

Efficacy of ozonized water for the management of endometritis in Jersey crossbred cows and factors influencing pregnancy

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ABSTRACT

Endometritis, a major reproductive disorder, adversely affects fertility and productivity in cows. Ozone has been explored as a potential non-antibiotic alternative against endometritis. This study evaluated the therapeutic efficacy of intrauterine ozonized water (O₃W) in Jersey crossbred cows suffering from endometritis and investigated factors influencing pregnancy outcome. A total of 60 consecutive spontaneous estrous cycles, (pre-treatment [C-1], treatment [C-2], and post-treatment [C-3] phases) were monitored in 20 endometritis cows. At C-1 estrus, endometritis was confirmed by the presence of $\geq 1\%$ polymorphonuclear (PMN) cells in endometrial cytology. During C-2 estrus, cows received intrauterine O₃W treatment (3300 μ g; 55 μ g/mL; 60mL) for two consecutive days. At enrolment, general parameters including age, parity, milk yield, number of previous inseminations without conception, and body condition score were recorded. Comparative assessment between C-1 and C-3 included evaluation of cervical discharge, preovulatory follicle, corpus luteum, estrous cycle parameters, hormonal profile, oxidative stress status, and reproductive performance. Microbial growth was detected in 75% of cows, predominantly involving *Escherichia coli* and *Staphylococcus aureus*. Following O₃W treatment, microbial burden significantly decreased, with 60% of cows exhibiting no detectable microbial growth. Improvements were observed in discharge quality, follicular size, estrus characteristics, progesterone profile, and oxidative stress markers. The first-service and overall (three inseminations in C-3) pregnancy rates were 50% and 70%, respectively. However, advanced age, a greater number of previous unsuccessful inseminations, higher PMN cell percentage, and smaller preovulatory follicle size at C-1 negatively influenced pregnancy outcomes. Overall, intrauterine O₃W was associated with reduced microbial burden and improved reproductive parameters, suggesting its potential as a non-antibiotic therapeutic option for bovine endometritis. However, further controlled studies are required to confirm its efficacy.

Keywords: Cows, Conception, Endometritis, Ozonized water

Endometritis remains a significant constraint in dairy cattle, adversely affecting reproductive performance and overall productivity (Várhidi *et al.* 2024) which could be attributed to compromised immune function in response to elevated malondialdehyde (MDA) concentrations (Kaya *et al.* 2018). Although a range of therapeutic options exists, antibiotics continue to be the mainstay. However, their application is associated with several important limitations, including the need for culture and sensitivity testing to ensure appropriate selection, the risk of antimicrobial resistance, persistence of drug residues in milk, reduced effectiveness in purulent uterine environments, potential allergic reactions, and limited efficacy against biofilm-forming pathogens (Kebede *et al.* 2014; Menoud *et al.*

2024). These challenges have driven interest in alternative, non-antibiotic therapies.

Ozone has emerged as a promising candidate due to its broad-spectrum antimicrobial activity against bacteria, viruses, and fungi, including those protected within biofilms (Bocci *et al.* 2009). In addition to its antimicrobial properties, ozone has been reported to exert immunomodulatory (Bocci 1999) and anti-inflammatory effects, promotes tissue healing (Napiórkowska-Baran *et al.* 2025), and does not result in antimicrobial residues in milk or meat (Rowen 2018). Such advantages have encouraged its exploration in the treatment of uterine infections in cattle (Kacar and Kaya 2014). While various ozone-based formulations have been investigated, many are not readily accessible in the Indian market (Sood 2024). In contrast, ozonized water (O₃W) offers a practical and easily prepared alternative for field application. Previous studies administering intrauterine O₃W at doses ranging from 2500 to 3300 μ g (50–55 μ g/mL) in volumes of 50–60 mL have reported improvements in conception rates by 10–33.8% in cycling or early postpartum cows with subclinical uterine infections (Escandón *et al.* 2020, Mali *et al.* 2020).

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Despite these encouraging findings, the therapeutic outcomes of O₃W remain inconsistent, especially in the cows that are ready to conceive. Therefore, identifying the factors influencing treatment efficacy is essential for optimizing its use. In this context, the present study aimed to evaluate the therapeutic effectiveness of intrauterine O₃W in endometritis cows, with particular emphasis on variables affecting the success of treatment.

MATERIALS AND METHODS

The study adhered to the guidelines of the College Animal Ethical Committee under the proceedings of Institutional Biosafety Committee meeting (IBSC/VGO/837; 18/06/25).

