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Less cytotoxic phthalocyanine derivative promotes in vitro wound healing compared to chlorhexidine

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Abstract

The use of adjunct chemical substances in the early postoperative period of periodontal surgical procedures is recommended due to the potential risk of trauma in the operated area. Chlorhexidine digluconate mouthwash is widely used but can cause adverse effects. Phthalocyanine derivatives are being studied as an alternative, demonstrating good antimicrobial activity, especially in the self-activated form, which does not require additional light or chemicals. The objective of this study is to compare the cytotoxicity of different concentrations of a phthalocyanine (PHY) with chlorhexidine (CHX) and assess their influence on fibroblast cell migration. Different concentrations of CHX and PHY (0.0075%–0.12%) were evaluated using NIH 3T3 fibroblasts. Cell viability was assessed by the MTT and crystal violet (CV) assay; CHX and PHY (0.0075% and 0.12%) were also evaluated by in vitro wound healing assay. PHY was less cytotoxic compared to CHX, based on cell viability assays. PHY did not interfere with experimental healing, allowing cell migration similar to the positive control with both concentrations (PHY 0.0075% and 0.12%) and only 0.0075% CHX allowed cell migration. In a comparative analysis, PHY showed less cytotoxicity than CHX and PHY concentrations of 0.0075% and 0.015% was non-toxic even after 48 h of contact with the cells. This in vitro evaluation demonstrated that PHY was less cytotoxic to NIH 3T3 fibroblasts compared to CHX. Furthermore, the different concentrations of PHY did not interfere negatively in the healing of experimental wounds.

Keywords

Photosensitizing agents, chlorhexidine, wound healing, cytotoxicity

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Introduction

The mechanical removal of dental biofilm using conventional and interdental brushes, and dental floss are essentials to maintain periodontal health. However, in early surgical postoperative periods, the patient may experience pain and discomfort during biofilm removal, with the potential risk of trauma. Therefore, chemical control of supragingival biofilm is mandatory.[1]

Among the available chemical substances, chlorhexidine (CHX) is the most prescribed after periodontal surgeries, as it is a broad-spectrum antiseptic agent and provides good control of biofilm and gingival inflammation. Studies

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demonstrated that CHX has antibacterial, antifungal, and antiviral effects, as well as the characteristic of substantivity for having the ability to bind to the surface of the dentin and maintain its effect for a long period, being released gradually, maintaining a sufficient quantity of molecules to create a bacteriostatic effect for a long period.[2–5] However, CHX may present adverse effects such as extrinsic tooth pigmentation, alteration of taste, burning sensation, mild desquamation, and mucosal ulceration.[6,7] Moreover, laboratory studies have demonstrated cytotoxic concentrations of CHX, which may inhibit fibroblast proliferation, reduce collagen synthesis, and negatively influence cell migration.[8–11]

Phthalocyanines or phthalocyanine derivatives (PHY) are chemical compounds that can be associated with cobalt, aluminum, zinc, or iron. This dye is widely studied due to its positive effects in antimicrobial photodynamic therapy (aPDT).[12] aPDT is based on the association of a light source and a photosensitizer that together produce lethal agents for microbial cells.[13] Another class of PHY, little explored in the literature, are the self-activated ones. These have a broad spectrum of action based on self-activation and continuous and localized formation of reactive oxygen in the absence of light, chemicals, or electricity, requiring only molecular oxygen.[14,15] The non-light activated PHY showed antimicrobial activity against a range of microorganisms, including viruses, and also has antibiofilm action.[14,15] When included in formulations for dental use, PHY reduced bacterial and fungal counts by 99.99% and promoted 90% or more viral inactivation.[16,17] In a clinical study using a 0.12% PHY mouthwash, there was a significant improvement in clinical symptoms caused by the coronavirus.[18,19] Regarding clinical studies, no adverse effects of PHY have been reported, an important observation corroborated by laboratorial studies that demonstrated non-cytotoxicity, bringing safety for future investigations.[15,19]

However, mechanisms that lead to all these benefits has not yet been fully clarified, revealing the scarcity of studies with PHY. There is lack of studies with different application protocols and PHY concentrations. Furthermore, considering the adverse effects of CHX and the promising studies of PHY mentioned, the objective of this in vitro study was to compare the cytotoxicity of CHX and PHY, as well as to evaluate the possible interference of the compounds in cell migration.

