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Artigo de Original

In vitro evaluation of the antimicrobial action of ozonated water on *Staphylococcus* spp. present in dental stainless-steel surfaces

Avaliação *in vitro* da ação antimicrobiana da água ozonizada sobre *Staphylococcus* spp. presentes em superfícies de inox de uso odontológico

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ABSTRACT

Purpose: This *in vitro* study investigated whether ozonated water has antimicrobial action on Staphylococcus spp. present on the surfaces of stainless-steel trays used in dentistry. **Methods:** The experiment consisted of 4 groups, two with mechanical disinfection with gauze soaked in ozonized water (OWG) or 70° ethyl alcohol (EAG), and two control groups without intervention (CG) or with simulated disinfection with 0.9% saline solution, placebo group (PG). Each group consisted of 10 stainless steel trays contaminated with strains of *Staphylococcus* spp. After microbiological reconstitution, serial dilutions between 10^{-1} and 10^{-5} of the samples were performed for seeding in Petri dishes and incubation at 37°C for 24 hours. Bacterial absorbance and growth were analyzed by spectrophotometer and the results were expressed in CFU/ml. **Results:** The EAG (1.60±2.5) and OWG (24±26.77) had lower CFU/ml counts than the two control groups (p=0.000). When comparing the EAG and OWG groups, the lowest counts were in the EAG (p=0.000). From the 10^{-3} dilution onwards, the OWG showed an antimicrobial effect similar to the EAG. **Clinical significance:** Considering the limitations of the present study, promising data were verified for the clinical use of ozonated water as a disinfectant agent since it has an antimicrobial effect on *Staphylococcus* spp. present on stainless steel surfaces for dental use.

Keywords: dental equipment disinfectants; ozone; water; *Staphylococcus*; *in vitro*.

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RESUMO

Objetivo: Este estudo in vitro investigou se a água ozonizada possui ação antimicrobiana sobre Staphylococcus spp. presente de bandejas de aço inox de uso odontológico. **Métodos:** O experimento foi composto por 4 grupos, dois com desinfecção mecânica com gaze embebida em água ozonizada (GAO) ou álcool etílico 70° (GAE), e dois grupos controle sem intervenção (GC) ou com desinfecção simulada com solução salina 0,9%, grupo placebo (GP). Cada grupo foi composto por 10 bandejas de aço inoxidável contaminadas com cepas de Staphylococcus spp. Após reconstituição microbiológica, foram realizadas diluições seriadas entre 10-1 e 10-5 das amostras para semeadura em placas de Petri e incubação a 37°C por 24 horas. A absorbância e o crescimento bacteriano foram analisados por espectrofotômetro e os resultados foram expressos em UFC/ml. **Resultados:** O GAE (1,60±2,5) e o GAO (24±26,77) apresentaram contagens de UFC/ml mais baixas que os dois grupos controle (p=0,000). Ao comparar os grupos GAE e GAO, as menores contagens foram no GAE (p=0,000). A partir da diluição 10-3, o GAO apresentou efeito antimicrobiano semelhante ao GAE. Significado clínico: Considerando as limitações do presente estudo, foram verificados dados promissores para o uso clínico da água ozonizada como agente desinfetante, uma vez que possui efeito antimicrobiano sobre Staphylococcus spp. presente em superfícies de aço inoxidável para uso odontológico.

Keywords: desinfetantes de equipamentos odontológicos; ozônio; água; *Staphylococcus*; *in vitro*.

INTRODUCTION

Dental equipment and instruments have direct contact with pathogens in the oral cavity through body fluids, such as saliva and blood. Dental instruments, such as high and low rotation pens, aggravate cross-contamination when there is production of aerosols represented by liquid particles and/ or solid suspended in the air, which promote the dissipation of microorganisms and consequent contamination of surfaces and equipment[1].

The control of pathogens in the dental environment is essential, mainly through the use of surface disinfection agents and among the most used are alcohols considered bactericidal, fungicidal and virucidal[2]. However, other disinfectant agents are poorly investigated regarding their antimicrobial effect on dental surfaces, such as ozone, which is an unstable gas activated by an electrical discharge and considered a potent oxidizing agent produced in the body in the process of activating antibodies, which is why it is considered a biological molecule and of safe therapeutic use[3].

