Heitor Ceolin Araujo

Efeitos antibiofilme e citotóxico de um novo nanosistema carreador de clorexidina e fluconazol

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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Ciências – Área de Saúde Bucal da Criança.

Orientador: Prof. Dr. Douglas Roberto Monteiro

Coorientador: Prof. Assoc. Juliano Pelim Pessan

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Dedicatória

Dedicatória

Dedico este trabalho

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"Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo".

Martin Luther King

Resumo

Resumo

ARAUJO H.C. **Efeitos antibiofilme e citotóxico de um novo nanosistema carreador de clorexidina e fluconazol**. 2022 79f. Tese (Doutorado em Ciências, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2022.

Os objetivos do presente estudo foram montar e caracterizar um novo nanocarreador dual de clorexidina (CLX) e fluconazol (FLZ), bem como avaliar seu efeito sobre biofilmes microcosmos e sua citotoxicidade sobre queratinócitos orais. Para montar o nanocarreador dual, CLX e FLZ foram adicionados a nanopartículas de óxido de ferro (NPsOF) previamente revestidas por quitosana (QTS), seguido de um processo de solubilização sob agitação magnética. O nanocarreador foi, então, caracterizado por microscopia eletrônica de transmissão, difração de raios X, espectroscopia no infravermelho por transformada de Fourier e análise termogravimétrica. A suscetibilidade de *Candida albicans* e *Candida glabrata* no estado planctônico ao nanocarreador dual foi determinada pelos valores de concentração inibitória mínima, utilizando o método da microdiluição em caldo. Um *pool* de saliva de 2 doadores saudáveis suplementado com espécies de *Candida* foi usado como inóculo para a formação de biofilmes microcosmos. Os biofilmes foram cultivados (72 h) sobre discos de vidro posicionados no *Amsterdam Active Attachment model* e tratados (24 h) com NPsOF-QTS carreando 39 µg/mL de CLX + 156 µg/mL de FLZ (NPsOF-QTS-CLX39-FLZ156), 78 µg/mL de CLX + 312 µg/mL de FLZ (NPsOF-QTS-CLX78-FLZ312) e 156 µg/mL de CLX + 624 µg/mL de FLZ (NPsOF-QTS-CLX156-FLZ624). NPsOF (218,5 µg/mL), QTS (218,5 µg/mL) e 156 µg/mL de CLX + 624 µg/mL de FLZ (CLX156-FLZ624) foram testados como controles. Posteriormente, foram realizadas as análises de quantificação das unidades formadoras de colônias (UFCs), produção de ácido lático (LA), composição da matriz extracelular (ME) e viabilidade celular por microscopia confocal de varredura a laser (MCVL). Para o ensaio de citotoxicidade, queratinócitos orais humanos (linhagem NOKsi) foram expostos a diferentes concentrações do nanocarreador dual, por 24 ou 48 h, e a viabilidade celular foi determinada pelo ensaio de redução de MTT. Os dados foram analisados por ANOVA ou teste de Kruskal-Wallis, seguidos dos testes de Fisher LSD ou Tukey (α = 0,05). Os testes de caracterização físico-química mostraram que um nanocarreador dual com dimensões em torno de 6 nm foi obtido, sem comprometer a propriedade cristalina

e a estabilidade de NPsOF. Os compostos que formam o nanocarreador estabeleceram uma interação sinérgica em relação ao efeito sobre células planctônicas de *Candida.* Para os ensaios de biofilme, NPsOF-QTS-CLX156-FLZ624 foi o composto mais eficaz na redução de UFCs de *Streptococcus mutans*, *Lactobacillus* spp., *C. albicans* e *C. glabrata*, diferindo significativamente dos outros grupos, e esses achados foram confirmados por MCVL. NPsOF-QTS-CLX39-FLZ156, NPsOF-QTS-CLX78-FLZ312 e CLX156-FLZ624 mostraram efeitos antibiofilme similares. O nanocarreador dual também reduziu significativamente a produção de AL e a quantidade de carboidratos e ácidos nucleicos da ME. Um efeito citotóxico dose-dependente sobre queratinócitos orais foi observado para o nanocarreador dual, independentemente do período de exposição testado (24 ou 48 h). NPsOF-QTS-CLX-FLZ e CLX-FLZ reduziram significativamente a viabilidade dos queratinócitos em concentrações de CLX e FLZ iguais ou superiores a 7,8 e 31,25 µg/mL, respectivamente. Por fim, a nanoterapia testada no presente estudo é promissora e constitui um grande avanço dentro dos métodos alternativos aos antimicrobianos tradicionais para o controle da candidíase oral.

Palavras-chave: Biofilmes; Clorexidina; Fluconazol; Nanopartículas magnéticas de óxido de ferro; Queratinócitos; Quitosana; Toxicidade.

Abstract

Abstract

ARAUJO H.C. **Antibiofilm and cytotoxic effects of a new nanocarrier of chlorhexidine and fluconazole**. 2022 79f. Tese (Doutorado em Ciências, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2022.

