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Article

Efficacy of copper sulphate on *Candida albicans* on heat-polymerized acrylic resin.

Eficacia del sulfato de cobre sobre *Candida albicans* en resinas acrílicas de termocurado.

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Abstract: The ageing of population is increasing, and a great percentage of these patients wear removable prostheses, and can suffer denture stomatitis, a condition that has been associated with candidiasis. **Aims:** To evaluate *in vitro* the effectiveness of Copper Sulfate against *Candida albicans* in samples of heat-polymerized acrylic resin, compared to nystatin, sodium hypochlorite and chlorhexidine. **Materials and Methods:** Initially, the minimum inhibitory concentration (MIC) of copper sulfate for *Candida albicans* was determined by microdilution. Then, 54 resin samples were divided into 6 treatment groups corresponding to Nystatin 100.000 UI, Sodium Hypochlorite 0.5%, chlorhexidine 0.12%, Copper Sulfate 4.7µg/ml, Copper Sulfate 9.4µg/ml and physiological saline solution, in which samples were submerged for 6 hours. Resin samples were then washed and cultured on solid media at 37°C for 72 hours. The number of colony-forming units was determined using a Quebec colony counter. The statistical analysis was performed using the Kruskal-Wallis test and the Mann-Whitney U test. **Results:** Copper sulfate at a concentration of 9.4µg/ml presented a similar effectiveness as the other control products regarding the reduction in the number of colonies of *Candida albicans* post-treatment. **Conclusion:** The effectiveness of copper sulfate against *Candida albicans* on acrylic resin was similar to that of nystatin, sodium hypochlorite and chlorhexidine.

Keywords: Candidiasis; stomatitis, denture; nystatin; sodium hypochlorite; chlorhexidine; copper sulfate.

Abstract: En las últimas décadas se ha observado un aumento de la población de adultos mayores, de los cuales un gran porcentaje es portador de prótesis removible, y dos tercios pueden sufrir estomatitis subprotésica, enfermedad que es asociada a infecciones como candidiasis. **Objetivo:** Evaluar la efectividad antimicótica *in vitro* del sulfato de cobre en placas de resinas acrílicas de termocurado inoculadas con *Candida albicans*, frente a Nistatina, Hipoclorito de Sodio y Clorhexidina. **Material y Métodos:** Inicialmente, y mediante microdilución del sulfato de cobre, se determinó la concentración mínima inhibitoria (CMI) para *Candida albicans*. En la fase experimental, 54 muestras de resina se dividieron en 6 grupos correspondientes a Nistatina 100.000 UI, Hipoclorito 0.5%, Clorhexidina 0.12%, Sulfato de Cu 4.7µg/ml, Sufato de Cu 9.4 µg/ml y suero fisiológico. Las muestras fueron sumergidas en estos agentes por 6 horas, para posteriormente ser lavadas y cultivada en medios solidos a 37°C por 72 horas. Luego se realizó el conteo de unidades formadoras de colonias mediante contador tipo Quebec. El análisis estadístico se realizó mediante la prueba de Kruskal-Wallis y la prueba U de Mann-Whitney. **Resultado:** El sulfato de cobre a una concentración de 9.4µg/

ml presentó una efectividad similar a los otros productos, en la reducción de colonias de *Candida albicans*. **Conclusión:** La efectividad del sulfato de cobre contra *Candida albicans* fue semejante a la de Nistatina, Hipoclorito y Clorhexidina.

Palabra Clave: *Candidiasis; estomatitis subprotética; nistatina; hipoclorito de sodio; clorhexidina; sulfato de cobre.*

INTRODUCTION.

