCC, and pH of clinical mastitic milk of cattle before and after three treatment regimens are depicted in Tables 1 to 3, respectively. Antibiotic susceptibility test results are shown in Table 5. There was a significant ($p<0.05$, $p<0.01$) reduction in CMT score, SSC of milk, pH of milk and even pathogens isolated on days 6 and 10 over day 0 following all three treatment regimens. Moreover, the decline in CMT score and SCC was significantly ($p<0.05$) faster in group B than group A, with intermediate values in Group C, by day 6 and/or both day 6 & 10, but no such trend was noted in milk pH (Table 1-3).

Table 1: Mean (±SE) CMT score of mastitis affected cattle on Day 0, 6 and 10

Days	Treatment			P-Value
	Group A (ozone)	Group B (antibiotic + ozone)	Group C (antibiotic)	
0	62.04 ± 8.23^{l}	62.01 ± 8.59^{l}	59.57 ± 9.74^{l}	NS
6	$26.44\pm4.64^{m,a}$	$8.81\pm1.51^{m,b}$	$23.47\pm5.07^{m,ab}$	<0.05
10	18.22 ± 6.27^{n}	3.00 ± 0.37^{n}	13.32 ± 5.23^{n}	NS
Mean ± SE	35.57±5.08	24.61±5.67	32.12±5.36	NS

Means bearing superscripts (l.m.n) within the column and (a, b) within the row differ significantly ($p<0.05$). NS=Non-significant.

Table 2: Mean (±SE) values of milk SCC of mastitis affected cattle on Day 0, 6 and 10

Days	Treatment			P-Value
	Group A (ozone)	Group B (antibiotic + ozone)	Group C (antibiotic)	
0	3.50 ± 0.17^{l}	3.50 ± 0.17^{l}	3.60 ± 0.16^{l}	NS
6	$2.40\pm0.22^{m,a}$	$1.30\pm0.15^{m,b}$	$2.1\pm0.23^{m,a}$	<0.05
10	$1.70\pm0.0.37^{n,a}$	$0.50\pm0.17^{n,b}$	$1.5\pm0.34^{n,ab}$	<0.05
Mean ± SE	2.53 ± 0.20^{a}	1.77 ± 0.25^{b}	2.40 ± 0.22^{a}	<0.05

Means bearing superscripts (l.m.n) within the column and (a, b) within the row differ significantly ($p<0.05$). NS=Non-significant.

Table 3: Mean (±SE) values of milk pH of mastitis affected cattle on Day 0, 6 and 10

Days	Treatment			P-Value
	Group A (ozone)	Group B (Antibiotic + ozone)	Group C (antibiotic)	
0	7.31 ± 0.19^{l}	7.36 ± 0.16^{l}	7.18 ± 0.11^{l}	NS
6	6.66 ± 0.16^{m}	6.76 ± 0.16^{m}	6.56 ± 0.10^{m}	NS
10	6.42 ± 0.13^{m}	6.35 ± 0.10^{m}	6.36 ± 0.12^{m}	NS
Mean ± SE	6.79±0.12	6.82±0.11	6.70±0.09	NS

Means bearing superscripts (l.m.n) within the column differ significantly ($p<0.05$). NS=Non-significant.

Ogata and Nagahata (2000) [11] reported the initial CMT score as 10±0.97, which decreased to 4.55±0.83 on the 3rd day after ozone treatment. In the present study, CMT scores were found to be significantly ($p<0.01$) reduced in group B (O_3 + AB combination) post-treatment when compared to pre-treatment (1.77±0.25 vs. 3.50±0.17) scores. They further reported SCC values decreased gradually towards normal range after 3 weeks of ozone and antibiotic treatment. In current study, SCC results were found to be decreased in line with Ogata and Nagahata (2000) [11], after therapy. In this study, post-treatment SCC values reduced to normal reference range when compared with pre-treatment values, *i.e.*, in group A (from 62.04±8.23 to 18.22±6.27) and in group B (from 62.01±8.59 to 3.00±0.37). The results of bacterial pathogens isolated from the clinical mastitic milk of cattle before and after three treatment regimens are depicted in Table 4. The most isolated bacteria among 30 mastitic milk samples before treatment were *Staphylococcus aureus* (12) and *E. coli* (8) followed by *Streptococcus* sp. (6), *Klebsiella sp.* (2) and mixed infection (2), which were reduced in post-treatment samples in all three groups, with complete absence of isolates particularly in Group B treated with Ozone + sensitive antibiotic (Table 4). *S. aureus* was also the most isolated bacteria (N=7) in study conducted by Ogata and Nagahata (2000) [11]. In place of using antibiotics, Shinozuka *et al.* (2008) [15] recommended the use of ozone therapy, which can reduce the endotoxin release from *E. coli* into the milk. They reported that ozone sterilization involves the destruction of microbial cell membranes.

