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Ozone use in the treatment of subclinical mastitis in dairy cows

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Research Article

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Abstract

This research communication paper addresses the hypothesis that the use of therapeutic alternatives for mastitis, such as intramammary ozone, can cure the disease with lower costs and without harmful residues for human consumption and without formation of microbial resistance like the ones caused by indiscriminate use of antibiotics in dairy farms. The study was performed in 36 mammary quarters from 12 dairy cows with subclinical mastitis grade three. The experimental units were randomly assigned into four groups and each group received a treatment. Treatments comprised (a) 20 µg/ml ozone gas; (b) 40 µg/ml ozone gas; (c) negative control treatment of 12.5 µg/ml ozonated saline and (d) positive control treatment of 100 mg of cephalexin + 100 mg of neomycin + 10 mg of prednisolone, all by intramammary injection. In all quarters, milk was collected before and after the application of treatments for California mastitis test and evaluation of milk composition, somatic cell count, and bacterial cultures. The results indicated that the use of intramammary ozone did have a therapeutic effect, and whilst this was less than that of antibiotics, ozone does confer some advantages. Treated milk had a good composition, the treatment cost was low, milk withdrawal may not be necessary and there is no risk of antibiotic resistance.

Mastitis is an inflammation of the mammary gland and its secretory tissues and is often regarded as the most common and most difficult disease to eradicate in dairy cows. The etiopathogenesis is usually infectious and classified as a multifactorial disease, and its prevalence depends on several risk factors such as the individual, environment, hygiene and causative agent. The treatment of this condition is done using antibiotics, which generates great costs, bacterial resistance, residues in milk, milk disposal and reduced production. More significantly, subclinical mastitis is still the leading cause of antibiotic use in herds, and the use of such milk with antibiotic residues is a potential problem in food production concerning human health (Dután Sanango, 2014).

Ozone-therapy is an integrative therapy that uses ozone gas. Ozone is generated by manipulation of O_2 molecules into O_3 through an electrical discharge. It is used in distinct ways such as the gas itself, autohemotherapy and ozonized saline (Bocci, 2011). Ozone's oxidizing properties allow it to act on the bacterial wall by attacking the olefinic bonds, dispersing the cellular cytoplasm and destroying the cell membrane. This effect may vary according to the concentration used, requiring different concentrations and time of contact with the bacterial wall to ensure efficacy against Gram-negative and Gram-positive bacteria. An important factor to be considered in the present study is the fact that ozone therapy does not cause bacterial resistance and, therefore the ozone-therapy is an effective, economic and ecological therapeutic method, with high antimicrobial potential (Bocci, 2011; Franzini *et al.*, 2016; Girgin Ersoy *et al.*, 2019).

We used ozone therapy as an alternative method in the treatment of subclinical mastitis, verifying its effect on the composition of the milk, its effectiveness against Gram-positive and Gram-negative bacteria of the affected quarters and comparing costs between the use of antibiotics and ozone-therapy.

Material and methods

This research project was performed on a dairy farm in the municipality of Batalha, Alagoas, Brazil, with approval from the Ethics Committee on Animal Use of the Federal University of Alagoas (CEUA-UFAL) under registration number 04/2020. The study was performed in 36 mammary quarters from 12 crossbred dairy cows (Holsteins × Gir) of varied ages from extensive breeding. To choose the quarters to be used, the California mastitis test (CMT) was performed on the entire herd during the milking hour at the barn and, thus, the groups to be treated were chosen based on the grade of subclinical mastitis (1–3). Cows in mid-lactation period, without previous history of subclinical mastitis with one or more quarters with grade 3 non-treated subclinical mastitis, were randomly assigned into four groups. Treatments comprised ozone gas at 20 and 40 µg/ml (G20 and G40), ozonated saline at 12.5 µg/ml (OS) and a positive control treatment of 100 mg of cephalexin + 100 mg of neomycin + 10 mg of prednisolone (PCA), all by intramammary injection. The tests and treatments were made by a veterinarian and assistants who were blinded to the treatment, following the milking order dictated by the husbandry staff. The sample size was calculated using the following formula:

$$\frac{N = z^2 \times p \ \times (1\text{-}p)}{E^2}$$

Z = critical value corresponding to the desired confidence level
p = population proportion (%)
E = desired margin of error