Demographic data in Chile show an increase in the elderly population. It is projected that the number of people over 65 years of age will rise from 10% in 2010 to 20% in the year 2038.[1] Of the total population older than 65 years, a high percentage wear removable prostheses, of which two thirds may suffer denture stomatitis.[2,3]

This disease of multifactorial etiology[4] is described as an inflammatory process of the hard palate mucosa, which underlies the mucosoported dental prostheses. It is mainly associated with infectious processes such as candidiasis, mainly due to *Candida albicans*.[5] It occurs mostly in elderly patients, being one of the most frequent oral mucosa lesions in this age group.[6]

The genus Candida comprise more than 350 yeast species, but only some are involved in human infections.[7] They are present in the skin microbiota, mucous membranes and gastrointestinal tract. *Candida albicans* is the most frequently isolated species from clinical samples in cases of denture stomatitis. It is capable of growing at pH ranging from 2 to 10, and its ability to withstand such a wide range of environmental pH is fundamental in its pathogenicity.[8] Due to the multifactorial etiology of denture stomatitis, its treatment involves the identification and removal the predisposing factors, before treating the condition. Infection management consists of eliminating the night use of the prosthesis, antifungal therapy and the control of bacterial plaque.[9]

The properties of copper have become well known and valued, with this substance being widely used in different areas. Its biocidal properties are useful in the prevention and control of the growth of a wide variety of microorganisms.[10,11] In contact with *Candida albicans*, copper damages the cytoplasmic membrane, producing depolarization and facilitating the entry of copper ions into the cell, inducing free radicals and increasing oxidative stress, a process called "death by contact".[12]

Copper sulfate ($CuSO_4$) is a chemical compound that occurs mainly in two forms: copper sulfate I (cuprous sulfate), and copper sulfate II (pentahydrate).[11] Copper sulfate II is used in the preparation for other copper compounds in the manufacture of different fungicidal products,[11,13] and also in the medical field. Although there are some studies researching the effects of copper on *Candida albicans*, there is little documentation about its activity on heat-polymerized acrylic resin inoculated with this yeast, using an approach to replicate the current clinical reality.

For this reason, the aim of this study was to evaluate the *in vitro* antifungal effectiveness of copper sulfate in heat-polymerized acrylic resin plates inoculated with *Candida albicans*, compared with nystatin, sodium hypochlorite and chlorhexidine.

MATERIALS AND METHODS.

Sample preparation

Fifty-four rectangular samples (15x15x2mm) of heat-polymerized acrylic resin were made (Marche termocurable, Santiago, Chile), from wax sheets of the same dimensions (Ecocera, Chile), using muffle furnaces (Quimis Q318s24, Diadema-SP, Brazil). Acrylic discs were polished with pumice stone and white rouge under running water. Finally they were autoclaved at 121°C for 15 minutes.

Determination of the Minimum Inhibitory Concentration (MIC) of copper sulfate

A broth microdilution methodology was employed adapting the method of The Clinical Laboratory Standards Institute (CLSI) for the assessment of antifungal susceptibility.

For the inoculum, a suspension of *Candida albicans* (ATCC 90028) in 0.9% NaCl at 0.5 McFarland turbidity was prepared from a 24-hour incubation culture.

Ten microliters of this suspension were transferred to a tube containing 10 ml of RPMI 1640 (Gibco, USA), with glutamine and sodium bicarbonate, buffered with MOPS 0.164M, adjusted to pH 7±0.1 and 0.2% glucose, thus achieving a 1:1000 dilution.

For the copper sulfate base solution, we started with a stock solution of 3010.56 µg/ml. From this, a series of dilutions were prepared according to the CLSI standard, using RPMI 1640 as the solvent. All tubes contained a final volume of 1ml.

Finally, a 1:5 dilution was made by adding 4ml of

RPMI to each tube. Ten microliters of copper sulfate at different concentrations were added to each well of the microplate. From column 1 to 10, wells were filled with decreasing concentrations, where column 1 corresponded to the concentration of tube 2 and so on.

Column 11 was the positive control (growth control), containing only the yeast inoculum. Column 12 was the negative control, containing only RPMI 1640 broth. The plate was incubated covered for 24 hours at 37°C, in the absence of light. This assay was performed in triplicate.

