Original Research Article

Ozonated water decreases *in vitro* contamination at dental implant interface in different connections

.

ABSTRACT

Aim: Cases of peri-implant diseases have increased, highlighting the need for preventive and therapeutic protocols. This study aimed to evaluate the effectiveness of ozonated water on in vitro decontamination of implant-abutment interfaces in external hexagon (EH) and morse cone (MC) connections.
Study design: *In vitro* study.
Place and Duration of Study: Sample: Department of Periodontics and Implant Dentistry of Ingá University Center – UNINGÁ, between June 2020 and December 2021.
Methodology: Twenty-four implant-abutments (IA) in EH (n=12) and MC (n=12) connections were divided into groups: negative control (NC) – sterile IA; positive control (PC) – IA contaminated with biofilm; and ozone (O) – IA contaminated with biofilm and decontaminated with ozonated water (60mg/L,1min). The effectiveness of ozonated water was evaluated by counting colony-forming units (CFU/mL), and both connection types were compared.
Results: There was a significant difference among groups in both connection types, outside and inside the implants (p=0.000). Group O showed a significant decrease in CFU/mL compared to group PC outside and inside the implants for EH and MC connections (p=0.000). Only group O in the EH connection presented a significative difference in CFU/mL compared to outside and inside the implants (2.475x103±0.320 and 1.775x103±0.125, respectively) (p=0.033). Comparison between connections was statistically different for groups PC and O, outside and inside the implants (p=0.000).
Conclusion: Ozonated water showed effectiveness *in vitro* decontamination of implant-abutment interfaces in both connection types.

Keywords: Dental implant-abutment interface. Ozone. Decontamination. Biofilm.

1. INTRODUCTION

Dental implant treatment is an admittedly successful reality, and this rehabilitation treatment modality has been widely used with the success of implants [1]. Consequently, it is natural that there is also an increase in problems related to this therapy [2].

Peri-implant mucositis is a complication frequently found in dental implant rehabilitation [3], reaching up to 80% of patients [4, 5]. It is a reversible inflammatory reaction that affects peri-implant tissues in which it is necessary to remove the biofilm to restore peri-

implant health conditions [6]. Although peri-implant mucositis is a more easily controllable condition once the primary etiologic agent is removed, biofilm [6, 7], it is considered a precursor of peri-implantitis [4, 7], a much more complex clinical condition that affects peri-implant tissues in a not reversible way, causing progressive loss of supporting bone [7].

Therefore, individuals diagnosed with peri-implant mucositis can evolve into peri-implantitis, especially in the absence of adequate control [7]. As stated before, peri-implantitis is an irreversible and complex condition [8]. Once there is no consensus about a gold-standard protocol to treat peri-implantitis [9, 10], peri-implant mucositis control and treatment become imperative to maintain peri-implant tissue health and implants in long-term function.

In this scenario, the implant-abutment interface deserves special attention due to it being a region that facilitates biofilm accumulation [11]. Despite advances in implant and component manufacturing methods that allow greater precision in interface adjustment, this region still has micro gaps that enable the entry of microorganisms in all connection types when submitted to mastication forces [12]. According to the literature, morse cone connection implants present less bacterial penetration than external and internal hexagon connection implants [13].

Peri-implant mucositis treatment depends on the debridement of the implant surface and implant-abutment interface using mechanical methods such as curettes, ultrasound devices, and abrasive powders jets [9, 14, 15], or antimicrobial agents such as chlorhexidine [9, 16], tetracycline, and antimicrobial photodynamic therapy [3, 10, 14].

Considering the peculiarities and characteristics of dental implants, there is a need to employ a decontamination therapy that reduces bacterial adherence without causing major changes to the implant surface and is effective in resolving inflammation and preserving peri-implant tissues [17]. Among the available antimicrobial agents, ozone has emerged as an alternative; however, few studies are related to peri-implant diseases.

