LYSOZYME ANTIMICROBIAL ACTIVITY AGAINST ENTEROCOCCUS FAECALIS – A PILOT STUDY

Atividade antimicrobiana da lisozima contra Enterococcus Faecalis – um estudo piloto

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RESUMO

O preparo químico-mecânico (PQM) do sistema de canais radiculares é essencial para eliminar tecidos infectados e garantir uma desinfecção adequada. O Hidróxido de Cálcio (HC) combinado com o propilenoglicol é frequentemente utilizado como uma pasta intracanal para desinfecção e medicação intraoperatória. No entanto, algumas bactérias, como o *Enterococcus faecalis* (*E. faecalis*), podem resistir aos efeitos do hidróxido de cálcio. O Ultracal® é uma medicação de hidróxido de cálcio de alta qualidade e radiopaco usado em procedimentos endodônticos. Já a lisozima é uma substância com propriedades antimicrobianas encontrada em várias partes do corpo humano e tem sido estudada como uma opção promissora para o tratamento de infecções endodônticas. O objetivo do presente estudo foi avaliar e comparar a atividade antimicrobiana do HC com propilenoglicol, Ultracal® e Lisozima contra *E. faecalis*. Foram realizadas escavações em placas de petri contaminadas com *E. faecalis*. Após, foi adicionado as medicações intracanais e as placas foram levadas a estufa a 37°C em aerobiose. Os halos de inibição formados foram medidos em 2, 4 e 7 dias. HC apresentou halos de inibição maiores quando comparado as outras medicações e com maior crescimento com o passar dos dias. A lisozima apresentou apenas ação nas primeiras 48 horas, perdendo seu efeito após esse período. Ambas as medicações com hidróxido de cálcio apresentaram valores crescentes. Baseado nos resultados obtidos, conclui-se que as medicações a base de hidróxido de cálcio demonstraram melhor ação contra *E. faecalis* em ação direta.

Palavras-chave: Ultracal®, lisozima, medicação intracanal, Hidróxido de Cálcio.

ABSTRACT

The chemical-mechanical preparation (CMP) of root canals system is essential to eliminate infected tissues and ensure adequate disinfection. Calcium hydroxide (CH) combined with propylene glycol is often used as an intracanal medication for intraoperative disinfection and medication. However, some bacteria, such as *Enterococcus faecalis* (*E. faecalis*), may resist the effects of calcium hydroxide. Ultracal® is a high-quality radiopaque calcium hydroxide medication used in endodontic procedures. Lysozyme, on the other hand, is a substance with antimicrobial properties found in various parts of the human body and has been studied as a promising option for the treatment of endodontic infections. The aim of this study was to evaluate and compare the antimicrobial activity of CH with propylene glycol, Ultracal®, and Lysozyme against *E. faecalis*. Petri plates contaminated with *E. faecalis* were excavated, intracanal medications were added, and the plates were incubated at 37°C in aerobic conditions. The

inhibition halos formed were measured at 2, 4, and 7 days. CH showed larger inhibition halos compared to the other medications and exhibited increased growth over the days. Lysozyme showed activity only in the first 48 hours, losing its effect after this period. Both medications with calcium hydroxide showed increasing values. Based on the results obtained, it is concluded that calcium hydroxide-based medications demonstrated better action against *E. faecalis* in direct action.

Keywords: Ultracal®, lysozyme, root canal dressing, Calcium hydroxide.

INTRODUCTION

The endodontic treatment of the root canal system aims to deliver irrigating solutions to the apical area, eliminate infected tissues, and create space for intracanal medication or obturation (COHEN; HARGREAVES, 2011). The root canal is a complex system of interconnected areas, especially in the apical 3mm COHEN; HARGREAVES, 2011). This intricate system promotes the growth of mixed microbial populations, including *Enterococcus faecalis* (*E. faecalis*). Which can migrate into dentinal tubules over time and survive in unfavorable conditions, possessing the ability to proliferate when a new substrate is made available (COHEN; HARGREAVES, 2011).

E. faecalis is the leading cause of healthcare-associated infections, including wound infections, endocarditis, catheter-associated urinary tract infections, and bloodstream infections (FIORE *et al*., 2019). Antibiotic resistance in enterococcal strains limits treatment options for these infections. *E. faecalis* is capable of surviving in various environmental conditions, including high temperatures and low pH, and could tolerate high levels of stress in response to host antimicrobial molecules, such as lysozyme, which acts on the cell envelope (FIORE *et al*., 2019).