The investigation comprised of 60 consecutive estrous cycles (C-1, pre-treatment; C-2, O₃W treatment; C-3, post-treatment) involving 20 semi-intensively managed Jersey crossbred cows. The selected cows exhibited regular estrous cycles, absence of reproductive disorders such as ovarian cysts, no systemic illnesses including mastitis or lameness, and no recent antibiotic or anti-inflammatory therapy for uterine infections. General parameters recorded included age (years), parity, milk yield (L/day), number of previous inseminations without pregnancy, and body condition score (BCS, Edmundson *et al.* 1989).

The study protocol was initiated during the C-1 estrus. Sequentially, each cow underwent a detailed trans-rectal palpation and ultrasonography of the genitalia to confirm estrus based on uterine tone (categorized as mild, moderate and good and assigned scores of 1, 2, and 3, respectively; Sood *et al.* 2009) and presence of a pre-ovulatory follicle (size [mm] using real-time B-mode, Mindray Z5; VETMODEL 75L50EAV).

Subsequently, cervical discharge was aspirated using a sterile insemination sheath attached to a syringe. It was evaluated for physical appearance, consistency, volume (Sood *et al.* 2009) and arborization pattern (Tsiligianni *et al.* 2011). Discharge appearance was scored as clear (3), presence of flecks of white to off-white pus (2), or purulent (1). Consistency was assessed according to the free-flowing nature of the discharge within the insemination sheath and classified as hanging (2) or breaking within 30 seconds (1). Based on the quantity aspirated, the discharge was categorized as scanty (≤ 1 mL; score 1) or copious (score 2).

A small aliquot of the collected discharge was smeared onto a glass slide, air-dried, and microscopically examined for arborization pattern. The branching patterns were classified as primary (1), secondary (2), tertiary (3), or quaternary (4). Thereafter, the Whiteside test was performed according to Polyantsev and Popov (1972). The test outcomes were categorized as normal (turbid or no color change; score 0), mild infection (light yellow; score 1), moderate infection (yellow; score 2), or severe infection (dark yellow; score 3). In addition to the Whiteside test, urine Test Strips 10SGL (YD Diagnostics Corp., Republic of Korea) (Sood *et al.* 2025b) were used for assessment of endometritis. According to the manufacturer's protocol, one

drop of cervical discharge was applied to each designated reagent segment for leukocyte count, pH, specific gravity, and protein estimation, and results were interpreted after one minute. Leukocyte concentration was categorized as negative, 25, 75, or 500 WBC/ μ L. The pH values were classified into five categories (5.0, 6.0, 7.0, 8.0, and 9.0), specific gravity into six categories (1.000, 1.005, 1.010, 1.020, 1.025, and 1.030), and protein concentration into six categories (negative, 0.1, 0.3, 1.0, 3.0, and 10.0 g/L).

The remaining discharge sample was transferred aseptically into sterile 5 mL conical tubes and maintained in a cold insulated container until transportation to the laboratory for aerobic and anaerobic qualitative isolation using streak plate method (Bergey 1994) and quantitative enumeration using serial dilution method followed by spread plate method (CFU/mL; Salfinger and Tortorello 2001). BHI agar plates were used and the plates were incubated at 37°C for 24 hours. Preliminary characterization was done using gram staining and further biochemical identification was done using Catalase test, Oxidase test, Indole test, Methyl Red (MR) test, Voges-Proskauer (VP) test, Citrate utilization test, Urease test, Sugar fermentation tests, Decarboxylation of amino acids test, Nitrate reduction test, Sulphide-Indole-Motility (SIM) test, and Triple Sugar Iron (TSI) test.

Finally, the cows were evaluated for endometrial cytology using a specially fabricated sterile 'Bovine endometrial cytotaping catheter' (Sood *et al.* 2025a). Samples were aseptically collected from the dorsal endometrial surface of the uterine body and stained with Leishman's stain (Sigma-Aldrich; EC Number 235-732-1) for determination of polymorphonuclear (PMN) cell percentage. As the cows in the present study were regularly cycling, a threshold of $\geq 1\%$ PMN cells was used to diagnose endometritis (Pascottini *et al.* 2017). This contrasted with the higher PMN thresholds (3–18%) commonly used to diagnose subclinical endometritis in acyclic cows between 21 and 62 days postpartum. The higher thresholds applied in postpartum cows is due to the physiological uterine involution occurring during this period, which is accompanied by a greater degree of inflammatory cell infiltration (Sood 2024).