Ozone (O3) is a potent oxidizing agent with antimicrobial activity due to releasing reactive oxygen species (ROS). The rapid microorganism's inactivation is one of its outstanding characteristics [18], proving effective in dental treatments [19, 20]. Ozone has an antimicrobial effect on bacteria, viruses, and fungi, as well as immunomodulatory, anti-hypoxic, biosynthetic, and anti-inflammatory properties [21]. In times of bacterial resistance, it emerges as an attractive antimicrobial option that does not present toxicity or side effects unless aspirated [22].

Ozone is available in some presentation forms [23], and ozonated water is considered the most predictable alternative to gas or oil due to the greater practicality of use, application control, and lower risk of toxicity [24].

Similar efficiencies were found in both therapies when compared to antimicrobial photodynamic therapy (association of a low-level laser and a photosensitizer that also generates ROS) in periodontal treatment, concluding that ozone is a valuable adjuvant in non-surgical periodontal treatment [25]. In a clinical study, peri-implantitis treatment was performed with regenerative therapy, and ozone was used in gaseous form as an adjunct treatment after mechanical decontamination methods, with significant clinical and radiographic improvements [26]. Also, in a recent systematic review [23], despite the heterogeneity of the studies, ozone showed encouraging therapeutic effects with regard to periodontal and peri-implant diseases.

Therefore, the present study aimed to evaluate the effectiveness of ozonated water on *in vitro* decontamination of implant-abutment interfaces in external hexagon and morse cone connections.

2. MATERIAL AND METHODS

This study was approved by the Research Ethics Committee of the Inga University Center (protocol number 4.444.416) for the use of subgingival biofilm. The biofilm was frozen and stored at the Inga University Center Microbiology Laboratory [14].

A sample size calculation was performed based on an alpha significance level of 5% (0.05) and a beta of 20% (0.20) to achieve 80% power of the test to detect a minimum difference of $3.5x10^6$ CFU/mL with a standard deviation of 1.49x106 for the colony-forming units (CFU/mL) [14, 15], which verified the need of twenty-four implant-abutments (IA) used in this study.

Twelve grade IV titanium implants, external hexagon connection (EH), surface treated with double acid etching, cylindrical, measuring 4.1 mm in diameter and 13 mm in length (Classic-CI-Systhex® – Curitiba/Brazil), and twelve EH abutments, straight, measuring 4.1 mm in diameter and 2 mm in transmucosal height (Systhex® – Curitiba/Brazil); twelve grade IV titanium implants, morse cone connection (MC), surface treated with double acid etching, cylindrical, measuring 4.3 mm in diameter and 13 mm in length (Attract-Systhex® – Curitiba/Brazil), and twelve MC abutments, straight, measuring 4.5 mm in diameter and 2.5 mm in transmucosal height (Systhex® – Curitiba/Brazil). Both implants and abutments were sterilized at the factory.

In a flow chamber, using gloves and sterile instruments, the abutments were installed in the respective implants and screwed with torque recommended by the manufacturer, 32 N for EH and 20 N for MC. Then, IA was distributed in the following experimental groups:

- NEGATIVE CONTROL (NC): sterile IA in EH (n=4) and MC (n=4) connections.
- POSITIVE CONTROL (PC): IA contaminated with subgingival biofilm in EH (n=4) and MC (n=4) connections.
- OZONE (O): IA contaminated with subgingival biofilm and treated with ozonated water in EH (n=4) and MC (n=4) connections.

After unfrozen, biofilm was cultivated in sterile brain heart infusion (BHI) broth (Kasvi – São Jose dos Pinhais/Brazil). The IA from groups PC and O were contaminated in vitro in test tubes containing 10 mL of broth containing subgingival biofilm and maintained for seven days in a 37°C oven for biofilm formation on interfaces. All procedures were performed in a laminar flow chamber, using gloves and sterile instruments to avoid contamination outside the experiment. The AI from group NC remained sterile and were placed in a sterile broth in an oven at 37°C for seven days for factory sterility certification.

