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Antimicrobial evaluation of copper sulfate (II) on strains of *Enterococcus faecalis.* In vitro study.

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Abstract: Introduction: Controlling *Enterococcus faecalis* is of vital importance in endodontics, as this pathogen is associated with endodontic failure. Experimental evidence has shown that copper has antibacterial activity against other pathogens with similar characteristics. The objective of this study was to determine the antimicrobial activity of copper (II) or cupric (SC-II) sulfate on strains of *Enterococcus faecalis* and to compare it with the most commonly used antimicrobials. Methodology: We used 33 strains of *Enterococcus faecalis* isolated from different clinical pictures in different Chilean hospitals. The minimum inhibitory concentration (MIC) of SC-II, chlorhexidine and calcium hydroxide was determined by the broth microdilution technique, following the recommendations given by the Clinical and Laboratory Standards Institute. Results: The MIC for CHX varied in the range of 5-10 µg/ml; SC-II from 1.5 to 12 µg/ml, and HC was >32 mg/ml. The geometric mean of SC-II was 6 µg/ml, lower than that of CHX, which was 7.29 µg/ml. Conclusions: SC-II showed antimicrobial activity at lower concentrations than CHX. HC (which could have been affected by the buffer effect of the broth microdilution technique) showed high values, not comparable to other compounds. We suggest carrying out further studies on the properties of SC-II, such assessing its biocompatibility and reaction with other materials to be used clinically in endodontic therapy. Keywords: "Enterococcus faecalis"[MeSH], "Copper sulfate"[MeSH], "Chlorhexidine"[MeSH], "Calcium Hydroxyde"[MeSH], "Endodontics"[MeSH].

Evaluación antimicrobiana del sulfato de cobre (II) sobre cepas de *Enterococcus faecalis.* Estudio in vitro.

Resumen: Introducción: El control de *Enterococcus faecalis* es de vital importancia en endodoncia, ya que este patógeno está asociado al fracaso endodóntico. Evidencias experimentales que han demostrado que el cobre presenta actividad antibacteriana en otros patógenos de similares características. El objetivo de este estudio es determinar la actividad antimicrobiana del sulfato de cobre (II) o cúprico (SC-II) sobre cepas de *Enterococcus faecalis* y compararla con los antimicrobianos más usados en la actualidad. Metodología: Estudio in vitro. Se utilizó la técnica de microdilución en caldo según lineamientos del Clinical and Laboratory Standards Institute, incluyendo 33 cepas de *Enterococcus faecalis* obtenidas desde hospitales chilenos, para cada una de las cuales se determinó las concentraciones mínimas inhibitorias (CMI) de: SC-II, Clorhexidina (CHX) e Hidróxido de calcio (HC). Resultados: La CMI para CHX tuvo un rango de 5-10 µg/ml, el SC-II de 1,5-12 mM y el HC fue >32 mg/ml. Estas diferencias fueron estadísticamente significativas entre los 3 antimicrobianos utilizados ($p<0,001$). Conclusiones: El SC-II mostró actividad antimicrobiana a bajas concentraciones, superiores a CHX, pero menores a HC (que pudo ser afectado por el efecto tampón de la técnica de microdilución en caldo). Se sugiere seguir con los estudios de las propiedades del SC-II, como evaluación de biocompatibilidad y reacción con otros materiales para ser utilizados clínicamente en la terapia endodóntica. Palabras clave: Enterococcus faecalis, sulfato de cobre, clorhexidina, hidróxido de calcio, endodoncia.

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Introduction.

The ultimate goal of endodontic treatment is to achieve a tight and sterile seal through the root filling. However, a drug capable of completely eliminating *Enterococcus faecalis* from the root canal has not been found yet. This pathogen has been reported as the main cause of endodontic treatment failures[1].

Enterococcus faecalis is a commensal microorganism, part of the normal flora of the digestive system and of the oral cavity, but is a highly resistant potential human pathogen[1]. Among the virulence factors expressed by it are proteolytic enzymes, adhesins, and cell and capsular wall polysaccharides; foremost among them is the enzyme gelatinase[2, 3]. Its great resistance is enhanced by gene transfer, which determines the spread of antibiotic resistance genes, even to other species[4]. This makes it resistant to certain medications, including sodium hypochlorite[5]. Moreover, *Enterococcus faecalis* survives in alkaline media that inhibit the growth of other bacteria, including calcium hydroxide (CH); a pH of 10.5-11 retards its growth, and only a pH> 11.5 prevents it, allowing the bacteria to survive in the presence of this drug and to proliferate when its action ends[6].

