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Dissertação:

EFICÁCIA DE BOCHECHO COM ÁGUA OZONIZADA SOBRE A FORMAÇÃO DO BIOFILME BACTERIANO BUCAL E INFLAMAÇÃO GENGIVAL: UM ENSAIO CLÍNICO RANDOMIZADO CRUZADO

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RESUMO

A ozonioterapia tem ganhado destaque recentemente, porém a reatividade da molécula de ozônio faz dela muito instável, sendo necessário um protocolo de ozonização que garanta sua ação terapêutica. Além disso, os efeitos do ozônio sobre biofilmes bucais e a inflamação gengival ainda precisam ser elucidados. Sendo assim, foram realizados dois estudos experimentais para esclarecer os pontos citados anteriormente. O primeiro estudo é de natureza laboratorial, que teve como objetivo avaliar a viabilidade da água ozonizada em diferentes temperaturas e regimes de ozonização ao longo do tempo. Foram realizados três experimentos. No primeiro foi avaliada a temperatura da água, no segundo o tempo de ozonização e no terceiro foi realizada a confirmação do que foi encontrado nos experimentos anteriores. Após obtenção da água em cada experimento, procedeu-se a análise da concentração desse gás em meio aquoso através de kit específico nos períodos de 5, 10, 15, 30, 60 minutos e 2, 4 e 6 horas. A partir deste estudo foi realizado um protocolo de ozonização com água bidestilada em temperatura próxima de 0ºC gerada a 70µg/mL de ozônio por 10 minutos que duraria aproximadamente 2 horas para ser utilizado no ensaio clínico posterior. O segundo estudo foi um ensaio clínico, randomizado, cego e cruzado que teve como objetivo avaliar o efeito do bochecho de água ozonizada na formação de biofilme e inflamação gengival. Para tal, 42 alunos de Odontologia da Universidade Federal do Rio Grande do Sul foram divididos em dois grupos: Grupo Teste, que utilizou o bochecho de água ozonizada, e Grupo Controle, que recebeu o bochecho de água bidestilada. Para a investigação da formação inicial de biofilme subgengival foi utilizado o Índice de Zona Livre de Placa em 24, 48, 72 e 96 horas e para a inflamação gengival, o volume de fluido crevicular gengival no baseline e nas 96 horas. Os resultados não revelaram diferença estatisticamente significativa entre os grupos na conversão da placa supragengival para subgengival. Nas avaliações de frequência dos escores de Indice de Zona Livre de Placa e no volume de fluido crevicular também não foram observadas diferenças estatisticamente significativas. De acordo com os resultados obtidos, pode-se concluir que a água ozonizada parece não afetar a formação de placa supra e subgengival assim como a inflamação gengival.

Palavras-chave: Ozônio. Uso terapêutico. Administração e dose. Biofilmes. Gengivite.

ABSTRACT

Ozone therapy has gained prominence in recent years, however the reactivity of the ozone molecule makes it very unstable, requiring an ozonation protocol that guarantees its therapeutic action. In addition, the effects of ozone on oral biofilms and gingival inflammation have yet to be elucidated. Therefore, two experimental studies were carried out to clarify the points mentioned above. The first one was a laboratory study, which aimed to assess the viability of ozonated water at different temperatures and ozonation regimes over time. Three experiments were carried out. In the first, the water temperature was evaluated, in the second the ozonation time and in the third, the confirmation of what was found in the previous experiments was carried out. After obtaining the water in each experiment, the analysis of the concentration of ozone in aqueous form was carried out through a specific kit in the periods of 5, 10, 15, 30, 60 minutes and 2, 4 and 6 hours. From this study, an ozonation protocol was performed with bidistilled water at a temperature close to 0^{o}C generated at 70µg/mL of ozone for 10 minutes that would last approximately 2 hours to be used in the subsequent clinical trial. The second study was a randomized crossover clinical trial that aimed to evaluate the effect of ozonated water mouthwash on the biofilm formation and gingival inflammation. For that, 42 dental students from the Federal University of Rio Grande do Sul were divided into two groups: Test Group, which used the ozonated water mouthwash, and Control Group, which received the bidistilled water mouthwash. For the investigation of the initial subgingival biofilm formation, the Plaque Free Zone Index was used at 24, 48, 72 and 96 hours and for gingival inflammation, the volume of gingival crevicular fluid at the baseline and at 96 hours. The results show that there was no statistically significant difference between the groups in the conversion of the supragingival to subgingival plaque. In the frequency assessments of the Plaque Free Zone Index scores and in the volume of crevicular fluid, no statistically significant differences were observed. According to the obtained results, it can be concluded that ozonated water does not seem to affect the formation of supra and subgingival plaque or gingival inflammation.

Key words: Ozone. Therapeutic uses. Administrations and dosage. Biofilms. Gingivitis.

APRESENTAÇÃO

A presente dissertação de mestrado foi desenvolvida no Programa de Pós-Graduação em Odontologia da Universidade Federal do Rio Grande do Sul (UFRGS) a partir do projeto de número 37291, estando prevista a publicação de dois artigos sendo um deles em uma revista da área de Ozonioterapia e outro em revista da área de periodontia de circulação internacional. Trata-se de um estudo realizado nas dependências da Faculdade de Odontologia da UFRGS e envolveu a participação de docentes, pós-graduandos e de alunos de Iniciação Científica da área de Periodontia desta instituição. É um projeto que apresenta duas frentes de trabalho principais: uma delas, em nível laboratorial, propõe-se a determinar o tempo e temperatura ideais para obtenção de uma água ozonizada viável para uso no experimento principal. A outra frente refere-se ao estudo principal que objetiva avaliar a eficácia clínica da água ozonizada comparada ao veículo em um cenário clínico.

Assim, esta dissertação se divide em três sessões distintas: uma introdução, que objetiva nortear o leitor acerca da temática em questão; dois artigos científicos, um de natureza laboratorial e um ensaio clínico randomizado; e, por fim, considerações finais, que incluem uma avaliação atual sobre a perspectiva que este estudo contribui com a literatura da área.

SUMÁRIO

1 INTRODUÇÃO

No processo de formação do biofilme dental, microrganismos se aderem à superfície dos dentes e crescem como uma comunidade organizada e estruturada com diversas espécies e envoltas por uma matriz extracelular (Costerton *et al.*, 1999; Marsh, 2004). Biofilmes apresentam características únicas, como uma formação mais lenta, porém muito mais resistentes do que bactérias ou culturas isoladas por terem desenvolvido mecanismos de comunicação e proteção intercelulares (Armitage, 1999).

O acúmulo desse biofilme supragengival gera uma resposta inflamatória no tecido gengival do hospedeiro, a gengivite. No processo de formação deste biofilme supragengival ocorre a comunicação do mesmo com o ambiente subgengival. Isso ocorre aproximadamente após 72-96 horas de acúmulo, quando a zona livre de placa, espaço entre a formação de biofilme e a margem gengival, começa a desaparecer pelas projeções bacterianas que vão em direção a esta margem (Weidlich *et al.*, 2001). Assim, ocorre o início da formação de biofilme subgengival, que poderá levar ao desenvolvimento da periodontite, doença infecto-inflamatória que acomete os tecidos de sustentação, podendo levar à perda dentária.

Parte do tratamento da gengivite e da periodontite compreende a desorganização deste biofilme diariamente por parte do paciente. Para uma desorganização diária e efetiva, os instrumentos mais utilizados são as escovas multicerdas e o fio dental, além de colutórios, que, como adjuvantes, podem compensar limitações dos instrumentos citados anteriormente (Gunsolley, 2006). Estão presentes no mercado diversas formulações e concentrações de colutórios, como os óleos essenciais, com ou sem álcool, e o cloreto de cetilpiridínio, podendo estar associado ou não a outros agentes, apresentando efeitos antiplaca e antigengivite quando utilizados como adjuvantes (Sharma *et al.*, 2004; Haps *et al.*, 2008; Quintas *et al.*, 2017; Rosing *et al.*, 2017). Como agente substitutivo, a clorexidina é considerada padrão ouro, apresentando baixa toxicidade e alta substantividade.

Embora exista uma grande variedade de soluções utilizadas como colutórios, todas elas apresentam limitações importantes como baixa substantividade, percepção gustativa variável entre os indivíduos e reações adversas que incluem manchamento extrínseco, alteração gustativa e alergias. Desta maneira, são estudados outros

agentes terapêuticos que tenham potencial para obterem resultados tão bons ou melhores que os já existentes e que tenham menos limitações que estes.

O ozônio (O_3) é a forma triatômica do oxigênio, um gás presente na atmosfera terrestre, apresentando coloração azul, odor característico, grande solubilidade e alta instabilidade, que acaba se decompondo espontaneamente em oxigênio. O O_3 tem ganhado popularidade na área odontológica por suas características antimicrobianas e cicatrizadoras com doses relativamente baixas.