The objectives of the present study were to assemble and characterize a new dual nanocarrier of chlorhexidine (CHX) and fluconazole (FLZ), and evaluate its effect on microcosm biofilms and its cytotoxicity against oral keratinocytes. To assemble the dual nanocarrier, CHX and FLZ were added to iron oxide nanoparticles (IONPs) previously coated by chitosan (CS), followed by a solubilization process under magnetic stirring. The nanocarrier was then characterized by transmission electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, and thermogravimetric analysis. The susceptibility of *Candida albicans* and *Candida glabrata* in the planktonic state to the dual nanocarrier was determined by the minimum inhibitory concentration values, using the broth microdilution method. A saliva pool from 2 healthy donors supplemented with *Candida* species was used as an inoculum for microcosm biofilm formation. Biofilms were grown (72 h) on glass discs positioned in the Amsterdam Active Attachment model and treated (24 h) with IONPs-CS carrying 39 µg/mL CHX + 156 µg/mL FLZ (IONPs-CS-CHX39-FLZ156), 78 µg/mL CHX + 312 µg/mL FLZ (IONPs-CS-CHX78-FLZ312), and 156 µg/mL CHX + 624 µg/mL FLZ (IONPs-CS-CHX156-FLZ624). IONPs at 218.5 µg/mL, 218.5 µg/mL CS, and 156 µg/mL CHX + 624 µg/mL FLZ (CHX156-FLZ624) were tested as controls. Next, analyses of the quantification of colony-forming units (CFUs), lactic acid production (LA), composition of the extracellular matrix (EM), and viability by confocal laser scanning microscopy (CLSM) were performed. For the cytotoxicity assay, human oral keratinocytes (NOKsi lineage) were exposed to different concentrations of the dual nanocarrier, for 24 or 48 h, and cell viability was determined by the MTT reduction assay. Data were analyzed by ANOVA or Kruskal-Wallis test, followed by Fisher LSD or Tukey tests ($\alpha = 0.05$). The physico-chemical characterization tests showed that a dual nanocarrier with dimensions around 6 nm was assembled, without compromising the crystalline property and stability of IONPs. The compounds that form the nanocarrier established a synergistic interaction in relation to the effect on *Candida* planktonic cells. Regarding biofilm assays, IONPs-CS-CHX156-FLZ624 was the most

effective compound in reducing CFUs from *Streptococcus mutans*, *Lactobacillus* spp., *C. albicans*, and *C. glabrata*, differing significantly from the other groups, and these findings were confirmed by CLSM. IONPs-CS-CHX39-FLZ156, IONPs-CS-CHX78-FLZ312, and CHX156-FLZ624 showed similar antibiofilm effects. The dual nanocarrier also significantly reduced LA production and the amount of carbohydrates and nucleic acids from the EM. A dose-dependent cytotoxic effect against oral keratinocytes was observed for the dual nanocarrier, regardless of the exposure period tested (24 or 48 h). IONPs-CS-CHX-FLZ and CHX-FLZ significantly reduced keratinocyte viability at CHX and FLZ concentrations equal to or greater than 7.8 and 31.25 µg/mL, respectively. In conclusion, the nanotherapy tested in the current study is promising and constitutes a major advance in alternative methods to traditional antimicrobials for oral candidiasis control.

Keywords: Biofilms; Chitosan; Chlorhexidine; Fluconazole; Keratinocytes; Magnetic iron oxide nanoparticles; Toxicity.

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Sumário

SUMÁRIO

Artigo

Dual nanocarrier of chlorhexidine and fluconazole: physico-chemical characterization and effects on microcosm biofilms and oral keratinocytes

Abstract

This study assembled a dual nanocarrier of chlorhexidine (CHX) and fluconazole (FLZ) and evaluated its effects on microcosm biofilms and oral keratinocytes. CHX and FLZ were added to iron oxide nanoparticles (IONPs) coated by chitosan (CS), and the nanocarrier was physico-chemically characterized. Saliva pool from healthy donors supplemented with *Candida* spp. was used as inoculum for biofilm formation. Preformed biofilms (72 h) were then treated (24 h) with IONPs-CS carrying different concentrations of CHX (39, 78 or 156 µg/mL) and FLZ (156, 312 or 624 µg/mL). Next, biofilms were analyzed by colony-forming units (CFUs), lactic acid production (LA), composition of the extracellular matrix (EM), and confocal laser scanning microscopy (CLSM). Regarding cytotoxicity, human oral keratinocytes (NOKsi lineage) were exposed to different concentrations of the dual nanocarrier, for 24 or 48 h, and cell viability was determined by MTT reduction assay. Data were analyzed by ANOVA or Kruskal-Wallis test, followed by Fisher LSD or Tukey tests ($\alpha = 0.05$). A dual nanocarrier with dimensions around 6 nm was effectively assembled. IONPs-CS carrying 156 µg/mL CHX and 624 µg/mL FLZ was the most effective compound in reducing CFUs from microcosm biofilms, and these findings were confirmed by CLSM. The nanocarrier also reduced LA production and components from the EM. A dose-dependent cytotoxic effect against oral keratinocytes was observed for the nanocarrier, regardless of the exposure period evaluated. In conclusion, the dual nanocarrier tested here is promising and constitutes a major advance in alternative methods to traditional antimicrobials for oral candidiasis control.