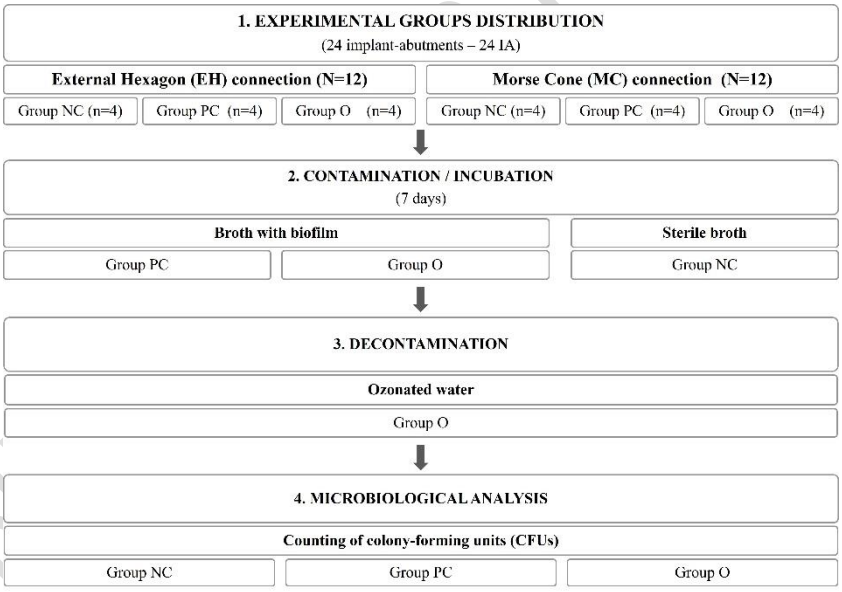
After seven days, sterile absorbent paper cones (Tanari® – Manacapuru/Brazil) were used to collect the biofilm from the implant-abutment interface region. After abutment removal, this collection was performed in the outer region of the implant/abutment interface (outside) and inside the implants (inside). The paper cones were used for seeding in Petri dishes containing culture medium (Laborclin® – Pinhais/Brazil).

In group O, decontamination was performed with ozonated water before collection. For ozonated water preparation, a Medplus ozone generator MX (Philozon® – Camboriu/Brazil) was used, with electronic regulation, medical oxygen (99.5%, White Martins® – Rio de Janeiro/Brazil), cooled double-distilled water (Sanobiol® – Pouso Alegre/Brazil) and the ozone was diluted in a specific glass column (Philozon® – Camboriu/Brazil). The equipment was calibrated at a concentration of 60 mg/L, its maximum capacity [27]. However, according to the manufacturer, only 20% of the ozone is fixed in the water. In this sense, it is estimated that the real concentration of ozone in the ozonized water used was around 12 mg/L [28].

The IA were then immersed in microtubes (Axygen® – São Paulo/Brazil) containing 2 mL of ozonated water, where it remained for 1 minute with the microtubes covered. Group O collection was also performed using sterile absorbent paper cones (Tanari® – Manacapuru/ Brazil), following the above-mentioned steps. As well as the collection of implants from groups PC and NC.

All seeded plates were stored in a CO_2 jar, simulating anaerobiosis and ensuring a microaerophilic condition. They were then waited 48 hours in an oven at 37˚C to allow colony growth. From this, the colony-forming units (CFU/mL) were counted with the naked eye by an experienced and calibrated examiner (M.A.L.O). Figure 1 illustrates the sequence of the experimental technique.