In endodontics, HC is frequently used as medication between sessions in infected canals due to its described antibacterial effect. Its antimicrobial effect comes from its bactericidal character against Gram-negative bacteria and its bacteriostatic character against Gram-positive bacteria[7].

Chlorhexidine (CHX) has bacteriostatic and bactericidal action, with some effectiveness on bacterial biofilms (much lower than sodium hypochlorite); it does not dissolve organic tissues and is inactivated in their presence[8]. It can be used in endodontics as irrigant and/or intracanal medication. Its association with HC is still under discussion[9]. Optimal antimicrobial activity is achieved at a pH between 5.5 and 7, and it works as a broad-spectrum antibacterial agent that can act against Gram-positive and Gram-negative bacteria. Its mechanism of action is based on its cationic molecular component, which adheres to areas of the negatively charged cell membrane, causing cell lysis[10].

It has been shown that copper surfaces, or surfaces coated with this metal, have a 90 to 95% lower bacterial load, reducing the transmission of nosocomial infections[11]. There are descriptions of its antimicrobial character against pathogens such as *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Clostridium difficile*, among others, but its effectiveness against *Enterococcus faecalis* has not been completely proven.

The advantage of copper over other metals such as silver and stainless steel have led to its use in hospital surfaces; its low surface energy is added to the intrinsic antibacterial effect of this material, which inhibits or causes the death of these microbial species[12]. Its antibacterial mechanisms are still being studied, but it is known to produce: inactivation of enzymatic pathways, formation of reactive oxygen species, precipitation of bacterial proteins, modification of their cell wall and destruction or alteration of the synthesis of nucleic acids, without being mutagenic[13].

These antibacterial properties could have a practical application in odontology, specifically against pathogens such as *Enterococcus faecalis.* The objective of this study was to determine the antimicrobial activity of copper (II) or cupric (SC-II) sulfate on strains of *Enterococcus faecalis* and compare it to that of CHX and HC.

Materials and methods.

We used 33 strains of *Enterococcus faecalis* isolated from patients from five Chilean hospitals with infectious pictures in different anatomical locations. All strains belong to the culture collection of the Laboratory for Research on Bacterial Agents (LIAA) of the Department of Microbiology, Faculty of Biological Sciences, University of Concepción. The strains were maintained at -80 °C in a mixture of trypticase broth and 50% glycerol. The ATCC 29212 strain of *Enterococcus faecalis* was used as control in the assays for determining the minimum inhibitory concentration (MIC) of antibacterial agents SC-II, HC and CHX.

The determination of the MIC was done by the broth microdilution method for clinical antibiotics, following the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI, 2010)[14]. Decreasing concentrations of the antimicrobial agent are placed on a microplate or microwell, which are then inoculated with the strain under study. The culture medium used in this experiment was the Mueller-Hinton broth (C-MH) (Oxoid).

Preparation of the antimicrobial solutions: The mass of HC was determined (Hertz ®, Chile) by putting in a tube and diluting it in 10 ml of sterile water to obtain a concentration of 64 mg/ml; complete dissolution was achieved using vortex. The SC-II (Merck ®, Germany) was diluted to a concentration of 24 µg/ml. The CHX (Oralgene, Maver ®, Chile), being in solution form, was diluted in sterile distilled water to a concentration of 40 µg/ml. The solutions were prepared to achieve 8 times the MIC determined in previous works for SC-II, CHX and HC[15-17].

Preparation of microdilution plates: The microtiter plates had 96 wells distributed in 12 columns (1-12) and 8 rows (A-H).100 µL of CM-H (Oxoid, lot. 489545) were added to each well, leaving the entire H row free, in the duplicate plates, where the inoculum was added. This medium was used because it is the one recommended by CLSI, as most pathogenic bacteria grow well in it and its concentration of divalent ions of Mg+2 and Ca+2 is regulated. Finally, 100 µL of the antimicrobial solution to be assayed were added to each well in row A, which was then serially diluted downwards on the microplate until the F row using a multichannel micropipette. No antimicrobial solution was added to column 12, which corresponded to the negative control. All the experiments were performed in duplicate.

