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Terapia Fotodinâmica em Cancro Oral

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Terapia Fotodinâmica em Cancro Oral

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Resumo

Introdução: O carcinoma espinocelular da cavidade oral (OSCC) oral é o sétimo cancro mais frequente no mundo. Em alguns casos de recidiva de OSCC ou quando não existe indicação para as terapias convencionais, a terapia fotodinâmica (PDT) pode representar uma opção de tratamento eficaz e mais conservadora. O fotossensibilizador (PS) clorina 5 tem apresentado excelentes resultados contra outros tipos de células tumorais. O presente trabalho tem como objetivo avaliar a eficácia da clorina 5 no tratamento de células de OSCC.

Métodos: Células humanas de OSCC (linha celular BICR 10) foram incubadas com clorina 5 em diferentes concentrações. Após 24 horas, a atividade metabólica foi avaliada, uma curva de dose-resposta traçada e o valor de IC_{50} calculado. O ensaio SRB foi realizado para avaliar o conteúdo proteico e o ensaio de cristal violeta para determinar o conteúdo de DNA. A morfologia celular foi avaliada com a coloração de May-Grünwald-Giemsa.

Resultados: O valor de IC^{50} da clorina 5 em células de OSCC é de 100.5 nM. Uma diminuição significativa no conteúdo proteico e de DNA foi evidente em todos os grupos de tratamento. Adicionalmente, células em apoptose e necrose foram identificadas nos grupos.

Discussão: A PDT tem um enorme potencial para tratar casos de OSCCs em que as terapias convencionais falharam ou ocorreu recorrência da doença. Esta é uma opção conservadora e mais seletiva do que as terapias atualmente disponíveis. Existe a necessidade de PSs mais eficazes, e a clorina 5 apresenta-se como um PS promissor com atividade fotodinâmica de ordem nanomolar. Estudos *in vitro* adicionais permitirão uma melhor caracterização dos efeitos da clorina.

Palavras-chave: terapia fotodinâmica; clorinas; carcinoma espinocelular da cavidade oral; tratamento.

Abstract

Introduction: Oral squamous cell carcinoma (OSCC) is the seventh most frequent cancer in the world, and photodynamic therapy (PDT) may represent an effective and more conservative treatment option in some OSCC cases. The photosensitizer (PS) chlorin 5 has exhibited excellent results against other cancer cell types, so the present study aims to evaluate the efficacy of chlorin 5 in PDT treatment on OSCC cells.

Methods: Human BICR 10 OSCC cells were treated with chlorin 5. After 24 hours, the metabolic activity was evaluated, a dose-response curve traced, and the IC_{50} determined. The SRB assay was performed to assess protein content and the crystal violet assay to determine DNA content. The cellular morphology was evaluated after treatment with the May-Grünwald-Giemsa staining.

Results: Chlorin 5 IC_{50} in OSCC cells is 100.5nM. A significant protein and DNA content decrease was evident in all treatment groups. In addition, apoptosis and necrosis were identified in the PS-treated cells.

Discussion: PDT has huge potential to treat OSCC cases where conventional therapies have failed or recurrence occurred. It is a conservative and selective option. There is a need for improved PSs, and chlorin 5 presents as a promising PS with nanomolar photodynamic activity. Further *in vitro* studies will allow characterizing chlorin effects better.

Keywords: photochemotherapy; photodynamic therapy; chlorins; oral squamous cell carcinoma; therapeutics.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the seventh most frequent cancer in the world and constitutes about 5% of all malignant conditions in humans. (1,2) The tongue, buccal and alveolar mucosa, and the floor of the mouth and ventral tongue are the most common affected sites, although lesions may be located in any site of the oral mucosa. (1–3) Tobacco and alcohol consumption are etiologic factors of OSCC. (1,4–6) Most recently, an association between HPV infection and the development of OSCC lesions has also been described. (1,4,7,8) The mechanism is not yet entirely understood, but better treatment responses are reported in HPV-associated cancer lesions. (1,4,8) Men and/or immunosuppressed patients have a higher risk of developing OSCC. (1) The oral cancer incidence is also reported to be higher in older patients; however, it has been increasing in females and young adults, associated with the described risk factors and representing up to 10% of OSCC cases. (1)