Keywords: Biofilms; Chitosan; Chlorhexidine; Fluconazole; Keratinocytes; Magnetic iron oxide nanoparticles; Toxicity.

Introduction

The oral cavity harbors a diverse range of at least 2000 microorganisms, and approximately 100 species are routinely found [1,2], either in the planktonic state or forming biofilms on biotic and abiotic surfaces [3]. Oral biofilms are complex, heterogeneous, highly organized microbial consortia protected by an extracellular matrix of polysaccharides, proteins, and nucleic acids [4]. Biofilm formation is a growing challenge in the biomedical area since in dysbiosis situations it can lead to chronic tissue infections, such as cases of oral candidiasis.

Denture stomatitis is a type of oral candidiasis induced by the use of removable dentures and characterized by the presence of both erythemas under the denture support areas and fungal colonization of the prosthetic material and mucosal tissues [5]. *Candida albicans* is the main fungal species associated with this pathology [6], however, non-*albicans Candida* species such as *C. glabrata*, *C. tropicalis,* and *C. parapsilosis* may also be related to the disease pathogenesis [7]. The ability to form biofilms [8] together with the production of phospholipases and proteinases, besides the hemolytic activity [9,10], are important virulence traits of *Candida* species. These species also form inter-kingdom polymicrobial biofilms with other bacterial species, which makes their control and eradication difficult [11].

For oral candidiasis management, topical or systemic antifungals may be used. Treatment with these medicines is aimed at curing oral lesions but may lead to side effects such as gastrointestinal and liver disorders [10]. Although fluconazole (FLZ) is the most widely used systemic antifungal, some *Candida* species are resistant to azoles [12,13], particularly *C. krusei* and *C. glabrata* [14-16]. Furthermore, its fungistatic activity can lead to *C. albicans* resistance, especially if this species is forming biofilms [8] or if the antifungal agent is used for long periods of time, with consequent recurrence of infection [17]. This resistance may be intrinsic to antifungal therapy and/or acquired during treatment [18]. Factors such as impaired immune function, underlying diseases, reduced drug bioavailability, and increased drug metabolism may be responsible for *Candida* resistance to conventional antifungals [18]. Another concern related to

FLZ refers to its cytotoxic potential, as previously reported for human fibroblasts and lung epithelial lineage cells [19].

In addition to antifungals, antimicrobial mouthwashes are used to control oral candidiasis, such as chlorhexidine (CHX). This antimicrobial agent acts against bacteria, fungi, and hyphae [20-22]. For *C. albicans* oral isolates in the planktonic state, CHX is able to act as a fungicidal agent [23], causing damage to microbial cell membranes/walls [24]. However, when tested on biofilms, CHX has a limited effect due to the difficulty of reaching the deeper layers [25]. Staining of oral tissues and rehabilitative materials, as well as taste disturbances [22] are among the main disadvantages of CHX. This antimicrobial at 0.2 and 2% is also highly cytotoxic to human gingival fibroblasts [26] and human endothelial cells [27].

In view of the aforementioned context, topical formulations based on carrier nanoparticles have been investigated as alternative nanotherapies in the control of oral infections, aiming both to increase the antibiofilm effects and to reduce the side effects of conventional antimicrobials. In this sense, iron oxide nanoparticles (IONPs) have been used as carriers of different classes of medicines because they have a greater area of reactivity and ability to cross cells and physical barriers of the biofilm and tissues, delivering drugs directly to specific targets [28]. In addition, IONPs may be coated with natural polymers, such as chitosan (CS), to stabilize and functionalize the set of nanoparticles with medicines [29,30]. This use of CS as a shell of IONPs is related to the favorable biological properties of CS, such as biocompatibility, antimicrobial action [31], and mucoadhesiveness [32].

In the medical field, the use of IONPs as dual carriers of chemotherapeutics has been recently described [33,34]. Cisplatin and metformin [34], as well as doxorubicin and cisplatin [33] were simultaneously carried by IONPs, showing that dual nanocarriers are more effective than medicines carried alone for cancer treatment. In Dentistry, CS-coated IONPs carrying CHX [35,36] or FLZ separately [37-39] showed similar or superior antibiofilm effects compared with the medicines applied alone (without nanocarrier) on *Streptococcus mutans*, *Enterococcus faecalis*,

and *Candida* species in single, dual-species or polymicrobial biofilm models. Despite the potential and promising results reported above, the effects of a dual nanocarrier of CHX and FLZ on *Candida* species in polymicrobial biofilms and on oral keratinocytes remain unknown. Consequently, the aims of this study were to prepare and characterize a novel dual nanocarrier of CHX and FLZ based on CS-coated IONPs, as well as to evaluate its effect on *Candida* species in salivary microcosm biofilms and its cytotoxicity on oral keratinocytes.