É um oxidante extremamente potente que demonstrou ser um agente confiável contra bactérias, fungos, protozoários e vírus, sem gerar resistência microbiana (Restaino *et al.*, 1995; Kim *et al.*, 1999; Arita *et al.*, 2005). A oxidação causada pelo ozônio danifica a parede celular e a membrana citoplasmática nestes microrganismos, aumentando a permeabilidade dessas membranas e permitindo que as moléculas de ozônio entrem nas células causando uma desordem funcional e estrutural, degradando-as (Bocci, 2006; Smith *et al.*, 2017). Estudos demonstram que além de interferir no funcionamento de células de bactérias isoladas, o ozônio também age sobre biofilmes *in vitro* (Nagayoshi *et al.*, 2004).

Além de atuar contra microrganismos, a molécula tem sua ação também sobre as células do indivíduo. Tais reações de oxidação mediadas por radicais livres causadas pelo ozônio geram um estresse oxidativo agudo e controlado. Sua alta solubilidade faz com que seja consumido na sua totalidade, não deixando resíduos tóxicos para o organismo (Cardile *et al.*, 1995; Smith *et al.*, 2017). As espécies reativas de oxigênio formadas reagem com componentes sanguíneos, células endoteliais e sistema vascular, provocando respostas biológicas que afetam positivamente o metabolismo do oxigênio, a energia celular, a imunomodulação e o sistema de defesa antioxidante (Shiratori *et al.*, 1993; Verrazzo *et al.*, 1995; Huth *et al.*, 2006; Seidler *et al.*, 2008; Kazancioglu *et al.*, 2014).

A utilização do ozônio de forma terapêutica começou há mais de 150 anos, quando a obtenção artificial do gás passou a ser viável a partir de um aparelho que era capaz de sintetizar o gás por meio de descargas elétricas nos átomos de hidrogênio. O uso medicinal deste gás no Brasil começou somente na década de 1970, e tem seu uso principal atualmente em operações sanitárias e na indústria alimentícia como desinfetante de água e alimentos para armazenamento diminuindo quantidade bacteriana e os níveis de compostos orgânicos tóxicos pelo seu grande poder oxidativo (Kim *et al.*, 1999).

As formas mais comuns para sua administração são o gás e a água ozonizada. A forma gasosa pode diminuir a viabilidade das células e levar a intoxicação, enquanto a forma aquosa apresenta um nível de biocompatibilidade adequado às células orais, sugerindo ser o modelo mais apropriado para aplicação (Ebensberger *et al.*, 2002; Nagayoshi *et al.*, 2004; Huth *et al.*, 2006). A água ozonizada, contudo, apresenta rápida degradação quando exposta ao oxigênio e a outros fatores ambientais como temperatura, luminosidade e composição da água, devendo ser utilizada nos primeiros cinco ou dez minutos após o preparo em temperatura ambiente (Staehelin e Hoigne, 1985). Apesar destes achados, estudos avaliando a viabilidade da água ozonizada em diferentes cenários incluindo temperatura, concentração e tempo de ozonização da água e ainda são necessários para um melhor entendimento das reais possibilidades de utilização do ozônio como um agente adjuvante ao controle mecânico do biofilme.

Existem diversos estudos analisando o papel do ozônio em diferentes áreas da odontologia. Ensaios clínicos utilizando a ozonioterapia no tratamento da hipersensibilidade dentinária, na cicatrização de lesões e feridas pós-operatórias, na desinfecção de lesões cariosas, de canais radiculares e como adjuvantes aos tratamentos das doenças periodontais existem e estão publicados, porém apresentam metodologias e resultados controversos (Baysan e Lynch, 2004; Azarpazhooh e Limeback, 2008; Patel *et al.*, 2011; Hayakumo *et al.*, 2013; Katti e Chava, 2013; Safwat *et al.*, 2017). Essas características dos estudos citados anteriormente demonstram que os mecanismos de ação do ozônio contra o biofilme dental, assim como suas concentrações, métodos de aplicação, entre outros, ainda precisam ser elucidados.

Assim, o potencial efeito em processos infecciosos e as características imunomoduladoras que a ozonioterapia apresenta, indicam uma possível ação antiplaca e antigengivite que precisa ser esclarecida. Além disso, a água ozonizada apresenta biocompatibilidade com tecidos gengivais e pode ser administrada como colutório a fim de utilizada como adjuvante ao controle mecânico do biofilme. Diante da limitada literatura disponível avaliando a viabilidade da água ozonizada em diferentes cenários, e da inexistência de estudos associando o bochecho de água ozonizada à formação inicial de biofilme subgengival e à inflamação, este estudo se justifica.

2 OBJETIVOS

2.1 GERAL

Avaliar o efeito do bochecho de água ozonizada na formação do biofilme e na inflamação gengival.

2.2 ESPECÍFICOS

- ✓ Avaliar a viabilidade da água ozonizada para otimizar seu uso clínico, através de um estudo laboratorial, contemplando eventuais influências de tempo e temperatura
- ✓ Avaliar o efeito de bochecho com água ozonizada sobre a formação inicial do biofilme bacteriano subgengival mensurado através do Índice de Zona Livre de Placa;
- ✓ Avaliar o efeito de bochecho com água ozonizada sobre o volume de fluido crevicular gengival.

2.3 HIPÓTESE

A hipótese a ser testada é que o uso da água ozonizada como bochecho resultará em diferenças na formação do biofilme bacteriano bucal e inflamação gengival.

3 ARTIGO CIENTÍFICO 1

EFFECT OF OZONATION TIME AND TEMPERATURE ON OZONATED WATER VIABILITY FOR THERAPEUTIC PURPOSES IN DENTISTRY

Runing Title: Time and temperature on viability of ozonated water

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ABSTRACT

Background: The literature still lacks clinical protocols for water ozonation considering the instability of the molecule and the need to maintain the proper concentration so that antimicrobial properties are guaranteed.

Objective: To evaluate the viability of ozonated water at different temperatures and ozonation regimes over time.

Methodology: Three experiments were carried out. First, the water was ozonated at 55µg/mL for 10 minutes and kept in two different places: at room temperature (22ºC) and under refrigeration. In the second experiment, the water was kept only under refrigeration and the concentration of O_3 in the device was increased to 70 µg/mL. In this phase, the ozonation process lasted 5 and 10 minutes in different samples. Finally, the last experiment generated two samples of ozonated water with a device set at 70 µg/mL with an ozonation time of 10 minutes, but with an even lower refrigerator temperature. After obtaining the water in each experiment, the analysis of the concentration of this gas in aqueous form was carried out through a specific kit in the periods of 5, 10, 15, 30, 60 minutes and 2, 4 and 6 hours.

Results: When evaluating the influence of ambient temperature in relation to its stability, the samples of ozonated water that were kept under refrigeration showed greater stability over time when compared to samples that were kept at room temperature. The ozonation time also influenced the stability of the solution, since the ozonated water for 10 minutes remained viable for a longer time, when compared to the ozonated water for 5 minutes. When evaluating an even lower temperature of the refrigerator (close to 0ºC) in an optimized concentration of ozonated water 70µg/mL, the viability of the solution was observed for a period of 2 hours.

Conclusion: Ozonation with bidistilled water at a temperature close to 0ºC at 70µg/mL for 10 minutes seems to favor the maintenance of the concentration with potential antimicrobial properties, enabling the viability for its use for therapeutic purposes.

Key Words: Ozone, Therapeutic uses, Administration and dosage

INTRODUCTION

Ozone (O_3) is an allotropic oxygen gas (O_2) naturally found on the surface of the Earth. The rupture of the existing bond between the O_2 atoms, caused by the interaction of this molecule with the ultraviolet radiation of solar origin, causes the oxygen released from this reaction to join the other integral O_2 molecule, thus generating ozone gas. Regarding the physicochemical properties, ozone has a sky-blue color and a characteristic odor, great water solubility and high instability (WFOT Scientific Advisory Committee, 2015)

The administration of ozone in the body can occur through contact with a gas mixture (95% O_2 and 5% O_3), ozonated water or ozonated oils, which can be obtained through specific generators for each case. Regarding stability, according to the literature, the ozone concentration remains more stable in the form of oil when compared to water and gas, respectively (Bocci, 2006). On the other hand, although water is not the solution that guarantees better gas stability, the use of O_3 through this vehicle is the one that has the greatest biocompatibility with human tissues. It should be noted that factors such as temperature, storage location, pH and water composition are directly related to the stability of the molecule in its different vehicles (Kim, Yousef, & Dave, 1999; Viebahn-Hänsler, León Fernández, & Fahmy, 2012).

Considering the chemical reactions that involve decomposition of ozone are endothermic, at lower temperatures this gas increases its life span, with an inversely proportional relationship between its stability and temperature (Batakliev, Georgiev, Anachkov, Rakovsky, & Zaikov, 2014). Regarding the pH of water, it is known that basic means accelerate its decomposition (Batakliev et al., 2014; Smith, Wilson, Gandhi, Vatsia, & Khan, 2017). In relation to the type of water used, it is known that the greater its impurity, the faster the disintegration of the ozone molecule. Finally, the contact of ozonated water with polymeric surfaces should be avoided, especially regarding its storage, since this molecule has high reactivity with this type of material (Viebahn-Hänsler et al., 2012).

In this context, ozone is a highly unstable and water-soluble gas and that its concentration must be at levels sufficiently capable of triggering cellular and microbiological responses so that it is possible to exert its therapeutic effect, studies capable of measuring the concentration of this gas and how it behaves over time in

ozonated water are needed. Thus, the present study aimed to assess the viability of ozonated water at different temperatures and ozonation regimes over time.