Fig. 1. Flowchart of experimental groups distribution and technique

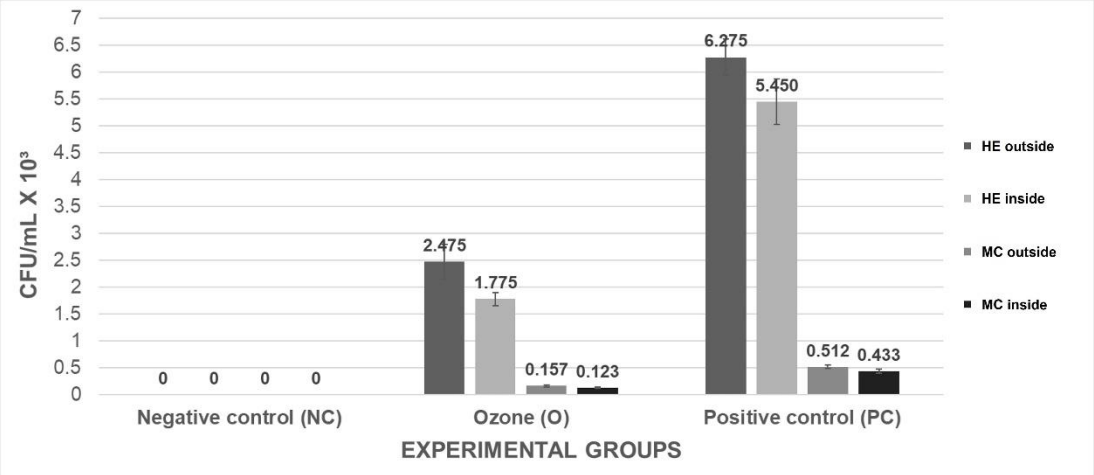


As the EH and MC implants had different diameters, the circumference of these implants was calculated (Formula: $C=2\pi r \rightarrow$ HE = 12.874 mm e MC = 13.502 mm), and the results of the MC implants were parameterized with the EH implants. For all groups, a Shapiro-Wilk normality test was performed. The intergroup comparison was performed by one-way analysis of variance (ANOVA) and Tukey's post-test. Comparisons between connection types and inside and outside the implants were done using the independent t-test, with a significance level of 5%.

3. RESULTS

Intergroup comparison showed a statistically significant difference among all groups in two connection types outside and inside the implants (*P* = .00). Group O showed a significant decrease in CFU/mL compared to group PC outside and inside the implants (*P* = .00), showing values of 2.475x10^3 (outside), 1.775x10^3 (inside), and 0.157x10^3 (outside), 0.123x10^3 (inside) for EH and MC connections, respectively. (Figure 2)

Fig. 2. Distribution of CFU/mL means and standard deviations obtained in the experimental groups for each connection type, outside and inside the implants (ANOVA and Tukey's post-test, p<.05)



In intragroup comparison, outside and inside the implants, in the same connection type, only group O in EH connection showed a statistically significant difference in the number of CFU/mL (*P* = .03) (Table 1). The comparison between the two connection types was statistically different for groups PC and O, outside and inside the implants (*P* = .00) (Table 2), demonstrating the greater contamination in EH connections and the potential for decontamination of the ozone outside and inside the implants on IA interface.

Table 1. Mean values and standard deviations of CFU/mL on comparison outside and inside the implants in the same connection type (independent t-test)

GROUPS	OUTSIDE	INSIDE	*P*
PC (EH)	6.275x10^3 ± 0.340	5.450x10^3 ± 0.420	0.08
O (EH)	2.475x10^3 ± 0.320	1.775x10^3 ± 0.125	0.03*
PC (MC)	0.512x10^3 ± 0.031	0.433x10^3 ± 0.036	0.09
O (MC)	0.157x10^3 ± 0.022	0.123x10^3 ± 0.015	0.17

* Statistically significant at *P* < .05

Table 2. Mean values and standard deviations of CFU/mL on comparison between the two connection types, outside and inside the implants (independent t-test)

GROUPS	EH	MC	*P*
PC (outside)	$6.275x10^3 \pm 0.340$	$0.512x10^3 \pm 0.031$	0.00*
PC (inside)	$5.450x10^3 \pm 0.420$	$0.433x10^3 \pm 0.036$	0.00*
O (outside)	$2.475x10^3 \pm 0.320$	$0.157x10^3 \pm 0.022$	0.00*
O (inside)	$1.775x10^3 \pm 0.125$	$0.123x10^3 \pm 0.015$	0.00*

* Statistically significant at $P < .05$

4. DISCUSSION

This study demonstrated that ozonated water decreased the number of CFU/mL at the implant-abutment interface in both connection types and also reduced the number of CFU/mL inside the external hexagon connection implants. These findings signal the potential of ozone as an adjunctive method for the prevention and treatment of peri-implant diseases.

