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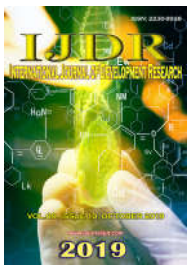

REVIEW ARTICLE OPEN ACCESS

OZONIZED WATER AS A DENTIN CLEANING SOLUTION

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ABSTRACT

This work aims to evaluate the antibacterial activity of ozonized water (OW) on the Streptococcus mutans and Enterococcus faecalis microorganisms and also to verify its influence on adhesive bond strength (BS), both conditions compared to 17% Trisodium EDTA; Sodium hypochlorite (HP) 2.5%; Chlorhexidine (CHX) 2% and Physiological solution (PS). The antibacterial evaluation was carried out through the Bacterial Viability Test (BVT) on the microorganisms E. faecalis and S. mutans, in the following times: T0 (soon after the bacterial inoculum placement); T10 (10min), T20 (20min), T30 (30min) and T60 (60min). For BS, 50 crowns of bovine teeth were cut and divided according to the cavity cleaning solution. Three bulk fill resin cylinders were made for each bovine crown and the test was performed after 24 hours. The failure modes were evaluated with stereoscopic loupes with a 40x magnification. The data were analyzed statistically by the ANOVA test and Tukey's post-test with a $p < 0.05$ significance level. All the tested solutions showed antibacterial activity, except for physiological solution. For BS there were no significant differences among the groups. It can be concluded that ozonized water may be an option as a dentin cleaning solution without interfering in bond strength.

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INTRODUCTION

Dental caries is still a major public health problem throughout the world with high prevalence and significant social impact. Although there are other bacteria involved in the dental caries pathogenesis, Streptococcus mutans plays a central role in the development of cariogenic biofilms, mainly due to their acidogenic characteristics. This microorganism uses dietary sucrose to synthesize extracellular polysaccharides which are functional structures that mediate their adhesion to dental surfaces.

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The carbohydrate fermentation process creates low pH microenvironments favoring the enamel and dentin demineralization (De Luca *et al*., 2017). Enterococcus faecalis is another microorganism found in the oral cavity, a facultative anaerobic gram-positive bacteria. Resistance to Enterococcus faecalis treatment is explained by its potential for penetrating into dentinal tubules and its prolonged survival may be due to its adhering capacity to the collagen matrix existing in the dentin, as well as to its inadequate response to the irrigation with antimicrobial solutions (Camacho-Alonso *et al*., 2017). Pretreatment of dental surface with an antibacterial agent is indicated for eliminating the harmful effects caused by bacterial infiltration (Ersin *et al*., 2016). Such procedure would

avoid caries recurrence and favor restorative materials retention to the dental structures, reducing the microleakage in the restorations (Franco *et al*., 2007). There are different solutions for this purpose, such as EDTA, sodium hypochlorite, chlorhexidine and recently, ozonized water. Ozonized water is known as a strong antimicrobial agent for bacteria, fungi, viruses and protozoa. With antimicrobial action, the use of ozone is confirmed as a new alternative cleaning solution. In the aqueous phase the ozone has advantages such as being biocompatible to dental structures, antimicrobial, lack of mutagenicity and rapid in microbicidal effects (Goztas *et al* 2014). Its antimicrobial action may eliminate resistant oral microorganisms. One of the crucial aqueous ozone properties is its non-toxicity to oral cells in vitro. However, there is no consensus in the literature on the influence of ozonized water in the bond strength of direct restorative materials (Oznurhan *et al*., 2015; Nohammadi *et al*., 2013). In addition to satisfactory microbicidal properties, suitable dentin cleaning solutions for the restoration process should be harmless or enhance the bond between tooth and adhesive systems. Since the durable bonding between polymeric restorative materials and hard dental structures is an important parameter for the dental restorations success, in order to determine the performance of an adhesive system, its mechanical strength is usually measured by determination of the shear bond strength (Escribiano *et al*., 2003). In this sense, bond strength tests are the most frequently used to assess the adhesive quality of the interface dentin structure and adhesive systems. Thus, this work aimed to compare the influence of the 16 ppm ozonized water on the S. mutans and E. faecalis microorganisms, and on the bond strength, in comparison to other solutions in the market.

Preparation of solutions: The selected solutions were used in the liquid state, exactly as in the original packaging. The description of the materials used is shown in Table 1.

