Ozone Use in the Treatment of Subclinical Mastitis in Dairy Cows.

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SUPPLEMENTARY FILE

Materials and methods

This research project was developed 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 x Gir) 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) (supplementary figure 1). The cows in mid lactation period with one or more quarters with grade 3 subclinical mastitis, were randomly assigned into four groups (CT, G40, G20, OS), as described in Supplementary Table 1. The tests and treatments were made by a veterinarian and assistants initially blinded, following the milking order according to the workers on the farm.

The sample size was calculated using the following formula:

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

WHERE:

Z = critical value corresponding to the desired confidence level

p = population

proportion (%)E =

desired margin of error

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

Note: Since it is not possible to have a fraction of a quarter, 35 quarters were defined as the finalN.

Z = 1.96

P= 10%

E= 10%

The sample size (N = 30) is based on an equitable division into three groups of 10 participants each,which is essential for our study. Although the sample size is limited, it was chosen based on considerations of statistical power and the need to maintain practical feasibility, as outlined below:

- Equitable Distribution: The division into three equal-sized groups ensured a fair comparisonamong the evaluated ozone concentrations.
- Statistical Power Consideration: Despite its limitation, three groups of 10 each allowed for a reasonable statistical analysis to detect significant differences.
- Acknowledgment of Limitations: The limited sample size could have affected the detectionof small effects or generalizability to a broader population. However, given the exploratorynature of this study to lay the foundation for future research, it was deemed an acceptablelimitation.

The 10% margin of error used is considered high in many research contexts. However, it was considered acceptable in this study for several reasons:

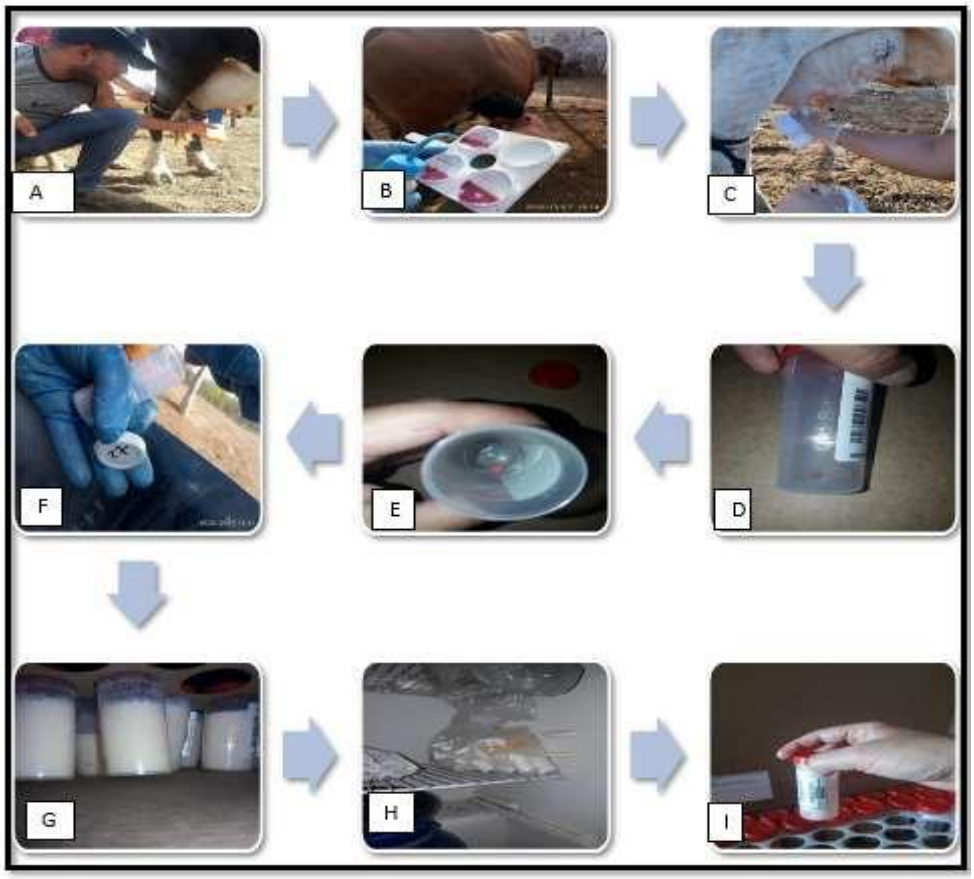
Nature of the Research: This study had an exploratory purpose to establish a baseline for new techniques, where very high precision was not essential.

Study Objectives: The primary aim of the study was to generate knowledge about ozone administration routes and effective concentrations, necessitating a baseline before more precise future investigations.

Available Resources: Logistical limitations, such as the availability of cows and farms during the pandemic, made obtaining a larger sample size impractical.

Results and Conclusions: Despite the limited sample size, the results provided valuable information regarding the effect of ozone on subclinical mastitis in dairy cows within the scope of the evaluated concentrations.

Supplementary Figure 1- CMT and storage of milk samples



Source: Authors.

Caption: Pre-dipping to perform CMT (A); CMT test (B); Antisepsis for sampling (C); Tubes for sample collection (D); Milk preservative in tube for samples (E); Sample collection (F); Samples ready and stored under cooling for later shipment to the laboratory (G, H and I).