MATERIAL AND METHODS

Study Design

The present study used an experimental design *in vitro* and was carried out in two different environments with controlled temperatures, one being air-conditioned and the other refrigerated.

This study was divided in three experiments. At first, the aim was to evaluate the impact of room temperature on water stability. Subsequently, an attempt was made to evaluate the possible influence on the increase in ozonation time and the amount of O_3 applied during the preparation of the water. Finally, based on the best results observed in experiments 1 and 2, a new assessment of the O_3 concentration was carried out, aiming at the establishment of an ozonation protocol. Figure 1 shows a summary flowchart of the experiments carried out in this study.

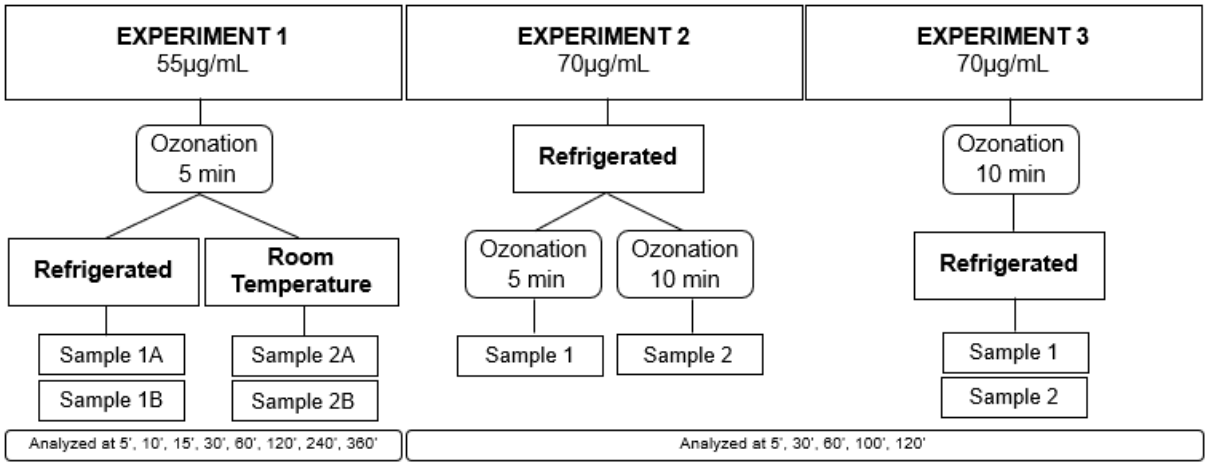


Figure 1. Study Flowchart.

Ozonation Protocol

To obtain ozonated water, bidistilled water (Linhamax®, Eurofarma Laboratórios S.A., São Paulo, Brazil), an ozone generator (Model O & L1.5 RM, Ozone&Life, São Paulo, Brazil) and medicinal oxygen were used. For the ozonation process, 500ml of

bidistilled water was added to a glass column with a lid (Ozone&Life, São Paulo, Brazil) and the liquid bubbled during a specific period according to each experiment. In the end of the ozonation procedure, the liquid was transferred to a glass container with a lid, sealed and stored according to the samples and experiments. The analysis of the concentration of O_3 in the obtained water were carried out in standardized times. In addition, the temperature of the storage location and each sample of the ozonated solution were measured and recorded using specific thermometers at the time of each analysis.

Experimental Procedures

In the first experiment, the device was programmed to generate with a concentration of 55µg/mL of O_3 and the tests were made in duplicate. The analyses in this group were performed in the periods of 5, 10, 15, 30, 60, 120, 240 and 360 minutes. In the group in which the water was kept at room temperature, 500mL of bidistilled water was added to the column of the apparatus at first and, after the process of obtaining the ozonated water, the solution was transferred to a glass bottle and stored at the experiment site, at room temperature (22ºC). In the group where the water was stored in a refrigerator, the experimental protocol was the same used in the previous group, except that, 24 hours prior to the test, the water was kept under refrigeration and after obtaining the solution, it was transferred to a glass bottle with a sealable lid and stored in a fridge (3ºC).

For the second experiment, the ozone generator was programmed in a concentration of 70µg/mL of O_3. After obtaining the solution, they were transferred to the glass container and kept refrigerated. The analysis in this group were performed in periods of 5, 30, 60 and 120 minutes. In this protocol, bidistilled water stored under refrigeration 24 hours before the tests were performed. Thus, for one of the samples, the bidistilled water was ozonated for 5 minutes; in the other, ozonated during the period of 10 minutes.

In the third experiment, the water was ozonated for 10 minutes with an ozone generator programmed in 70µg/mL of O_3 and were made in duplicate. After water preparation, it was transferred to the container and kept under refrigeration, at a temperature lower than that of experiment 2. The analyses in this experiment were carried out in the periods of 5, 30, 60, 100 and 120 minutes.

Ozonated Water Analysis

The analysis was performed using an Ozone colorimetric kit (Ozone – CHEMets Visual Kit K-7404®, CHEMetrics Inc., Virginia, USA). The test consisted of analysis by means of visual comparison using a color scale. The preparation for the analysis of the samples proceeded as follows: two study investigators were responsible for the standardized preparation of the ozonated water, after one investigator performed the step of making the samples, which consisted of the following preparation: 5 drops of reagent solution (A-7400, CHEMetrics) were added to the flask that makes up the kit, 20mL of sterile bidistilled water and 5mL of ozonated water were also added to the flask. Both ozonated and bidistilled water were collected by the pipetting method so that the quantities needed for the test were accurate. Immediately after, a vacuum ampoule (R-7404, CHEMetrics) was inserted into the vial and its tip broken, in order to allow the reaction between the solution present and the sample in the vial. When the reaction occurred, the liquid inside the ampoule turned pink, in different gradations, and was mixed until the entire solution in the ampoule was homogeneous for 1 minute. The ampoule went through a drying process with paper towels and then the analysis was carried out by a single examiner, using a colorimetric scale. If necessary, a reference examiner was requested. When performing the analysis, according to the comparators, the indicated concentration value was multiplied by 5, since the ozonated water was initially diluted in bidistilled water, in order to match the values found in the study with the real values.

RESULTS

Experiment 1

Experiment 1 aimed to evaluate the influence of water temperature on ozonation in different scenarios. Figure 2 illustrates the temperature variation in the environments where the water was kept at room temperature (samples 1-A and 1-B) and cooled in a refrigerator (samples 2-A and 2-B). A difference in temperature was observed when the 2 scenarios were compared, with higher water temperatures observed in the air-conditioned environment. For the acclimatized environment, little variation occurred in the temperature, which oscillated between 21 and 23ºC. In the environment where the water was previously cooled, this variation was greater and oscillated between 5

and 10ºC.

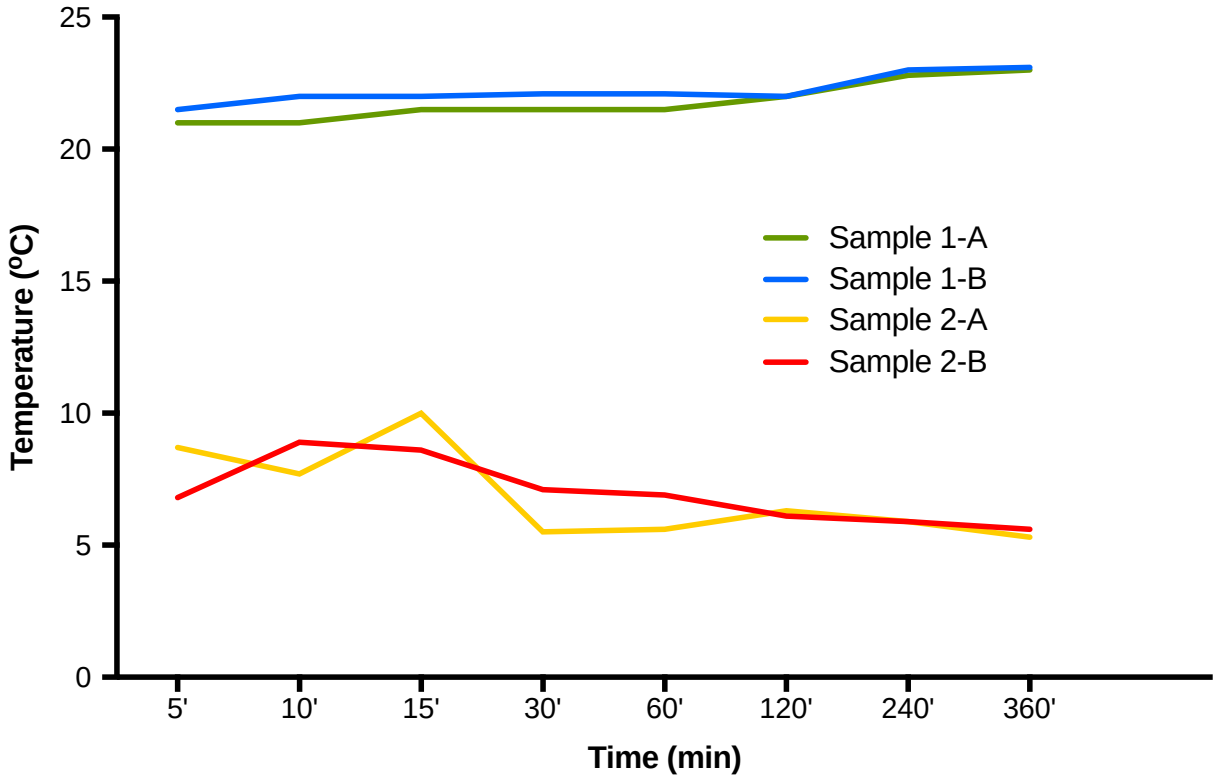


Figure 2. Variation of temperature according to the different time periods and samples evaluated.