Calcium Hydroxide (CH) in combination with propylene glycol is commonly used as an intracanal medication (VATANKHAH *et al*., 2022). It is applied inside the root canal during endodontic treatment, specifically in the stages of disinfection and intraoperative medication. CH possesses antimicrobial properties and aids in the disinfection of the root canal, inhibiting the growth of bacteria and other microorganisms (ESTRELA *et al*., 2023). Additionally, it stimulates the formation of mineralized tissue and may promote the repair of tissue damage caused by infection. Propylene glycol also assists in the gradual release of calcium hydroxide over time, prolonging its therapeutic effect within the canal (RAHDE *et al*., 2006).

Ultracal® is a high-quality radiopaque calcium hydroxide with antimicrobial properties that reduce bacterial load in the root canal. It also possesses alkaline characteristics that stimulate the formation of mineralized tissue and regeneration of periapical tissue. Its radiopacity enables easy visualization in dental radiographs, facilitating the monitoring of proper canal filling and assessment of periapical tissue response (VILLA *et al*., 2020).

Lysozyme is a component of the innate immune system that breaks the b-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine residues in peptidoglycan, leading to cell lysis (PEREIRA, 2019). Lysozyme can be found in various parts of the human body, including the skin, saliva, tears, urine, milk, and respiratory and cervical secretions. Additionally, lysozyme can act as a cationic antimicrobial peptide (CAMP) that destabilizes the bacterial cell membrane (RUAS, 2010). Due to its specificity for the bacterial cell wall and apparent lack of toxic effects on humans, lysozymes from various sources are ideal candidates for use as antimicrobial components not only in pharmaceuticals but also as preservatives in pharmaceutical and cosmetic formulations (RUAS, 2010; PEREIRA, 2019; PEDRON, 2022).

Considering the foregoing, the objective of this study is to evaluate the potential antimicrobial activity of lysozyme, CH, and Ultracal® against *E. faecalis*.

MATERIAL AND METHODS

SAMPLE SIZE CALCULATION

Sample size calculation was performed using G*Power 3.1.9.4 software (Heinrich-Heine University, Düsseldorf, Germany), utilizing the "a priori" calculation parameter for repeated measures ANOVA. The statistical power was set at 80%, with an alpha level of 5%, and effect size of 1.0. Accordingly, the minimum stipulated number for each group was 8 wells.

MICROBIOLOGICAL ANALYSIS

E. faecalis strains (ATCC 29212) were cultured in Brain Heart Infusion (BHI) Agar (Kasvi, São José dos Pinhais, PR, Brazil) at 37°C for 24 hours. Subsequently, the strains were transferred to Falcon tubes containing 25 mL of sterile BHI broth (Kasvi, São José dos Pinhais, PR, Brazil) and again incubated at 37°C for another 24 hours. After this period, the bacterial concentration of the inoculum was adjusted to 1.0 on the McFarland scale (3 x 10^8) using an LGI-VS-721N spectrophotometer (LGI Scientific, São Paulo, SP, Brazil), and 20 µL of each

sample were streaked on 90x150 mm Petri Plates containing BHI Agar as the culture medium, using a sterile swab.

Each plate was perforated with a sterile 4 mm diameter punch. Then, the medications from the tested groups were added to the perforations until each one was filled. After a 30-minute diffusion for the medication in the created holes, the plates were placed in an aerobic incubator at 37°C. The inhibition halos formed after 24 hours, 72 hours, and 7 days were measured using a digital caliper. Measurements were taken both vertically and horizontally, with the center of the perforation as the reference point. These evaluations were performed by two previously calibrated evaluators.

Ultracal®, being a ready-to-use paste, was used according to the manufacturer's recommendations. The CH paste was prepared in a ratio of four parts of CH powder (Biodinâmica, Ibiporã, PR, Brazil) to 22 drops of propylene glycol, being manipulated on a glass slab until homogenization of the components was achieved (CH+Prop). Lysozyme (Sigma-Aldrich, St. Louis, Missouri, USA), being a lyophilized powder, needed to be prepared following these steps: 20 mg of lysozyme were diluted and vortexed in 1 mL of sterile distilled water. Subsequently, an aliquot of 100 µL was transferred to another tube containing 900 µL of propylene glycol and vortexed for 10 seconds. A new aliquot containing 100 µL was diluted in another 900 µL of propylene glycol, completing the dilutions with a final lysozyme concentration of 0.2 mg/mL, which was used in this study.