Table S1- Division of treatment groups with grade 3 (1-3) of subclinical mastitis.

Treatment	Total Quarters	Mastitis
Antibiotic (CT)	4	Subclinical grade 3
Ozone gas 40 µg / mL (G40)	10	Subclinical grade 3
Ozone gas 20 µg / mL (G20)	10	Subclinical grade 3
Ozonated saline at a concentration of 12.5µg/mL (OS)	12	Subclinical grade 3

After this delimitation of the quarters to be treated, the order of the treatments to be made was randomly chosen (through simple randomization method). Then, the treatments were performed at the end of milking, after antisepsis with 70% alcohol. Immediately before the first application of each treatment, the cows were identified and, two milk samples were collected from the affected quarters, one sample was sent for analysis of milk composition and another sample sent for microbiological culture to identify bacteria associated with the presented mastitis.

Once the samples were collected from each quarter, the milking was conducted normally and, at the end of the milking, the proper treatments were applied. After the treatments, the cows were allocated on their usual pasture.

All treatments were applied every 24 hours for three consecutive days after milking. On the fourth day (24 hours 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 G40 (40 µg/mL) and G20 (20 µg/mL), ozone 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% ozonated Sodium Chloride was used, which was subjected to bubbling for 10 minutes with the 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 (Supplementary figure 2).

Suppementary Figure 2- Methodology of preparation and therapeutic execution.

Source: Authors.

Caption: Materials used in the field (A); Saline ozonation (B); Filling syringes (C); Syringes ready for application (D); Insertion of the intramammary cannula (E); Infiltration of the ozonized suspension (F); Teat antisepsis (G); Control treatment antibiotic syringe (H).

For the analysis of milk composition, the following variables were considered: fat, protein, lactose, total dry extract, total defatted dry extract (TDS), Somatic Cell Count (SCC). The samples were sent to the Instituto Clinica do Leite® laboratory in Piracicaba, São Paulo. The Laboratory provided the previously requested tubes with the Brononata preserving agent. 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 the culture in chromogenic medium, using materials, equipment and procedures patented by OnFarm® based in Piracicaba, São Paulo. However, these analyzes were conducted by the representative of OnFarm®, Plantel Agropecuária, based in Batalha, Alagoas. The technique was performed an average of 4 hours after collections, and the culture plates were inoculated and placed in an incubator for reading 24 hours after inoculation.

Information on milk constituents, fat, protein, lactose, total dry extracts, total nonfat dry extract (TDS), Somatic Cell Count (SCC) of the experimental units were submitted to descriptive and variance analysis. Analysis of variance was performed by ANOVA, one-way ANOVA, with treatment as the only effect at levels 4 [control treatment (CT);

ozonized gas solution at 40 µg/mL (G40); 20 µg/mL ozonized gas solution (G20) and 12.5 µg/mL ozonated saline solution (SO)]. Means were compared using the Student Newman Keuls test (SNK) 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 as follows.

$$TSCC=\log_{10}SCC$$

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 ($\chi 2$) was used, at the 5% probability level, chi-square test ($p<0.05$). Statistical analyzes were performed using the R® statistical program (R Core Team, 2022).

Results

Variable	Average	Standard Error	Standard deviation	VC (%)	Minimum	Maximum
Fat	2.99	0.21	1.21	40.48	1.02	5.76
Protein	3.32	0.06	0.36	10.97	2.76	4.00
Lactose	4.21	0.08	0.49	11.53	2.95	4.74
Total dry extract.	11.58	0.26	1.52	13.10	8.98	14.34
Total defatted dry extract	8.58	0.12	0.67	7.81	6.87	9.79
SCC	2,004.61	471.58	2,709.00	135.14	271	9999
TSCC	6.97	0.19	1.06	15.25	5.60	9.21

Supplementary Table S2. Descriptive statistics of variables fat, protein, lactose, total dry extracts, Total defatted dry extract, SCC and TSCC (96 hours after treatments).

SCC - somatic cell counts;
TSCC - SCC transformed to log in base 10
VC – Variation coefficient

Supplementary Table S3. Sensitivity of bacteria to treatments.

Bacteria	Treatments			
	PCA	G20	G40	OS
Non-typed Gram Negatives	+	N/R	N/R	R
Escherichia coli	+	N/R	N/R	N/R
Enterococcus spp	N/R	N/R	N/R	N/R
Klebsiella/ Enterobacter	N/R	N/R	N/R	+
Lactococcus spp	N/R	N/R	N/R	N/R
Another negative gram not typified	+	N/R	N/R	R
Another gram positive non-typed	N/R	N/R	+	+
Prototheca/ Yeast	N/R	N/R	N/R	R
Pseudomonas spp	N/R	N/R	N/R	N/R
Serratia spp	N/R	N/R	N/R	N/R
Staphylococcus non-typed	+	+	R	+
Staphylococcus aureus	+	+	+	+
Streptococcus agalactiae/dysgalactiae	N/R	+	R	N/R
Streptococcus uberis	+	+	R	+
+=Sensitive to treatment				

R=Showed resistance to treatment

N/R= No resistance to treatment

References

R Core Team (2022) R: A language and environment for statistical ## computing.

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.