Additional parameters evaluated included ultrasonographic measurement of corpus luteum (CL) size on day 11 following C-1 estrus, serum progesterone (P₄; Acculite® CLIA kits, Japan) and serum MDA (TECAN Austria GmbH, Austria) concentrations using enzyme-linked immunosorbent assay in the blood samples collected at C-1 estrus and again 11 days later. The estrus duration (Ruthwal 2010) was also recorded.

During C-2 estrus, all cows received intrauterine administration of O₃W at a dose of 3300 μ g (55 μ g/mL; 60 mL) for two consecutive days. O₃W was generated in the laboratory using a Lab Ozonator (LO-2.5; Ozone Engineers, Coimbatore, Tamil Nadu, India) connected to an oxygen cylinder. As per the manufacturer's instructions, oxygen feeding was regulated at 0.5 liters per minute so

as to generate 55 µg of O₃/mL. The generated ozone was collected in equal volumes and concentrations in two glass syringes sealed with a three-way valve. The prepared O₃W was transported to the farmers' doorstep in an insulated ice box to preserve stability and was administered within 20 minutes of preparation as it decomposes rapidly and readily.

Except for endometrial cytology, all parameters evaluated during C-1 were reassessed during C-3 using the same schedule and methodology. All cows were inseminated by the same inseminator during the C-3 estrus according to the A.M.-P.M. rule using 0.25 mL frozen-thawed semen from three different certified bulls. Animals returning to estrus were re-inseminated, with a maximum of three consecutive inseminations included in the analysis. Parameters calculated included pregnancy rate to the three inseminations and overall pregnancy rate. Pregnancy diagnosis in non-return cows was confirmed by ultrasonography 45 days after insemination.

The inter-estrus intervals (IEI) between C-1 and C-2, as well as between C-2 and C-3, were also recorded.

The determinants recorded during C-1 and C-3 were expressed as Mean ± SEM, tested for normality (Shapiro-Wilk test) and compared using the paired Student's t-test. The ordinal parameters viz. physical appearance, consistency, volume, arborization, Whiteside test and uterine tone, were compared using Wilcoxon signed-rank test. A correlation matrix between the different diagnostic determinants related to discharge was ascertained. Pregnancy rates across three inseminations were analyzed using Fisher's exact test. A binary logistic regression was performed to identify the independent predictors of pregnancy and one-way analysis of variance (ANOVA) was utilized to compare determinants among pregnancy outcome groups, including cows pregnant after the first insemination, overall pregnant cows to three inseminations, and non-pregnant cows. Statistical significance was set at $p \leq 0.05$; tendencies at $0.05 \leq p \leq 0.10$. Analysis was performed using SPSS version 24 (IBM Corporation, NY, USA).

RESULTS AND DISCUSSION

The general characteristics of the enrolled cows are

Table 1. Comparison (Mean ± S.E.M.) of endometrial cytology, general, and genital discharge related determinants (first [C-1] versus third [C-3] – cycle; pregnant versus non-pregnant in light of C-1) in endometritis cows treated with intrauterine ozonized water