The tests were repeated three times, and the analysis was conducted by calculating the mean diameters of the halos formed at each time point. After data tabulation, homogeneity was verified, and statistical analysis was conducted using the SPSS software through the specific test.

STATISTICAL ANALYSIS

The normality of the data was initially checked using the Shapiro-Wilk test, showing normality for all data. Subsequently, a two-way repeated measures Analysis of Variance (ANOVA) was performed. Statistical tests were conducted using SPSS software with a significance level of 5%.

RESULTS

Observing the results, it can be noted that in the Lysozyme group, at the evaluation time points of 2 days, 4 days, and 7 days, the average inhibition halo sizes were 12mm, 0mm, and 0mm, respectively. There was a statistical

difference between day 2 and day 4, as well as between day 2 and day 7, indicating a decline in its antimicrobial action.

In the Ultracal® group, at the evaluation time points of 2 days, 4 days, and 7 days, the averages were 22.12mm, 23.50mm, and 25.06mm, respectively. A progressive increase in the average over time is observed, indicating a statistically significant difference between the evaluation time points within the Ultracal® group, specifically from day 4 to day 7.

Table 1. Mean and Standard Deviation in millimeters of measurements over time.

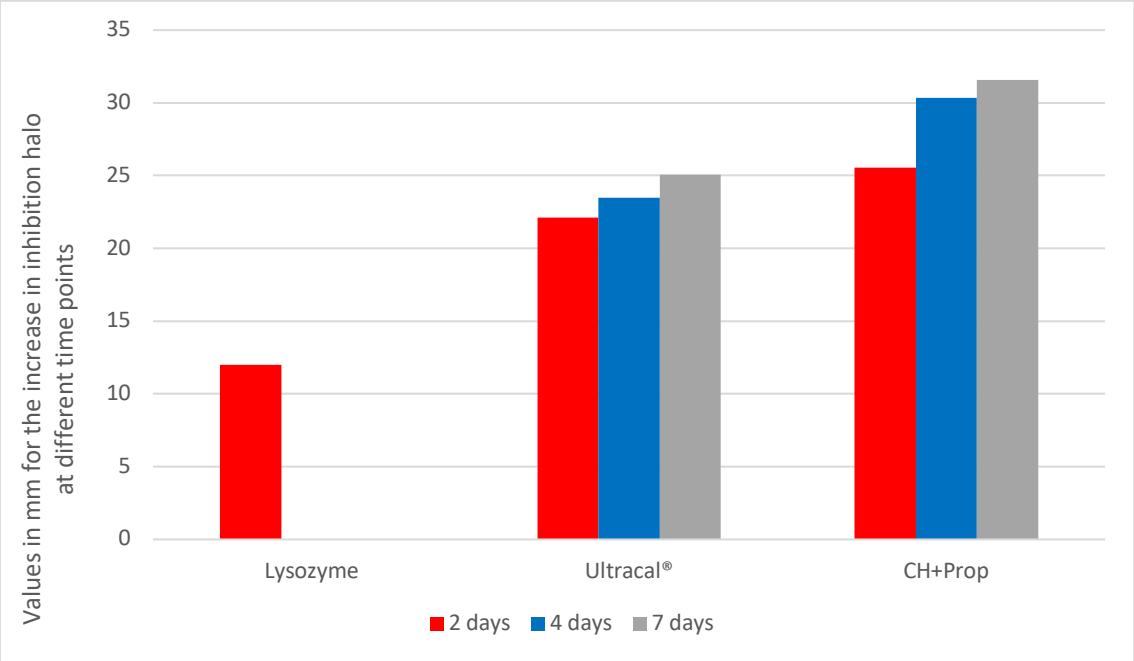
Group	2 days	4 days	7 days
Lysozyme	12 (±0,18) Aa	0 (±0) Ab	0 (±0) Ab
Ultracal®	22.12 (±0,47) Ba	23.50 (±0,70) Ba	25.06 (±0,76) Bb
CH+Prop	25.56 (±0,53) Ca	30.35 (±0,69) Cb	31.56 (±0,76) Cc

Different uppercase letters indicate differences between groups at the same time. Different lowercase letters indicate statistical differences between time points within the same group.

In the CH+Prop group, at the evaluation time points of 2 days, 4 days, and 7 days, the averages were 25.56mm, 30.35mm, and 31.56mm, respectively. Once again, there is a progressive increase in the average over time, indicating a statistically significant difference between the evaluation time points within the CH+Prop group at all measurement times. These results suggest that the CH+Prop group exhibited the highest averages among all samples at the three evaluation time points, indicating a statistically significant difference between this group and the others.