$$\frac{N = (1.96)2 \times 0.10 \ \times (1\text{-}0.10) = 34.5744}{(0.1)^2}$$

Since it is not possible to have a fraction of a quarter, 35 quarters were defined as the final number. After this delineatation of the quarters to be treated, the order of the treatments to be made was randomly chosen (through a simple randomization method) where G20 and G40 were used in 10 quarters each, PCA was used in 4 quarters and OS in 12 quarters (Supplementary Table S1). Before the first treatment was done, the cows were identified, and two pre-milking milk samples were collected from the affected quarters using aseptic technique with application of 70% alcohol. One sample was taken into a tube with Brononata preserving agent and sent for analysis of milk composition and another sample was sent for microbiological culture to identify bacteria associated with the mastitis. Once the samples were collected, the milking was conducted normally. The treatments were then performed at the end of milking, again using aseptic technique with application of 70% alcohol. After the treatments, the cows were returned to their usual pasture. All treatments were applied once every 24 h for three consecutive days. On the fourth day (24 h after the last treatment), another CMT test was performed and, again, two samples were taken to repeat the tests and compare the changes in the milk before and after the treatments.

In the case of G20 and G40 ozone gas, 20 and 40 µg/ml respectively of the gas in 20 ml disposable syringes were used, to which a sterile stainless-steel intramammary cannula was adapted. In the case of OS, 0.9% ozonized sodium chloride was used, which was subjected to bubbling for 10 min with a generator (Ozone & Life ozone generator model O&L PORTABLE), calibrated to obtain a gas concentration equal to 50 µg/ml, resulting in a final concentration of 12.5 µg/ml (Yoldi *et al.*, 2019). Once the saline was ozonized, the doses were prepared in 10 ml disposable syringes, to which a stainless-steel intramammary cannula previously sterilized for each syringe was attached. A new ozonized saline was prepared daily, discarding the excess portion.

For the analysis of milk composition, the following variables were considered: fat, protein, lactose, total dry extract, total defatted dry extract, somatic cell count (SCC). The samples were sent to the Instituto Clinica do Leite® laboratory in Piracicaba, São Paulo. The collected samples were kept under refrigeration at an average temperature of 5°C until arrival at the laboratory. For the microbiological analyses, the samples were kept at an average temperature of 5°C and sent directly to the laboratory for processing on the same day. The microbiological evaluation method was culture in chromogenic medium, using materials, equipment and procedures patented by OnFarm® based in Piracicaba, São Paulo. However, these analyses were conducted by the representative of OnFarm®, Plantel Agropecuária, based in Batalha, Alagoas. The technique was performed an average of 4 h after collections, and the culture plates were inoculated and placed in an incubator for reading 24 h after inoculation.

Milk composition data were submitted to descriptive and variance analysis. Analysis of variance was performed by one-way ANOVA, with treatment as the only effect. Means were compared using the Student–Newman–Keuls test to compare means at a 5% probability level ($P < 0.05$). To approximate the distribution of the SCC variable to the normal distribution, the SCC data were log-transformed in base 10.

For data analysis of CMT and microbiological growth, non-parametric data analysis was used due to the behavior of these variables. Then, the chi-square test (χ^2) was used, at the 5% probability level, chi-square test ($P < 0.05$). All statistical analyses were performed using the R® statistical program. Further details of the materials and methods are provided in the online Supplementary File

Results

At the end of the treatments, there was a loss of two samples in the G40 treatment, as the milk from the quarters presented alterations that made it impossible to read the samples according to laboratory standards. These losses were considered in the statistical analysis. Descriptive statistics for the different variables are presented in online Supplementary Table S2 and treatment means are given in Table 1. Statistically, there was no significant difference between treatments in any of the components evaluated in the physicochemical analysis of the milk, with the exception of total dry extract which was lower for the G40 and G20 treatments and lowest in G40 (Table 1).

In the microbiological results (Table 1), bacterial growth was eliminated in 3 of the 4 PCA (antibiotic) quarters (75% recovery), 3 of the 11 G20 quarters (27% recovery) and 5 of the 12 OS quarters (42% recovery). None of the G40 quarters recovered, with 22% of quarters showing new growth and 78% sustained growth. New growth was present in 18% of the G20 quarters, the remaining 55% of infected quarters having sustained growth. No new growth was present in PCA quarters.

Figure 1 shows the isolates identified after culture analysis, and Supplementary Table S3 shows data for bacterial resistance to treatment. In PCA quarters the predominant bacteria present before treatment were Gram-positive *Streptococcus uberis, Staphylococcus aureus* and non-aureus *Staphylococci*. After treatment the greatest effect was obtained on Gram negatives, specifically *E. coli*, and amongst Gram positive specifically non-aureus *Staphylococci*, where at the end of the treatment there was no growth. In the case of *Staphylococcus aureus* and *Streptococcus uberis*, there was growth, but it was smaller than the initial growth.