Methods

Cell culture

Mouse cells of the NIH 3T3 fibroblast lineage, obtained from the American Type Culture Collection (ATCC) CRL-1658 and stored in the cell bank of the Biochemistry discipline, Department of Biological Sciences, at the Bauru School of Dentistry – University of São Paulo, were used. The NIH 3T3 cells have been used in biocompatibility/cytotoxicity studies since the 1960s,[20] and were standardized as one of the first cell lines for testing new solutions, materials, compounds, among others. Thus, choosing this lineage allows comparisons with studies in the literature, as these cell types are extensively used for this purpose.

Cell manipulation was carried out under sterile conditions and cell cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2. NIH 3T3 fibroblasts were cultured in 75 cm^2 bottles containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics. The cells were grown in 96-well culture plates at a density of 5×10^3 cells per well for MTT and crystal violet assays, and in 12-well culture plates at a density of 1×10^5 cells per well for wound healing assay.

Experimental groups

A pilot study was conducted to analyze the solubility and concentrations of the chemical agents: Chlorhexidine Digluconate (CHX) (Bauru Formulas, Bauru, Brazil) and Phthalocyanine Derivative (PHY) (TRIALS – Oral Health and Technologies, Bauru, Brazil), in culture medium. The initial concentrations were based on the clinical use commercial concentration of CHX of 0.12%. After the pilot study, the concentrations were defined as 0.0075%, 0.015%, 0.03%, 0.06%, and 0.12%.

Cell viability analysis

To analyze cell viability, MTT and crystal violet tests were performed. For the MTT assay, cells were incubated with the described concentrations of both chemical agents (CHX and PHY) for 1 min and 24 h. In the PHY group, viability evaluation was also performed at 48 h. For the crystal violet assay, cells were in contact with both agents for 1 min.

The positive control group used was the culture with DMEM + 10% FBS. After each experimental period, cells were incubated for an additional 24 h (37°C, 5% CO_2) with DMEM containing 10% FBS for stabilization prior to performing the MTT and crystal violet assays.

MTT assay

Cell viability was evaluated by the mitochondrial activity of the cells using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction method. This method involves the conversion of the tetrazolium salt to a purple dye called formazan. The tetrazolium salt is yellow and soluble in water and light reactive. This salt undergoes a reduction reaction promoted by the succinate dehydrogenase enzyme, present inside the mitochondria, transforming the salt into formazan, which has a purple/dark blue color and is insoluble in water, accumulating in the cytoplasm of viable cells.

After each period, the treatment culture medium was removed and 110 μL of MTT solution (0.5 mg/mL

in culture medium) was added per well. The cells were incubated at 37°C for 4 h. After this period, the MTT solution was removed, and the insoluble formazan crystals generated were solubilized using 200 µL of DMSO (Dimethyl sulfoxide). As this reaction occurs only in living cells, the more crystals produced, the more purple the solution appears, indicating a higher number of viable cells. The suspension absorbance was measured using a spectrophotometer with a 550 nm filter (Synergy H1 multi-mode reader—BioTek Instruments, Winooski, VT, USA). For statistical analysis, the optical density (OD) data obtained for each group were presented as mean and standard deviation, previously converted into cell viability percentage using the following equation: (Treatment absorbance/control absorbance) × 100. The mitochondrial function of viable cells was calculated in relation to the positive control group, in biological triplicates.

Crystal violet assay

The crystal violet dye was also used to determine cell viability due to its ability to bind to the cell's DNA. After each cell treatment period, the culture medium was completely removed to carry out the staining with crystal violet in 96-well plates, where 50 µL of 0.5% crystal violet staining solution was added to each well and kept for 20 min at room temperature. Subsequently, the crystal violet solution was removed and the plate was washed four times with running water. After washing, the plate was inverted over filter paper and gently tapped to remove any remaining liquid. The plate was dried without the lid for at least 2 h at room temperature and protected from light. Then, 200 µL of methanol was added to each well for 20 min at room temperature to dilute the crystals. The absorbance of the suspension was measured using a spectrophotometer at 570 nm (Synergy H1 multi-mode reader—BioTek Instruments, Winooski, VT, USA). The results were converted to cell viability in percentage using the equation: (Treatment absorbance/control absorbance) × 100.