The most important result of this experiment is shown in Figure 3 It is noted that for ozone to be considered viable and with its properties maintained, it must have a concentration equal to or greater than 8µg/mL. Thus, there was a very large influence of the temperature of the environment on the concentration of ozone in the water. In the group in which the water was kept in the refrigerator, viability was maintained for approximately 30 minutes. In the group in which the water was kept at room temperature, the ozone concentration did not reach the minimum values necessary to show clinical efficacy from the beginning of the experimental period.

Figure 3. Ozone concentration according to the different times and environments evaluated.

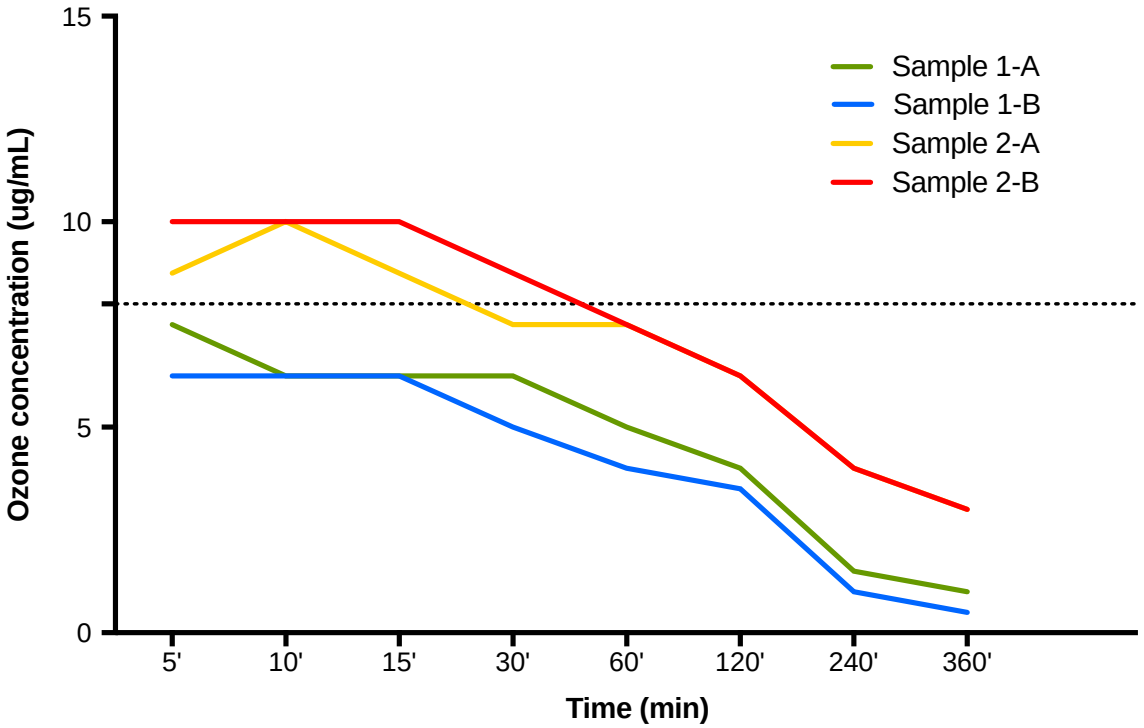


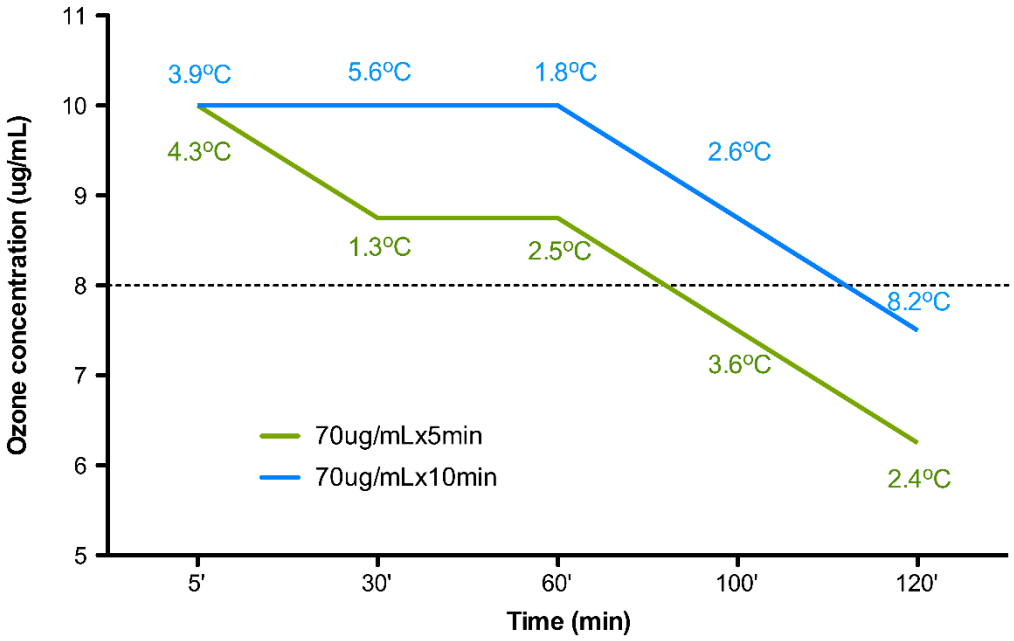
Figure 3. Ozone concentration according to the different times and environments evaluated.

Experiment 2

Experiment 2 aimed to evaluate the influence of increased ozonation time, and the amount of ozone applied during water preparation. In this sense, and based on the findings of Experiment 1, it was decided to increase the amount of ozone injected into the water to 70µg/mL instead of 55µg/mL, with the water ozonation time being the variable under study. Figure 4 illustrates the comparison of water ozonation of 70µg/mL in 5 and 10 minutes, as well as the temperature in each of the samples. Considering these ozone injection amounts and times, the initial concentrations observed in the water were 10µg/mL for both samples.

An increase in the viability of ozonated water was observed as the ozonation time increased. In the sample in which the water was ozonated for a longer time, water viability was observed after 100 minutes while in the sample with ozonation time of 5 minutes this did not occur. However, after 120 minutes, both samples showed ozone concentrations that were not viable for clinical use, thus losing their antimicrobial

properties. In this experiment, a refrigerator temperature ranging between 1.3 and 8.2ºC is observed.

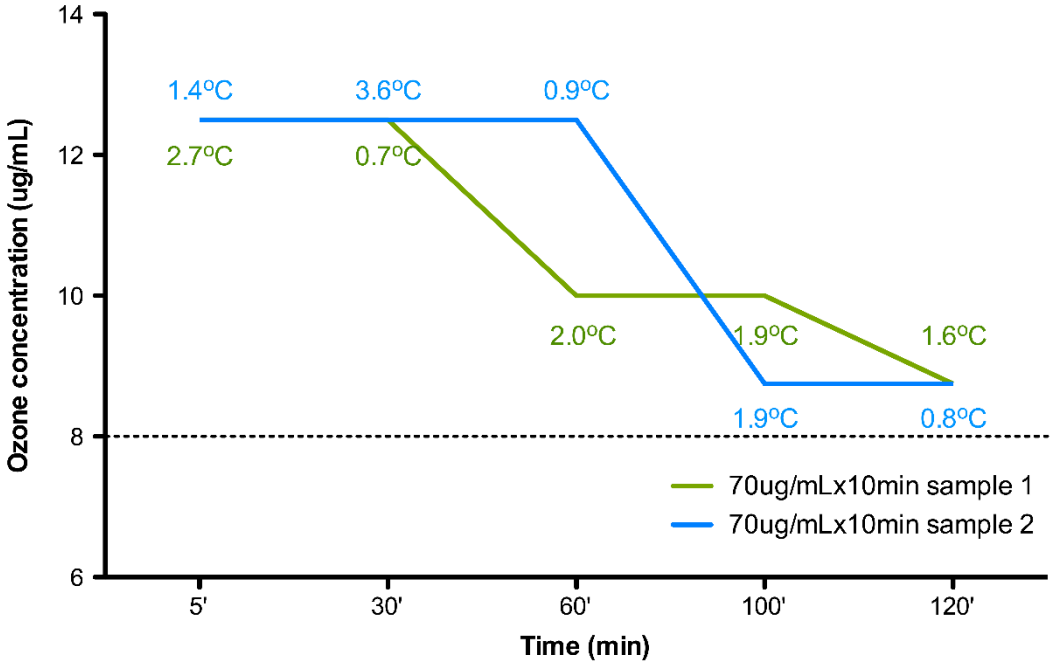


Dotted line indicates viable ozone concentration.
Values above each line represent the fridge temperature according to experimental periods.