The growth of *Candida albicans* was assessed by a vi-sual examination, with a Zeiss Stemi 2000-C ste-reoscopic microscope at 40X, determining that the MIC was the lowest concentration at which no yeast growth was observed. Additionally, 3 µl of sample were taken from one of the wells and inoculated onto Sabouraud agar and incubated, to confirm that the yeast observed corresponded to *Candida albicans*.

Inoculation

An inoculation test was performed, where the samples were immersed in 30ml of Sabouraud broth with *Candida albicans* strain (ATCC 90028) at a turbidity of 1 McFarland. They were incubated for 72 hours at 37°C in a Memmert IN110 culture oven.

Then, they were removed from the oven and rinsed with 100 ml of 0.9% NaCl for 30 seconds. Each sample body was deposited in an individual test tube with 15ml of 0.9% NaCl, and was mixed using a Vortex Dragonlab MX-F for 60 seconds. 1ml of each tube was extracted, and inoculated on Sabouraud agar at 37°C for 72 hours in a Memmert IN110 culture oven. It was visually checked with a Zeiss Stemi 2000-C stereoscopic microscope at 40X to confirm if there was growth of *Candida albicans* in the Petri dishes.

Experimental phase

Fifty-four heat-polymerized acrylic resin samples were inoculated with *Candida albicans* (ATCC 90028) at 1 McFarland in triplicate and incubated for 72 hours at 37°C, were then rinsed with 100ml of 0.9% physiological serum for 30 seconds.

The acrylic samples were divided into individual test tubes in a 10ml volume as follows: nine samples in 0.12% chlorhexidine, nine with 100000 IU nystatin, nine in 0.5% sodium hypochlorite, nine in 4.7µg/ml copper sulfate, nine in 9.4 µg/ml copper sulfate, and three in 0.9% NaCl (negative control). After 6 hours they were removed and rinsed in groups of 3 with 100ml of 0.9% NaCl for 30 seconds.

Then each sample was shaken with Vortex Dragonlab MX-F for 60 seconds in an individual test tube with 15ml of 0.9% NaCl, 1ml of each tube was evenly spread on Sabouraud agar for 37°C for 72 hours in a Memmert IN110 culture oven.

Visual Test

The counting of colony forming units was performed with an American Optical Darkfield Quebec 3330 colony counter, with the aid of a 1.5X amplifying lens, by visual-analog count.

Statistics

Statistical analysis was performed with statistical software SPSS (version 19). First, an analysis was carried out of the assumptions, of which randomness and independence were met; homoscedasticity and normality were not met.

After analyzing the normality of the data, the Mann-Whitney U test was used for comparison between independent individual samples, and then the Kruskal-Wallis test was used for the analysis of the groups. The established level of significance was $p \leq 0.05$.

RESULTS.

Due to the experimental nature of copper sulfate, a minimum inhibitory concentration (MIC) was calculated, where the lowest concentrations at which no growth of *Candida albicans* was observed were 4.7 µg/ml and 9.4 µg/ml (Table 1).

A concentration of 9.4 µg/ml was more effective in reducing colonies of *Candida albicans* compared to copper sulfate at 4.7 µg/ml during the resin sample assay. The results also showed that nystatin, hypochlorite and chlorhexidine produce complete inhibition of *Candida albicans* in all phases of the study where they were used (Figure 1).

The Kruskal-Wallis test shows that there is a significant difference between the studied groups (p=<0.001). The Mann-Whitney U test determined that there is a significant difference in the inhibition of *Candida albicans* between copper sulfate at 4.7 µg/ml and nystatin 100,000 IU (p=0.002), chlorhexidine 0.12% (p=0.002), and 0.5% hypochlorite (p=0.002).

A significant difference was also observed between the negative control group with all the studied compounds (p=<0.001). There was no significant difference in the inhibition of *Candida albicans* between copper sulfate at 9.4 µg/ml, Nystatin, Hypochlorite and Chlorhexidine (p=0.166) (Figure 1).