Graph 1 illustrates the performance of the medications used in this study over the days. It is visually evident that only the calcium hydroxide-based medications showed an increase in inhibition halos at different intervals. Lysozyme, on the contrary, only exhibited activity in the first 24 hours of testing. After this period, it completely lost its effectiveness.

Graph 1. Mean of the inhibition halos obtained at different measurement times.



DISCUSSION

When analyzing the results obtained in this study, we can compare them with findings present in the scientific literature. According to Pereira (2019) *E. faecalis* exhibits intrinsic resistance to the bactericidal action of lysozyme. This resistance is related to the extracytoplasmic sigma factor SigV activation through the intramembrane-regulated proteolysis of the corresponding anti-sigma RsiV (7). These findings support the information that the Lysozyme group in the study did not show significant variation over time, indicating the intrinsic resistance of this enzyme in *E. faecalis.*

E. faecalis is known for its remarkable persistence and resistance, demonstrating surprising survival abilities in hostile environments, where it shows resilience against adverse conditions such as high concentrations of antibiotics and endodontic treatments (COHEN; HARGREAVES, 2011; FIORE *et al*., 2019; ZANCAN *et al*., 2019). This resistance represents a significant clinical challenge, as it can lead to persistent and recurrent infections. Additionally, *E. faecalis* is one of the main bacterial species associated with endodontic infections, especially in cases of treatment failure or when endodontic retreatment is necessary. Therefore, studying *E. faecalis* is imperative to understand its behavior and develop more effective treatment strategies (SEDGLEY *et al*., 2005; SASSONE *et al*., 2007).

Another important characteristic is the ability of *E. faecalis* to form biofilms, which are structured communities of bacteria adhered to various surfaces, including root canals (ROSEN *et al*., 2016; SEDGLEY *et al*., 2005). These biofilms pose a challenging obstacle to treatment due to their protective nature, shielding bacteria against antimicrobial agents and the host's immune system. The ease of culturing and manipulating *E. faecalis* in laboratory settings also reinforces its suitability for scientific investigation (SASSONE *et al*., 2007). This bacterium can be easily cultivated under controlled conditions, allowing for more controlled and reproducible studies. Its manipulation in in vitro and in vivo experiments is relatively straightforward, facilitating the evaluation of various treatment modalities and medications. Furthermore, *E. faecalis* holds notable clinical significance, as infections caused by this bacterium can result in serious complications such as periapical abscesses, periapical lesions, and failures in endodontic treatment (SASSONE *et al*., 2007; SEDGLEY *et al*., 2005; KHALIFA *et al*., 2016).

Ultracal® is a calcium hydroxide cement used in endodontic procedures, temporarily filling the empty space in the root canal as an intracanal paste. The usage period is typically one to four weeks, depending on the clinical situation, aiding in canal disinfection and healing (VILLA *et al*.; 2020). On the other hand, Lysozyme is an enzyme with natural antibacterial properties found in various bodily secretions, used as an adjunct in infection treatment, especially when susceptible bacteria are involved (RUAS, 2010; PEREIRA, 2019). The usage period varies depending on the specific clinical condition, prescribed for several weeks to control the infection. As for CH+Prop, it is a mixture commonly used in endodontics as an intracanal paste, applied in endodontic treatment to assist in root canal disinfection. The usage period may vary, but it is generally recommended to leave it in the canal for one to four weeks, contributing to disinfection and preparation before the definitive canal filling (VILLA *et al*., 2020; VATANKHAH *et al*., 2022; ESTRELA *et al*., 2023; RAHDE *et al*., 2006). Regarding the comparison between the Ultracal® group and CH+Prop group, the results found in this study align with previous research.

Lysozyme is a substance that can have its action potentiated when used in combination with other vehicles. These vehicles can include special formulations, carrier agents, or controlled release systems that help optimize the effectiveness and stability of lysozyme. This combined approach can enhance lysozyme ability to combat infections, especially when applied in a targeted and controlled manner (RUAS, 2010; PEDRON, 2022; ROUCHON *et al*., 2022). In our study, when combining lysozyme with the vehicle, it exhibited antimicrobial activity only on day 2. According to the literature, lysozyme proved to be less effective in terms of antibacterial activity when compared to Ultracal®, which has well-known antibacterial properties (PEDRON, 2022). This information is supported by the

results of the Ultracal® group, where a progressive increase in the average evaluated activity over time was observed.