Figure 4. O_3 concentration over time according to different periods of ozonation and fridge temperatures.

Experiment 3

Experiment 3 aimed to evaluate the ozone concentration at 70µg/mL for 10 minutes in water with a temperature still lower than that of experiment 2. Figure 5 illustrates the ozone concentration measured when the water was ozonated at 70µg/mL for 10 minutes. In this experiment, efforts were made to keep the refrigerator at even lower temperatures, which varied between 0.7 and 3.6ºC. Considering these amounts and time of ozone injection, the initial concentrations observed in the water were 12.5µg/mL for both samples. The observed results show that when the temperature is maintained at that level, the viability of the ozonated water remains even after 120 minutes of ozonation.



Dotted line indicates viable ozone concentration.
Values above each line represent the frdge temperature according to experimental periods.

Figure 5. O_3 concentration over time and temperature of the fridge in each ozonated sample as 70µg/mL x 10min.

DISCUSSION

The present study aimed to evaluate the influence of different temperatures and time elapsed after water ozonation on the ozone concentration. In addition, the influence of ozonation time on the concentration of this gas in an aqueous form was verified.

There are reports of the use of ozone in dentistry for disinfection of root canals, treatment of carious lesions, as an adjunct to periodontal and peri-implant therapy, as an aid in soft tissue healing and action on fungal infections (El Hadary, Yassin, Mekhemer, Holmes, & Grootveld, 2011; Huth et al., 2009; Makeeva, Turkina, Margaryan, Paramonov, & Polyakova, 2017; Noites et al., 2014). However, the literature that supports the different possible uses of ozone therapy in dental practice is scarce and of limited quality (Azarpazhooh & Limeback, 2008). Systematic searches in the literature point to a limited number of studies, whose methodologies are quite heterogeneous and not very detailed. Those who use ozone therapy in dentistry do so

strictly following anecdotal information or advertising material, or even only on the recommendation of the manufacturers.

The research question involved in this work is linked to the fact that an agent for use in patients' needs, in addition to efficacy/effectiveness, to be easy to use. The information regarding the stability of residual ozone concentrations in ozonated bidistilled water is up to 10 hours (Viebahn-Hänsler et al., 2012). However, maintaining ozonated water in concentrations of O_3 sufficiently adequate for it to be able to exert antimicrobial properties is somewhat challenging, since the amount of ozone present after 24 hours is already insufficient to exert an antimicrobial therapeutic role (WFOT Scientific Advisory Committee, 2015). In this study, the maximum time of stability of ozonated water in a concentration capable of exerting antimicrobial action was 2 hours when kept under the refrigeration regime and ozonated at 70µg/mL for 10 minutes. This time is quite restricted, especially since the use of ozone is limited for use in the office or in places where it is possible to ozonate water. Therefore this study sought to verify the influence of water temperature and the environment on the stability of ozone concentration.

Studies in which ozonated water is used as monotherapy or as an adjunct to a treatment lack information regarding its obtaining procedures. In view of the several variables on which water stability is dependent to present required properties, the preparation of ozonated water must be carried out in a standardized manner. Factors that must be considered are temperature, water storage location, pH and water composition (Kim et al., 1999; Viebahn-Hänsler et al., 2012). The water used for ozonation must be as pure as possible, since ozone is highly reactive, when it comes into contact with impurities present in tap water or even distilled water, it may have its disintegration in advance (Bocci, 2006; Restaino, Frampton, Hemphill, & Palnikar, 1995; Staehelin & Hoigne, 1985).

When the ozone storage container is considered, the literature again refers to the fact that ozone is a highly reactive molecule, so water must be stored in a container that is resistant to this reactivity such as glass, silicone or Teflon (Viebahn-Hänsler et al., 2012). This fact was the object of concern in this study as a glass container with a lid was used to store the after ozonation process. In addition, some authors recommend that the container be closed and stored in a refrigerator (Bocci, 2006), pointing another fundamental point for the stability of ozonated water: the temperature. As this factor was a subject of analysis in this study, temperature showed to have a

great influence on the stability of ozonated water. For this reason, it is reasonable to say that the half-life of ozone in water is temperature-dependent. The water ozonation time is another factor that must be considered, the period for water saturation in the ozonation process must be at least 5 minutes and it can reach up to 15 minutes of bubbling (Azuma et al., 2014).

The present study analyzed two ozonation times and we obtained satisfactory results in the longer period of water bubbling. It has been clearly demonstrated that the ozonation of ice water prolongs the shelf life of the product. An easy and low-cost procedure makes it possible for ozone therapy to increase its possibility of real use. While an alternative that provides greater stability is not available to the dentist, this study highlights the need for ozonation of a previously cooled bidistilled water and shows that maintaining the solution in an environment with a temperature close to 0ºC is mandatory. It can be considered a limitation of this study the temperature's variation of the refrigerator that could decrease the viability of the ozonated water once it variated more than expected.

Finally, the present study aimed to support ozone therapy in terms of ensuring the presence of ozone as a therapeutic agent, mainly warranting its antimicrobial properties. Other tests must be carried out, since the use of ozone therapy, although important gains in practicality have been obtained with the results of these experiments, still lacks other strategies to increase the validity of the products. The expansion of stability will allow, for example, the home use of ozone therapy, which would be potentially interesting for the management of the most prevalent oral diseases. In conclusion, ozonation with bidistilled water at a temperature close to 0ºC at 70µg/mL for 10 minutes seems to favor the maintenance of the concentration with antimicrobial properties, enabling the viability for its use for therapeutic purposes.

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4 ARTIGO CIENTÍFICO 2

EFFICACY OF AN OZONATED WATER MOUTHWASH ON EARLY PLAQUE FORMATION AND GINGIVAL INFLAMMATION: A RANDOMIZED CONTROLLED CROSSOVER TRIAL

Runing Title: Ozonated water and early plaque formation

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ABSTRACT

Backgroud: Ozone therapy has antimicrobial and healing characteristics, however these effects on oral biofilms and gingival inflammation need further elucidation.

Objective: To evaluate the effect of ozonated water in early plaque formation and gingival inflammation.

Methodology: This randomized, controlled, double-blind, crossover clinical trial was performed in two experimental periods of 96 hours each, with 10 washout days between them. The sample consisted of students from the Faculty of Dentistry of the Federal University of Rio Grande do Sul, with 42 participants divided into: Test Group, which used the mouthwash of ozonated water, and Control Group, which received the bidistilled water mouthwash. The participants were instructed not to perform oral hygiene on the upper teeth and used the assigned mouthwash under supervision once a day during the two experimental periods. For the investigation of the initial subgingival biofilm formation, the Plaque Free Zone Index was used at 24, 48, 72 and 96 hours. The volume of gingival crevicular fluid was assessed at baseline and after 96h. Questionnaire for taste perception assessment and analysis of the adverse effects were also carried out. Outcome assessors and participants were blinded to group allocation. The main outcome of this study was the conversion of supragingival plaque to subgingival by the Plaque Free Zone Index.

Results: The percentage of the conversion scores 0 and 1 to 2 of PFZ Index from 24 to 96 hours for all dental surfaces showed no statistically difference between Test and Control groups, with 19.07 and 19.79, respectively. Also, there was not a statistically difference of score frequencies at each time point, with a frequency of 19.1 in Test and 19.8 in Control group of scores 2 in all surfaces at 96 hours. When separating buccal from proximal sites, statistically significant difference between the groups was also not observed. The evaluation of GCF demonstrated that both groups had an increase of volume during the experimental periods and at 96 hours there was no statistically significant difference among groups. The Test group had worse evaluation of taste perception and more adverse effects.

Conclusion: Ozonated water seems not to affect the formation of supra and subgingival biofilms, as well as gingival inflammation.

Key Words: Ozone, Biofilms, Inflammation, Gingivitis

INTRODUCTION

Ozone is a highly oxidative gas found in the atmosphere that has been used in dental practice on the basis of its supposed healing and antimicrobial properties. The mechanism of action of ozone is based on the fact that free radicals formed as it reacts cause an acute but controlled oxidative stress in human cells modulating antioxidant defense, the oxygen metabolism and cellular energy leading to positive biological responses (Huth et al., 2006; Kazancioglu, Kurklu, & Ezirganli, 2014; Seidler et al., 2008; Shiratori et al., 1993; Verrazzo et al., 1995). Ozone high solubility and instability guarantees its total consumption, not generating any kind of toxic residue in the body (Cardile et al., 1995; Smith, Wilson, Gandhi, Vatsia, & Khan, 2017). These characteristics support the potential action to modulate gingival inflammation.

Besides the action in host cells, ozone damages the bacterial cell wall and the cytoplasmatic membrane, allowing the free radicals in and generating a functional disorder with progressive degradation of proteins, destroying them (Bocci, 2006; Smith et al., 2017). This effect does not lead to antimicrobial resistance. In addition to the ozone activity in bacteria, viruses, protozoa and fungi also appear as targets, even biofilms seem to be affected in *in vitro* studies (Arita et al., 2005; Kim, Yousef, & Dave, 1999; Restaino, Frampton, Hemphill, & Palnikar, 1995).