The first-line treatment for early to moderate stages of OSCC is surgery. (1,9) However, depending on the existence of positive lymph nodes and/or lymph nodes with metastasis extending beyond the lymph node capsule, the therapeutic approach may differ, and radiotherapy combined or not with chemotherapy can be necessary. (1,9) In advanced OSCC cases, to improve disease control, prolong survival, and maintain an acceptable patient's quality of life, multidisciplinary non-surgical therapeutic approaches are being used with increasing frequency. (1,4,9) Despite the therapeutic effectiveness of surgery, radiotherapy, and chemotherapy, the literature reports recurrence rates for oral cancer higher than 50% in patients after primary treatment. (1,10) Furthermore, poor postoperative recuperation, psychosocial repercussion, disfigurement of the face, xerostomia, inflammation of the mucosa, and fibrosis constitute hugely mutilating consequences of these conventional treatments. (1,11) Therefore, alternative treatments for better management of OSCC are needed. (1)

Photodynamic therapy (PDT) is based on the administration of a photosensitizer (PS) followed by visible light irradiation, with wavelengths from 600 to 800 nm. (1,12,13) The PS molecules are selectively absorbed by the tumor cells, and suitable light exposure of the tumor region leads to the formation of cytotoxic reactive oxygen species (ROS). (1,12,13) Reactive oxygen species induce cancer cell death, damage tumor-associated vasculature, and activate an antitumor immune response. (1,12–14) Phototherapeutic window light activation of PS molecules bears enough energy to produce ROS, reach deeper into the tissues, avoid excitation of endogenous chromophores, and induce cell death. (1,12–15) These features provide PDT with a valued dual selectivity, inducing tumor cell death and limiting damage to healthy tissue. (1,12,15)

The strong absorption within the phototherapeutic range is one of the desirable characteristics of the PS. (12–16) Several PS are commercially available, such as Foscan®, Photofrin®, or 5-aminolevulinic acid (5-ALA). These molecules present interesting results, but PSs with improved characteristics are needed to expand PDT use. Chlorins are hydroporphyrins that present typically intense absorption bands in the red and near-infrared (NIR) regions, with good yields for singlet oxygen generation. (12,16) Therefore, chlorins are an efficient and preferable PDT PS. (12,16) Chlorin 5 (4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-fused chlorin) presents high stability, intense absorption bands at 650 nm, and impressive photosensitizer ability against melanoma cancer cells. (16) Chlorin 5 formula is presented in Figure 1.

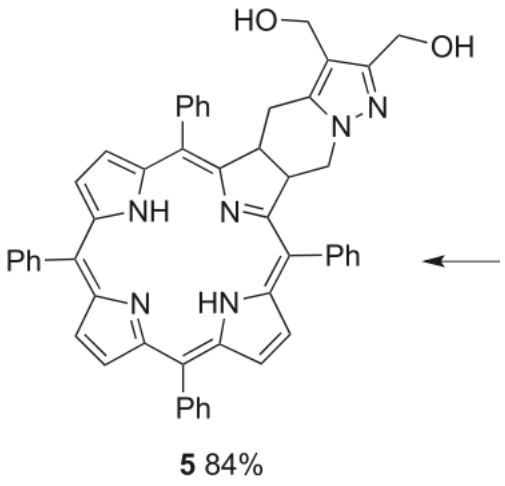

Figure 1. Chlorin 5 formula.

In the European Union, PDT indications are choroidal neovascularization associated with age-related macular degeneration and pathological myopia, mild to moderate actinic keratosis of the face and scalp, advanced prostate adenocarcinoma, and head and neck squamous cell carcinoma. (1,17) Additionally, PDT applications are described for lung and pleural cancers, high-grade cervical dysplastic lesions and menorrhagia, glioblastomas or their recurrence, esophageal cancers, and cholangiocarcinoma, and superficial basal cell carcinomas and Bowen´s disease. (1,18)

Current evidence does not point to the use of PDT as a first-line option for OSCC treatment. (1) However, PDT may represent an effective and more conservative treatment option in OSCC cases without surgical eligibility and recurrent OSCC that did not respond to conventional treatments. (1,11) PDT conservative treatment approach presents few side effects, excellent healing and cosmetic results, preservation of organ function, and the possibility of being performed before and after other treatments. (1)

The oral cavity is readily accessible to visible light irradiation, which represents a potentiality for PDT application on OSCC treatment. (1) Since chlorin 5 has revealed an impressive potential as PDT PS against other cancer cell types, the present study aims to evaluate the efficacy of chlorin 5 as PDT PS on OSCC cells.