Additionally, the addition of propylene glycol to calcium hydroxide resulted in a synergistic effect, further enhancing the evaluated activity (PEDRON, 2022; NALAWADE *et al*., 2015). This finding is in line with previous studies indicating that the combination of propylene glycol with calcium hydroxide can optimize the compound's release or absorption, expanding its beneficial effects. However, it is essential to highlight that conclusions should be interpreted with caution, considering the study's limitations and unaddressed variables. Further research is needed to validate and deepen these findings, considering the specific composition of the materials used, the employed methodology, and the interaction with the biological environment.

CONCLUSION

Based on the results of this study, it can be concluded that the use of CH in combination with propylene glycol is more effective against *E. faecalis* when compared to Ultracal® and lysozyme. The action is even more relevant when used in a 7-day protocol. However, additional studies are needed to confirm and further explore these promising results.

REFERENCES

1. COHEN S, HARGREAVES KM. Caminhos da polpa. 10º ed. Rio de Janeiro: Elsevier; 2011.

2. Fiore E, Van Tyne D, Gilmore MS. Pathogenicity of Enterococci. Microbiol Spectr. 2019;7(4).

3. Vatankhah M, Khosravi K, Zargar N, Shirvani A, Nekoofar M, Dianat O. Antibacterial efficacy of antibiotic pastes versus calcium hydroxide intracanal dressing: A systematic review and meta-analysis of ex vivo studies. J Conserv Dent. 2022;25(5):463.

4. Estrela C, Cintra LTA, Duarte MAH, Rossi-Fedele G, Gavini G, Damião Sousa-Neto M. Mechanism of action of Bioactive Endodontic Materials. Braz Dent J. 2023;34(1):1–11.

5. Rahde N de M, Figueiredo JAP, Oliveira EPM. Influence of calcium hydroxide points on the quality of intracanal dressing filling. J Appl Oral Sci. 2006;14(3):219–23.

6. Villa N, Santos VV Dos, Costa UM da, Mendes AT, Duarte PHM, Rosa RA da, et al. A New Calcium Silicate-Based Root Canal Dressing: Physical and Chemical Properties, Cytotoxicity and Dentinal Tubule Penetration. Braz Dent J. 2020;31(6):598–604.

7. Pereira OFG. Concentrações Inibitória E Bactericida Mínimas Na Atividade Antimicrobiana Da Lisozima. Florianópolis: Universidade Federal de Santa Catarina; 2019.

8. Ruas GW. Avaliação da atividade antimicrobiana e citotóxica de lisozimas. São Paulo: Universidade de São Paulo; 2010.

9. Pedron J. Avaliação da ação antimicrobiana da lisozima sobre Enterococcus faecalis. Florianópolis: Universidade Federal de Santa Catarina; 2022.

10. Zancan RF, Cavenago BC, Oda DF, Bramante CM, Andrade FB de, Duarte MAH. Antimicrobial Activity and Physicochemical Properties of Antibiotic Pastes Used in Regenerative Endodontics. Braz Dent J. 2019;30(6):536–41.

11. Sedgley CM, Lennan SL, Appelbe OK. Survival of Enterococcus faecalis in root canals ex vivo. Int Endod J. 2005;38(10):735–42.

12. Sassone L, Fidel R, Figueiredo L, Fidel S, Faveri M, Feres M. Evaluation of the microbiota of primary endodontic infections using checkerboard DNA-DNA hybridization. Oral Microbiol Immunol. 2007;22(6):390–7.

13. Rosen E, Goldberger T, Taschieri S, Del Fabbro M, Corbella S, Tsesis I. The Prognosis of Altered Sensation after Extrusion of Root Canal Filling Materials: A Systematic Review of the Literature. J Endod. 2016 2022;42(6):873–9.

14. Khalifa L, Shlezinger M, Beyth S, Houri-Haddad Y, Coppenhagen-Glazer S, Beyth N, et al. Phage therapy against Enterococcus faecalis in dental root canals. J Oral Microbiol. 2016;8(1).

15. Rouchon CN, Harris J, Zubair-Nizami Z, Weinstein AJ, Roky M, Frank KL. The Cationic Antimicrobial Peptide Activity of Lysozyme Reduces Viable *Enterococcus faecalis* Cells in Biofilms. Antimicrob Agents Chemother. 2022;66(5).

16. Nalawade T, Bhat K, Sogi SumaHP. Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. J Int Soc Prev Community Dent. 2015;5(2):114.