Ozone therapy has several different forms of administration, being gas and aqueous forms the most common ones. Gas can decrease the cell viability and lead to intoxication while the ozonated water is biocompatible to oral cells and still has the mentioned properties (Ebensberger, Pohl, & Filippi, 2002; Huth et al., 2006; Nagayoshi et al., 2004). These characteristics lead to suppose that aqueous form is an appropriate model to application of ozone, having studies using this agent as an irrigating solution and as mouthwashes to treat gingivitis and periodontitis (Hayakumo, Arakawa, Mano, & Izumi, 2013; Katti & Chava, 2013).

Different formulations of mouthwashes are available, even with chlorhexidine as gold standard, to allow a continuous use with fewer adverse events acting as adjuvants in plaque control (Haps, Slot, Berchier, & Van der Weijden, 2008; Quintas et al., 2017; Rosing et al., 2017; Sharma et al., 2004). New formulations of adjuncts try to compensate the limitations of those that already exist and ozonated water has the

potential to act as an adjuvant to mechanical biofilm control based on its characteristics. Also, an eventual anti-inflammatory property is foreseen.

Ozone is biocompatible to gingival tissues, can be used as a mouthwash and has a potential effect in infectious and inflammatory processes that must be enlightened. Its antimicrobial property could be useful for chemical control of biofilm and its anti-inflammatory action could modulate gingivitis. In this context, and in the face of inexistence of clinical trials assessing the modification of biofilm formation with ozone, the objective of this study was to evaluate the effect of ozonated water in early plaque formation and gingival inflammation. The hypothesis is that ozonated water could help to retard the formation of subgingival biofilm, leading to less subclinical inflammation.

MATERIAL AND METHODS

Study design

A randomized, controlled, double-blind, superiority, crossover clinical trial was registered under the protocol RBR-57pgws (ensaiosclinicos.gov.br) and reported following the recommendations of CONSORT Statement. Participating individuals read and signed an informed consent and the protocol was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul, Brazil.

Description of the Sample

Dental students of the Federal University of Rio Grande do Sul participated in the trial. The following inclusion criteria were used: 18-40 years old, in good general health, non-smokers, with at least 20 natural teeth. Individuals with restorations at vestibular marginal sites on upper premolars, canines and incisors, orthodontic appliances, anatomic irregularities, gingival recession, gingivitis and/or periodontitis, those submitted to antibiotic or anti-inflammatory therapy up to 30 days prior to the start of the study, pregnant or breastfeeding were not included. Invitation to participate in the trial was conducted by an investigator not involved in group allocation.

Sample size calculation was performed with data from a similar study (Santos et al., 2017). The main outcome was the percentage of conversion of scores 0 and 1 to score 2 of the Plaque Free Zone Index (PFZ Index). A difference of 24% was

considered between the experimental groups for the main outcome. Considering 5% and 80% alfa and beta errors respectively, 39 participants were estimated. Based on 10% attrition rate from previous studies, 42 participants were included. All calculations were performed using G*Power 3.1 Software.

Experimental procedures

This trial was conducted in November 2019 and occurred in two experimental periods with 96 hours each (Figure 1). After signing the informed consent, the participants were randomly assigned to one of the following groups in Experimental Period 1 (P1): Test Group - ozonated water mouthwash, or Control Group - bidistilled water mouthwash. In the Experimental Period 2 (P2) the groups were switched.

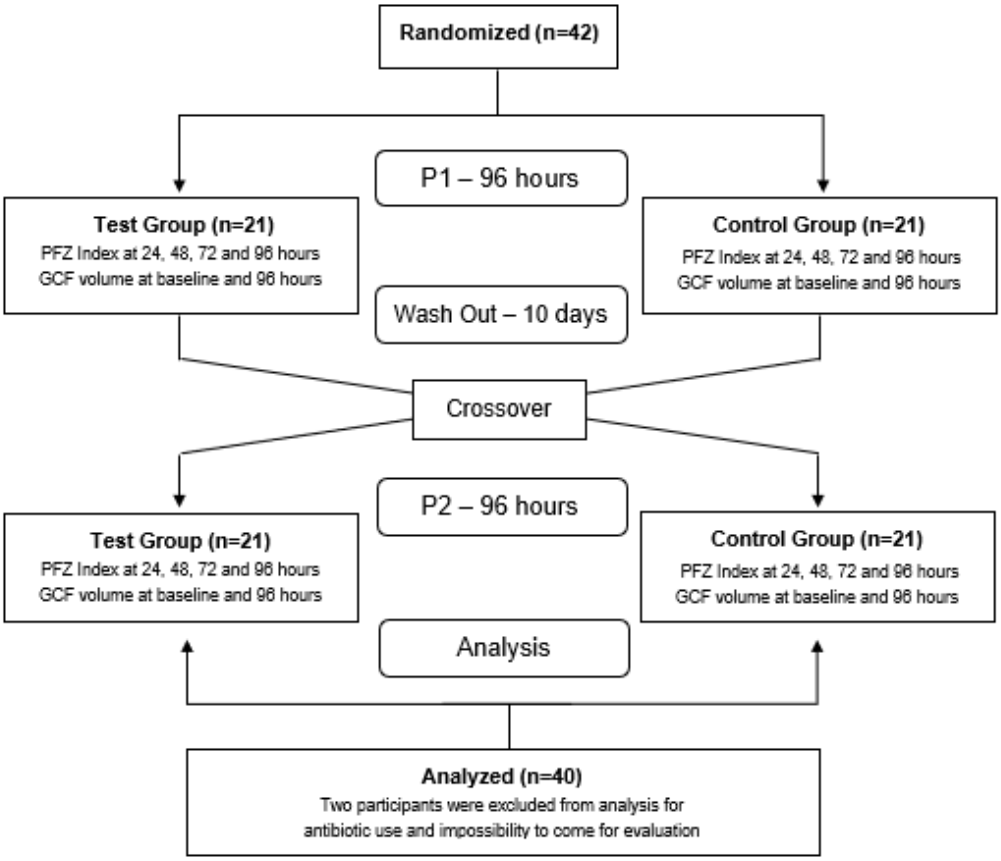


Figure 1. Study flowchart.

Randomization of the order of product use was performed in a website (sealedenvelope.com) in a permuted blocks design with randomly varying sizes by an investigator (JC) not involved in the examination. The same investigators in charge of random allocation were also responsible for the mouthwash distribution and supervision. Allocation of each group was concealed and opened right before the first supervised mouthwash after participant information already recorded. Examiners and participants were kept blinded to allocation.

An *in vitro* study was conducted previously to this trial to establish the conditions and dose of the ozonated water. According to this study, ozonated water was obtained utilizing bidistilled water (Linhamax®, Eurofarma Laboratórios S.A., São Paulo, Brazil), a tower and an ozone generator (O&L1.5 RM®, Ozone&Life, São Paulo, Brazil) calibrated in 70ug/ml for 10 minutes and medicinal oxygen regulated at 1/8, been produced every 2 hours and remaining stored in an amber glass bottles in ice (7,5ºC).

Upper premolars, canines and incisors were considered experimental teeth. At P1 baseline, biofilm was stained using basic fuchsine (ReplanicT®, Iodontec, São Paulo, SP, Brazil) and experimental teeth undergone polishing for complete biofilm removal. Participants were instructed to refrain from mechanical cleaning of their upper teeth. They were advised not to use any other oral hygiene product during the experimental periods. All these instructions were handed to each individual in written and remembered every visit by the investigators.

For the recording of early plaque formation, teeth were washed and dried and fuchsine was applied with a cotton ball to the experimental teeth. After thirty seconds, the agent was washed, and the buccal surfaces were divided with a hectograph copying pencil into vertical thirds: mesiobuccal, buccal and distobuccal. PFZ Index was recorded at 24, 48, 72 and 96 hours (Maliska, Weidlich, Gomes, & Oppermann, 2006). The Index is composed by the score 0 indicating the absence of biofilm and the PFZ, the score 1 showing the presence of biofilm and the PFZ and the score 2 indicating the presence of biofilm but the absence of the PFZ. To assure the application quality of the fuchsine and for better visualization during the exam, the participants used lip retractors.

At baseline and 96h, before the examination procedures, gingival crevicular fluid

(GCF) was collected with a paperstrip (Periopaper®, Oraflow, New York, USA) placed 30 seconds in the upper incisors' and canines' sulcus according to manufacturer instructions. In three of those teeth, the paperstrips were placed in the buccal sites and in the other three, in the proximal sites. Volume of GCF was measured with a calibrated equipment (Periotron 8000, Oraflow, New York, USA). All the PFZ Index evaluations were performed by the same examiner (ACN), trained and calibrated by a gold standard examiner (PW). The Kappa for the interexaminer reproducibility was 0.75.

Each participant received 15ml of the experimental solution in glasses and rinsed for 1 minute, once a day, under supervision. After the 96h evaluations at P1, polishing was performed and a 10-day washout period with the participants returning to their usual oral hygiene was given. After the last rinsing in each experimental period, the participants answered a questionnaire about taste perception using a visual analogue scale (VAS) and closed questions about their experience with the rinse.