Therefore, what differentiates ozone from other agents is the mechanism of destruction of microorganisms due to its superior oxidation capacity that promotes direct action on the cell wall with rupture and death in a reduced time, making it impossible for microorganisms to recover[4]. The antimicrobial effect of ozone results from its action on the bacterial cytoplasmic membrane and oxidative effect on the intracellular content, specifically on amino acids[5].

Ozone in aqueous form has demonstrated an antimicrobial effect on bacteria, fungi and viruses since ozone quickly dissociates in water and releases reactive oxygen species (ROS), that can oxidize the cells, which characterizes its antimicrobial action. Furthermore, one of the main properties of aqueous ozone is its lower toxicity when compared to its gaseous form[5,6]. However, in aqueous form, its antimicrobial effect has a half-life of a maximum of one hour due to its rapid degradation in oxygen[7].

Ozone rapidly dissociates in water and releases a reactive form of oxygen that can oxidize cells, showing antimicrobial efficacy, especially against persistent organisms. In addition, it has advantages over other disinfectant agents such as chlorine, alcohols, aldehydes, among others, due to a superior or similar bactericidal action without presenting risks and side effects when in contact with the skin or mucous membranes.[8,9,10] The oxidative effect of ozone makes it a safe and low-cost option for antimicrobial control of surfaces.[11]

The applicability of ozone has been investigated in different dental clinical situations, such as a disinfectant agent for dental prostheses with a significant reduction of *Candida albicans*[12]; in the adjuvant treatment of carious lesions with a reduction in the number of microorganisms present[13,14]; and in endodontic treatments reducing bacterial strains of *Staphylococcus, Streptococcus, Pseudomonas, Enterococcus* and *Escherichia coli*[15]. *In vitro* studies have demonstrated that ozonated water was effective for killing gram-positive and gram-negative oral microorganisms and oral *Candida albicans* in pure culture and showed bactericidal activity against the bacteria in dental plaque biofilm[16]. Furthermore, *in vitro* studies have reported the action of ozonated water as an option for sanitizing dental instruments by reducing the CFU/mL of *Escherichia coli, Staphylococcus aureus, Candida albicans* and *Bacillus atrophaeus* spores[5].

Therefore, the aim of the present study was to investigate whether the ozonated water used as a disinfectant agent has an antimicrobial effect on strains of *Staphylococcus* spp. present on surfaces of stainless- steel trays for dental use.

MATERIAL AND METHODS

Study design and location

The *in vitro* study by microbiological analysis was conducted at the laboratories of the Fishing Institute of the State of São Paulo and Biological Institute the SP, Brazil.

Calculation and sample size

Infinite population calculation was used for quantitative variables with the following formula:

$$n = \left(\frac{Z_{\frac{\alpha}{2}}.\delta}{E}\right)^2$$

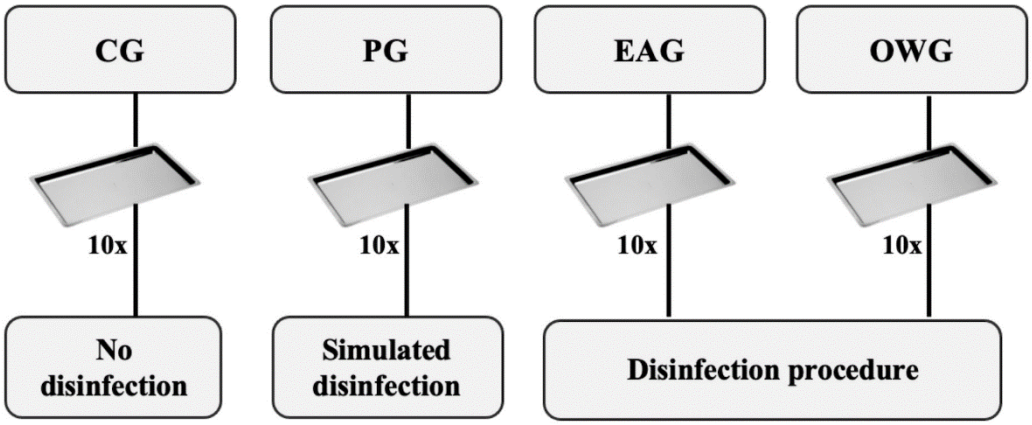
Where “n” represents the sample size; “$Z_{\alpha/2}$” or critical value for the confidence level of 1.96 (95%); “δ” or population standard deviation of the variable of 0.08 and “E” or ±5% standard error. Thus, the required sample size was 10 trays per group.