Preparation of inoculum: The samples were preserved at -80 °C in 35% glycerol. To activate their metabolism they were cultured for 18 to 24 h in Trypticase Soy Broth (Oxoid, lot.36397).Subsequently, the concentration was adjusted to 0.5 McFarland standard (1.5-2 × 10^8 CFU/mL) using a turbidimeter. Finally, the inoculum was diluted 1/10 in order to reach a final concentration of 10^7 CFU/mL in the microplates.

Inoculation and incubation of the plates: After standardization, 5 µL of the of the diluted inoculum were added to all wells of the plate except for the sterility control, which contained only CM-H, obtaining a final concentration per well of 4-5x10^5 CFU/mL.

Subsequently, the plates were incubated in an oven at 37 °C for 24 h.

Reading and determination of MIC: MIC determination for each of the antimicrobials was done visually. Microbial growth was considered as positive in the wells that showed any increase in turbidity or growth at the bottom. The wells containing completely translucent CM-H were considered as negative microbial growth. The MIC was defined as the lowest dilution with negative growth. When growth was observed in the dilution with the highest concentration of the antibacterial, the MIC was considered as the highest dilution and was indicated by the sign "greater than (>)". When no growth was observed in the lowest dilution but was observed in the negative control, its MIC was considered as the lowest dilution and was indicated by a sign "less than or equal to (<)".

Statistical analysis. The MIC values were considered as discrete quantitative variables. We considered the highest value of the ranges.

Results.

The MIC of various antimicrobial agents for the 33 strains of Enterococcus faecalis studied is shown in Table 1. The MIC values for CHX varied in a range of 5 to 10 µg/ml; for HC it was >32 mg/ml for all strains. In the case of SC-II, there was a range of 1.5 to 12 µg/ml, and the value of 1.5 µg/ml was obtained in a single strain, indicating a specific susceptibility versus SC-II; in the other strains, the MICs ranged from 6 to 12 µg/ml.

The MIC50 was 3.7 µg/ml for CHX and 3.1 µg/ml for SC-II, indicating the value at which 50% of the strains were inhibited. The MIC90 was 6.6 µg/ml for CHX and 5.6 µg/ml for SC-II, indicating the value at which 90% of the strains were inhibited. Finally, the geometric mean for CHX was 7.29 µg/ml and 6 µg/ml for SC-II, which allowed us to obtain an average without the result being affected by extreme values. The values of three parameters are lower for SC-II, showing that the studied strains are more sensitive to this compound (Table 2).

Discussion.

The results of this in vitro study showed that SC-II is an effective compound against *Enterococcus faecalis* at low concentrations. This is true for CHX as well, but at an even lower concentration, showing a statistically significant difference. At the concentrations used in this study, HC showed to be ineffective against this microorganism.

Previous experiments that determined the MIC for HC found a value of approximately 16 mg/ml[15]. Other studies which determined the MIC of HC, Iodoform, Ciprofloxacin and iodine-potassium iodide for *Enterococcus faecalis* ATCC 29212 and other bacteria commonly found in the root canal system report a value of 16.0 mg/ml[16] for HC, while another experiment yielded a value of 1562

Strain ATCC 29212.	Chlorhexidine (µg/ml)	Copper sulfate (II) (µg/ml)	Calcium hydroxide (mg/ml)
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	1.5	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	5	12	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	3	> 32
Enterococcus faecalis	10	12	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	6	> 32
Enterococcus faecalis	10	6	> 32
Enterococcus faecalis	5	12	> 32
Enterococcus faecalis	10	6	> 32

Table 1. Minimum inhibitory concentrations of copper sulfate II, chlorhexidine and calcium hydroxide against 33 strains of *Enterococcus faecalis*.

	Range	MIC50	MIC90	Geometric mean
Chlorhexidine (µg/ml)	5-10	3.7	6.6	7.29
Copper sulfate (II) (µg/ml)	1.5-12	3.1	5.6	6

Table 2. Ranges of minimum inhibitory concentrations, MIC50, MIC90 and geometric mean for chlorhexidine and copper sulfate (II).