Figure 1. Colony forming units (CFUs) of *Candida albicans* obtained following treatment with Chlorhexidine, Nystatin, Sodium Hypochlorite, Copper Sulfate and normal saline in the experimental phase.

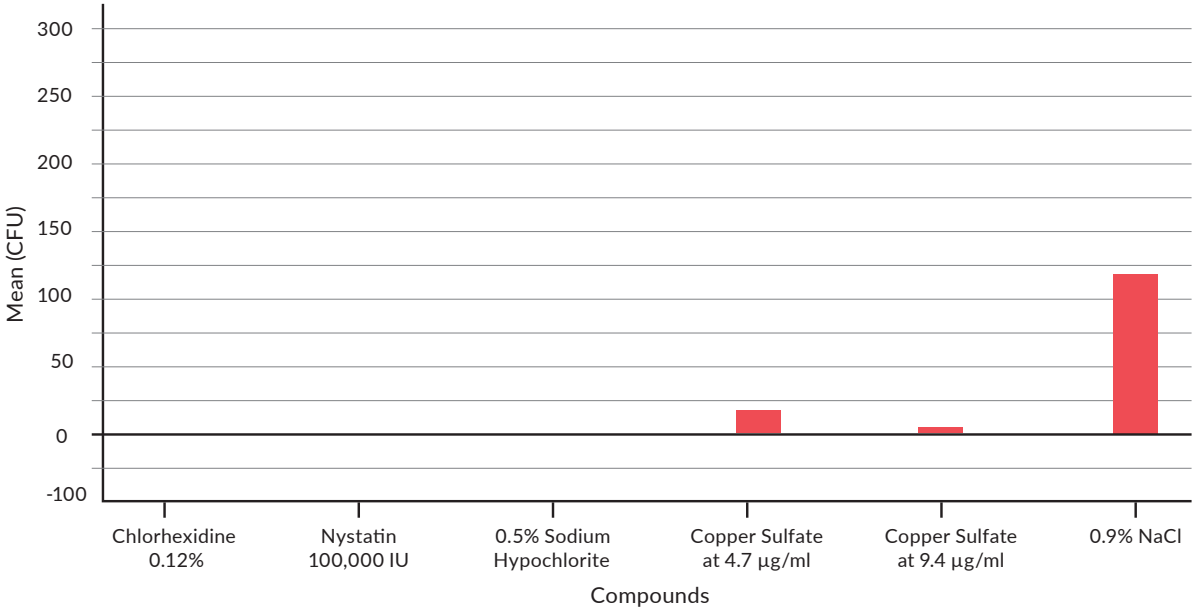

Table 1. Minimum inhibition concentration of copper sulfate on *Candida albicans* according to the broth microdilution for antifungal testing.

Well No.	$CuSO_4$ [µg/ml]	*Candida albicans* growth
1	150.5	No
2	75.3	No
3	37.6	No
4	18.8	No
5	9.4	No
6	4.7	No
7	2.4	Yes
8	1.2	Yes
9	0.6	Yes
10	0.3	Yes

DISCUSSION.

The increase in life expectancy brings an increase in the population of older adults and a greater concern for the diseases that affect them. Consequently, the lack of teeth in older adults, their prosthetic rehabilitation and associated oral pathologies have become a public health issue.[3] Denture stomatitis is one of the most frequent complications associated with the use of acrylic prostheses.

The main etiological agent is *Candida albicans*, so the study of agents with antifungal activity, which have the least amount of side effects and a low cost, is of increasing interest.[14,15]

The use of copper as an antifungal agent has been reported by several authors, who especially highlight its effectiveness against *Candida albicans*, as it damages the cell surface,[12,16,17] and the entry of copper ions induces free radicals and oxidative stress.[18,19] It has been shown that mechanical cleaning of the prosthesis (brushing) is not enough and a chemical cleaning adjuvant is essential to prevent associated infections.[20,21]

Chlorhexidine is an effective antiseptic agent that, however, has a series of unwanted side effects, such as causing brown stains on the acrylic resin, affecting its hardness and texture, as well as causing stains on mucous membranes and teeth, dry mouth, desquamation and