2. Material and Methods

2.1. PS preparation

Chlorin 5 was synthesized according to the previously described methodology (16).

The photosensitizer powder was solubilized in DMSO (Fisher Chemical, 200-664-3) at 1 mg/mL. Consecutive dilutions were performed with DMSO to obtain the desirable PSs concentrations to be tested.

2.2. Cell Culture

The human BICR 10 OSCC cell line was acquired from the American Type Culture Collection. BICR 10 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma D-5648) supplemented with 0.4 µg/mL hydrocortisone (#H0888 Sigma) and 10% fetal bovine serum (FBS, Sigma F7524). Cell culture was performed in standard conditions, and the cells were maintained at 37 °C in a humidified incubator (HeraCell 150) with 95% air and 5% CO_2.

Trypsin-EDTA solution at 0,25% (Gibco) was used to detach cells and prepare cell suspensions before experiments. Cells were seeded at 125,000 cells/ml and left overnight to allow cell attachment before the experimental procedures were performed.

2.3. Photodynamic Treatment

The experimental controls consisted of untreated cell cultures and cultures treated with 1% DMSO, which is the PS vehicle of administration. The PS was administered in concentrations between 1 nM and 10 µM, and cells were left for 24 h in the incubator. After this, phosphate-buffered saline (PBS; in mM: 137 NaCl (JMGS), 2.7 KCl (Sigma), 10 Na2HPO4 (Merck), and 1.8 KH2PO4 (Sigma); pH 7.4) was used to wash cells, and the medium was replaced by fresh

PS-free DMEM. The irradiation of plates was performed with a fluence rate of 7.5 mW/cm^2 and a maximum of 10 J. The light source was equipped with a red filter.

2.4. Photocytotoxicity Analysis

2.4.1. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine the metabolic activity of BICR 10 cells after chlorin 5 administration and 24 hours after irradiation. Cells were washed with PBS and a solution of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (0.5 mg/mL, Sigma M5655) was added. Then, cells were maintained in the incubator at 37 °C in humidified air with 95% air and 5% CO_2 overnight. A 0.04 M solution of hydrochloric acid (Merck Millipore100317) in isopropanol (Sigma 278475) was added to dissolve formazan crystals. An EnSpire Multimode Plate Reader (Perkin Elmer) was used to quantify absorbance at 570 nm, with a reference filter at 620nm. Expression of chlorin 5 photocytotoxicity was established as the percentage of metabolic activity relative to cell cultures treated only with DMSO. Using GraphPad Prism 9, a dose-response curve was traced, and the derivation of chlorin 5 concentration that inhibits the cellular proliferation at 50% (IC_{50}) was obtained.

2.4.2. Sulforhodamine B (SRB) assay

The correlation between cell viability and protein content was assessed with the SRB assay. After a post-irradiation period of 24 hours, the assay was performed according to a previously described protocol. (19) Briefly, cells were washed with cold PBS, fixed with an acetic acid solution in 1% methanol, and stained with 0.5% SRB solution. TRIS-NaOH (pH 10.0) was added to solubilize the protein-bound dye for quantification. Absorbance was read at 540 nm and using a reference filter at 690nm using the EnSpire Multimode Plate Reader. The expression of chlorin 5 photocytotoxicity was determined as the percentage of protein content relative to cell cultures treated with DMSO.

2.4.3. Crystal Violet assay

After 24 h of the plates´ irradiation, DNA content was determined with crystal violet according to the protocol previously described. Briefly, cells were fixed in methanol and later stained with crystal violet dye. Next, hydrochloric acid in isopropanol was used to solubilize the day and allow quantification. Absorbance was quantified at 570 nm with a reference filter at 620 nm using the EnSpire Multimode Plate Reader. The photosensitizer photocytotoxicity was expressed as the percentage of DNA content relative to cell cultures treated with DMSO.