Table 4: Treatment group wise bacterial isolates from milk samples of mastitis affected cattle pre-treatment and bacterial cure after-treatment

Sr. No.	Treatment Group	*Staphylococcus sp.* (N=12)	*E. coli* (N=8)	*Streptococcus sp.* (N=6)	*Klebsiella sp.*(N=2)	Mixed infection (N=2)
Bacteria isolated from mastitis affected cattle under different treatment groups on 'Day 0'(pre-treatment)						
1.	Group A	2	5	3	0	0
2.	Group B	5	2	1	1	1
3.	Group C	5	1	2	1	1
Bacterial cure in different treatment groups (post-treatment)						
1.	Group A	1	0	3	0	0
2.	Group B	0	0	0	0	0
3.	Group C	1	1	0	0	1

In the present study, majority of isolates were sensitive to ceftriaxone + tazobactum, cefoparazone, amoxicillin + sulbactum, and gentamicin (70.00-76.67%), with moderate sensitivity to others, and the least (33.33%) to cefotaxime + clavulonic acid (Table 5). The present results were in line with Kaliwal *et al.* (2011) [8] and Kumar *et al.* (2020) [9].

Table 5: Antibiotic sensitivity of bacterial isolates from mastitis affected cattle

Sr. No.	Name of Antibiotics	No of sensitive isolates (30)	Sensitivity (%)
1	Ceftriaxone + Tazobactum	23	76.67%
2	Amoxicillin + Sulbactum	22	70.00%
3	Ampicillin + Sulbactum	20	66.67%
4	Gentamicin	21	70.00%
5	Amikacin	16	53.33%
6	Ciprofloxacin	15	50.00%
7	Enrofloxacin	19	66.67%
8	Cefotaxime + Clavulonic acid	10	33.33%
9	Cefoparazone	21	73.33%

In the current study, every treatment group demonstrated clinical cure in terms of improving clinical signs, milk parameters (SCC, CMT, improvement in milk's colour, consistency and pH), udder consistency and inflammatory signs. Out of 10 cattle in treatment group A, 6 cows (60%) showed clinical cure and improvement in milk pH, SCC level and CMT score and biological cure. Treatment group B showed cure of 10 out of 10 cattle (100%) that received treatment with ozonated normal saline (@ 60 µg/mL) intramammary along with the most sensitive antibiotic in terms of all above criteria, while in Group C, 7 (70%) cattle out of 10 recovered, which received the most sensitive antibiotic alone.

According to the statistical analysis in the current study, the most efficient treatment regime was group B when compared to other groups (A and C) and was found statistically significant. The efficacy of group A and C was found significant when compared to group B in cattle for clinical mastitis.

Rilling and Viebahn (1990) [12] found that the effectiveness and convalescence of AT and OT therapies were nearly identical. Ogata and Nagahata (2000) [11] found changes in the

EC and SCC of cows in the O_3 and antibiotic groups of similar patterns in terms of inflamed quarter recovery after each therapy. The changes observed in O_3 and antibiotic were similar, indicating that O_3 had the same efficacy as antibiotic. The current study also showed similar results. According to Ogata and Nagahata (2000) [11] and Enginler *et al.* (2015) [6] *Streptococcus uberis* has a high resistance to ozone treatment in mastitis. The current results were consistent with these reports.

Conclusion

Clinical cases of mastitis in dairy cattle can be effectively treated with intramammary infusion of ozone alone or together with antibiotics. The results showed that ozone insufflation is a successful treatment regimen for curing clinical mastitis. However, the animals that received ozone therapy in addition to antibiotic therapy showed the most favourable results, leading to the conclusion that ozone therapy can be beneficial as a complementary approach in cases of clinical mastitis in dairy cattle.

Acknowledgement

This work was supported by all staff of KNPCVS, Shirwal, Dist. Satara, and Maharashtra.

Conflict of Interest

Not available

Financial Support

Not available

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How to Cite This Article

Wakade AS, Ambore BN, Wankhede GD, Borikar ST, Rangnekar MN, Mhase PP. Evaluation of Intramammary ozone therapy for clinical mastitis in dairy cows. International Journal of Veterinary Sciences and Animal Husbandry. 2025;10(1):226-229.