As reported in previous studies [21, 25, 26, 29, 30], ozone has antimicrobial potential; however, as far as we know, studies related to peri-implant diseases are still scarce. Therefore, this study focused on the decontamination of the implant-abutment interface, which is a critical region for biofilm accumulation [11]. This accumulation leads to the development of peri-implant mucositis which, if not treated correctly [7], can evolve and compromise peri-implant bone [7, 11].

In this sense, finding a method that is effective and easy to apply not only in peri-implant mucositis treatment but also that can be used in peri-implant supportive therapy is important, aiming the maintenance of peri-implant tissue health and as a way of preventing peri-implant diseases.

In this study, ozone showed good antimicrobial action, reducing the number of CFU/mL compared to group PC, although it did not eliminate microorganisms from the interfaces. These results agree with Hauser-Gerspach et al. and Isler et al., who obtained good efficacy from ozone in gas form when decontaminating implant surfaces [26, 29]. Indeed, it is difficult to perform complete decontamination on these surfaces due to, among other factors, implants' macro and microgeometry.

In spite of ozone has some forms of application [23, 31, 32], in this study, ozonated water was chosen due to its easy application and greater safety than gas form [23, 31], for example. From a clinical point of view, ozonated water would be more interesting for peri-implant diseases treatment since this form can be used as an irrigation agent, inside the peri-implant sulcus, as an adjunct therapy in peri-implant mucositis treatment, or even applied in surgical procedures during peri-implantitis treatment, avoiding the dissipation that would occur with the use of the gas form [27].

Considering decontamination by ozone, concentration is also an important parameter. The literature says that the greatest antimicrobial effect of ozone is related to its concentration;

the greater the concentration, the greater the effect [32]. The increase in application time does not appear to improve its antimicrobial effects [32]. Considering this information and based on previous studies from our research group [27, 28], this study used a concentration of 60 mg/L, the maximum concentration offered by the used equipment.

Furthermore, according to the manufacturer, only 20% of the ozone that leaves the equipment is fixed in the water [28]. Therefore, it is estimated that the concentration applied on the interfaces was 12 mg/L. Tonon et al. used a final concentration of 7.24 µg/mL and obtained good antimicrobial action against biofilms, even with a lower ozone concentration in the water [32]. This limitation can be important in clinical use due to the difficulty in maintaining ozone concentration to deliver it to a specific site or needed area.

Temperature and pH also have an important effect on ozone concentration in water. Gas degradation rates increase with increasing temperature and pH of the medium [32]. For this reason, the water used was stored in a refrigerator at 4°C for the experiment, and the ozonated water was used immediately after preparation for the decontamination of the interfaces. The water used in this study has a pH of around 5 to 7, as informed by the manufacturer.

As expected, the EH connection was more contaminated. The microorganism's capacity of penetration inside the implants was also higher in the EH connection compared to the MC connection. These results agree with those found by Canullo et al. in their study with patients [12].

As for the ozone penetration potential decontaminating the interior of the implants, only the EH connection had a good result, significantly reducing the number of CFU/mL inside the implants. Although this difference may have occurred due to the greater contamination in EH, this finding is quite interesting since it can improve the predictability of this implant in the oral cavity, offsetting a possible disadvantage compared to the morse cone connection in terms of microorganism accumulation and penetration.

As an *in vitro* study, some limitations must be stated to collaborate with research in the field. First of all, in vitro, this study demonstrated a good antimicrobial effect of the ozonated water with the possibility of being tested in a clinical scenario. However, that is future research, and with the results presented here, it is not possible to state its effects in a clinical context. Also, this study did not compare ozonated water with other decontaminating agents, which is important in future in vivo research.

5. CONCLUSION

Considering this proposal's limitations and pioneering spirit, it was possible to conclude that the ozonated water presented good efficacy on in vitro decontamination of implant-abutment interfaces in external hexagon and morse cone connections. The good results presented here encourage future research in order to develop a truly effective clinical application protocol using ozone for treating and preventing peri-implant mucositis.