Wound healing assay

The wound healing assay was performed by assessing the migration capacity of NIH 3T3 mouse fibroblasts. The cells were plated at a density of 1×10^5 cells/well in 12-well plates and kept in the incubator for 72 h to achieve complete confluency. Then, the wells were treated with 5 µg/mL of mitomycin C (antiproliferative) for 15 min. The pre-treatment with mitomycin C ensured that the cells were migrating and not proliferating. The wells were washed with 1X PBS (Phosphate buffered saline) and a "wound" was made in the monolayer using a 1000 µL pipette tip, forming a vertical scratch. Sequentially, the wells were washed three times with 1X PBS and culture medium supplemented with 10% SFB with the CHX and PHY compound groups defined by viability assays were added, using concentrations of 0.0075% and 0.12%. Complete medium without the addition of compounds was used as a control. Images of the scratches were captured at time zero and 24, 48, and 72 h after treatment, using an Olympus U-TV0.5XC-3 inverted optical microscope (Olympus, Tokyo, Japan). The closure of the wound was calculated by comparing the area at the initial experimental period (0 h) with those at 24, 48, and 72 h and statistical comparison was made in relation to the control group (no treatment). Biological triplicates were performed and for each experiment, a total of three scratches were measured per group, and the results were analyzed using the Image J software (Research Services Branch, National Institute Of Health Image—NHI, Bethesda, MD, USA).

Statistical analysis

The data were presented as mean and standard deviation (SD) from three independent experiments. The normality test was performed, showing normal distribution parameters, and ANOVA test was used. Significant differences among the groups were determined by post-hoc tests of Dunnett, Bonferroni, or Tukey at $p < 0.05$. All statistical tests were performed using GraphPad Prism 7.04 software (GraphPad, San Diego, CA, USA).

Results

Cell viability analysis

MTT assay. In the 1-min time point (Figure 1), for the CHX group, all concentrations showed high inhibition of cell viability compared to the positive control ($p < 0.05$). Only the lower concentrations (CHX 0.0075% and 0.015%) had detectable cell viability. In the PHY group, the concentration of 0.0075% showed an increase in viability ($p < 0.05$), and the concentration of 0.015% showed no statistical difference compared to the positive control. The higher concentrations (0.03%, 0.06%, and 0.12%) showed inhibition of viability compared to the positive control ($p < 0.05$). When comparing the groups (CHX and PHY), higher percentages of cell viability were observed in the PHY group ($p < 0.05$) at different concentrations.

At the 24-h time point, inhibition of viability was observed in all CHX concentrations compared to the positive control ($p < 0.05$). PHY concentrations of 0.0075% and 0.015% showed no statistical difference compared to the positive control. PHY concentrations of 0.03%, 0.06%, and 0.12% showed inhibition of viability compared to the positive control ($p < 0.05$).

After 48 h, no viable cells were observed in any CHX concentrations, only in the PHY group. In this group, the results were similar to the 24-h period. Concentrations of 0.0075% and 0.015% showed no statistical difference compared to the positive control, while PHY 0.03%,

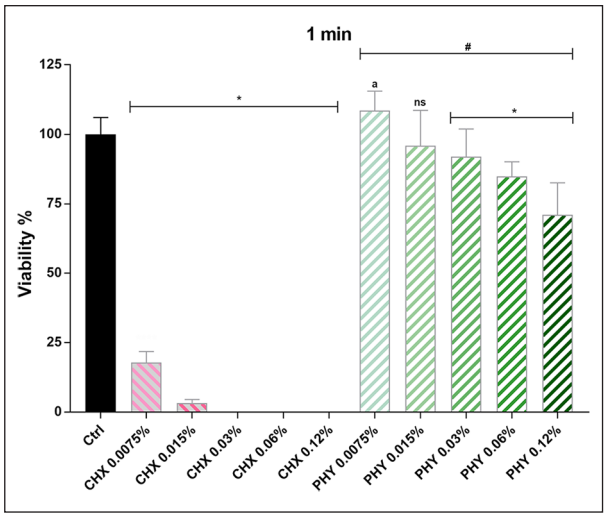

Figure 1. Representative graph of the 1-min MTT assay of cell exposure with the experimental groups.
Viability %: percentage of viable cells; Ctrl: control group; CHX: chlorexidin group (0.0075%–0.12%); PHY: phytalocianine derivative group (0.0075%–0.12%); ns: no statistically significant difference compared to Ctrl.
[a]Higher cell viability compared to Ctrl.
*Lower cell viability compared to Ctrl.
[#]Higher cell viability compared to CHX Group. PHY in all concentrations presents greater viability than all CHX group ($p < 0.05$).