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temporary alteration in the sense of taste.[22,23]

On the other hand, sodium hypochlorite works as a disinfectant when it is used as a solution to submerge the prostheses in case of denture stomatitis, because it reduces the ability of Candida to adhere to the prosthesis material. It has been used for a long time as a prosthetic disinfectant, since it decreases microbial growth on the surfaces of the prosthesis. The immersion of the prosthesis in 0.5% NaOCl for five minutes is sufficient to eliminate *Candida albicans*.[24] However, if the prosthesis is not well rinsed, its intake can produce side effects such as nausea and vomiting.[25]

When using 0.12% chlorhexidine, 0.5% sodium hypochlorite and 100000UI nystatin, complete inhibition of *Candida albicans* growth on Sabouraud agar surface is observed, presenting a statistically significant difference in the reduction in the growth of *Candida albicans* with respect to the control group.

These results are in agreemnet with those obtained in other studies, which have shown that there is a high reduction in the number of colonies of *Candida albicans* followed treatment with Chlorhexidine,[26,27] Sodium Hypochlorite,[24,28] and Nystatin.[29] In the present study, when determining the minimum inhibitory concentration (MIC), no growth of *Candida albicans* was observed at a concentration of 4.7 µg/ml.

However, in the experimental phase, only the concentration of 9.4 µg/ml produced a reduction of more than 90% of CFUs compared to the control group. The lower reduction of CFUs of *Candida albicans* with copper sulfate at a concentration of 4.7 µg/ml showed statistically significant differences *versus* Nystatin, Sodium Hypochlorite and Chlorhexidine; although it did result in a reduction in the number of colonies when compared with the control group.

On the other hand, the effectiveness of copper sulfate at a concentration of 9.4 µg/ml is similar to that obtained with nystatin, sodium hypochlorite and chlorhexidine. Studies that analyze the effect of copper sulfate on *Candida albicans* in heat-polymerized acrylic resins were not found in the literature.

However, there are several studies about the effectiveness of copper as a disinfectant that reinforce the results obtained in this study.[11,12,17] Anand *et al.*,[11] determined a MIC of 1mg/ml for copper sulfate, which is a substantially higher value than that obtained in this study, suggesting that the effectiveness of copper sulfate could have a dose-dependent component.

On the other hand, there is the issue of the toxicity of copper in humans at high doses, described in individuals who accidentally have ingested this metal.[30] The doses used in this study would not present a risk of toxicity in case of accidental intake.

The present results demonstrate that effectiveness is achieved at a concentration lower than that reported in other similar studies (9.4 µg/ml *versus* 1mg/ml), which would be associated with an evident decrease in potential side effects. It has been shown that pH is a factor that can influence the properties of copper sulfate.[31]

In very acidic media containing sulfuric acid, the concentration of sulfate ion decreases, reducing its effectiveness. In this study, copper sulfate solutions at pH of 7.5 and 7.9 were used, so the effect would not be altered. On the other hand, *Candida albicans* is able to grow in pH ranging from 2 to 10, demonstrating that the growth inhibition is due to the antifungal capacity of copper sulfate as such and not to pH8. Because copper sulphate at 9.4 µg/ml has a similar effectiveness to Nystatin, Sodium Hypochlorite and Chlorhexidine in inhibiting the growth of *Candida albicans*, it could be considered as an potential adjuvant in disinfection of removable prostheses and in the treatment of denture stomatitis.

Although at present it is only commercially available for other uses, in the future it could be used as a product for dental practice, having as advantages its low cost and ease of use. Despite this, future studies are required, where the action of copper sulfate on the prosthetic structure over time and in contact with human tissue is evaluated.

CONCLUSION.

The results of this study indicate that copper sulfate at a concentration of 9.4 µg/ml is a potentially useful agent for the antifungal disinfection of heat-polymerized acrylic resin prostheses, as it inhibits the growth of *Candida albicans* using an acrylic resin model.

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