Material and methods

Preparation and characterization of the dual nanocarrier

CHX, FLZ, and CS were purchased from Sigma-Aldrich (Saint Louis, MO, USA), while the colloidal suspension of IONPs at 5000 µg/mL was kindly provided by *nChemi* (Engenharia de Materiais, São Carlos, São Paulo, Brazil). The preparation and characterization steps of the dual nanocarrier were based on the protocols previously described by Vieira et al. [35] and de Lima et al. [37], with minor modifications. Briefly, CS was solubilized in 2% acetic acid under constant stirring for 24 h at room temperature. Subsequently, the coating of IONPs with CS was achieved by mixing equal volumes and concentrations (1400 µg/mL) of both compounds. To obtain the dual nanocarrier IONPs-CS-CHX-FLZ, 500 and 2000 µg of CHX and FLZ, respectively, were added to the compound IONPs-CS (700 µg/mL), followed by a 1 h solubilization process under magnetic stirring at room temperature. The physico-chemical characterization of the nanocarrier was performed by transmission electron microscopy (TEM; JEOL JEM 2100, Tokyo, Japan), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and thermogravimetric analysis (TGA). The mean particle size of IONPs, IONPs-CS, and IONPs-CS-CHX-FLZ was calculated from the XRD results, using the Debye Scherrer equation, as previously detailed [40].

Candida strains and growth conditions

American Type Culture Collection (ATCC) strains of *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 90030) were used in the present study. Both *Candida* species were cultivated on Sabouraud dextrose agar (Difco, Le Pont de Claix, France) at 37 °C for 48 h. Then, colonies of each species were individually inserted into polypropylene tubes containing 30 mL of Sabourand dextrose broth (Difco) and incubated overnight (37 ºC) in an orbital shaker (120 rpm) under aerobic conditions. Finally, the tubes were centrifuged (8000 rpm, 5 min), the cell pellets were washed 3× with phosphate-buffered saline (PBS; 0.1 M, pH 7.0), and the fungal cell concentration was adjusted for 1×10^7 cells/mL in human saliva with the assistance of a Neubauer chamber.

Determination of the minimum inhibitory concentration (MIC)

The broth microdilution method was employed to determine the MICs of the dual nanocarrier IONPs-QTS-CHX-FLZ against *Candida* strains, following the document M27-A3 of the Clinical Laboratory Standards Institute [41], with minor modifications. The nanocarrier colloidal suspension was diluted in geometric order in deionized water and subsequently in Roswell Park Memorial Institute culture medium (RPMI 1640; Sigma-Aldrich) (1:5). Cell suspensions of *C. albicans* and *C. glabrata* were adjusted to standard 0.5 on the McFarland scale and diluted in 0.85% saline (1:5) and in RPMI 1640 culture medium (1:20). Next, 100 μL of each yeast suspension were added to the wells of a 96-well U-bottom plate (OLEN-KASVI, São José dos Pinhais, PR, Brazil) containing 100 μL of each previously diluted nanocarrier concentration. The final concentrations of CHX and FLZ in the nanocarrier ranged from 0.09-50 μg/mL and 0.19-100 μg/mL, respectively. IONPs (0.97-500 μg/mL), CS (0.54-140 μg/mL), CHX (0.09-50 μg/mL), and FLZ (0.19-100 μg/mL) were separately tested as controls. After incubation of the plates for 48 h at 37 °C, the MIC values were visually determined (100% inhibition of fungal growth). In addition, the fractional inhibitory concentration index (FICI) was calculated based on the MIC values to ascertain the type of interaction established among the components of the dual nanocarrier, as previously detailed [42].

Human saliva collection

This study was approved by the local Ethics Committee (CAAE:10330919.4.0000.5420) and the volunteers signed the Free and Informed Consent Form before saliva collection. Two healthy adult volunteers, non-smokers, without systemic diseases and who did not use systemic antibiotics (in the last 6 months) or oral antimicrobials in the form of mouthwash (in the last month) participated in the study [43]. Donors were instructed not to consume alcoholic beverages and not to brush their teeth the night before and on the day of collection. At the time of collection (morning period), volunteers were fasting for at least 2 h [43] and chewed a flexible film (Parafilm® M, Sigma-Aldrich) to boost saliva production [44], which was stored in polypropylene tubes (on ice). Samples from the two participants were dispensed into a single tube, forming a saliva pool [45]. This pool was then diluted (1:1) with 60 % glycerol, aliquoted into centrifuge microtubes, and stored at - 80 ºC until use [45].

Microcosm biofilm formation and treatment with the dual nanocarrier

The microbial inoculum for microcosm biofilm formation was prepared by diluting (1:50) the saliva pool in McBain medium [46] supplemented with 0.2% sucrose [45]. This previous inoculum was then supplemented with *C. albicans* and *C. glabrata*, both at 1×10^7 cells/mL, forming the final microbial inoculum. The purpose of this supplementation was to reproduce a microcosm of oral candidiasis [38]. A 1.5 mL volume of the final inoculum was added to the wells of a 24-well plate (Falcon®; Corning Incorporated-Life Sciences, New York, NY, USA), which was closed with the stainless steel lid of the Amsterdam Active Attachment model (AAA). Glass discs (12 mm in diameter; Menzel, Braunschweig, Germany) were fixed in the inner position of this steel lid, which served as surfaces for biofilm formation [45]. The plate + steel lid assembly was anaerobically incubated (Anaerobac; Probac do Brasil Produtos Bacteriológicos Ltda., São Paulo, Brazil) at 37 °C for 8 h. After this period, the AAA model lid was placed on a new 24-well

plate containing 1.5 mL of fresh McBain supplemented with 0.2% sucrose, followed by anaerobic incubation for 16 h. The culture medium was renewed daily, until biofilm formation for 72 h.