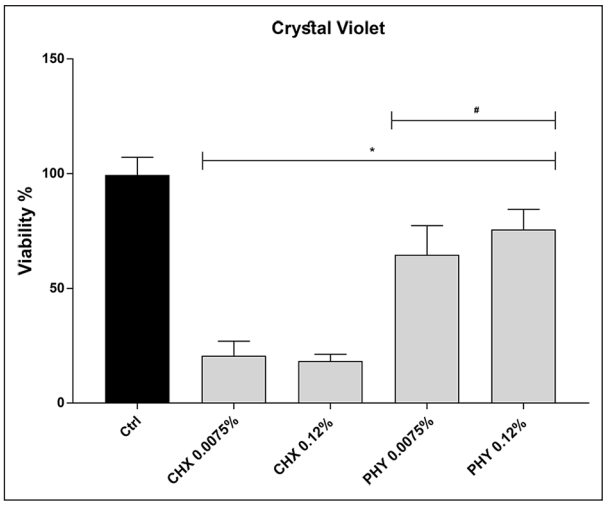

Figure 2. Graph of the cristal violet assay.
Viability %: percentage of viable cells; Ctrl: control group; CHX: chlorexidine group (0.0075% and 0.12%); PHY: phytalocianine derivative group (0.0075% and 0.12%).
*Lower cell viability compared to Ctrl.
[#]Higher cell viability compared to CHX. PHY is less cytotoxic compared to both CHX groups.

0.06%, and 0.12% had lower percentages than the positive control ($p < 0.05$). In the 24-h and 48-h periods, cell viability in the PHY group was higher than in the CHX group at different concentrations studied ($p < 0.05$).

From the experiment's results, it was possible to calculate the IC_{50} of the PHY group at 24 and 48 h. This value represents the PHY concentration that maintains 50% cell viability in the analyzed periods. At 24 h, the IC_{50} was 0.081% (±0.006), and at 48 h, 0.054% (±0.004).

Crystal violet assay. For this assay, concentrations of 0.12% and 0.0075% of both compounds previously evaluated in the MTT assay were selected. The therapeutic dose of CHX used clinically is 0.12%, and the concentration of 0.0075% was the only one that maintained some cell viability for CHX. In contrast, for PHY, the lowest concentration apparently stimulated proliferation with statistical significance, which could potentially enhance the healing effect.

Therefore, in the Crystal Violet assay, both the CHX and PHY groups showed lower percentages of cell viability compared to the positive control ($p < 0.05$) (Figure 2). After 1 min of contact, concentrations of 0.0075% and 0.12% of PHY still maintained cell viability above 50%, which did not occur in the CHX group.

Wound healing assay. The concentrations of PHY (0.0075% and 0.12%) did not negatively influence cell migration during the analyzed periods. In the CHX group, only the concentration of 0.0075% allowed wound closure. The wound closure percentages (Figure 3) in the PHY group at both concentrations were similar to the positive control from 0 to 72 h, with no statistically significant difference between the concentrations, both allowing wound closure at the end of the experiment. The CHX group at a concentration of 0.0075% showed no statistical difference compared to the positive control, also allowing experimental wound closure at the end of the analyzed period. However, the concentration of CHX 0.12% did not allow cell migration in any of the analyzed periods, showing a statistically significant difference compared to the positive control. In the comparison between groups, PHY (0.0075%) at 48 h showed better wound closure ($p < 0.05$) compared to the CHX group (0.0075%).

The photomicrographs of the experiment (Figure 4) allow visualization of the positive control, in which cells migrated in 24 h. After 72 h, there was experimental wound closure compared to the initial 0-h period. At the lower concentration of 0.0075%, both the CHX and PHY groups showed experimental wound closure in 72 h, with no differences compared to the control group. When cells were exposed to a concentration of 0.12% in the CHX group, CHX negatively interfered with cell migration in all periods, with no cell migration observed even at 72 h, with profuse presence of dead cells. On the other hand, the PHY 0.12% group did not negatively interfere with the cell migration process, with cell migration observed at 24 h and wound closure observed at the end of 72 h.