Sr. No.	Determinants	C-1	C-3	p-value	Pregnant to		Non-pregnant
					First insemination (n=10)	All inseminations (n=14)	All Inseminations (n=6)
Endometrial cytology							
1.	Polymorphonuclear cells (%)	2.35 ± 0.28			1.50 ± 0.22 ^y	2.00 ± 0.28	3.17 ± 0.60 ^x
General							
2.	Age (years)	6.25 ± 0.84			4.30 ± 0.50 ^y	5.14 ± 0.73	8.83 ± 0.92 ^x
3.	Parity	2.20 ± 0.39			1.60 ± 0.43	1.93 ± 0.41	2.83 ± 0.87
4.	Milk yield (L/day)	5.15 ± 1.05			9.43 ± 1.21 (n=7)	8.40 ± 0.97 (n=10)	3.80 ± 1.51 (n=5)
5.	Number of previous inseminations without pregnancy	5.45 ± 0.89			3.40 ± 0.34 ^b	3.93 ± 0.38 ^a	9.00 ± 2.34 ^a
6.	Body Condition Score	3.04 ± 0.13			3.38 ± 0.15	3.18 ± 0.15	2.71 ± 0.19
Genital discharge							
7.	Physical appearance	2.05 ± 0.15	2.80 ± 0.09	0.00 (1.0 to 2.0)	3.00 ± 0.00	2.93 ± 0.07	2.50 ± 0.22
8.	Consistency	1.55 ± 0.11	1.65 ± 0.11	0.52 (-1.0 to +1.0)	1.90 ± 0.10	1.71 ± 0.13	1.50 ± 0.22
9.	Volume	1.70 ± 0.11	1.75 ± 0.10	1.00 (0.0 to 1.0)	1.90 ± 0.10	1.71 ± 0.13	1.83 ± 0.17
10.	Arborization pattern	2.80 ± 0.09	2.75 ± 0.10	0.77 (-1.0 to +1.0)	2.90 ± 0.10	2.86 ± 0.10	2.50 ± 0.22
11.	Whiteside test	1.45 ± 0.15	0.25 ± 0.10	0.00 (-2.0 to +1.0)	0.00 ± 0.00 ^b	0.07 ± 0.07 ^b	0.67 ± 0.21 ^a
12.	Leucocytes(WBC/μL)	68.75 ± 33.34	2.50 ± 1.72	0.05	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b	8.33 ± 5.27 ^a
13.	pH	7.82 ± 0.09	7.22 ± 0.07	0.00	7.15 ± 0.08	7.25 ± 0.09	7.17 ± 0.11
14.	Specific Gravity	1.02 ± 0.00	1.01 ± 0.00	0.00	1.01	1.01	1.01
15.	Protein (g/L)	1.46 ± 0.67	1.11 ± 0.68	0.00	0.06 ± 0.02	0.77 ± 0.71	1.90 ± 1.63
16.	Microbiology (CFU/mL [× 10 ⁷])	4.99 ± 2.01	2.60 ± 1.23	0.01	1.96 ± 1.59	1.57 ± 1.13	5.05 ± 3.09

Parenthesis alongside p-value is Hodges Lehmann Estimate depicting 95% confidence interval

presented in Table 1. Endometrial cytology demonstrated a PMN cell percentage of 2.35 ± 0.28 in all cows, confirming the presence of endometritis. A PMN threshold of 1%, as adopted in the present study, is considered diagnostic of endometritis in cyclic cows (Pascottini *et al.* 2016).

Qualitative and quantitative microbiological analyses of uterine discharge at C-1 revealed microbial growth in 15 of 20 cows (75.0%), closely aligning with the 72.0% prevalence reported previously (Pascottini *et al.* 2020). The pathogens identified, in decreasing order of occurrence, were *Escherichia coli*, *Staphylococcus aureus*, *Bacillus licheniformis*, and *Micrococcus luteus*. Following treatment, 12 cows (60.0%) exhibited no microbial growth, accompanied by a significant reduction in microbial burden (Table 1). An important finding was the presence of PMN cell positivity in 25.0% of cows despite negative microbial culture results. This inherent limitation necessitates the use of advanced molecular approaches, such as next-generation sequencing and meta-transcriptomics, for detection of low-biomass or non-culturable uterine microorganisms (Stoyancheva *et al.* 2025). These findings suggested that microbiologically negative animals might remain untreated,

whereas empirical antibiotic administration without antimicrobial sensitivity data may be inappropriate. Non-antibiotic interventions, such as intrauterine O_3W , may therefore provide a valuable therapeutic alternative.

Elimination or reduction of uterine pathogens following O_3W therapy may explain significant improvements ($p < 0.05$, at least) in discharge characteristics, Whiteside test scores, test-strip parameters, MDA concentrations, pre-ovulatory follicle size, and estrus duration during C-3 compared with C-1 (Table 2). Uterine infection impairs follicular growth by suppressing estradiol synthesis and disrupting follicular development (Sheldon *et al.* 2002; Williams *et al.* 2007). It is also associated with elevated P_4 concentrations during estrus, either due to incomplete luteolysis resulting from reduced prostaglandin $F_{2\alpha}$ secretion (Kaneko and Kawakami 2008) or premature luteinization of the preovulatory follicle caused by impaired steroidogenesis (Robinson *et al.* 2006). These endocrine alterations may delay or suppress the preovulatory luteinizing hormone surge, thereby prolonging estrus duration (Duchens *et al.* 1995). Conversely, resolution of uterine infection is associated with improved mid-luteal

Table 2. Comparison (Mean $\pm$ S.E.M.) of ultrasonography, estrous cycle, and hormonal and oxidative stress marker determinants (first [C-1] versus third [C-3] – cycle; pregnant versus non-pregnant in light of C-1) in endometritis cows treated with intrauterine ozonized water