Ozonized water preparation: The OW solution was prepared by the ozone generator (Ozone & Life Ltda, Model O & L1.5 RM, São José dos Campos, SP, Brazil) minutes before its use. Medicinal ozone was used associated with distilled water. The aqueous ozone was prepared by passing ozone gas through the water into a beaker with one end of the silicon tube connected to the ozone generator's end and the other end placed inside the beaker containing water. The ozone concentration was 16 ppm, confirmed by the iodometric titration test (Oznurhan *et al*., 2015).

Evaluation of Antibacterial action

Bacterial viability test: Bacterial sensitivity was assessed by formation of microbial growth inhibition in sterile Petri dishes, with Chromium agar plates for E. faecalis and blood agar plates for S. mutans. Initially, strains of E. faecalis and S. mutans were reactivated in Trypticase Soy Broth (TSB), incubated in a bacteriological oven at 37°C (± 1) for 24 hours. After reactivation, strains of E. faecalis and S. mutans were respectively plated in nutrient agar and blood agar plates, and incubated under the same conditions described above. After colony growth, 3 to 4 colonies of each microorganism were transferred into tubes containing 0.9% physiological solution in order to obtain a turbidity corresponding to 0.5 of the McFarland scale, that is, approximately 1.5 x 10^8 CFU/mL (colony forming units/mL). Then, by using a calibrated pipette, 0.5 mL of the E. faecalis inoculum and 0.5 mL of S. mutans were added to 0.5 mL of the EDTA, HP, CHX and OW dentin solutions as well as 0.5 mL of PS and plated with the aid of a 10 µl disposable sterile calibrated loop on the surfaces of the repetition plates containing Chromium agar for E. faecalis and blood agar for S. mutans in different times distributed as follow: T0 (soon after the placement of the bacterial inoculum); T10 (10min), T20 (20min), T30 (30min) and T60 (60min), each plate being divided into 10 areas for each solution and time analyzed in a total of 10 repetitions for each analysis, as shown in the diagrams below:

After the plating they were placed in a bacteriological oven at 37ºC ± 1 for 48 hours for further bacterial growth analysis.

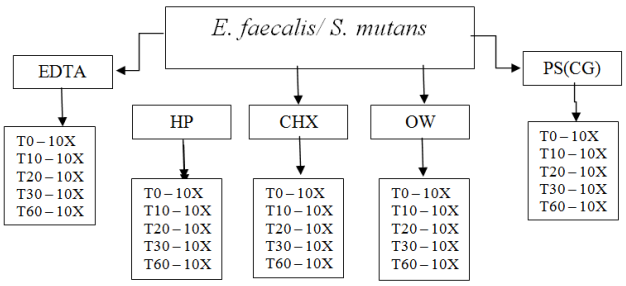
Table 1. Origin of the materials used in the research

Materials (Abbreviation)	Composition	Manufacturer
EDTA trisodium 17% (EDTA)	Trisodium ethylenediaminetetraacetic acid; sodium hydroxide; distilled water.	Biodinâmica (Ibiporã, Paraná, Brazil)
Sodium hypochlorite chlorine 2.5% (HP)	Sodium hypochlorite, alkalinizers and water	Ciclo Forma (Serrana, São Paulo, Brazil)
Chlorhexidine digluconate 2% (CHX)	2% Gluconate; Water; glycerin; ethanol; polysorbate 20; sodium saccharinate; FD & C Blue, No. 1.	Maquira (Maringa, Paraná, Brazil)
Ozonized Water (OW)	Distilled water + 16 ppm ozone	
Physiological solution (PS)	0.354g of Na^+; 0.546g of Cl^-; pH=6.0.	
Condac 37%	Orthophosphoric acid 37%	FGM - (Joinville, Santa Catarina, Brazil)
Ambar Universal	Methacrylic monomers; photoinitiators; co-initiators; stabilizer; Inactive ingredients: inert filler: silica nanoparticles. Vehicle: ethanol.	FGM - (Joinville, Santa Catarina, Brazil)
Opus Bulk Fill Flow	Active ingredients: uretanadimetacrylic monomers, stabilizers, camphorquinone and co-initiator. Inactive ingredients: inorganic silicon dioxide (silica) fillers, stabilizers and pigments.	FGM - (Joinville, Santa Catarina, Brazil)

MATERIALS AND METHODS

Sample Calculation

The sample calculation was based on family F probability distributions, with a design of repeated families and interaction within and among the factors. The effect size used was 0.15, type 1 error (α) of 0.05, analysis power of 0.85 guaranteed a minimum of 50 sample units, of which 10 samples per experimental group, both for the antimicrobial action tests and the micro-shear bond strength test. The sample calculation was performed in GPower program (3.1.9.2 version - University of Düsseldorf, Düsseldorf - Germany).



