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Research Article

Intramammary ozone therapy in Candida mastitis

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ABSTRACT

This study investigated the effect of intramammary ozonated distilled water on treatment success rates for mastitis caused by Candida spp., which resulted from intensive antibiotic use. The study material involved 60 Holstein udder quarters infected with Candida spp., which were divided into an ozone treatment group (n=30) and a control group (n=30). To conduct the study, 25 µg/ml of ozonated distilled water was applied intramammary to each udder quarter from which the causative agent had been isolated. Milk samples were collected on days 6 and 18 after application, and microbiological and mycological analyses were performed on them. The analysis results showed no statistically significant difference in recovery rates between days 0-6 after treatment, but a significant difference emerged between days 6-18 ($P<0.05$). A significant statistical difference also existed in the overall recovery rates between the ozone and control groups ($P<0.05$). Consequently, the conclusion drawn was that utilizing ozonated distilled water may prove effective for treating mastitis caused by Candida spp. and may increase recovery rates.

INTRODUCTION

Mastitis denotes an inflammatory reaction occurring in mammary tissue, typically triggered by microorganisms or physical trauma. It is widely acknowledged as a prevalent issue in dairy farming, leading to notable economic losses attributed to diminished milk yield and quality (Cheng & Han, 2020). Regarding the etiology of udder infections in cattle, bacteria stand as the primary causative agents, with viruses, mycoplasma, algae, yeast, and fungi being less frequently implicated (Costa et al., 2012). While bacterial infections are most commonly associated with mastitis, studies have shown that the prevalence of yeast-induced mastitis varies from 1% to 12%. However, in specific herds, this prevalence may exceed 50% (Kirk et al., 1986).

In several research studies, mycotic infections of the mammary gland induced by yeast are predominantly attributed to Candida spp. Instances of mastitis infections resulting from Candida spp. have been documented in animals over an extended period. The earliest documentation of mycotic mastitis caused by Candida spp. dates back to Fleischer in 1930 (Du Pereez, 2000; Sartori et al., 2014). Additionally, yeast species such as Cryptococcus, Rhodotorula, and Trichosporum have also been implicated in mastitis among dairy cattle (Costa et al., 2012).

Yeasts are commonly found in humid environments abundant in organic materials and can be easily identified on teats and milking equipment (Akdouche et al., 2014). Prolonged use of antibiotics can lead to a decrease in vitamin A levels, consequently compromising the integrity of the mammary epithelium. This, in turn, facilitates the invasion of fungi and yeasts into the mammary glands (JasmMohammed and Yassein, 2020; Kalinska et al., 2017). Additionally, inadequate sanitation in the milking parlor and with milking equipment, improper handling of the cannulas used for intramammary treatment, and insufficient hygiene practices can result in severe yeast and fungal infections (Abd-El Razik et al., 2011).

While spontaneous recovery is possible in mastitis caused by fungi and yeasts, persistent yeast or fungal infections can endure for 6 to 12 months (Bourtzi-Hatzopoulou et al., 2003). Antifungal medications and supportive treatment approaches are typically utilized to manage mastitis resulting from yeast and fungi. Moreover, implementing management protocols and culling chronically infected animals can aid in preventing outbreaks (da Costa et al., 2012).

Diverse alternative therapeutic modalities have been employed for the treatment of both clinical and subclinical mastitis. These modalities encompass ozone therapy, acupuncture, saline, glyoxylate, homeopathic remedies, and the use of lactobacilli (probiotics). Among these options, ozone therapy has emerged as one of the more favored approaches. Ozone possesses bactericidal, fungicidal, and virucidal properties due to its oxidative effects on microorganisms (Sciorsci et al., 2020). However, it is noteworthy that yeasts and fungi exhibit greater resistance to ozone compared to bacteria (Varga & Szigeti, 2016).