ETHICAL APPROVAL

Approved by the Research Ethics Committee of the Inga University Center, number 4.444.416.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

Option 2:

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc have been used during writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology

Details of the AI usage are given below:

1.

2.

3.

REFERENCES

1. Quirynen M, Herrera D, Teughels W, Sanz M. Implant therapy: 40 years of experience. Periodontology 2000. 2014;66(12):7-12.

2. Dreyer H, Grischke J, Tiede C, Eberhard J, Schweitzer A, Toikkanen SE, Glockner S, Krause G, Stiesch M. Epidemiology and risk factors of peri-implantitis: A systematic review. J Periodontal Res. 2018;53(5):657-81.

3. Mongardini C, Pilloni A, Farina R, Di Tanna G, Zeza B. Adjunctive efficacy of probiotics in the treatment of experimental peri-implant mucositis with mechanical and photodynamic therapy: a randomized, cross-over clinical trial. J Clin Periodontol. 2017;44(4):410-7.

4. Froum SJ, Gonzalez de la Torre E, Rosen PS. Peri-implant Mucositis. Int J Periodontics Restorative Dent. 2019;39(2):e46-e57.

5. Lindhe J, Meyle J, Group DoEWoP. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. J Clin Periodontol. 2008;35(8 Suppl):282-5.

6. Heitz-Mayfield LJA, Salvi GE. Peri-implant mucositis. J Clin Periodontol. 2018;45 Suppl 20:S237-S45.

7. Berglundh T, Armitage G, Araujo MG, Avila-Ortiz G, Blanco J, et al. Peri-implant diseases and conditions: Consensus report of workgroup 4 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018;89 Suppl 1:S313-S8.

8. Lee CT, Huang YW, Zhu L, Weltman R. Prevalences of peri-implantitis and peri-implant mucositis: systematic review and meta-analysis. J Dent. 2017;62:1-12.

9. Figuero E, Graziani F, Sanz I, Herrera D, Sanz M. Management of peri-implant mucositis and peri-implantitis. Periodontol 2000. 2014;66(1):255-73.

10. Salmeron S, Rezende ML, Consolaro A, Sant'ana AC, Damante CA, Greghi SL, Passanezi E. Laser therapy as an effective method for implant surface decontamination: a histomorphometric study in rats. J Periodontol. 2013;84(5):641-9.

11. Tallarico M, Canullo L, Caneva M, Ozcan M. Microbial colonization at the implant-abutment interface and its possible influence on periimplantitis: A systematic review and meta-analysis. J Prosthodont Res. 2017;61(3):233-41.

12. Canullo L, Penarrocha-Oltra D, Soldini C, Mazzocco F, Penarrocha M, Covani U. Microbiological assessment of the implant-abutment interface in different connections: cross-sectional study after 5 years of functional loading. Clin Oral Implants Res. 2015;26(4):426-34.

13. Carinci F, Lauritano D, Cura F, Lopez MA, Andreasi Bassi M, Confalone L, Pezzetti F. Prevention of bacterial leakage at implant-abutment connection level: an in vitro study of the efficacy of three different implant systems. J Biol Regul Homeost Agents. 2016;30(2 Suppl 1):69-73.

14. Batalha VC, Bueno RA, Fronchetti Junior E, Mariano JR, Santin GC, Freitas KMS, Ortiz MAL, Salmeron S. Dental Implants Surface in vitro Decontamination Protocols. Eur J Dent. 2020.

15. Junior EF, Zubek MJG, Mazucatto Queiroz P, Salvatore Freitas KM, Lopes Ortiz MA, Salmeron S. Sodium Bicarbonate Jet Reduces Contamination of Dental Implants In Vitro Without Causing Visible Surface Changes. Int J Oral Maxillofac Implants. 2022;37(3):587-92.

16. Verardi G, Cenci MS, Maske TT, Webber B, Santos LR. Antiseptics and microcosm biofilm formation on titanium surfaces. Braz Oral Res. 2016;30.