Legend: EDTA: EDTA Trisodium Liquid 17%; HP: Sodium hypochlorite Chlorine 2.5%; CHX: Chlorhexidine 2%; OW: Ozonized water; PS (CG): Physiological solution (control group).

Figure 1. Distribution diagram of the groups for evaluation of antibacterial action on E. Faecalis and S. mutans

Bond Strength Evaluation

Preparation of sample units (SUs): Fifty bovine incisor teeth recently extracted and free of periodontal tissue and stored in 1% thymol solution were selected. The teeth were cut in order to separate the crown from the root, in high rotation on abundant cooling with diamond tip nº 4138 (KG Sorensen, São Paulo, SP - Brazil). Then, the crowns were included in PVC tubes with acrylic resin, leaving the vestibular surface free. Dental enamel was worn on the vestibular face with a gypsum trimmer until reaching dentin, then the elements were stored in physiological solution.

Adhesive procedure: The teeth were randomly divided according to the cavity cleaning material used (n = 10): EDTA; HP; CHX; OW and PS, as shown below:

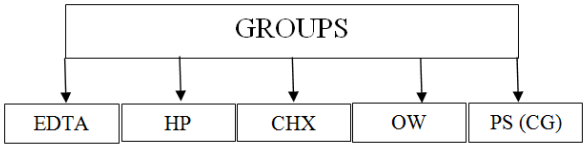


Figure 2. Diagram distribution of the groups for evaluation of bond strength

Legend: EDTA: EDTA Trisodium Liquid 17%; HP: Sodium hypochlorite Chlorine 2.5%; CHX: Chlorhexidine 2%; OW: Ozonized water; PS (CG): Physiological solution (control group).

The bulk fill flow composite resin cylinders were made using a Tygon matrix (Tygontubing, TYG -030, Saint-Gobain Performance Plastic, MaimeLakes, FL, USA) with an internal diameter of 1mm and 2mm in height. The adhesive procedure was performed according to the manufacturer's instructions.

After the adhesive technique, the matrix was placed with a clinical clamp on the surface and the Opus Bulk Fill Flow resin (FGM - Joinville, SC, Brazil) was inserted. The photo activation was done with Bluephase light apparatus (Ivoclar Vivadent - Liechtenstein, Austria) with 900 mW/cm^2 (Demetron-Demetron research Radiometer, Danbury, CT, USA) for 40 seconds. Three bulk fill composite resin cylinders were made for each bovine crown, totaling 30 SUs for each evaluated group. The matrix was then sectioned and removed with a nº11 scalpel blade and the SUs were stored for 24 hours in physiological solution at 37ºC ±1.

Micro-shear bond strength test: The resin cement cylinders were tested in a universal testing machine (EMIC DL-200 MF). The shear loading was applied at the base of the cylinders at a speed of 0.5mm/min until the union break. The micro-shear bond strength was calculated and expressed in MPa.

Fracture analysis: The failure modes were evaluated with a stereoscopic loupes with a 40× magnification (Lambda LEB-3 n° 18233, São Paulo, Brazil) and the classification followed the recommended criteria by the International Organization for Standardization (TR 11405.18) 9 (ISO, 1994):

- Cohesive in Dentin (CD): failure exclusively within the dentin;
- Cohesive in Resin (CR): failure exclusively within the resin;
- Adhesive (A): failure of the cement/dentin interface;
- Mixed (M): failures of the adhesive/dentin/cement interface, which include cohesive failure on close substrates.

Statistical analysis

The tests were performed using the Bioestat 5.3 program (Instituto Mamirauá, 2007). The data obtained were preliminarily submitted to the evaluation of the distribution type by the Shapiro-Wilk test. Considering that the data adhered to the normality curve, ANOVA (one-way) test was used for union strength test and antibacterial activity, $p < 0.05$, for the comparisons among the experimental groups.

RESULTS

Evaluation of antibacterial action: The absence of bacterial growth was observed for all solutions in the two culture media used for the bacterial activity test and at all times, except for the control group, according to Table 2.