Distribution of groups

The experiment consisted of 4 groups (Figure 1). Control group (CG), composed of 10 contaminated trays and without disinfection procedure; placebo group (PG), consisting of 10 contaminated trays and simulated disinfection by manual rubbing of sterile gauze soaked in 0.9% saline solution; 70º hydrated ethyl alcohol group (EAG), consisting of 10 contaminated trays and disinfection by manual rubbing of sterile gauze soaked in 70º ethyl alcohol; and ozonated water group (OWG), consisting of 10 contaminated trays and disinfection by manual rubbing of sterile gauze soaked in

ozonated water.

Figure 1. Flowchart of distribution of groups and procedures.

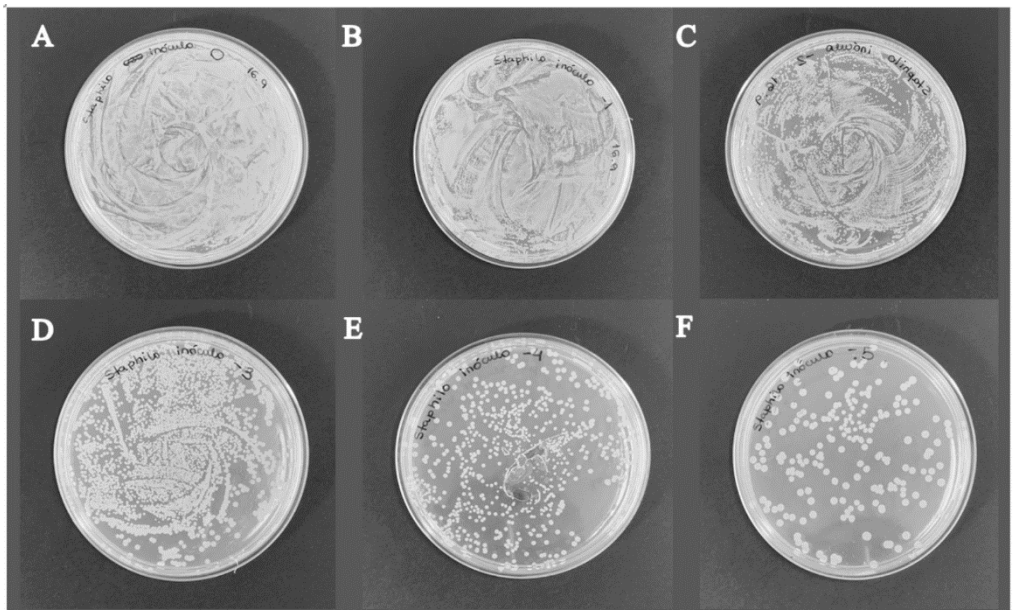


Legend: CG (Control Group); PG (Placebo Group); EAG (Ethyl Alcohol Group); OWG (Ozonized Water Group).

Obtaining the bacterial strain of *Staphylococcus* spp.

The strain of *Staphylococcus* spp. was isolated from dental instruments contaminated after clinical use. The strain of *Staphylococcus* spp. was individually resuspended in test tubes with lids containing 5 ml of Mueller Hinton broth and incubated in a bacteriological oven at 37°C for 48 hours. Later, with a spectrophotometer, the absorbance and bacterial growth were measured by the turbidity of the culture medium. The expression of the inoculum result was in CFU/mL obtained from plates seeded in the serial dilution at base 10 (Figure 2), related to the absorbance in a spectrophotometer with a wavelength of 600nm used for strains of *Staphylococcus* spp.

Figure 2. Petri dishes containing Muller Hinton agar after seeding and incubation of *Staphylococcus* spp. a) without dilution, b) 10^{-1}, c) 10^{-2}, d) 10^{-3}, e) 10^{-4} e f) 10^{-5}.