2.4.4. May-Grünwald-Giemsa staining

May-Grünwald-Giemsa was used to evaluate the cells' morphology. Twenty-four hours after irradiation, the cells medium was collected, and cells detached from plates. Cell suspensions were centrifugated at 2500 rpm for 5 min. Cells were resuspended in 15 µL of FBS. Cells were smeared on the slides and left to dry for 90 min. All smears were covered by May-Grünwald for 3 min. Milli-Q water was added over May-Grünwald for 1 min. Giemsa dilution was added to cover the slides for 15 min. The excess stain was removed with water and left to dry in the air. Cells were photographed using a Nikon OS-Fi2 (Nikon, Tokyo, Japan) camera at 100× and 500× magnifications.

3. Results

3.1. MTT assay

The photocytotoxicity of chlorin 5 against the human BICR 10 OSCC cell line was evaluated. The dose-response curve is presented in Figure 2. Chlorin 5 revealed high phototoxicity against BICR 10 OSCC cell line, presenting an IC_{50} value of 100.5 nM, R^2 of 0.95, and CI_{95} values between 84.0 and 119.9 nM.

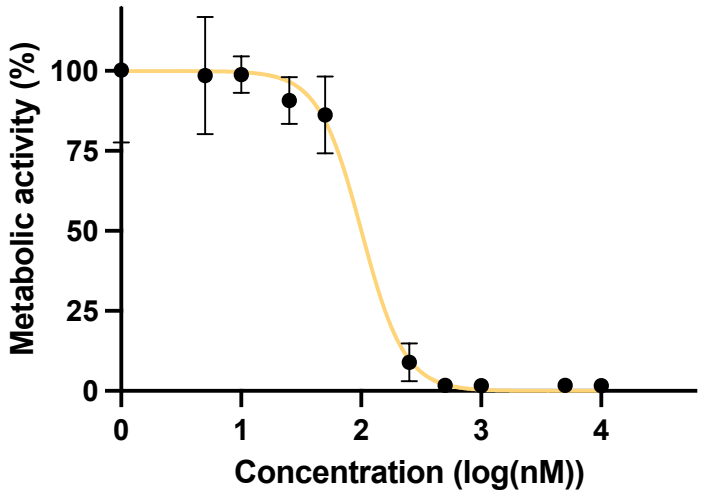

Figure 2. Dose-response curve of BICR 10. Metabolic activity evaluation was performed 24 h after PDT treatment with chlorin 5. Data points correspond to the mean ± SD of at least eight replicates and three independent experiments.

3.2. SRB assay

The correlation between cell viability and protein content was analyzed with the SRB assay. A significant protein content decrease of about 70% was evident in all treatment groups, as seen in Figure 3.

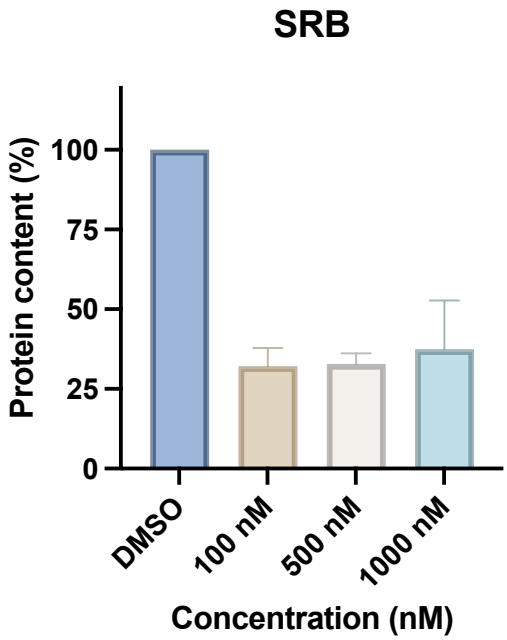

Figure 3. Protein content was evaluated using the SRB assay 24 h after PDT treatment with chlorin 5. Each column presents the mean ± SD of at least two replicates and one independent experiment.