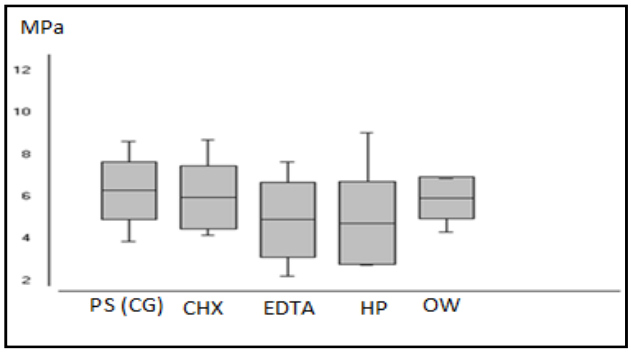
Table 2. Viability of the microorganism against the solutions

	T0	T1	T2	T3	T4
EDTA	0	0	0	0	0
HP	0	0	0	0	0
CHX	0	0	0	0	0
OW	0	0	0	0	0
PS(CG)	$>10^3$ CFU/mL	$>10^3$ CFU/mL	$>10^3$ CFU/mL	$>10^3$ CFU/mL	$>10^3$ CFU/mL

Legend: EDTA: EDTA Trisodium Liquid 17%; HP: Sodium hypochlorite Chlorine 2.5%; CHX: Chlorhexidine 2%; OW: Ozonized water; PS (CG): Physiological solution (control group)

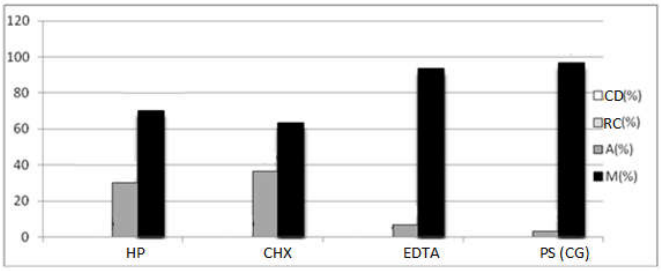
Evaluation of Bond strength: Figure 3 represents the result of the statistical analysis for the union resistance variable, it was possible to observe the absence of significant differences between the evaluated groups, p≥. 0.05.

Evaluation of fracture analyses: Regarding the fracture analysis, a higher incidence of the samples rupture in mixed fracture was observed, followed by adhesive fracture. The other types of fractures (cohesive in dentin and cohesive in resin) were not found.



Legend: EDTA: EDTA Trisodium Liquid 17%; HP: Sodium hypochlorite Chlorine 2.5%; CHX: Chlorhexidine 2%; OW: Ozonized water; PS (CG): Physiological solution (control group)

Figure 3. Box plot of the experimental groups according to the evaluated solution. Absence of significant differences, p > 0.05



Legend: EDTA: EDTA Trisodium Liquid 17%; HP: Sodium hypochlorite Chlorine 2.5%; CHX: Chlorhexidine 2%; OW: Ozonized water; PS (CG): Physiological solution (control group); Cohesive in Dentin (CD): failure exclusively within the dentin; Cohesive in Resin (CR): failure exclusively within the resin; Adhesive (A): failure of the cement/dentin interface; Mixed (M): failures of the adhesive/dentin/cement interface, which include cohesive failure on close substrates.

Figure 4. Bar chart of the experimental groups according to the fracture type

DISCUSSION

This study's results highlighted that for the bacterial viability test a positive response was obtained for all the solutions used. These results are probably due to the favorable conditions for the use of ozone in this test, in a temperature of 37ºC ± 1 during all the analysis steps (T0, T10, T20, T30 and T60), respecting the pH ideal conditions, temperature and half life of the solution (Herrera, *et al*., 2010). These results corroborate with the findings of Estrela *et al*. (2007) and Ercan *et al*. (2009), which demonstrate the antimicrobial action of both sodium hypochlorite and chlorhexidine. These solutions, like ozone, act through the high oxidizing power, presenting powerful antimicrobial action, as demonstrated by the studies of Huth *et al*. (2009) and Kustarci *et al*. (2009). The antibacterial activity found in ozonized water may be the result of its oxidation potential capable of destroying the cell wall and cytoplasmic membrane of bacteria and fungi. When dissociated from oxygen, the ozone creates an oxygen rich environment, thus disturbing the normal ecosystem of the plate. The enzymatic control system of the cell is blocked by the inhibition of glycoproteins, glycolipids and other amino acids resulting in functional cessation and death of the microorganism (Celiberti *et al*., 2006).