Sr. No.	Determinants		C-1	C-3	p-value	Pregnant		Non-pregnant
						First insemination (n=10)	All inseminations (n=14)	All Inseminations (n=6)
Ultrasonography								
1.	Pre-ovulatory follicle size (mm)		11.21 ± 0.40	11.77 ± 0.31	0.03	12.74 ± 0.39 ^x	12.16 ± 0.39	10.89 ± 0.25 ^y
2.	Corpus luteum size (mm)		20.81 ± 0.75	21.17 ± 0.67	0.45	23.06 ± 0.68 ^x	21.65 ± 0.81	20.07 ± 1.17
Estrous cycle								
3.	Estrus duration(h)	C-1	49.80 ± 3.05	40.30 ± 2.94	0.00	45.60 ± 2.99	48.86 ± 2.94	52.00 ± 8.00
		C-3			36.00 ± 4.00	39.57 ± 3.46	42.00 ± 6.00	
4.	Inter-estrus interval (days)	C-1 to C-2	21.60 ± 0.71*	21.30 ± 0.22**	0.65	22.50 ± 1.20	21.71 ± 0.99	21.33 ± 0.67
		C-2 to C-3			21.60 ± 0.31	21.29 ± 0.29	21.33 ± 0.33	
5.	Uterine tone		2.45± 0.14	2.5 ± 0.14	0.78 (-1.0 to +1.0)	2.60 ± 0.16	2.50 ± 0.17	2.50 ± 0.22
Hormonal and oxidative stress marker								
6.	Progesterone (ng/mL)	Day 0	1.21 ± 0.51	0.28 ± 0.04	0.09	0.32 ± 0.08	0.30 ± 0.06	0.26 ± 0.05
		Day 11	7.62 ± 0.93	13.12 ± 0.96	0.00	13.15 ± 0.91	13.97 ± 2.95	
7.	Malondialdehyde (nmol/mL)	Day 0	19.18 ± 2.72	9.53 ± 0.45	0.00	9.12 ± 0.67	9.36 ± 0.52	9.94 ± 0.96
		Day 11	19.12 ± 3.04	12.87 ± 0.64	0.05	13.32 ± 0.88	13.54 ± 0.66	11.34 ± 1.43

*between C-1 and C-2; ** between C-2 and C-3; Day 0 is the day of estrus; ^{x,y} $p \leq 0.05$; ^{a,b} $p \leq 0.01$; parenthesis alongside p-value is Hodges Lehmann Estimate depicting 95% confidence interval

Table 3. Pearson correlation matrix among various determinants investigated at C-1 estrous cycle