3.3. Crystal Violet assay

The DNA content results are presented in Figure 4. The DNA content decreased with the increase in PS concentration. A significant decrease from 40% to 50% was evident in all treatment groups, as seen in Figure 4.

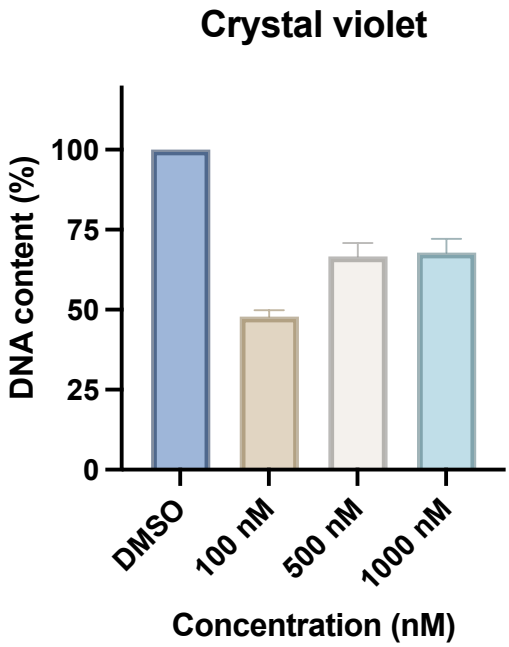

Figure 4. Protein content was evaluated using the crystal violet assay 24 h after PDT treatment with chlorin 5, using the crystal violet assay. Each column presents the mean ± SD of at least four replicates and one independent experiment.

3.4. May-Grünwald-Giemsa staining

Cellular morphology was evaluated using May-Grünwald-Giemsa staining. Representative photographs at 100× magnification are presented in Figure 5, and 500× magnification photographs are presented in Figure 6. Chlorin 5 concentrations of 100 nM, 500 nM, and 1 μM revealed phototoxicity against BICR 10 OSCC cell line. Morphological evidence of both apoptosis and necrosis cell death were identified.