Subsequently, the dual nanocarrier was diluted in McBain medium to obtain 39, 78, and 156 µg/mL of CHX and 156, 312, and 624 µg/mL of FLZ generating the nanocarriers IONPs-CS-CHX39-FLZ156, IONPs-CS-CHX78-FLZ312, and IONPs-CS-CHX156-FLZ624. These concentrations are equivalent to 100-, 200-, and 400-fold the lowest MIC value of the nanocarrier. For biofilm treatment, 1.5 mL of each nanocarrier suspension was added to the wells of a new 24-well plate. This plate was then closed with the AAA model lid containing the glass discs with the biofilms, and incubated in anaerobic conditions for 24 h (37 ºC). Biofilms were also treated with IONP and CS alone, both at 218.5 µg/mL and with 156 µg/mL CHX + 624 µg/mL FLZ (CHX156-FLZ624) without nanocarrier. The exposure of biofilms for 24 h to pure McBain medium corresponded to the negative control group (NC).

Biofilm quantification assays

To quantify the colony-forming units (CFUs), the glass discs were removed from the AAA model lid, washed 3 times with PBS, and inserted into polypropylene tubes with 1 mL of PBS. The microcosm biofilms were detached from the glass discs by sonication (2 min) and vortexing (1 min). After, the obtained polymicrobial suspension was diluted in PBS and plated on specific culture media to quantify total aerobic and anaerobic organisms, *C. albicans*, *C. glabrata*, *Streptococcus mutans*, and *Lactobacillus* spp., as detailed by Caldeirão et al. [38]. The plates were incubated at 37 °C for 48-72 h and the results were presented as Log_{10} CFU/mL.

The components of the extracellular matrix (proteins, carbohydrates, and nucleic acids) were also quantified. For this, the resulting biofilms after treatment with the different compounds were processed as detailed in a previous study [38], and the liquid phase of the matrix was separated from the cell pellet by filtration (0.22 µm filter). The tubes containing the cell pellets were stored in an oven until the complete drying, and a constant dry weight of the biofilm was obtained. The

protein content of the extracellular matrix was quantified by the bicinchoninic acid method (BCA kit; Sigma-Aldrich), using a standard curve of bovine serum albumin [47]. Total carbohydrate concentration was measured by the sulfuric acid-phenol method [48], based on a standard curve with different glucose concentrations. In turn, the total nucleic acid concentration was evaluated by a NanoDrop spectrophotometer (Eon Microplate Spectrophotometer; Bio Tek, Winooski, USA) at wavelengths of 260 nm and 280 nm [49]. The values for each component of the extracellular matrix were normalized by the biofilm dry weight and expressed as mg/g dry weight.

For lactic acid quantification, the AAA model lid was transferred to a new 24-well plate containing buffered peptone water (BPW; 1.5 mL/well) supplemented with 0.2% glucose [45]. After incubation of this plate in anaerobic conditions for 3 h at 37 °C, the enzyme lactate dehydrogenase (Sigma-Aldrich) was used to establish the lactate concentration in the BPW solution, with absorbance reading at 340 nm [50]. A standard curve of sodium L-lactate (Sigma-Aldrich) was constructed with concentrations ranging from 0 to 10 mM.

Structural and viability analyses of the biofilm by confocal laser scanning microscopy (CLSM)

The resulting microcosm biofilms after treatment with nanocarriers and controls were washed with PBS and exposed to the fluorescent dyes SYTO9 and propidium iodide from the FilmTracer™ Live/Dead™ Biofilm Viability Kit (Invitrogen, Life Technologies Corporation, Eugene, OR, USA), following the protocols recommended by the manufacturer. Next, the biofilms were examined under a confocal microscope (Nikon C2/C2si, Tokyo, Japan) at × 20 magnification, according to previously reported parameters [51]. For each experimental group, three 2D images were obtained, which were processed using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA). Cell viability was also evaluated by CLSM. For this, the red fluorescence intensity (dead cells) was divided by the total fluorescence intensity (live and dead cells), and the results were represented as % dead cells/total cells [38,39].

Cell cytotoxicity assay

Human oral keratinocytes of the NOKsi lineage were used in the cytotoxicity assay of the nanocarriers, and the cultivation of these cells was based on previously described protocols [36,52], with some modifications. In summary, this cell line was cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C under microaerophilic conditions (5% CO_2) until reaching a confluence of 90-100%. Next, the cells were seeded in 24-well plates at a concentration of 1×10^5 cells/mL/well and incubated for 24 h. After this period, the pure DMEM medium was replaced by 500 µL of fresh DMEM containing the dual nanocarrier with CHX and FLZ concentrations ranging from 0-250 µg/mL and 0-1000 µg/mL, respectively, and the plates were incubated for 24 or 48 h. DMEM containing IONPs (0-700 µg/mL) and CS (0-700 µg/mL) alone, as well as the combination of CHX (0-250 µg/mL) with FLZ (0-1000) without nanocarrier were also placed in contact with the oral keratinocytes. Cells exposed to pure DMEM medium were considered as negative control (NC). Cell viability after each exposure period (24 or 48 h) was evaluated by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) reduction assay, following the protocol described by Mosmann [53]. Results were presented as a percentage of cell viability in relation to NC.