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The present study aims to investigate the efficacy of intramammary administration of ozonated distilled water in the treatment of Candida spp.-induced mastitis in dairy cows with a history of mastitis due to intensive antibiotic usage. The study will evaluate the therapeutic outcomes based on California Mastitis Test (CMT) scores and mastitis type following the ozone therapy intervention.

MATERIALS and METHODS

Animal

The study was conducted on 240 Simmental cows aged 2-7 years in 2012-2014 province, Burdur. Medical records indicated the intensive use of systemic and intramammary antibiotic therapy for the treatment of clinical mastitis cases, with an average of 45 days elapsing between the antibiotic treatment and the commencement of the present study. Subsequent to bacteriological and mycological analyses, the animal material comprised 60 udder quarters (from 38 Simmental cows) exhibiting clinical or subclinical mastitis.

Collection of milk samples

Prior to sample collection, the teats were cleaned with 70% ethanol solution. After discarding the initial few streams of milk into a strip cup, milk samples were aseptically collected into sterile 10 ml syringes. All milk samples were properly labelled and transferred to the laboratory for microbiological and mycological analyses under cold chain conditions.

Mastitis determination and California Mastitis Test

The presence of clinical mastitis was assessed based on symptoms such as redness, edema, pain, induration in the udder, and the presence of visually abnormal milk. Udder quarters exhibiting any of these signs were recorded as clinical mastitis cases. Two ml of milk was sampled from each udder quarter. The California Mastitis Test (CMT) was performed according to the standard procedure, and results were scored as +1, +2, +3, +4, or +5 following the Scandinavian system (Klastrup, 1975).

Microbiological and mycological analyzes

Udder quarters milk samples with clinical mastitis, CMT>+1 score and Candida spp. growth were included in the study. Each milk sample was inoculated onto sheep blood agar and MacConkey agar and incubated at 37°C for 48 hours. Standard biochemical methods and tests were employed for the isolation and identification of bacterial isolates (Quinn, 2011). For mycological culture, each milk sample was inoculated in duplicate onto Sabouraud's dextrose agar containing 0.05 mg/ml chloramphenicol and incubated at 37°C and 25°C for 3-5 days. Yeast isolates were identified based on morphological and microbiological characteristics. The standard procedure was followed for the isolation and identification of Candida spp. (Jasm Mohammed & Yassein, 2020).

Groups and application of intramammary ozonated distilled water

The study comprised two separate groups: the ozone treatment group (n=30 udder quarters) and the control group (n=30 udder quarters). In both groups, the mammary quarters were completely milked out prior to the treatment applications. For the ozone group, 25 µg/ml ozonated distilled water (100 ml) was administered intramammarily at 12-hour intervals for a duration of 3 days. Similarly, in the control group, only non-ozonated distilled water (100 ml) was administered intramammarily.

Ozone production

A mobile ozone generator (Medozon Compact, Germany) was transported to the study site on the scheduled treatment days. Subsequently, 5 liters of distilled water were added to the bubbler accessory of the device, and the ozone generator was activated. The distilled water was ozonated at a concentration of 100 µg/ml for a total duration of 5 minutes under manometer control. As a result of the ozonation process, a gas/liquid mixture was prepared at a concentration of 25 µg/ml (Vertini, 2004). The intramammary administration of ozonated water was completed within an average of 15 minutes.

Statistical analyzes

Statistical analyses were performed using the Minitab 16 software package. The chi-square test was employed to evaluate differences in CMT scores and recovery rates between the groups. Statistical significance was set at the cut-off point as $P<0.05$.

RESULTS

Microbiological and mycological analysis

According to the results of microbiological and mycological analyses of CMT-positive milk samples, Candida spp. was isolated in combination with coagulase-negative staphylococci (CNS), S. aureus, or Streptococcus spp. in 7 udder quarters. In the remaining 60 mammary quarters, pure Candida spp. was isolated. The study focused solely on the 60 mammary quarters from which Candida spp. was isolated.