Procedures for bacterial isolation and contamination of trays

From the isolation of the *Staphylococcus* spp., an optical density (OD) of 0.447nm was obtained, which corresponded to 3.47 x 10^7 CFU/mL. Afterwards, stainless steel trays measuring 22.5 x 10 cm, unused and autoclaved, were contaminated with 100µl of culture medium containing 3.47 x 10 CFU/ml of *Staphylococcus* spp. Contamination was carried out by spreading with the aid of a glass Drigalski loop, which was flamed after each use. After the total drying of the inoculum at room temperature, the disinfection procedures of the trays in the EAG and OWG groups and a simulation of disinfection with a 0.9% sodium chloride solution (saline solution, Needs®), in the PG were carried out.

In the EAG, 70º hydrated ethyl alcohol was produced in the laboratory with PA absolute alcohol, and sterile reverse osmosis water (30%), with the aid of a Gay Lussac alcoholometer. In the OWG, the ozonated water was obtained by a generator (MedPlus MX, Philozon®, Eletroterapia, SC, Brazil), with 1 liter of sterile reverse osmosis water at 12°C added to the glass tower coupled to the ozone generator for 5 minutes with continuous flow with gas bubbling in the water to release medicinal oxygen (White Martins®), which resulted in the production of 40 ppm of ozonated water at an initial concentration of 40 mg/L. Thereafter, the ozonated water was stored in a light-blocking bottle (Corning, NY, USA) to maintain the ozone gas concentration for use in the OWG within 20 min of obtaining it.[9,17]

In all groups, 10ml of each corresponding solution, individually packaged in a sterile Becker, was soaked in sterile gauze folded into four parts and fixed by a sterile hemostat and rubbed in vertical movements in a single direction and across the entire surface of the stainless-steel trays. After complete drying at room temperature, microbiological reconstitution was performed individually with the application of 5ml of sterile 0.85% saline solution with the aid of a single-channel pipette (1000µl) and spread with a flamed glass Drigalski loop after each use.

For microbiological recovery, the same saline solution used in the reconstitution process was placed in sterile screw-top test tubes for further serial dilution at base 10 and seeding in a Petri dish containing sterile Muller Hinton agar. In the CG, only the reconstitution process was performed to count the CFU/mL.

Serial dilution procedures and tray incubation

To make it possible to count the colonies of *Staphylococcus* spp. on the plates, serial dilution was performed using 6 microtubes of 1.5ml. The first microtube contained 1ml of saline solution from the microbiological recovery without dilution and the others with dilutions between 10^{-1} and 10^{-5} with 900µl of sterile 0.9% saline solution used as diluent.

For each tube, 100µl of the microtube solution was added and homogenized with a sterile pipette at each dilution. Subsequently, 100µl of each dilution was homogenized with the aid of a pipette in a Petri dish containing Muller Hinton agar and spread with a flamed glass Drigalski loop after each use. Afterwards, the plates were incubated at 37°C for 24 hours and CFU/mL counts were performed.

Data analysis

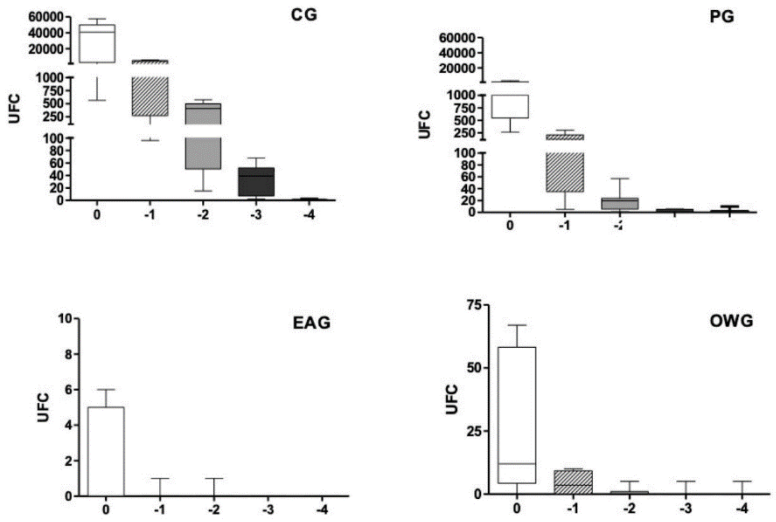
The Shapiro-Wilk test showed non-normal data distribution, with the Kruskal-Wallis test being used to analyze the CFU/mL variable in the independent groups. The post hoc Conover-Iman test was used to verify differences between the groups regarding the reduction of CFU/mL and the corresponding p values were adjusted by the Benjamini-Hochberg correction test. Differences were considered statistically significant when $p < 0.05$.