Most G20 quarters showed the presence of Gram-positive bacteria, *Staphylococcus aureus,* non-aureus *Staphylococci*, *Streptococcus agalactiae, S. dysgalactiae* and *S. uberis*. The best results in controlling bacterial growth were found with *Streptococcus agalactiae* and *S. dysgalactiae*, in which there was

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Table 1. Adjusted means of milk components, California mastitis test (CMT) results and microbiological evaluation of quarters submitted to the different treatments

		Milk components						California mastitis test				Microbiological evaluation			
	No of quarters	Fat	Protein	Lactose	TDE	TDDE	TSCC	CMT (−)	CMT (+)	CMT (−) %	*P value*	IQ	IQ%	BG (−)	*P value*
PCA	4	3.17	3.40	4.47	12.14[a]	8.97	6.97	4	0	100	–	1	25	3	–
G40	10	2.16	3.07	4.07	10.34[b]	8.19	7.52	0	10	0	0.0002	10	100	0	0.3932
G20	11	2.91	3.21	4.25	11.39[ab]	8.48	6.93	1	10	9.09	0.001	8	73	3	0.6797
OS	12	3.56	3.53	4.20	12.36[a]	8.80	6.65	3	9	25	0.008	7	58	5	0.5510

PCA, positive antibiotic control; G40 and G20, ozone gas at 40 and 20 μg/ml; OS, ozonated saline at 12.5 μg/ml.
TDE, total dry extract; TDDE, total defatted dry extract; TSCC, somatic cell count transformed to log base 10.
IQ, Infected Quarters; BG(−), quarters without bacterial growth.

no growth after treatment. In the case of *Streptococcus uberis,* there was an improvement of more than 50%, while non-aureus *Staphylococci, Staphylococcus* untyped and Gram-positive untyped also showed improvement. In G40 quarters only other Gram positives were sensitive and *E. coli*, with recovery of 1 treated quarter and, in the case of *Staphylococcus aureus*, recovery of 2 quarters out of 7 treated (Fig. 1C). The other bacteria showed greater growth after the application of the treatment.

For quarters treated with ozonized saline, the most sensitive bacteria were untyped Gram positive bacteria, *Klebsiella, Enterobacter, Staphylococcus aureus* and non-aureus *Staphylococci* as well as *Streptococcus uberis*. In turn, bacteria non-sensitive to treatment were untyped Gram negative, prototheca and yeasts.

The costs involved within the treatments shows a considerable difference between control treatment and ozone treatment. The cost of each dose of control treatment was (US) $1.86 ($5.58 for four quarters). Taking into account all materials used in G20 and G40 ozone treatments (1L oxygen refill for 1000 doses, US $ 0.01 per dose, syringes $0.19 per unit) the per dose cost for ozone was $0.20 and total cost of $0.60 for the whole treatment. For OS treatment, the smaller syringe costs $0.13 per unit and the physiological solution costs $0.16 per dose, resulting in $0.30 per dose and a total treatment cost of $0.90.

Discussion

Our results indicate that the use of ozone in the form of gas does not generate changes in milk composition (when compared to the use of antibiotics and excluding a small change in total dry extract) and does to some extent reduce the prevalence of infection. This agrees with Ogata and Nagahata (2000) and Argudo and Soria (2017), in the first of which an improvement of 60% was obtained after a single application of ozonized gas by intramammary insufflation and, in the second, three applications of gas resulted in an improvement relative to ozonized saline. In both cases the positive control treatment with antibiotics had better results compared to the treatments with ozone, and it would be of interest to consider the combined treatment of ozone with antibiotics in lower doses or reduced treatment time in the future.

There appeared to be no dependence between the application of ozone and the CMT test, therefore the values obtained did not suffer a direct effect from the application of ozone. However, it should also be considered that the CMT reading was taken in the 3-day interval between the first and the last test, when the bacterial presence can be affected by the ozone effect, but the total effect in terms of inflammation has not yet been achieved, so it would be relevant for future research to perform a third CMT test in about 5–7 d and evaluate its residual effect in terms of the inflammatory process.