CMT scores and clinical presentation

The CMT score of +1 was considered negative for mastitis, while scores of +2, +3, +4, and +5 were considered positive. Subclinical Candida spp. mastitis was identified in 26 isolated mammary quarters, and clinical mastitis was present in 34 mammary quarters. Mammary quarters with clinical symptoms and CMT score +4 and higher were considered as clinical mastitis. The distribution of Candida spp.-isolated mammary quarters according to CMT scores is presented in Table 1. Mild redness, increased body temperature, and coagulation-like structures were observed in the udder quarters diagnosed with clinical mastitis. There was no significant difference in the incidence of subclinical and clinical mastitis based on CMT scores ($P>0.05$).

Recovery rates

The gold standard diagnostic method for mastitis is microbiological analysis. For this purpose, milk samples that yielded negative results in the microbiological analysis were considered indicative of recovery. Recovery rates increased at both

sampling time points in the ozone and control groups. However, no statistical difference in recovery rates was observed between the two groups after 6 days and 18 days of treatment. group. However, according to the data obtained at the end of the study, a statistically significant difference in recovery rates was found in the group with the CMT score of +5 (P<0.05).

Table 1. Distribution of mastitis according to CMT scores

	CMT score	Number of isolated udder quarters
Subclinical mastitis[a]	2	10
	3	16
Clinical mastitis[a]	4	15
	5	19

Statistical analysis was performed within the columns only. Different letters indicate statistically significant difference.

Table 2. A total number of healed udder quarters on the 0-6. and 6-18 days and at the end of the study by groups

Groups	Number of negative udder quarters on the 6. day after treatment	Number of negative udder quarters on the 18. day after treatment	Total number of negative udder quarters (Healing rate)
Ozone group	14[a]	9[a]	23[a] (%76,67)
Control group	8[a]	3[b]	11[b] (%36,67)

Statistical analysis was performed within the columns only. Different letters indicate statistically significant difference.

Table 3. Number of microorganism negative udder quarters on days 6 and 18 according to groups and mastitis scores

Distribution of groups according to CMT scores	CMT scores	Pre-treatment Candida (+)	0-6. days Candida (-)	6-18 days Candida (-)	Total Candida (-)
Ozone group	2	6	3	2	5
Control group		4	0	1	1
Ozone group	3	9	4	2	6
Control group		7	3	0	3
Ozone group	4	6	1	2	3
Control group		9	1	1	2
Ozone group	5	9	6	3	9[a]
Control group		10	4	1	5[b]

Statistical analysis was performed within the columns only. Different letters indicate statistically significant difference.

There was a significant difference in recovery rates between the two sampling time points (P<0.05). Additionally, a significant statistical difference was found in the overall recovery rates between the ozone and control groups (P<0.05) (Table 2). Recovery rates according to treatment groups and are presented in Table 3. When evaluating recovery rates based on CMT scores, the causative pathogen was not detected in almost all udder quarters across the groups. In fact, during the study period of 0-6 days and 6-18 days, complete recovery was not observed in either the ozone-treated group or the control

DISCUSSION

Yeasts are considered opportunistic pathogens of the mammary tissue. The types of mastitis caused by yeasts are closely related to environmental hygiene (Akdouche et al., 2014). The excessive use of antibacterial drugs (Crawshaw et al., 2005), contaminated antibiotic solutions, infected syringes, or other materials used in intramammary treatment may predispose the mammary tissue to yeast proliferation (Zaragoza et al., 2011). Additionally, the mammary defense mechanisms may be compromised due to potential immunosuppressive effects (Bekele

et al., 2019). Mycotic mastitis is classified into primary mycotic mastitis and secondary mycotic mastitis based on its mechanism of occurrence (Akdouche et al., 2014). In the present study, the yeast infections were defined as secondary mycotic mastitis. It was postulated that the resulting mastitis could have occurred due to the prolonged duration of antibiotic treatment or as a consequence of inappropriate manipulations within the mammary canal.