Parameters	Age	Parity	MY	Prev. A.I.	BCS	Uterine tone	Phy. app.	Consist.	Vol.	Arb.	WST	Leu.	pH	S.G.	Protein	CFU/ml	PMNCs (%)	POFD	CL size	P4 D0	P4 D11	MDA D0	MDA D11	Estrus duration (h)	IEI duration (C1-C2)
Age	1.00																								
Parity	0.86**	1.00																							
MY	0.19	0.43	1.00																						
Prev. A.I.	0.44*	0.25	-0.22	1.00																					
BCS	-0.41	-0.35	0.27	-0.11	1.00																				
Uterine tone	-0.12	0.06	-0.08	-0.15	0.18	1.00																			
Phy. app.	-0.05	0.12	0.06	-0.24	0.13	0.58**	1.00																		
Consist.	-0.19	0.05	0.16	-0.31	0.37	0.86**	0.52*	1.00																	
Vol.	0.04	0.27	0.07	0.16	0.09	0.68**	0.54*	0.50*	1.00																
Arb.	0.38	0.50*	0.15	0.09	0.26	0.59**	0.60**	0.55*	0.49*	1.00															
WST	-0.11	-0.12	0.04	0.08	-0.11	-0.26	-0.72**	-0.14	-0.54*	-0.41	1.00														
Leu.	-0.20	-0.16	-0.22	0.22	-0.15	0.00	-0.59**	-0.06	-0.20	-0.28	0.83**	1.00													
pH	-0.02	0.09	0.32	0.15	0.09	0.02	-0.16	0.23	-0.01	-0.06	0.49*	0.37	1.00												
S.G.	0.02	0.18	0.24	-0.19	0.14	0.66**	0.35	0.70**	0.41	0.53*	0.01	-0.02	0.02	1.00											
Protein	0.12	0.20	0.03	0.25	0.22	0.02	-0.26	0.27	-0.10	0.20	0.50*	0.46*	0.60**	0.13	1.00										
CFU/ml	-0.16	-0.15	0.14	-0.10	0.02	-0.36	-0.34	-0.17	-0.07	-0.53*	-0.03	-0.15	0.12	-0.24	-0.15	1.00									
PMNCs (%)	0.21	0.16	-0.20	0.42	-0.11	-0.08	-0.44*	-0.07	-0.08	-0.06	0.47*	0.44*	0.58**	-0.12	0.60**	0.16	1.00								
POFD	-0.23	-0.19	0.31	-0.27	0.47*	0.15	0.09	0.20	0.07	0.06	-0.08	0.01	0.01	-0.07	-0.25	0.16	-0.30	1.00							
CL size	-0.36	-0.35	0.30	-0.25	0.49*	0.07	0.23	0.08	0.11	-0.02	-0.16	-0.10	-0.04	0.01	-0.27	-0.07	-0.53*	0.72**	1.00						
P4 Day 0	0.11	-0.09	-0.15	-0.09	-0.14	-0.25	-0.40	-0.18	-0.50*	-0.39	0.27	0.02	-0.04	-0.24	0.17	-0.07	0.06	-0.36	-0.28	1.00					
P4 Day 11	-0.50*	-0.44	0.25	-0.51*	0.44*	0.01	-0.21	0.05	-0.08	-0.22	0.05	-0.04	-0.12	0.09	-0.17	0.22	-0.24	0.25	0.43	0.12	1.00				
MDA Day 0	-0.04	0.04	0.22	-0.09	0.23	-0.09	-0.20	0.06	-0.14	-0.13	0.02	-0.01	-0.09	0.06	0.08	0.40	-0.05	-0.04	-0.28	0.18	0.17	1.00			
MDA Day 11	0.13	0.16	0.27	0.22	0.26	-0.12	-0.14	-0.01	0.01	0.01	-0.08	-0.12	-0.11	0.10	0.11	0.31	-0.05	-0.15	-0.25	0.04	0.08	0.89**	1.00		
Estrus duration (h)	-0.05	-0.15	-0.13	0.23	-0.17	-0.33	-0.21	-0.24	-0.11	-0.27	0.04	0.07	0.17	-0.11	-0.03	0.52*	0.33	-0.16	-0.26	-0.34	-0.23	0.15	0.16	1.00	
IEI (C1-C2)	0.04	0.05	0.41	0.00	0.15	-0.40	-0.09	-0.18	-0.16	-0.15	-0.13	-0.38	-0.04	-0.16	-0.24	0.48*	-0.36	0.27	0.24	-0.02	0.11	0.08	0.15	-0.04	1.00
Conc.	-0.46*	-0.25	0.28	-0.60**	0.38	0.13	0.38	0.29	-0.19	0.22	-0.05	-0.16	-0.15	0.20	-0.18	-0.20	-0.43	0.30	0.20	-0.28	0.20	0.14	0.01	-0.11	0.06

*(p<0.05) ***(p<0.01)

(MY-Milk yield; Prev. A.I.- Previous A.I.; Phy. app.- Physical appearance; Consist.- Consistency; Vol.- Volume; Arb.- Arborization; WST- Whiteside test; Leu.- Leucocytes; S.G.- Specific gravity; POFD- Pre-ovulatory follicle diameter; Conc- Conception)

Table 4. Pearson correlation matrix among various determinants investigated at C-3 estrous cycle