Statistical analysis

All microbiological and cytotoxic assays were conducted in triplicate on at least three different days. The data showed normal distribution by the Shapiro-Wilk test, except for the CS cytotoxicity assay. Biofilm results were evaluated by one-way Analysis of Variance (ANOVA) and Fisher LSD’s post hoc test, while cytotoxicity data were submitted to two-way ANOVA and Tukey's post hoc test. The CS cytotoxicity results were analyzed by the Kruskal-Wallis and Tukey tests. The statistical program used in data analysis was SigmaPlot (version 12.0; Systat Software Inc. San Jose, USA), with a significance level of 5%.

Results

Characterization of the dual nanocarrier

For IONPs alone, TEM analysis showed a cluster of nanoparticles formed by particles smaller than 20 nm (Fig. 1A). This cluster made it impossible to accurately characterize the IONPs shape. For IONPs-CS-CHX-FLZ, however, the particles were more dispersed and with a predominantly spherical shape, maintaining a diameter smaller than 20 nm (Fig. 1B).

IONPs, IONPs-CS, and IONPs-CS-CHX-FLZ were also analyzed by XRD, and the results are represented in Figure 1C. The six typical peaks of magnetite (Fe_3O_4) with spinel structure were observed at 30.1, 35.5, 43.1, 53.5, 57, and 62.6°, representing the indices (220), (311), (400), (422), (511), and (440), respectively (JCPDS 86-1352). The crystallographic planes symbolized by the diffractograms of the three compounds were similar, indicating that the functionalization of IONPS with CS, CHX, and FLZ did not change the crystalline structure of IONPs. According to the Debye-Scherrer equation, the estimated sizes of IONPs, IONPs-CS, and IONPs-CS-CHX-FLZ were 5.69, 6.15, and 6.07 nm, respectively.

Figure 1 D shows the FTIR spectra for IONPs, IONPs-CS, and IONPs-CS-CHX-FLZ. For IONPs, the absorption peaks at 582 cm^{-1} and 638 cm^{-1} indicate the binding of iron with oxygen (Fe-O; stretching vibration), confirming the formation of IONPs [54]. The coating of IONPs with CS was also confirmed since peaks at 1382 and 1636 cm^{-1} were detected in the IONPs-CS sample, which may correspond to C-O and C=O bonds (amide group of CS), respectively [55]. Furthermore, peaks at 1413 and 1636 cm^{-1} may also represent the stretching vibration of C-H present in the pyranoside ring of CS [35,55]. For IONPs-CS-CHX-FLZ, the representative bands of IONPs-CS were maintained, while the presence of CHX was confirmed by its characteristic peaks in the range from 1100 to 1600 cm^{-1}, which refer to the stretching vibrations C=N and C-N-C [56]. The peak around 1093 cm^{-1} indicates the binding of Cl to the aromatic functional group of

CHX [35]. In turn, the presence of FLZ in the nanocarrier sample may be attributed to the C-F bond represented by the peaks close to 1000 cm^{-1} [37].

Regarding the thermal behavior of the samples, IONPs lost approximately 42% of the mass in the temperature range from 25 to 800 °C (Fig. 1E), which may be linked to the decomposition of the polyethylene glycol present on the nanoparticle surfaces. On the other hand, when IONPs were coated with CS, the total mass loss of the IONPs-CS compound was higher (73%) compared to IONPs in their free form (Fig. 1E). From this result, it was possible to infer that CS effectively bound to IONPs, generating a compound with approximately 31% CS. The thermal curve of IONPs-CS-CHX-FLZ showed the highest mass loss among the tested compounds (~83%), confirming the effective conjugation of CHX and FLZ to the CS-coated IONPs (Fig. 1E). For the dual nanocarrier sample, mass losses between 100-250 °C and above 250 °C reflected the evaporation of adsorbed water molecules and decomposition of CS, CHX, and FLZ, respectively (Fig.1E).

MIC determination

When IONPs, CS, CHX, and FLZ were tested in combination, forming the dual nanocarrier, the MIC values were reduced compared to those obtained for each compound alone, for both *Candida* species tested (Table 1). The compounds that form the nanocarrier established a synergistic interaction in relation to the effect on *Candida* planktonic cells (Table 1).