Discussion

PHY was less cytotoxic compared to CHX in cell viability assays (MTT and CV). Furthermore, PHY did not interfere with experimental wound healing, allowing similar cell

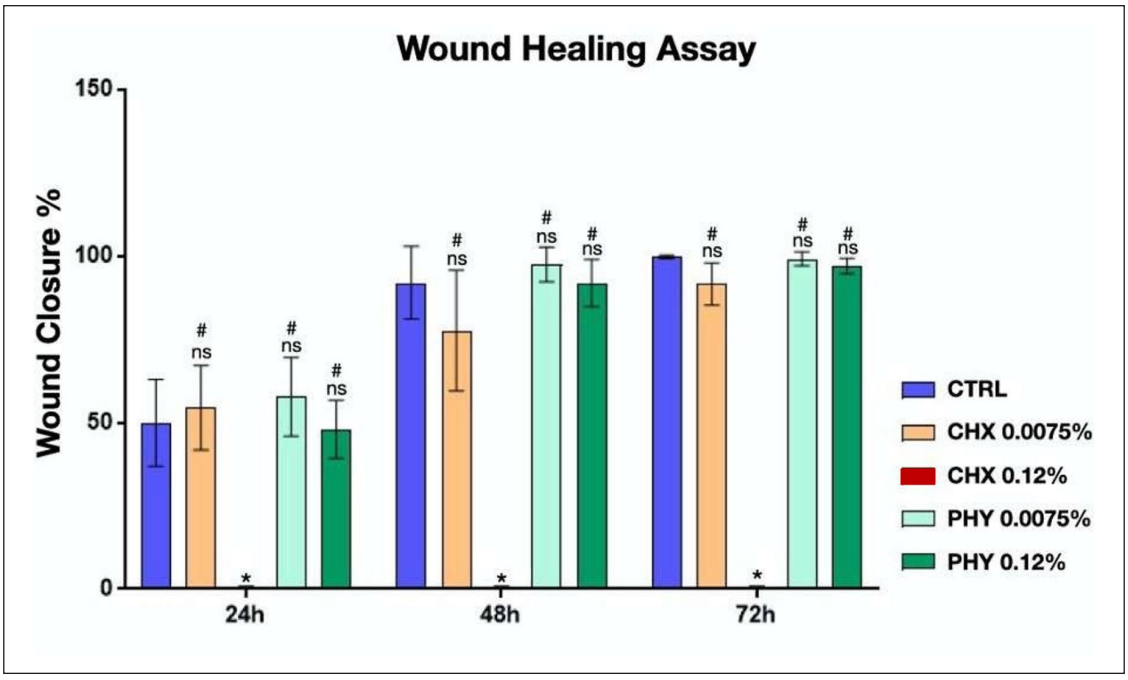

Figure 3. Graph of the wound healing assay.
Wound closure %: percentage of wound closure; Ctrl: control group; CHX: chlorexidine group (0.0075% and 0.12%); PHY: phytalocianine derivative group (0.0075% and 0.12%); ns: no statistically significant difference compared to Ctrl.
*Lower wound closure compared to Ctrl.
#Higher wound closure compared to CHX 0.12%. PHY allows wound closure similar to Ctrl at both concentrations, as only CHX 0.0075% is similar to Ctrl.