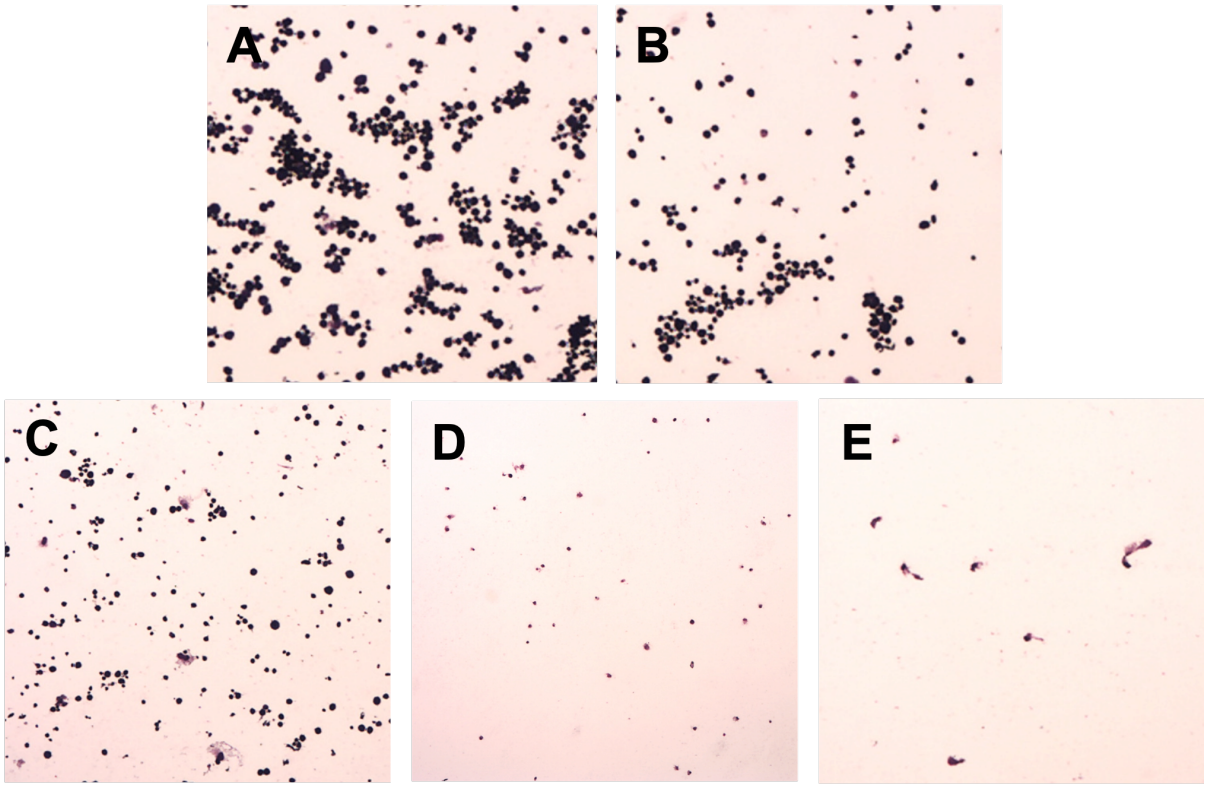

Figure 5. BICR 10 OSCC cells stained with May-Grünwald-Giemsa. Experimental controls of untreated cell cultures (A), cultures treated with 1% DMSO (B), and after 100 nM (C), 500 nM (D), and 1 μM (E) chlorin 5 administration are presented. Images at 100× magnification.

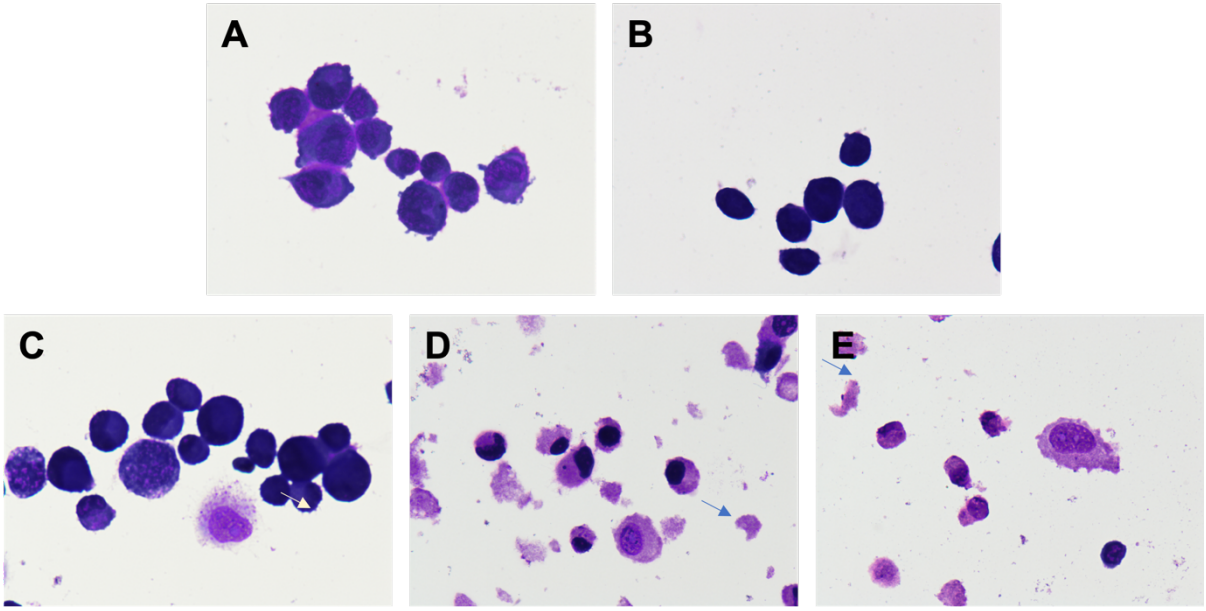

Figure 6. BICR 10 OSCC cells stained with May-Grünwald-Giemsa. Experimental controls of untreated cell cultures (A), cultures treated with 1% DMSO (B), and after 100 nM (C), 500 nM (D), and 1 μM (E)

chlorin 5 administration are presented. Evidence of cellular debris (blue arrows) and blebs (yellow arrow). Images at 500× magnification.