Microcosm biofilm quantification assays

For the CFU quantification assay, the IONPs-CS-CHX156-FLZ624 nanocarrier showed the best antibiofilm effect against the evaluated microorganisms, significantly differing from the other compounds (Fig. 2). When compared to NC, IONPs-CS-CHX156-FLZ624 led to reductions in the number of CFUs of 4.81-, 3.38-, 4.79-, 1.9-, 4.54-, and 4.40-log_{10}, respectively for total anaerobes, total aerobes, *Streptococcus mutans*, *Lactobacillus* spp., *C. albicans*, and *C. glabrata* ($p < 0.001$;

Fig. 2). For total anaerobes, total aerobes, *C. albicans*, and *C. glabrata*, treatments with CHX156-FLZ624, IONPs-CS-CHX39-FLZ156, and IONPs-CS-CHX78-FLZ312 did not differ from each other and led to significant reductions in CFUs ranging from 1.25- to 2.94-$\log_{10}$ (Fig. 2A), 2.02- to 2.34-$\log_{10}$ (Fig. 2B), 1.87- to 2.12-$\log_{10}$ (Fig. 2E), and 1.68- to 1.87-$\log_{10}$ (Fig. 2F), respectively, compared to NC. Regarding the cultivable cells of *Streptococcus mutans* and *Lactobacillus* spp., CHX156-FLZ624 and IONPs-CS-CHX78-FLZ312 behaved similarly and promoted significant reductions ranging from 2.75- to 2.99-$\log_{10}$ (Fig. 2C) and 0.46- to 0.98-$\log_{10}$ (Fig. 2D), respectively, compared to NC.

For the quantification of extracellular matrix proteins, all compounds were statistically similar to NC, except CHX156-FLZ624, which led to a significant 3.6-fold increase (Table 2). For carbohydrate content, however, IONPs, CS, and the dual nanocarrier led to significant reductions compared to NC (Table 2). The greatest reductions in carbohydrate levels compared to NC were obtained after treatment with IONPs-CS-CHX39-FLZ156 (70.7%; $p < 0.001$), IONPs-CS-CHX78-FLZ312 (78.4%; $p < 0.001$), and IONPs-CS-CHX156-FLZ624 (77.4%; $p < 0.001$). On the other hand, CHX156-FLZ624 promoted an approximately 2-fold increase in carbohydrate content compared to NC ($p < 0.001$; Table 2). For the extracellular matrix nucleic acid content, treatments with CS, IONPs-CS-CHX78-FLZ312, and IONPs-CS-CHX156-FLZ624 promoted reductions of 28.1% ($p = 0.032$), 37.8% ($p = 0.005$), and 60.7% ($p < 0.001$), respectively, compared to NC (Table 2).

All compounds tested also significantly reduced lactic acid production compared to NC, with reduction percentages ranging from 78.2 to 89.4% ($p < 0.001$; Fig. 3).

Structural and viability analyses of the biofilm by CLSM

The CLSM images showed biofilms with a structural arrangement formed by clusters of microbial cells (bacteria and fungi) partially covering the surfaces of the glass discs, regardless of the analyzed group (Fig. 4). It was also possible to observe greater intensity of red fluorescence,

which is indicative of dead cells, in the resulting biofilms after treatment with CHX156-FLZ624 (Fig. 4D), IONPs-CS-CHX39-FLZ156 (Fig. 4E), IONPs-CS-CHX78-FLZ312 (Fig. 4F), and IONPs-CS-CHX156-FLZ624 (Fig. 4G). In turn, the quantitative analysis showed that treatments with CS, CHX156-FLZ624, IONPs-CS-CHX39-FLZ156, and IONPs-CS-CHX78-FLZ312 were similar to each other and led to increases in the percentage of dead cells ranging from 34.6 to 45.1%, compared to NC ($p < 0.001$; Fig. 4H). The highest percentage of dead cells (76.3%) was observed after treatment of the biofilm with IONPs-CS-CHX156-FLZ624, significantly differing from all other treatments (Fig. 4H).

Cell cytotoxicity assay

For IONPs, only the concentration of 700 µg/mL was cytotoxic, reducing cell viability by 60.8 and 50.6% ($p < 0.001$) compared to NC, respectively for 24 and 48 h of exposure (Fig. 5A). For CS, however, concentrations greater than 87.5 µg/mL drastically reduced (~ 90%; $p < 0.001$) cell viability compared to NC, regardless of the exposure period evaluated (Fig. 5B). Comparing 24 and 48 h, CS at 87.5 and 350 µg/mL was significantly less toxic for the shortest exposure period (Fig. 5B). The combination of CHX/FLZ at concentrations ranging from 15.6/62.5 to 250/1000 µg/mL significantly reduced the viability of oral keratinocytes compared to NC, for all exposure periods evaluated. These reductions ranged from 38.7 to 94.6%, with a dose-dependent cytotoxic effect (Fig. 5C). Specifically, for the concentration range from 7.8/31.25 to 250/1000 µg/mL, the CHX/FLZ compound was more cytotoxic when in contact with keratinocytes for 48 h compared to the 24 h exposure period (Fig. 5C). For the dual nanocarrier, significant reductions in cell viability compared to NC were also observed for CHX/FLZ concentrations in the range of 15.6/62.5 to 250/1000 µg/mL, for both exposure periods (Fig. 5D). However, the cytotoxic effect of the nanocarrier was more abrupt compared to that found for CHX/FLZ without nanocarrier, considering that the reduction in cell viability reached values above 77% for the concentration of 15.6/62.5 µg/mL, for both exposure periods (Fig. 5D). Comparing the exposure periods, the

nanocarrier at 7.8/31.25 µg/mL was significantly more cytotoxic after 48 h of exposure. Significant but less marked differences were also observed for concentrations of 15.6/62.5 and 125/500 µg/mL, with greater cytotoxicity after 48 h of exposure (Fig. 5D).