Statistical analysis

The primary outcome of this study was the percentage of scores 0 and 1 converting to 2 of the PFZ index. Between-groups comparisons were made using Generalized Estimating Equations with identity link and exchangeable correlation and a robust variance estimator. The between-groups distribution of PFZ scores at each time point was compared using the Chi-square test. For the analysis of GCF, the comparison among groups were made using Wilcoxon test. A carryover effect assessment was made comparing the frequencies of PFZ Index score 1 in different time points during each experimental period using Wilcoxon test. The comparison between groups on VAS for the taste perception was evaluated using paired T-test. The significance level was set at 5%. Analyzes were performed using Stata 14 for Macintosh.

RESULTS

Forty-two dental students were enrolled, matching the inclusion criteria, after 2 months of recruitment (September and October 2019). Two participants were excluded due to reasons not related to the protocol (antibiotic use and impossibility to come for evaluation) (Figure 1). The mean age of the sample was 23.43 (± 3.63) years, and 24 participants were females.

Table 1 demonstrates that both groups presented a very similar pattern of plaque formation according to PFZ in all time points. At 24 hours, most of the surfaces remained biofilm free in Test and Control groups (67.1% and 66.4%, respectively). At 48 and 72 hours, the number of surfaces harboring supragingival plaque raised, with no statistically significant difference between groups. The scores 0, 1 and 2 showed no statistically significant differences in their distribution at 96 hours between the mouthwashes, with an increasing number of sites presenting supragingival and subgingival biofilm observed in both groups. The buccal surfaces presented a higher percentage of score 2 (Table 2) than the proximal surfaces (Table 3), demonstrating that the localization of the site differs in the PFZ evaluations in this study. However, no statistically significant differences were observed among the groups when the sites were separated. An analysis of the carryover effect was made, showing no statistically significant difference between the experimental periods and the groups.

Table 1. Percentage of scores 0, 1 and 2 for all tooth surfaces according to experimental groups.

	Test Group	Control Group	*p* value
24 hours			
0	67.1	66.4	0.9
1	32.9	33.6	0.7
2	0	0	1
48 hours			
0	27.8	26.1	0.5
1	72.2	73.9	0.5
2	0	0	1
72 hours			
0	5.6	4.0	0.2
1	86.6	87.4	0.7
2	7.8	8.6	0.8
96 hours			
0	1.2	1.5	0.4
1	79.7	78.7	0.7
2	19.1	19.8	0.7

Wilcoxon Signed-Rank Test ($p<0.05$)

Table 2. Percentage of scores 0, 1 and 2 for buccal surfaces according to experimental groups.

	Test Group	Control Group	*p* value
24 hours			
0	75.3	77.2	0.6
1	24.7	22.8	0.6
2	0	0	1
48 hours			
0	33.4	32.2	0.7
1	66.6	67.8	0.7
2	0	0	1
72 hours			
0	6.6	6.0	0.7
1	82.8	82.2	0.4
2	10.6	11.8	0.5
96 hours			
0	2.2	1.9	0.8
1	70.9	73.7	0.3
2	26.9	24.4	0.3

Wilcoxon Signed-Rank Test ($p<0.05$)

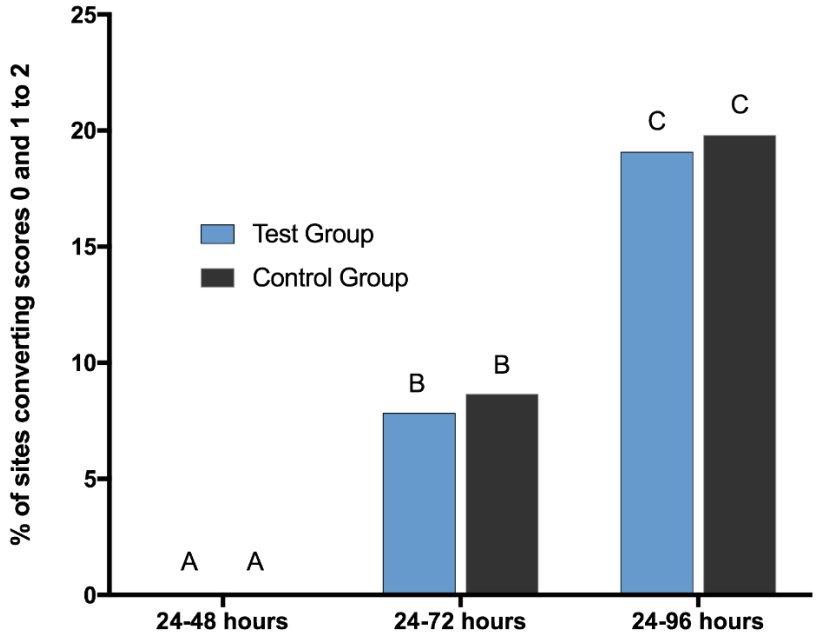
Table 3. Percentage of scores 0, 1 and 2 for proximal surfaces according to experimental groups.

	Test Group	Control Group	*p* value
24 hours			
0	63.0	60.9	0.6
1	37.0	39.1	0.6
2	0	0	1
48 hours			
0	25.0	23.1	0.3
1	75.0	76.9	0.3
2	0	0	1
72 hours			
0	5.2	3.0	0.1
1	88.4	90.0	0.3
2	6.4	7.0	0.9
96 hours			
0	0.7	1.2	0.1
1	84.1	81.3	0.09
2	15.2	17.5	0.1

Wilcoxon Signed-Rank Test ($p<0.05$)

For the evaluation of the main outcome, conversion of scores 0 and 1 to 2 were used to represent transformation from supragingival to subgingival biofilm. Figure 2 exhibit the results of this conversion comparing the 24 hours with the periods of 48, 72 and 96 hours between ozonated water and bidistilled water. No statistically significant difference was observed in any of the time points. Also, in Figure 2 the difference among the initial and final periods of biofilm accumulation are shown, demonstrating that in both groups subgingival plaque increased over time.

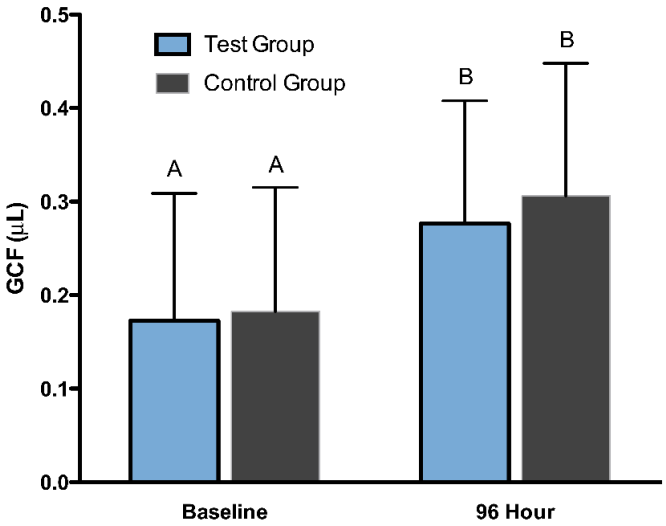
Figure 2. Percentage of the conversion scores 0 and 1 to 2 of PFZ Index for all dental surfaces according to experimental groups and time points comparison.



Different letters indicate within-group statistically significant difference over time.

Relating to GCF volume, no statistically significant difference was observed between Test and Control groups at baseline and at 96 hours (0.27µL and 0.30 µL, respectively). However, there was a significant difference in mean GCF volume when comparing baseline to 96 hours within groups, showing an increase of volume over a four-day period.

Figure 3. Mean (SD) of the GCF according to experimental groups and time points.



The ozonated water had the worst evaluation on VAS by the participants. Mean scores were 4.66 (±1.90) and 6.17 (±2.21) for Test and Control groups, respectively ($p<0.01$). Other variables assessed such as discomfort, taste disturbance, bitter taste after rinsing and opposing to use the mouthwash did not differ between the groups.

The analysis of adverse events revealed that burning mouth sensation was reported by two participants. Moreover, gingival erythema, gingival edema and reddish papules were also observed in one participant each. All the adverse events occurred in the Test Group at Experimental Period 1 and were resolved without interventions.

DISCUSSION

The present study aimed to evaluate the effect of ozonated water in plaque formation and gingival inflammation. The results showed no statistically significant differences between the groups for both PFZ Index and volume of GCF. Also, the Test Group had worst taste evaluation and more adverse events when compared to bidistilled water.

In a systematic review of the literature evaluating ozone therapy in dentistry, ozone in gas and aqueous forms appear to have potential to reduce biolfim formation and act as a disinfectant. However, controversial results were reported. Even with the supposed potential and biocompatibility with oral cells, there is insufficient evidence supporting the antimicrobial efficacy and the treatment of oral diseases, suggesting

that well designed studies with methodological accuracy are needed to sustain the use of ozone as a therapeutic agent (Azarpazhooh & Limeback, 2008). The studies published after the review also presented high risk of bias and did not add sound information substantially.