While bacteria are frequently reported as causative agents in mastitis cases, infections caused by yeasts and fungi are less common. However, these prevalence rates can vary according to farm conditions (Tel et al., 2009). In a study investigating the microorganisms responsible for mastitis, bacteria were identified in 64.10%, a combination of bacteria and yeast in 34.62%, and yeast alone in 1.28% of the 78 CMT-positive milk samples collected from 400 cows. In a separate study, Candida spp. was detected in 29.35% of a different herd experiencing a yeast mastitis outbreak due to inadequate hygiene conditions.

Onwuhafua et al. (2018) identified only 12.3% fungal isolates from 300 cows with subclinical mastitis across 26 dairy farms in Nigeria. As can be inferred from these studies, the prevalence of mastitis caused by yeasts and fungi can vary substantially depending on farm and hygiene conditions. The rates of yeast or fungal isolates reported in these studies are quite low compared to the findings of our study. This discrepancy can be attributed to the concurrent bacterial factors causing a relative decrease in the rate of yeast mastitis in those herds. In our study, the etiology of yeast-induced mastitis was associated with long-term antibiotic usage. Consequently, almost all of the isolated microorganisms consisted of Candida spp. Infections can sometimes occur as mixed infections originating from both yeasts and bacteria, while at other times, pure yeast colonies are observed (Crawshaw et al., 2005; Costa et al., 1993; Dudko et al., 2010; Zaragoza et al., 2011).

Candida spp. is the most frequently isolated species from mycotic isolates in different studies (Bourtzi-Hatzopoulou et al., 2003; Czernomysy-Furowicz et al., 2008). However, it is possible to encounter diverse yeast and fungal species in dairy herds (JasmMohammed and Yassein 2020). Various yeast species, such as Candida, Cryptococcus, Rhodotorula, and Trichosporon spp., have been associated with mastitis in dairy cows (Akdouche et al., 2014). Du et al. (2018) collected 482 milk samples from cows with clinical mastitis across 4 different herds, and microorganisms were isolated in 256 of these samples. A total of 60 isolates belonging to nine different Candida species were detected in 23.44% of these samples. In our study, all the yeast agents isolated were identified as Candida spp. The reason for this predominance is that Candida was likely transmitted throughout the herd via milking equipment, udder tubes used for intramammary applications, and other environmental contaminants during the long-term antibiotic treatment period.

Milanov et al. (2014) identified the presence of bacteria (73.49%) and yeasts (6.02%) in cows exhibiting both clinical and subclinical mastitis. Costa et al. (2012) reported the detection of Candida yeasts at a rate of 29.35% in a farm undergoing a yeast mastitis outbreak in Brazil and noted that the mastitis type comprised 6.8% clinical and 30.2% subclinical cases when considering all causative factors. Indeed, the manifestation of clinical symptoms in yeast and fungal mastitis might be linked to the causative agents or their virulence factors (Sukumar and James, 2012). Similarly, in the present study, both clinical and subclinical yeast mastitis were observed, yet no statistically difference was discerned between the two mastitis types. In most clinical instances, mastitis induced by yeasts and yeast-like fungi presents with udder induration and watery or coagulated milk (Sukumar and James, 2012; Şeker, 2010). In certain cases, systemic symptoms may also manifest (Bourtzi-Hatzopoulou et al., 2003). In our investigation, akin symptoms were observed in clinical cases, albeit no systemic issues were noted. The extensive antibiotic and supportive treatment administered 45 days prior to the study commencement is presumed to have mitigated more severe clinical disorders in the udder and milk.

Antifungal medications are commonly employed in the treatment of yeast mastitis. Furthermore, the utilization of probiotics and bioactive natural compounds in therapies has shown promising outcomes (Abd-El Razik et al., 2011). It is recognized that yeasts exhibit varying degrees of sensitivity and resistance to antifungal drugs, including miconazole, ketoconazole, amphotericin B, itraconazole, nystatin, fluconazole, fusidic acid, and voriconazole. Studies have indicated that intramammary administration of miconazole at a dosage of 100 mg/50 ml can yield effective results; however, systemic administration may lead to hepatic and cardiovascular side effects (Asfour et al., 2021). Nonetheless, numerous antifungal drugs can be toxic to udder tissue (Du Preez, 2010). In contrast, the ozone employed in this study did not induce any toxic or systemic adverse effects in the animals.