In the case of quarters infected by microorganisms, there is a correlation between the growth of microorganisms and the administration of G20 and OS but not G40. The OS treatment presented the best results for having a greater number of recovered quarters, in addition to maintaining stable bacterial growth after treatment. The most sensitive bacteria to OS were untyped Gram-positive bacteria so the treatment of Gram-positive infections with OS can be recommended. On the other hand, the treatment with the worst performance was G40, which did not show improvement, with about 1/5 of the quarters showing new bacterial growth and 3/4 of the remaining quarters with sustained bacterial growth. The G20 treatment was intermediate. Our results agree with those of Martins *et al.* (2020), with intramammary

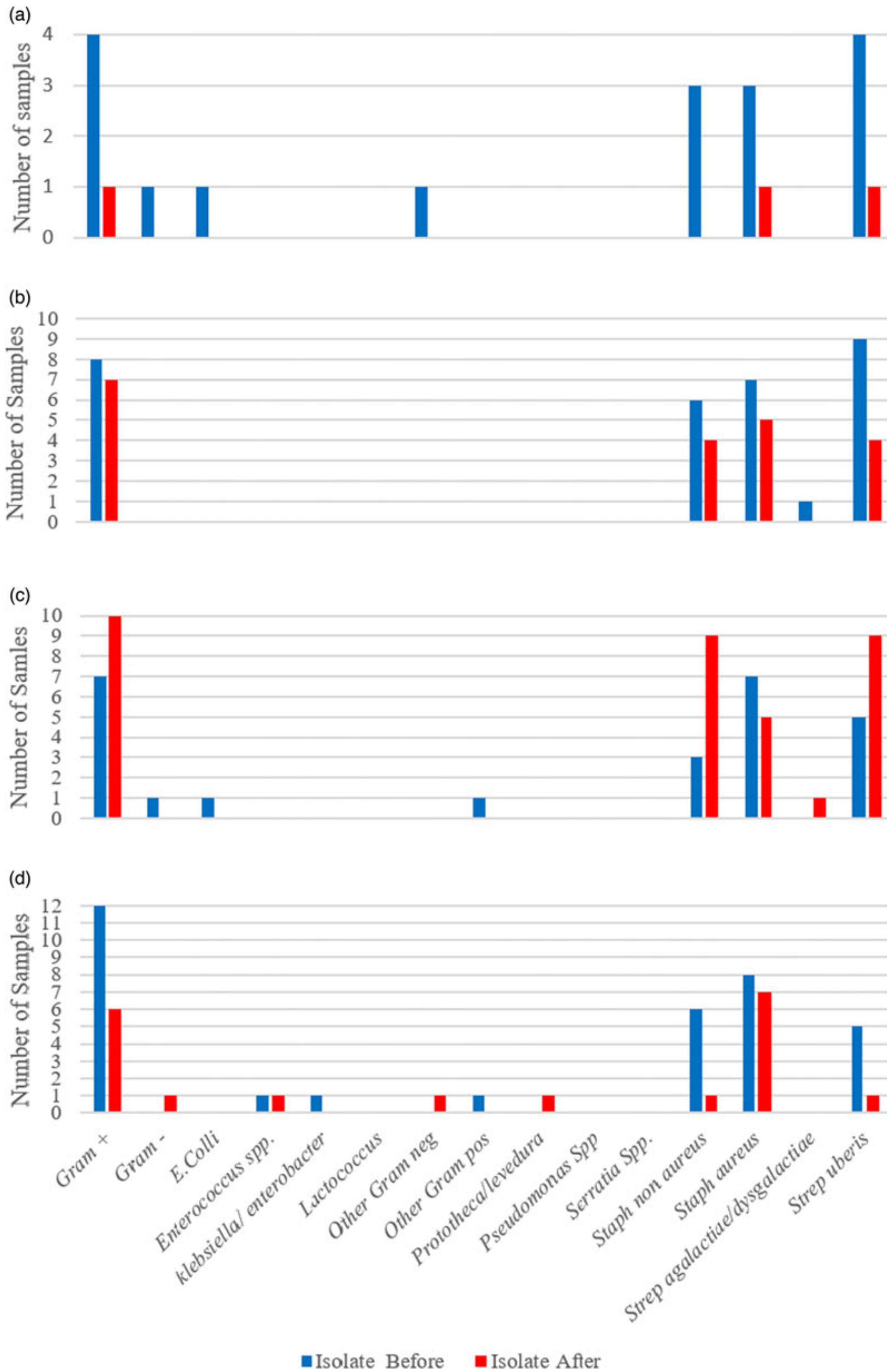

Figure 1. Bacterial isolates obtained before (blue bars) and after (red bars) treatment with (A) a positive antibiotic control (PCA), (B) 20 and (C) 40 μg/ml ozone gas (G20, G40) and (D) 12.5 μg/ml ozonized saline.

ozone in cows with positive mastitis where an improvement was obtained in the affected quarters, but the mastitis was not completely eliminated. An explanation could be that mastitis with Gram-negative bacteria requires longer exposure to ozone or more doses to achieve an adequate therapeutic effect (Girgin Ersoy *et al*., 2019).