As found in this study, Ebenezar *et al*. (2015) verified in an in vitro study that ozonized water completely inhibited the S. mutans and E. faecalis growth. These data disagree with the finding by Van Acker *et al*. (2014), who found the absence of the ozonized water antibacterial activity, which can be justified by the methodological difference of time and temperature of solution analysis, since when submitted to high storage temperatures, it allows to accelerate the decomposition process of the ozone (Lapoli *et al*., 2014). In addition, when used as a disinfectant, ozone has an immediate effect, allowing recontamination, since its residual effect is practically zero, being possible the decomposition of the solution, disabling the antibacterial action. In the gaseous form it is a selective oxidant and affects only certain compounds, however the aqueous form is highly unstable and decomposes rapidly through a complex series of chain reactions resulting in the formation of hydroxyl radicals (OH) which are among the most oxidant reactive species (Anumula *et al*., 2017). For the micro-shear test, bovine teeth were used, based on the work of Reis *et al*. (2004) which demonstrated similar performance in the BS tests on human teeth, both in enamel and dentin. This also corroborates with the findings of Munch et. al. (2000), who affirm that there are similarities in the general morphology and organic matrix of collagen compared to human dentin, which allows them to be used in adhesion tests. Regarding the specificities of each solution, this work agrees with Sauro *et al*. (2010) which state that the use of EDTA prior to acid conditioning was statistically similar to the other tested solutions and did not find differences among irrigation solutions when using water/ethanol based adhesive systems. However, such behavior differs from Gu *et al*. (2009), in which the EDTA presented statistically higher values than the control group, this difference can be attributed to the methodology, since the authors used root dentin and our study used coronary dentin. In the coronary dentin, the EDTA promotes only a slight removal of the smear layer, without increasing the surface roughness, not interfering in the retention; opposed to when applied to root dentin, resulting in a greater uniformity of TAGs (polymerized resin extensions in the dentin tubules), providing that the collagen fibers maintain a higher mineral content, becoming more stable and less susceptible to dehydration, improving the infiltration of the resinous monomers and resulting in high adhesive strength values (Habelitz *et al*., 2002).

The CHX adhesive systems' effect on dentin bond strength is a controversial issue. Some studies, such as Sharma *et al*. (2011) and Reddy *et al*. (2013) reported that pretreatment of the dentin surface with 2% CHX for 20s has adversely affected the bond strength of the self-etching adhesive system. Brackett *et al*. (2007) have demonstrated that the use of CHX prior to acid etching, as performed in this study, does not interfere with the bond strength of conventional adhesive systems. Chlorhexidine has an immediate bactericidal effect inside the cavity, the ability to modify the smear layer and an ionic bonding with the dentin surface. In addition to the antibacterial effect, it acts as an inhibitor of the matrix of metalloproteinases (MMP). This additional chlorhexidine effect can prevent degradation of collagen at the adhesive interface over time (Loguercio *et al*., 2016). Oznurhan *et al*. (2015) showed that pretreatment with CHX (2%) has increased the adhesive strength of the self-etching adhesive system. In this study, pretreatment with CHX did not interfere in the bond strength when compared to the other groups. Such differences may be justified because of the difference among the dentin of the teeth and the type of adhesive used, the regularizations of the dentin layer and the duration of disinfection. Regarding ozone, it corroborates with Schmidlin *et al*. (2005) and Pithon & dos Santos (2010) who did not observe reduction in

adhesion between the dental substrate and the composite resin when evaluating the effects of the ozone application on the dentin and enamel of the bovine teeth in the union resistance by the micro-shear test. In a different study, Oznurhan *et al*. (2015) found that aqueous ozone (3-4 ppm) increased the bond strength of the self-etching adhesive system. This dissimilarity has occurred due to its study methods (lower ppm of aqueous ozone, different test methods and use of deciduous teeth), since ozone can not alter the physical properties of the enamel and does not affect the modulus of elasticity and Vickers hardness of the dentin, besides not interfering in the sealing capacity of the adhesive systems15, which favors its use when compared to other solutions. Regarding the failure mode, there was a predominance of mixed failures for all groups. The occurrence of cohesive and mixed failures in bond strength tests with reduced adhesive area indicates bond strength values higher than the adhesive interface causing damage to dentin and resinous substrates, corroborating with a study by Dönmez *et al*. (2018). Despite the limitations of this study, it was possible to verify that the ozonized water presented similar results to the cleaning solutions already established in the literature, both in the immediate antibacterial action and the bond strength. Thus, freshly prepared ozonized water is an alternative oral antiseptic with high antibacterial activity against oral pathogens and fulfills cellular biological characteristics in terms of oral biocompatibility (Munch *et al*. 200). Further studies on the use of ozonized water in dentistry are needed to prove its efficacy and to stablish protocols for the ozone use.

Conclusion

Based on the results, it was possible to conclude that the ozonized water presented similar behavior to the other dentin cleaning solutions in both the antibacterial action and the bonding strength.

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