Parameters	Age	Parity	MY	Previous A.I.	BCS	Uterine tone	Phy. app.	Consist.	Volume	Arb.	WST	Leu.	pH	S.G.	Protein	CFU/ mL	POFD	CL size	P4 Day 0	P4 Day 11	MDA Day 0	MDA Day 11	ED (h)	IEI (C2-C3)	
Age (years)	1.00																								
Parity	0.86**	1.00																							
Milk yield (L/day)	0.19	0.43	1.00																						
Previous A.I.	0.44*	0.25	-0.22	1.00																					
BCS	-0.41	-0.35	0.27	-0.11	1.00																				
Uterine tone	-0.10	0.00	0.12	0.10	0.40	1.00																			
Phy. App.	-0.24	-0.09	0.13	-0.52*	-0.08	-0.21	1.00																		
Consist.	-0.27	-0.22	0.25	0.09	0.52*	0.44*	0.16	1.00																	
Volume	-0.02	0.14	-0.06	0.13	0.24	0.29	0.29	0.30	1.00																
Arb.	-0.18	-0.20	0.27	-0.41	0.35	0.10	0.00	0.54*	-0.33	1.00															
WST	-0.10	-0.27	-0.45*	0.56**	-0.24	0.10	-0.58**	-0.06	-0.20	-0.20	1.00														
Leu.	0.02	-0.04	-0.23	0.18	-0.10	0.00	-0.67**	-0.10	-0.19	0.19	0.58**	1.00													
pH	0.16	0.06	0.09	0.22	0.25	0.22	-0.47*	0.03	-0.34	0.24	0.15	0.03	1.00												
S.G.	0.29	0.35	-0.06	0.30	-0.25	-0.14	0.00	-0.27	0.00	-0.29	0.00	0.00	0.07	1.00											
Protein	0.09	0.17	0.02	0.15	0.20	0.30	-0.70**	-0.10	-0.18	0.16	0.22	0.44*	0.61**	0.28	1.00										
CFU/mL	-0.21	-0.22	0.03	-0.06	-0.06	-0.32	0.18	-0.25	-0.11	-0.36	0.19	-0.09	-0.34	0.21	-0.13	1.00									
POFD	-0.34	-0.19	0.44*	-0.38	0.64**	-0.01	0.36	0.56**	0.32	0.38	-0.48*	-0.30	-0.01	-0.28	-0.10	0.12	1.00								
CL size	-0.29	-0.21	0.25	-0.27	0.53**	0.11	0.21	0.50*	0.50*	0.35	-0.27	-0.12	-0.01	-0.49*	-0.12	-0.18	0.68**	1.00							
P4 Day 0	0.08	0.12	0.46*	0.01	0.22	0.23	0.09	0.13	0.14	0.01	-0.25	-0.06	0.13	0.08	-0.06	-0.16	0.07	0.10	1.00						
P4 Day 11	-0.47*	-0.47*	-0.18	-0.28	0.14	-0.10	-0.06	-0.24	-0.02	-0.03	0.34	0.28	-0.09	-0.11	0.12	0.44	-0.07	0.20	-0.14	1.00					
MDA Day 0	-0.10	-0.03	-0.37	-0.13	-0.24	-0.32	-0.14	-0.34	0.05	-0.05	0.26	0.44*	-0.20	-0.01	0.14	-0.05	-0.21	0.03	-0.46*	0.52*	1.00				
MDA Day 11	-0.14	-0.02	-0.06	-0.15	0.35	0.23	0.21	0.29	0.22	0.23	-0.39	-0.27	0.06	0.23	0.29	-0.14	0.19	0.08	-0.11	-0.08	-0.03	1.00			
ED (h)	0.37	0.30	0.23	0.25	-0.18	-0.19	0.15	-0.16	-0.26	-0.13	-0.09	-0.42	0.09	0.51*	-0.08	0.32	-0.22	-0.44*	0.09	-0.16	-0.35	0.01	1.00		
IEI (C2-C3)	-0.31	-0.32	-0.09	-0.08	0.14	-0.18	0.29	0.45*	0.18	0.18	0.06	0.07	-0.33	-0.35	-0.28	0.26	0.37	0.40	-0.27	0.17	0.03	0.14	-0.37	1.00	
Conc.	-0.46*	-0.25	0.28	-0.60**	0.38	0.00	0.49**	0.21	-0.13	0.38	-0.63**	-0.51*	0.13	-0.18	-0.17	-0.30	0.44*	0.25	0.10	-0.13	-0.13	0.36	-0.09	-0.02	1.00

*(p<0.05) ***(p<0.01)

(MY-Milk yield; Phy. App.- Physical appearance; Consist.- Consistency; Arb.- Arborization; WST- Whiteside test; Leu.- Leucocytes; S.G.- Specific gravity; POFD- Pre-ovulatory follicle diameter; ED- Estrus duration; Conc- Conception)

P_4 concentrations (Kasimanickam *et al.* 2025), consistent with the significant increase in day-11 P_4 concentrations observed in the present study.

A strong positive correlation was observed between discharge appearance and arborization pattern, discharge consistency, and volume, whereas strong negative correlations existed with the Whiteside test score, leukocyte count (WBC/ μ L), pH, and protein concentration (g/L) at both C-1 and C-3. Arborization score also showed positive and negative correlations with specific gravity and CFU/mL, respectively, at C-1 estrus (Table 3 and Table 4). These findings validate the diagnostic utility of these variables in addition to endometrial cytology, which was intentionally avoided at C-3 to prevent any adverse effects on ensuing conception.