All analyses were performed using the statistical program R, version 3.5.1 (*Copyrigh*t (C), 2018 *The R Foundation for Statistical Computing*).

RESULTS

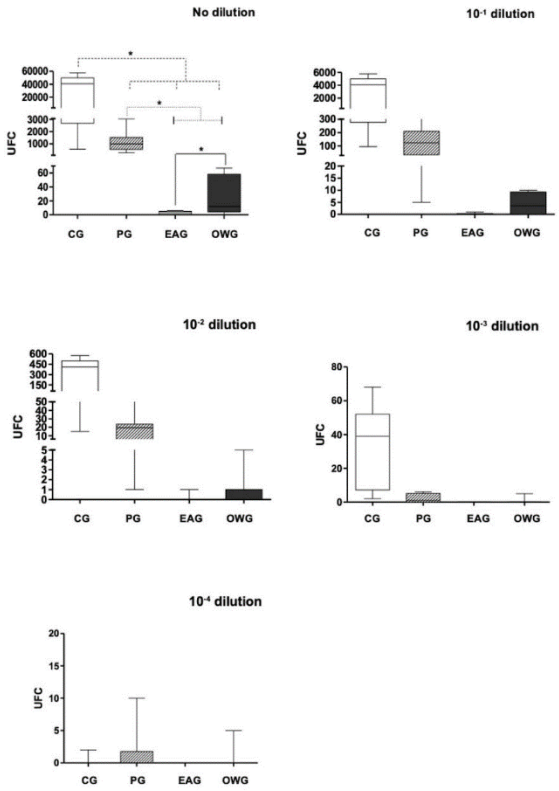
The CFU/mL count of *Staphylococcus* spp. in each group at different dilutions were analyzed for microbial reduction in median values and 95%CI (Figure 3).

Figure 3. Distribution of CFU/mL values according to each group at different dilutions.



The EAG and OWG groups presented lower CFU/mL dilutions than the control groups (CG and PG), with the lowest counts in the group that used 70º ethyl alcohol. However, the ozonized water group (OWG), showed an antimicrobial effect similar to the EAG from the 10^{-3} dilution.

Figure 4. Comparison of medians (95% CI) of CFU/mL, according to the groups evaluated in the different serial dilutions.



Comparing the groups (Figure 4), PG (1167±859.7 CFU/mL), EAG (1.60±2.5 CFU/mL) and OWG (24±26.77 CFU/mL), showed CFU/mL significantly lower (p=0.000) than the CG (29931±23762 CFU/ml). Likewise, the EAG and OWG groups compared to the PG (p=0.005). Furthermore, the EAG had lower CFU/mL counts than the OWG (p=0.003). These results were confirmed in the adjusted analysis.

DISCUSSION

The disinfection protocol with ozonated water used in the present study was able to reduce the CFU/mL of the *Staphylococcus* spp. strain on stainless steel surfaces for dental use. However, even though the antimicrobial action of ozonated water was not superior to that of 70º hydrated ethyl alcohol, from the 10^{-3} dilution onwards, the ozonated water presented similar antimicrobial action in terms of the decrease in CFU/mL.

Hydrated ethyl alcohol at 70º showed greater ability to reduce the CFU/mL of *Staphylococcus* spp. due to the action of 70º alcohol. It does not dehydrate the cell wall of microorganisms, but rather penetrates inside it with protein denaturation, which does not occur in other concentrations.[16,18] However, some studies point to limitations in the disinfection process with 70º hydrated ethyl alcohol, especially in the presence of organic remains, such as in saliva, making bacteria resistant to its disinfectant action[19].

Medical ozone has a high oxidative power and antimicrobial action in the oxidation of constituent lipids of the cytoplasmic membrane, destroying the functional capacity of the bacterial cell.[20] In the present study, we used the recommended criteria to achieve the best water quality, such as low temperature and purity of autoclaved reverse osmosis water. However, we know that disinfection with ozonated water has less oxidative power than ozone gas because it has a lower concentration of the

molecule and low substantivity, but the gas is toxic to the airways, making it difficult to use it *in natura* directly on surfaces[21].