17. Esposito M, Grusovin MG, Worthington HV. Interventions for replacing missing teeth: treatment of peri-implantitis. Cochrane Database Syst Rev. 2012;1:CD004970.

18. Baysan A, Whiley RA, Lynch E. Antimicrobial effect of a novel ozone- generating device on micro-organisms associated with primary root carious lesions in vitro. Caries Res. 2000;34(6):498-501.

19. Eick S, Tigan M, Sculean A. Effect of ozone on periodontopathogenic species--an in vitro study. Clin Oral Investig. 2012;16(2):537-44.

20. McKenna DF, Borzabadi-Farahani A, Lynch E. The effect of subgingival ozone and/or hydrogen peroxide on the development of peri-implant mucositis: a double-blind randomized controlled trial. Int J Oral Maxillofac Implants. 2013;28(6):1483-9.

21. Srinivasan S, Amaechi B. Ozone: A paradigm shift in dental therapy. Journal of Global Oral Health. 2019;2(1):68-77.

22. Kumar P, Tyagi P, Bhagawati S, Kumar A. Current interpretations and scientific rationale of the ozone usage in dentistry: A systematic review of literature. European Journal of General Dentistry. 2014;3(3):175.

23. D'Ambrosio F, Caggiano M, Acerra A, Pisano M, Giordano F. Is Ozone a Valid Adjuvant Therapy for Periodontitis and Peri-Implantitis? A Systematic Review. J Pers Med. 2023;13(4).

24. Salazar-Leal JLM-S, Gloria Oca, María de los Ángeles, Andrea Carvajal-Montes de Chapa-Arizpe MG, Martínez-González GI. Application of ozonated water in non-surgical periodontal treatment. International Journal of Applied Dental Sciences. 2019;5(2):353-6.

25. Ameyaroy DK, Ramabhadran BK, Emmatty R, Paul TP, Jose P. Comparative evaluation of the effect of Ozone therapy and Photodynamic therapy in non-surgical management of Chronic periodontitis: A split mouth longitudinal study. J Indian Soc Periodontol. 2020;24(5):447-53.

26. Isler SC, Unsal B, Soysal F, Ozcan G, Peker E, Karaca IR. The effects of ozone therapy as an adjunct to the surgical treatment of peri-implantitis. J Periodontal Implant Sci. 2018;48(3):136-51.

27. Agostini F, Faccini M, Fitarelli F, Ortiz MAL, Salmeron S, Oliveira RCG, Valarelli FP, Oliveira RC, Freitas KMS. In Vitro Comparison of Antibacterial Effect of Ozonated Water and Ozonated Gas. Ozone: Science & Engineering. 2020:1-7.

28. Rosado RB, Cruz EJ, Souza TP, Freitas KMS, Ortiz MAL, Salmeron S. Ozonated Water Promotes in vitro Decontamination of Dental Implants Surface. Ozone: Science & Engineering. 2024;46(1):6.

29. Hauser-Gerspach I, Vadaszan J, Deronjic I, Gass C, Meyer J, Dard M, Waltimo T, Stubinger S, Mauth C. Influence of gaseous ozone in peri-implantitis: bactericidal efficacy and cellular response. An in vitro study using titanium and zirconia. Clin Oral Investig. 2012;16(4):1049-59.

30. Vasthavi C, Babu HM, Rangaraju VM, Dasappa S, Jagadish L, Shivamurthy R. Evaluation of ozone as an adjunct to scaling and root planing in the treatment of chronic periodontitis: A randomized clinico-microbial study. J Indian Soc Periodontol. 2020;24(1):42-6.

31. Suh Y, Patel S, Kaitlyn R, Gandhi J, Joshi G, Smith NL, Khan SA. Clinical utility of ozone therapy in dental and oral medicine. Med Gas Res. 2019;9(3):163-7.

32. Tonon CC, Panariello BHD, Spolidorio DMP, Gossweiler AG, Duarte S. Anti-biofilm effect of ozonized physiological saline solution on peri-implant-related biofilm. J Periodontol. 2020.