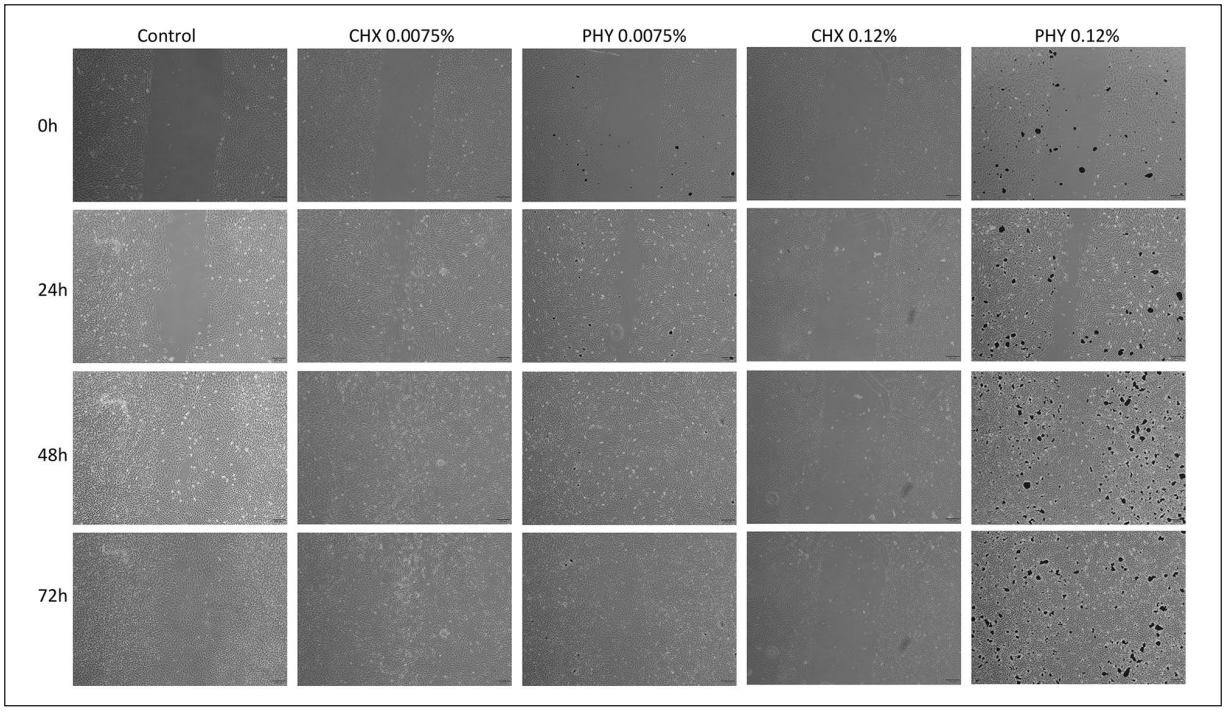

Figure 4. Photomicrograph illustrating the wound healing assay of all groups. At the end of the experimental period (72 h), complete wound closure was observed in the Control Group. Similarly, this outcome was noted for CHX 0.0075%, PHY 0.0075%, and PHY 0.12%. However, for CHX 0.12%, it was observed that this concentration inhibited cell migration, with dead cells being identifiable throughout the experiment.
Control: control group; CHX: chlorexidine group (0.0075% and 0.12%); PHY: phytalocianine derivative group (0.0075% and 0.12%).

migration to positive control at both concentrations studied. Concentrations of 0.0075% and 0.015% of PHY were non-toxic even after 48 h of contact with cells. At 24 h, the PHY concentration of 0.081% maintained at least 50% cell viability (IC_{50}), while at 48 h, the IC_{50} was with PHY 0.054%. In the CHX groups, all concentrations were inhibitors of more than 50% of cell viability. It is important to highlight that, to date, there are no published studies with the presented methodology comparing CHX to PHY derivatives.

Studies about the action of CHX are numerous but with controversial results. In vitro studies showed that CHX, even at lower concentrations than those used clinically, negatively influenced experimental wound healing. CHX 0.0032% and CHX 0.2% altered fibroblast migration by almost 80%. Lower concentrations (0.0016% and 0.0008%) allowed experimental wound closure.[21] A similar result was observed for CHX 0.00039%, with closure similar to the positive control after 48 h.[11] In another study, CHX 0.002% allowed fibroblast cell migration in the wound healing assay, but inhibited closure by myoblasts and osteoblasts.[10] Similarly, this study demonstrated that CHX at lower concentrations (0.0075%) allowed similar migration to the positive control, but at a higher concentration (0.12%), inhibited 100% of in vitro cell migration.