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After a complete treatment with antibiotics there was still the presence of *Staphylococcus aureus* and *Streptococcus uberis* (Fig. 1A), which would indicate that these bacteria already have resistance to the antibiotic used. From this, it is noted that treatment with ozone, in any of the presentations, is more recommended than the use of antibiotics, considering the reduction in the prevalence of quarters affected by microorganisms, with G20 and saline being the most recommended.

These studies have produced a variety of results, paving the way for generating new experiments that also evaluate the effectiveness of ozone in the form of combinations, as in the case of the study carried out by Girgin Ersoy *et al.* (2019), who evaluated different concentrations of ozone gas and a combination of gas with antibiotic, obtaining better results in the combined use of gas and antibiotic. Studies are also suggested to evaluate ozone oil combinations such as the study by Quintana *et al.* (2019), which showed improvements in oil treated cows. It is important to further investigate such factors as the ozone concentration and form of application, and measure efficacy both at a therapeutic and disinfectant level in the search for better protocols with synergism between doses, routes, forms and combined use with antibiotics. It would also be interesting to evaluate other aspects of the milking routine aligned with distinct types of ozone exposure.

A relevant point in conducting this research was to understand the costs involved with the treatments. It is noted that the costs of the treatments with antibiotics and with ozone were quite different. Ozone treatments would present a better cost-benefit ratio for any farmer who already had the ozone generator, and in cases where the farmer would have to buy the generator the cost–benefit ratio would still be better on a long-term investment. The therapeutic effectiveness of ozone was less than that of antibiotic, but there are advantages. Depending on legislation (which differs between countries), treated milk may not require disposal, the milk composition remains the same and ozone does not causes bacterial resistance, this being a relevant point in relation to human health as well (Franzini *et al.*, 2016).

Thus, we conclude that intramammary ozone in the form of ozonized gas or saline is an alternative that can be considered for the treatment of subclinical mastitis. It may help combat microbial resistance and indiscriminate use of antibiotics on dairy farms as well as yielding a better cost–benefit ratio, but more research is needed in a greater number of animals to support this in practice, evaluating different mechanisms of use, concentrations and exposure times, as well as ozone and antibiotic combinations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029923000808

References

Argudo D and Soria C (2017) La ozonoterapia como alternativa de tratamiento para la mastitis clínica en ganado de leche.

Bocci V (2011) *Ozone – A New Medical Drug*. Dordrecht: Springer Netherlands.

Dután Sanango AI (2014) *Determinación de la Eficacia de Ozonoterapia en el Tratamiento de Mastitis Clínica en Vacas*. Cuenca: Universidad Politecnica Salesiana.

Franzini M, Valdenassi L and Ionita G (2016) First evaluations of oxygen-ozone therapy in antibiotic-resistant infections. *Ozone Therapy* **1**, 5.

Girgin Ersoy Z, Barisci S and Dinc O (2019) Mechanisms of the *Escherichia coli* and *Enterococcus faecalis* inactivation by ozone. *LWT Food Science and Technology* **100**, 306–313.

Martins CV, Laudino N de J, Menezes ACJ and Blankenheim TM (2020) Tratamento de mastite clínica e subclínica em vacas leiteiras com gás ozônio intra-mamário. A Pesquisa Nos Diferentes Campos Da Medicina Veterinária 3. Atena Editora, pp. 173–182.

Ogata A and Nagahata H (2000) Intramammary application of ozone therapy to acute clinical mastitis in dairy cows. *Journal of Veterinary Medical Science* **62**, 681–686.

Quintana MCF, Domingues IM and Ribeiro AR (2019) Uso de óleo ozonizado no tratamento de mastite subclínica em vaca Jersey: Relato de caso. *Pubvet* **13**, 1–4.

Yoldi CF, Hidalgo Ó, Ramos JF and Sánchez R (2019) Medida de la concentracion del ozono en agua en dosis bajas. *Ozone Therapy Global Journal* **9**, 61–73.