4. Discussion

The potential of PDT treatment in OSCC and other cancers is recognized and worthy of more investigation due to its improved therapeutic action, selectivity, and decreased side effects, among other relevant characteristics.

Our group has previously reported impressive chlorin 5 PS ability against human melanoma cells. (16) Human melanocytic melanoma A375 cells revealed particularly sensitivity to chlorin 5 with an IC_{50} value of 31 nM, while human amelanotic melanoma C32 cells are more resistant, presenting an IC_{50} value of 231 nM. (16) In the present study, chlorin 5 revealed high phototoxicity against OSCC cells with an IC_{50} value of 100.5 nM. This nanomolar photodynamic activity supports chlorin 5 as a hugely effective low-dose photosensitizing agent against human BICR 10 OSCC cells.

The MTT colorimetric assay was used to evaluate BICR 10 cells' metabolic activity, which indicates cells' sensitivity to chlorin 5. (12,20,21) The metabolic activity was significantly reduced with higher concentrations of PS and allowed us to calculate the IC_{50}, as previously referred to. However, a decrease in the metabolic activity does not necessarily results from cell death and may be a consequence of a stimulus that limits cellular metabolism. (20) Thus, we performed additional evaluations to evaluate chlorin 5 photocytotoxicity, testing three chosen concentrations: 100 nM, 500 nM, and 1 μM. (20)

The SRB assay directly correlates cell viability and protein content, complementing the MTT results. (22,23) The decrease in SRB content is evident in all tested concentrations, supporting a protein loss associated with cell death. Additionally, the crystal violet assay was used to evaluate DNA content and better characterize the PS effects on cells. The DNA amount decreased to all tested concentrations, supporting the loss of cells after treatment. These results show that chlorin 5 concentrations of 100 nM, 500 nM, and 1 μM revealed phototoxicity against BICR 10 OSCC cell line.

To further characterize the PDT effect on cells, May-Grünwald-Giemsa staining was performed. In the experimental controls, cells present a rounded and well-defined morphology, characteristic of live cells. Apoptosis and necrosis were identified in all PS-treated cells. In addition, morphological evidence of augmented volume cells, blebbing, nuclei fragmentation, cytoplasmatic vacuolization, and cytoplasm extravasation compatible with cell death were identified (Figure 6). Thus, we confirmed chlorin 5 induces cell death in BICR 10 OSCC cells.

According to the obtained results, it may be affirmed that chlorin 5 presents effective low-dose phototoxicity against the human BICR 10 OSCC cell line. Noteworthy, SRB and crystal violet assays should be replicated to validate the preliminary results obtained.

Our results confirm chlorin 5 as an efficient and preferable PDT PS, with possible application in OSCC. (12,16) PDT represents a valid treatment option in OSCC cases without surgical eligibility and recurrent OSCC that did not respond to conventional treatments. PDT conservative approach is characterized by few side effects, excellent healing and cosmetic results, preservation of organ function, and the possibility of being performed before and after other treatments. (1) Moreover, the oral cavity is promptly accessible to visible light irradiation, which also represents a potentiality for PDT conservative treatment approach application to OSCC treatment. (1)

Interesting results were obtained in the present study; however, a more detailed evaluation should be performed to characterize PDT effects better. Further studies of viability and types of cell death with flow cytometry, cell cycle with flow cytometry, uptake, and ROS evaluation will provide relevant information. Also, since this is in an *in vitro* study, the clinical translation of the obtained results is not possible since *in vitro* studies cannot replicate the complexity and dynamic of in vivo processes. (24) Therefore, further in vivo studies are of great interest to mimic human situation and evaluate PDT efficacy in established OSCC lesions.

5. Conclusions

Chlorin 5 is hugely effective low-dose photosensitizing agent against human BICR 10 OSCC cells. Chlorin 5 reveals high phototoxicity against BICR 10 OSCC cell line, decreasing metabolic activity, protein and DNA contain and induces OSCC cells death.

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