The clinically recommended concentration of CHX in mouthwash solutions varies from 0.12% to 0.2%. In the present study, it was observed that CHX 0.12% in a short interval (1 min) of contact with NIH 3T3 fibroblasts was cytotoxic and inhibited cell viability. Consistent with these results, other studies[22–24] demonstrated that CHX 0.12% (1 min) induced cell death and mitochondrial dysfunction. These analyses of CHX cytotoxicity in a short period of time (1 min) may not show what actually happens to cells, since cells do not die immediately. The 24-h incubation time after treatment allows for the mimicry of what happens with the patient's mucosal monolayer. Despite these negative results with higher concentrations of CHX, the option to maintain the evaluation of this concentration in the present study was precisely for comparison with the same concentration of PHY.

Animal studies have also shown controversial results of CHX in the healing process. One study showed, after histometric analysis, that with the use of CHX 0.2% after periodontal surgical procedures, wound closure occurred with lower levels of inflammatory infiltrate. The dental surface also showed less biofilm compared to the control.[25] When used in higher concentrations (CHX 0.5%) in mucosal-bone wounds in rats, there was a significant delay in wound healing compared to the control.[26]

In clinical studies, CHX 0.2% has been shown to promote better wound healing and less plaque accumulation compared to placebo mouthwash in areas undergoing periodontal surgical procedures.[27] Other authors also support that CHX 0.12% does not exert negative influence and may induce early wound healing, as well as its effective activity in controlling plaque in the absence of adequate post-surgical mechanical oral hygiene.[1,28]

In addition to the controversy surrounding CHX results, its clinical adverse effects should also be considered, justifying the constant search for products and alternatives that present benefits but with less cytotoxicity and side effects. When comparing different agents, it was found that the enzyme mouthwash (Biotène) was less cytotoxic than CHX 0.2%.[29] Another study compared the cytotoxicity of a mouthwash with octenidine (Octenidol) and CHX. Octenidine had less influence on cellular metabolism and greater cellular viability, concluding that this active ingredient could be an alternative to CHX with less cytotoxic effect.[30] When comparing antibacterial concentrations of CHX 0.1% and curcumin 0.003%, this alternative product presented lower cytotoxicity compared to CHX. The product also showed bactericidal effects, allowing adequate experimental wound healing.[22]

The present study demonstrated positive and promising results of PHY compared to CHX. The non-light-activated PHY used in the present study showed antimicrobial and antibiofilm activity.[14,15] Like the broad-spectrum action of CHX, PHY presented antibacterial action against a range of microorganisms, including viruses. When included in formulations for dental use, PHY reduced bacteria and fungi counts by 99.99% and revealed a 90% viral inactivation percentage.[16,17]

However, the mechanism related to the potential of PHY in wound healing has not yet been elucidated, and literature on the subject is scarce. It is suggested that PHY benefits the healing process by its ability to generate ROS (reactive oxygen species) when activated. These molecules are responsible for promoting cell proliferation through the activation of stem cells that regulate inflammatory factors and collagen remodeling. Additionally, they are responsible for preventing the development of infections by inactivating microorganisms.[31]

Clinical studies using PHY during the SARS-CoV-2 pandemic period showed promising results without adverse effects. The 0.12% PHY mouthwash reduced coronavirus-related clinical symptoms more quickly.[18,19] In the same context, PHY incorporated into a toothpaste formulation significantly reduced the viral load of SARS-CoV-2 in the oral cavity.[32]

In periodontal supportive therapy, a PHY-containing toothpaste was used to assist in controlling stain formation in a smoking patient, reducing the frequency of calculus accumulation and deposition during 6 months of follow-up.[33] According to these studies, the results provide safety for future investigations with this class of PHY.[15,19]

Thus, the present study presents unprecedented and promising results of PHY. Considering the limitations of an in vitro study and knowing that PHY is clinically safe, a randomized clinical study comparing PHY with CHX is

suggested, thus verifying its antimicrobial benefits and possible potential in improving wound healing.

Conclusion

PHY is less cytotoxic for NIH 3T3 fibroblasts compared to CHX. Different concentrations of PHY did not negatively interfere with in vitro wound healing.

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Contributorship

CAS: Conceptualization, Methodology, Writing – Original Draft. ASP: Methodology, Validation, Formal analysis. FVV: Investigation, Resources, Funding acquisition. RCO: Methodology, Resources. PSSS: Visualization, Project administration. CAD: Writing – Review and Editing, Formal analysis. MSRZ: Writing – Review and Editing, Supervision. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Declaration of conflicting interests

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