According to this need in the literature, this is the first clinical trial evaluating if the aqueous form of ozone can in fact change the pattern of biofilm formation and, subsequentially, gingival inflammation. As a proof of concept study, the intention was not to treat a disease, but to see the effect on what causes the disease. In order to demonstrate a high level of evidence with this study, methodological principles such as randomization, blinding and allocation concealment were established *a priori* and followed to the end of experimental periods (Schulz, Altman, Moher, & Group, 2010).

To evaluate the early plaque formation, the Plaque Free Zone Index was used in the present study once the biofilm behavior is well known during the first 96 hours of accumulation by this index, based on that higher increments of biofilm formation can be seen in the first 4 days of growth. In the first 24 hours, a continuous thin line of stained plaque is present, separated from the gingival margin by the Plaque Free Zone. Following the periods of evaluation, the biofilm grows in the incisal direction and in the 72-96 hours, projections to the gingival margin are visualized in some areas where the PFZ is absent (Weidlich, Lopes de Souza, & Oppermann, 2001). The absence of the zone shows that the plaque starts to migrate in direction of the gingival margin and this margin also shifts incisally as part of the edema.

There are studies with this methodology both in extracted teeth and in clinical trials showing a specific pattern of accumulation, having also trials comparing mouthwashes using this Index (Brady, 1973; Friedman, Barber, Mordan, & Newman, 1992; Santos et al., 2017; Weidlich, Lopes de Souza, & Oppermann, 2001). This pattern is observed in this study, confirming that the index was correctly executed and pointing out that there were no differences between the groups regarding the distribution of scores in each time of assessment neither in the percentage of supragingival converted to subgingival plaque, the groups were practically identical, showing that ozonated water offered no change in the process of biofilm formation.

The volume of gingival crevicular fluid was measured in order to show the

inflammation in the tissues after 96 hours of biofilm build-up following the use of one of the rinses. The volume of GCF is a reliable method to assess gingival inflammation, used in several studies for this purpose, managing to indicate even very low levels of subclinical inflammation (Stoller, Karras, & Johnson, 1990). As the trial reveals, there was a significant increase between baseline and the 96 hours, with this last one presenting higher volume of fluid, as expected, due to the increased gingival inflammatory process. Among the groups, no statistically significant differences were observed at baseline or 96 hours, indicating that, in addition to not having an effect in the biofilm, the ozonated water does not modulate inflammation.

The dose of the ozone in the water used in this trial was previously checked by an *in vitro* study performed by the same investigators. To assure the dose utilized in the trial was going to be in a therapeutic dose, different environments were tested, reaching the conclusion that in order to have the ozonated water viable for more than 1 hour and 40 minutes, the ozone generator had to be calibrated in 70ug/ml for 10 minutes in addition to using medicinal oxygen, utilizing bidistilled water for its purity and storing the water in amber glass bottles at the lowest possible temperature. The analysis of the viability of the water were made with a colorimetric kit (Ozone – CHEMets Visual Kit K-7404®, CHEMetrics Inc., Virginia, USA). All this caution was taken once the aqueous form of ozone is very unstable, depending on environmental factors as temperature, luminosity, pH, water composition (KIM, 1999; VIEBAHN-HÄNSLER, 2012). It is a challenge to get the concentration of ozone in water superior to 8ug/ml to obtain the desired properties.

The ozonated water's instability made unfeasible to deliver the product for participants to use at home, what would decrease the time between the doses. The supervised rinse performed only every 24 hours can be insufficient for ozone to show its properties and change the course of biofilm formation, however in order to a patient to use this resource it has to be in a dental office, where the time between the doses are even longer. This characteristic also makes the home use of ozonated water unlikely. Possibly, ozone has a lower substantivity than what is evaluated in this study and closest dosages could reveal a difference among groups as demonstrated in *in vitro* and *in vivo* studies, still to show an actual benefit of using ozonated water in a way that cannot be reproduced because of the instability of it makes little sense.

The results of this trial demonstrate significant differences between the experimental groups on VAS assessment of taste perception, with lower scores attributed to the mouthwash containing ozone. This is probably the first study that included the outcome of taste perception assessments involving mouthwashes containing ozone in its formulation. Studies of this nature are essential as greater adherence to antiseptic agents has been related to the product's flavor (Hepso, Bjornland, & Skoglund, 1988; Laugisch et al., 2016). This may constitute an important issue since ozone has a possible capacity to react to flavoring agents reducing the product efficacy (Staehelin & Hoigne, 1985).

Dental students composed the sample in order to facilitate their displacement and motivation during the study and to assure that the participants had a previous proper oral hygiene, not having gingivitis and/or periodontitis. The inflammation of the protection and supporting tissues of gingiva can change the process of biofilm formation with a higher number of bacteria in saliva and with the greater amounts of gingival crevicular fluid volume. This selection of the sample ensures a higher internal validity of this trial once this variable is well controlled, but the external validity diminishes. As a proof of concept study, its preferable to have the control of this and other variables, making sure that the protocol is followed accordingly to expected, than extrapolating this method to other populations. If a difference cannot be seen in a study like this, is highly unlikely that in a context less supervised the difference will appear.

This trial has strengths and limitations. As the major strengths, previously cited, are the methodological rigor followed in the conception, experimental periods and analysis of this study. Besides, the crossover design decreases the variability between groups, once the participants are their own controls, requiring a smaller sample, but with the same statistical power of a parallel design. The use of a crossover design might have a potential carryover effect, not being observed in this study. As limitations, the reduction of external validity in order to originate a proof of concept study and the daily ozonated water mouthwash dose, that can be insufficient to provide the expected action of ozone, even if it is not possible to hand the participants to rinse at home, can be cited. Another limitation is the fact that zone has a characteristic odor and taste that differ from the bidistilled water. Even with the participants blinded to the allocation, it was possible to identify the mouthwashes by these characteristics, still the implication

of this feature on the primary outcome of this study is limited, once the mouthwashes were supervised.

In the present study, the absence of statistically significant differences among the groups both in the pattern of early plaque formation and in volume of gingival crevicular fluid reveals that the clinical application of ozonated water does not have support for the treatment of infecto-inflammatory oral diseases. It cannot modify the causal factor of these diseases, the biofilm, in addition to cause a considerable number of adverse events. Therefore, the pre-established hypothesis of the present study was not confirmed. After these results, the application of an ozone generator in office with the finality of delaying biofilm accumulation or decreasing inflammatory response can be questioned. In conclusion, ozonated water seems not to affect the formation of supra and subgingival biofilms, as well as gingival inflammation.

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5 CONSIDERAÇÕES FINAIS

A presente dissertação visou avaliar o efeito da água ozonizada sobre a formação inicial de biofilme e sobre a inflamação gengival. O crescente uso do produto em consultórios odontológicos e o aumento do número de cursos e palestras sobre o assunto, além de estudos publicados mostrando os possíveis efeitos benéficos do ozônio despertaram o interesse na área.

Diante da instabilidade da água ozonizada, causada pela grande reatividade da molécula de ozônio, o primeiro artigo desta dissertação foi um estudo *in vitro* onde a temperatura e o tempo de ozonização da água foram testados. O principal objetivo deste estudo foi estabelecer por quanto tempo a água ozonizada estaria viável, com uma quantidade de ozônio que tivesse sua ação terapêutica assegurada, para ser utilizada no ensaio clínico que viria a seguir. Neste estudo, foi observado que a temperatura da água tem grande influência na viabilidade da mesma, assim como o tempo de ozonização. Deste modo, o protocolo de ozonização do ensaio clínico randomizado foi estabelecido como 10 minutos de ozonização de água bidestilada, com o gerador de ozônio regulado em 70µg/mL, utilizando oxigênio medicinal, mantendo a água refrigerada e tendo uma duração aproximada de 2 horas.

Um ensaio clínico randomizado cruzado foi conduzido seguindo as informações obtidas no estudo anterior, para de fato demonstrar se há um efeito do ozônio no biofilme e na inflamação. Esse possível efeito seria de grande utilidade na periodontia, já que o ozônio une propriedades antimicrobianas e cicatrizadoras, podendo se tornar um importante adjuvante no tratamento de doenças infecto-inflamatórias como a gengivite, periodontite e doenças periimplantares. Neste sentido, foi avaliada a formação inicial de biofilme por meio do Índice de Zona Livre de Placa e a inflamação gengival pelo volume do fluido crevicular gengival. Os resultados mostraram que não há diferenças estatisticamente significativas entre o grupo que recebeu o bochecho

de água ozonizada e o grupo que recebeu o bochecho de água bidestilada para nenhum dos desfechos citados anteriormente. Ainda, o grupo que recebeu a água ozonizada apresentou mais efeitos adversos e teve pior avaliação na percepção gustativa pelos participantes, com diferença estatisticamente significativa.

A partir dos resultados obtidos nesta dissertação e do que foi discutido anteriormente, a ausência de diferença estatisticamente significativa entre os grupos tanto na formação inicial de biofilme quanto em volume de fluido crevicular gengival mostra que a aplicação clínica de água ozonizada não se justifica para o tratamento de doenças infecto inflamatórias. A utilização de um gerador de ozônio em consultório com a finalidade de retardar o acúmulo de biofilme ou diminuir a resposta inflamatoria pode ser questionada. Conclui-se, portanto, que a água ozonizada não é capaz de influenciar a formação de biofilme e não modula inflamação gengival.

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