µg/ml, equivalent to 1.562 mg/ml[17], for these compounds. The latter value is different to the results obtained in this study. Although the methods were similar, other growing media were used in those studies, such as brain heart agar instead of the Mueller-Hinton broth used in our study. Furthermore, those studies used only the ATCC 29212 strain of *Enterococcus faecalis*, which is used for quality control in antibiotic susceptibility studies, unlike the bacteria used in this study, which were isolated from the infectious processes of patients in different hospital compounds, and were thus highly resistant.

It is worth noting that the presence of calcium hydroxide precipitate in the wells of the microplate used in this study made it difficult to read the results, and thus the use of other methods, such as the agar diffusion technique, could be considered more appropriate to evaluate this antimicrobial agent. It is also noteworthy that the experiment was performed in 24 hours; however, there is still controversy in the literature about the time calcium hydroxide needs to be in contact to have antibacterial effect[9]. Agar diffusion studies of calcium hydroxide associated with inert substances (distilled water, saline solution or glycerin) showed that it was not effective in inhibiting the growth of several strict and facultative anaerobic bacteria[18]. This is due to the presence of buffer solution in the formulation of culture media. This could explain the presence of bacterial growth at all the concentrations of HC used in this study.

Regarding CHX, the literature shows MIC values between 3.22 µg/ml and 4 µg/ml. A study of the susceptibility of microorganisms to CHX determined the CIM for various endodontic pathogens by the microdilution method, obtaining a value of 4 µg/ml for *Enterococcus faecalis* ATCC 29212[19]. Another study of the susceptibility of microorganisms to CHX and paramonochlorophenol also determined a MIC of 4 µg/ml for *Enterococcus faecalis* ATCC 29212[17]. This study found a range between 5 and 10 µg/ml, which is consistent with the values reported in the literature.

Several investigations showed that the use of CHX gel is significantly more effective against *Enterococcus faecalis* than formulations of HC and HC+CHX. For this, strains of *Enterococcus faecalis* were incubated in canals of extracted human teeth and were subsequently treated with CHX, showing that, in most cases, no growth was evident[20]. This has been corroborated by other reports showing that the use of CHX alone is significantly better than HC alone, but not significantly better than the combination CHX + HC[21]. Previous studies have further determined that 2% CHX is efficient in short periods of time[22]. Still other studies have found that CHX has better antimicrobial efficacy than metronidazole within the root canal[23]. All this points to the great effectiveness of CHX against *Enterococcus faecalis* compared to HC, which is corroborated by the data obtained in this investigation. In addition, other studies have shown that CHX, at low concentrations, is effective against the microorganisms most commonly found in infected canals such as anaerobic bacteria and *Candida albicans*[24].

However, the use of intracanal CHX, either as mediation or even as irrigant, has begun to be discussed. The combination of CHX with sodium hypochlorite, a widely used irrigant due to its high disinfecting and dissolving power in organic tissue, forms a brown precipitate called para-chloroaniline, which is described as having the capacity to stain the teeth structure, occlude dentinal tubules and interfere with the adhesion of sealants. Previous studies indicate that p-chloroaniline has the capacity to induce mutagenesis in animal models, thus questioning the combination of both irrigants. Even when using means for drying the root canal or using other irrigants such as citric acid or EDTA as a rinse between NaOCl and CHX, this precipitate will always be present[25]. That's why despite its great activity against a broad spectrum of bacteria, new antimicrobial agents should be considered for replacing it during endodontic therapy.

The present study found that the MIC of SC-II for *Enterococcus faecalis* strains varied in a range of 1.5 to 12 µg/ml. Previous experiments made in a veterinary environment showed values ranging between 2 and 24 µg/ml, with a mean of 10.5 µg/ml, values which are similar to those obtained in this investigation[26]; however, there no other reports in endodontics.

Conclusion.

This research may establish a starting point for the eventual clinical use of SC-II to disinfect the root canal system, either in a new compound or added to some existing material in order to improve its antibacterial properties.

We recommend carrying out ex vivo studies of the effectiveness of SC-II, due to the ability of Enterococcus faecalis to form a biofilm and penetrate dentinal tubules. These studies should use *Enterococcus faecalis* strains isolated from infectious processes of endodontic origin (in this research they came from other body regions), as the results may differ due to the different susceptibility of the strains involved, and to standardize the presentation of the antimicrobial and its measurement unit.

Finally, we suggest to continue studying the properties of SC-II, evaluating its biocompatibility and reaction with other materials used clinically.

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