Unlike other studies, the ozone concentration used in the present study (40ppm) may have resulted in greater efficiency in decontaminating surfaces.[22] Furthermore, according to the findings of the study by Pinheiro et al. (2018)[17], the properties of ozone in aqueous solution make it an important disinfectant due to its oxidative potential to induce the destruction of cell walls and the bacterial cytoplasmic membrane. Ozone acts on glycoproteins, glycolipids and amino acids, inhibiting the cell's enzymatic control system resulting in greater membrane permeability, which allows ozone molecules to easily penetrate the cell and induce microbial lysis.[17]

However, the action of ozonated water at a concentration of 40 mg/L on *Staphylococcus* spp. demonstrated antimicrobial effect, which was also reported in another study that used ozonated water between 10 and 30 minutes to decontaminate diamond burs for dental use contaminated with strains of *Staphylococcus* aureus, *Escherichia coli, C. albicans* and spores of *Bacillus atrophaeus*[5]. However, ozonated water maintains its antimicrobial activity during the first 20 minutes, but after 30 minutes this activity decreased substantially due to the instability of ozone gas[7]. In the present study, ozonated water was used within 20 min after obtaining it and the exposure time of ozonated water on stainless steel surfaces was shorter because the mechanical disinfection technique was used. Therefore, the length of stay of disinfectant agents on surfaces can be a determining factor for the reduction of microorganisms.

Ozonated water has advantages due to its ease of use, rapid antimicrobial effects and suitability for use as a disinfectant solution for dental instruments[5,12,23]. Furthermore, the results obtained in the present study demonstrate potential for the clinical use of ozonated water as a disinfection agent on strains of *Staphylococcus* spp. However, some limitations of the present study must be considered, such as the analysis of other species of bacterial strains common to the oral cavity, as well as microbiological analysis methods to expand knowledge of the antimicrobial action of ozonated water, in addition to the need for clinical studies to investigate its real effectiveness. Moreover, the need to conduct clinical studies of equivalence and non-inferiority of ozonated water in relation to the antimicrobial effect of 70° ethyl alcohol becomes evident.

Studies have shown conflicting results on the antimicrobial efficacy of ozone. However, when we analyze the methods used in studies on the antimicrobial efficacy of ozonated water, we notice methodological limitations in the studies, especially when they report non-efficacy of the ozonated water.[24] Studies have used an extremely low dose of ozone in their experiments.[25] Ozonated water will not have a successful action if it is used in low concentrations of ozone or with inadequate exposure time. The concentration of ozone in water is directly related to its antimicrobial action.[10,26] This concentration drops immediately after its production, and will have decreased even further at the time of its use.[25,27]

Several studies have shown ozonated water to be a solution with good applicability, low toxicity, and no adverse events, with very promising results. In addition, the ozone generator is extremely economical and easy to operate and can be a valuable tool for clinical use in a wide range of medical areas. In addition, ozone has a high oxidation potential, being 1.5 times more effective than chloride as an antimicrobial agent against various microorganisms, and can also stimulate blood flow and the immune response, providing several benefits to its use.[17,28]

The production of ozonated water can be considered viable because it has several clinical applications and also antimicrobial effect. However, we must emphasize that technological advances in ozone generators are still needed to obtain a higher concentration of the residual molecule in order to improve its antimicrobial capacity.

CONCLUSION

Considering the limitations of the present *in vitro* study, promising data were verified for the clinical use of ozonated water as a disinfectant agent since it has an antimicrobial effect on *Staphylococcus* spp. present on stainless steel surfaces for dental use. Furthermore, the sensitivity of *Staphylococcus* spp. to ozonated water was comparable to that of 70° ethyl alcohol in the 10^{-3} dilution.

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Conflict of interests

The authors have no conflicting interests to declare that are relevant to the content of the present article.

Authors Contributions

Todos os autores contribuíram para a concepção e delineamento do estudo.

CMB, LLC e GFK: preparação do material, a coleta e análise dos dados

LT e CMI: análise microbiológica

PAGH e SAQMJ: revisão final do artigo Miguens-Jr. Todos os autores leram e aprovaram o manuscrito final.

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