The first-service and overall pregnancy rates were 50.0% and 70.0% ($n=14$), respectively, with no significant differences ($p=0.24$) among the three insemination attempts. Previous studies administering intrauterine O_3W (2500 to 3300 μ g; 50-55 μ g/mL in 50-60 mL) reported 10-33.8% improvements in conception rates in cows with subclinical uterine infections (Escandón *et al.* 2020; Mali *et al.* 2020). The first-service conception rate exceeded the 24.4% and 42.0% reported by Denis-Robichaud and Dubuc (2015) and Kasimanickam *et al.* (2005), respectively, but remained lower than the 62.2% documented by Ahmadi *et al.* (2015) in postpartum cows treated with cephalixin. Nevertheless, a 30.0% treatment failure rate highlights the need to identify factors affecting therapeutic response (Table 1).

The score statistics from the logistic regression model identified age, number of previous unsuccessful inseminations, Whiteside test score, leukocyte counts, and the physical appearance of discharge as significant predictors of pregnancy outcome. These findings underscore the importance of reproductive history and uterine health being the primary determinants of pregnancy following intrauterine ozonized water therapy (Table 5).

Notably, age, number of prior unsuccessful inseminations, PMN cell percentage, and pre-ovulatory follicle diameter differed significantly ($p \leq 0.05$, at least) between pregnant and non-pregnant cows at treatment initiation (Table 1). These observations are consistent with the 31.1% decline in conception rate reported in older cows (Khan *et al.* 2015), likely due to reduced fertilization efficiency, diminished pituitary gonadotropin secretion, and depletion of ovarian follicular reserves (Fuerst-Waltl *et al.* 2004, Takeo *et al.* 2017). Older cows (>7 years) may also accumulate greater endometrial and glandular damage due to repeated uterine infections, reducing the uterine receptivity, despite microbial clearance. The number of prior inseminations may be indicative of the chronicity of uterine pathology. Earlier, cows with clinical endometritis frequently developed subclinical endometritis or persisted with inflammation after treatment, which also reduced conception rates even in bacteriologically negative uteri (Galvão *et al.* 2009, Barański *et al.* 2022, Valdmann

et al. 2022). Consequently, complete histological and functional endometrial recovery may require additional time even after microbial elimination (Herath *et al.* 2006). A higher PMN percentage ($>3\%$) at enrolment reflected a more severe inflammatory burden and advanced uterine infection (Jakupov *et al.* 2024), supported by elevated Whiteside scores and leukocyte counts (WBC/ μ L). This warrants evaluating repeated O_3W treatment across subsequent estrous cycles in such cows. Although follicular growth improved following O_3W treatment, cows with smaller follicles at treatment initiation may still experience compromised fertility because of compromised oocyte competence and CL function (Williams *et al.* 2007; Echternkamp *et al.* 2009, Sood *et al.* 2022, Arrayás *et al.* 2025).

Table 5. Score statistics for variables associated with pregnancy outcome in the initial binary logistic regression model

Variables	Score	df	p-value
Endometrial cytology	5.363	4	0.25
Endometrial cytology (1)	1.266	1	0.26
Endometrial cytology (2)	0.060	1	0.80
Endometrial cytology (3)	0.317	1	0.57
Endometrial cytology (4)	2.260	1	0.13
BCS	2.956	1	0.08
Milk yield	1.603	1	0.20
Age	4.305	1	0.03
Parity	1.202	1	0.27
Estrus duration	0.151	1	0.69
Uterine tone	0.682	2	0.71
Uterine tone (1)	0.451	1	0.50
Uterine tone (2)	0.357	1	0.55
Previous artificial inseminations	7.227	1	0.00
Interestrus interval	0.010	1	0.91
Physical appearance (1)	4.821	1	0.02
Consistency (1)	0.848	1	0.35
Volume (1)	0.317	1	0.57
Whiteside Test (1)	7.937	1	0.00
Leucocytes count	5.185	1	0.02
pH	0.336	1	0.56
Protein	0.606	1	0.43
Specific gravity	0.680	1	0.41
Fern pattern (1)	2.857	1	0.09
Ovulatory follicle	3.794	1	0.05
Corpus luteum	1.219	1	0.27
Micro	1.780	1	0.18
P_4 Day 0	0.197	1	0.65
P_4 Day 11	0.342	1	0.55
MDA Day 0	0.359	1	0.54
MDA Day 11	2.527	1	0.11

In conclusion, despite the limited sample size, the study provides preliminary evidence regarding the beneficial effects and the cow-related factors dictating the usefulness of O₃W against endometritis and pregnancy set up. However, larger studies are warranted to endorse these findings.

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