The medical application of ozone relies on its antioxidant, immunostimulant, and antimicrobial properties. Particularly, when ozone is administered to exert its antibacterial, antiviral, antifungal, anti-yeast, and antiprotozoal effects, no residue is left in tissues and biological fluids post-application (Sciorsci et al., 2020). Ozone has demonstrated effectiveness in treating various forms of mastitis in recent years. Studies have aimed to enhance healing rates, reduce bacterial load, improve clinical symptoms, and decrease the number of somatic cells in bacterial mastitis cases (Ogata and Nagahata, 2000; Jo et al., 2005). However, there is a scarcity of studies investigating the use of ozone in treating yeast mastitis. Research concerning yeast and contamination primarily focuses on food products and storage conditions.

Numerous studies have explored ozone application using various methods and doses. Jo et al. (2005) utilized ozonated oil in cows with chronic mastitis, observing a decrease in somatic cell counts in the ozonated groups, although no statistical difference was found compared to the control group. Conversely, Sertkol et al. (2018) investigated the efficacy of intramammary ozone gas application on bacterial mastitis, concluding that it had no curative effect in acute clinical mastitis caused by Streptococcus spp. and yeast. Enginler et al. (2015) assessed the effectiveness of different doses of ozone gas in combination with antibiotics in cows with clinical

mastitis. They found that ozone insufflation was effective in treating clinical mastitis, with the best results achieved in the group using high-dose ozone (70 µg/mL) alongside antibiotic treatment. In our study, we administered a volume of 100 ml of ozonated water into the mammary lobe, aiming to cover the majority of the glandular area in the udder and thereby achieve effective treatment. The ineffectiveness of ozone on yeast forms observed in other studies may be attributed to the ozone mixture administered in gas form failing to reach a sufficient concentration in all glandular tissues of the udder. Hassan et al. (2016) conducted ozone fumigation on yeast and fungal isolates collected from buffaloes with mycotic mastitis. They demonstrated that low-dose (20ppm) ozone application against Fusarium and Candida spp among fungal isolates effectively reduced fungal growth in a short time. Similar results were obtained in our study concerning mycological recovery against Candida spp. The homogeneous distribution of ozonated water in the udder is presumed to have contributed to this outcome. It can be inferred that an adequate amount of ozone was reached in the isolate in our study, conducted in both the culture medium and tissue.

Recovery rates in yeast mastitis are closely intertwined with the type of causative agent. Yeasts inherently exhibit greater resistance to ozone compared to bacteria, owing to their thicker cell walls. They are also more susceptible to ozone than fungi (Varga and Szigeti, 2016). Moreover, varying sensitivities between yeast species have been reported. Debaryomyces spp. are noted to be more prevalent than other species such as (Vallone and Stella, 2014; Varga and Szigeti, 2016). The isolation of Candida spp. in the study conferred an advantage in treatment.

CONCLUSION

In conclusion, intramammary application of ozonated distilled water did not impact Candida spp. It has been deduced that this method is effective in treating mastitis as it enhances recovery rates and presents itself as an alternative to antibiotic use. Additionally, ozone does not cause harm to intramammary structures, rendering it safe for intramammary applications.

DECLARATIONS

Ethics Approval

The experimental procedures were approved by the Committee of Animal Experiments of Burdur Mehmet Akif Ersoy University. Approval number:MAKU-HADYEK 2013/35

Conflict of Interest

The author declare that they have no conflict of interests.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: A.K.

Data collection and analysis: A.K.

Drafting of the manuscript: A.K.

Critical review: A.K.

Data Availability

The data used to prepare this manuscript are available from the corresponding author when requested.

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