Discussion

The physico-chemical characterization tests employed in the current study showed that a dual nanocarrier with dimensions around 6 nm was actually assembled and that the coating of IONPs with CS and the binding of CHX and FLZ to the core-shell nanostructure did not compromise the crystalline property and stability of the core (IONPs). In fact, it was possible to observe by TEM that the nanoparticles were more dispersed in the nanocarrier sample compared to those present in the pure colloidal suspension of IONPs (Fig. 1A and B). For the successful assembly of the dual nanocarrier, it is important that the compounds establish interactions between atoms or electrostatics. In this sense, the multiple positively charged amine groups on the surface of the CS are the true connecting links responsible for nanocarrier formation. These groups interact with the oxygen from IONPs [36] and are also attracted by negative groups from nanoparticles [57]. In addition, CS has a high capacity to chelate metal ions [58]. The presence of CS coating the IONPs then prevents the aggregation of nanoparticles, considering that this functionalization promotes electrostatic repulsion between nanoparticles coated by positive charge [28]. The amine group from CS also establishes a chemical bond with the nitrogen of the imine group from CHX [36] and with the -OH or -F groups present in FLZ [37,40], thus forming the nanocarrier. Consequently, CS stabilizes IONPs and still serves as a scaffold for CHX and FLZ anchorage, protecting medicines from degradation.

Regarding the antimicrobial effect on *Candida* planktonic cells, the MIC values for IONPs, CS, CHX, and FLZ forming the dual nanocarrier were lower than those found for their counterparts in the free form (without nanocarrier), resulting in a synergistic antifungal effect (Table 1). A similar trend was noted in a previous study comparing the MIC values of FLZ alone or carried by

CS-coated IONPs [37]. However, when this same core-shell structure carried only CHX, an indifferent antimicrobial effect in comparison to the medicine alone was observed on *C. albicans*, *S. mutans*, *C. glabrata*, and *E. faecalis* in the planktonic state [35,36]. Given these findings, it seems reasonable to assume that the presence of FLZ is essential for the synergistic effect of the dual nanocarrier tested in the present study, potentiating the antimicrobial effect of the other compounds. Thus, this synergistic antifungal effect may be a reflection of the sum of the antimicrobial action mechanisms of IONPs, CS, CHX, and FLZ, which may act on different targets. IONPs inhibit the expression of the ERG11 gene involved in the biosynthesis of ergosterol in the *Candida* plasmatic membrane [59] and generate intracellular reactive oxygen species (ROS) that cause DNA and protein damage in bacterial and fungal cells [60,61]. The antimicrobial effect of CS, however, is related to an interaction caused by electrostatic adsorption between its positive charge and the negative charge of microbial cells, which favors the entry of CS inside the cells, preventing DNA transcription [62]. The positive charge of CHX is also attracted by the negative microbial charge, leading to the formation of pores in the cell membrane followed by cytoplasmic coagulation and escape of this content to the extracellular environment [24,63]. On the other hand, FLZ blocks the ergosterol biosynthetic pathway, inhibiting the enzyme CYP45O lanosterol 14-α-demethylase and preventing the conversion of lanosterol to ergosterol in the fungal cell membrane [17,64].

After the planktonic cell susceptibility assay, the dual nanocarrier at different concentrations was evaluated on polymicrobial biofilms simulating a microcosm of oral candidiasis, following the same biofilm formation protocol proposed by Caldeirão et al. [38]. For CFU quantification of all microorganisms tested and for cell viability analysis by confocal microscopy, the nanocarrier IONPs-CS-CHX156-FLZ624 overcame the reducing effects promoted by the combination of CHX with FLZ (without nanocarrier) and significantly differed from all groups, resulting in a synergistic antibiofilm effect (Figs. 2 and 4). These effects are also related to the sum of the different mechanisms of antimicrobial action of the compounds present in the

nanocarrier, as previously discussed for MIC results. It is also believed that the core-shell nanostructure (IONPs-CS) has diffused through the different layers of the microcosm biofilms, facilitating the arrival of medicines (CHX and FLZ) in the deeper portions. This hypothesis is based on previous evidence reporting the visualization of Fe atoms by energy-dispersive X-ray spectroscopy in the basal layers of biofilms exposed to nanocarriers formed by CS-coated IONPs [35,37]. Probably, the transport of CHX with FLZ through the layers of the microcosm biofilm was also facilitated by the electrostatic attraction between CS and microbial cell membranes. Additionally, CS may increase the action time of medicines administered topically in the oral cavity as it is a mucoadhesive compound [65].

For all microorganisms quantified from the microcosm biofilm, the results also showed that it is possible to halve the concentrations of CHX and FLZ in the nanocarrier (IONPs-CS-CHX78-FLZ312) and obtain the same antibiofilm effect generated by CHX156+FLZ624 (Figs. 2 and 4). These findings are relevant from a cytotoxic point of view, as they may directly reflect the reduction of side effects associated with the administration of CHX and FLZ. In this way, it is evident that nanocarriers have advantages compared to the administration of medicines in their free form, including a modification in pharmacokinetics, protection against rapid deterioration after administration, and improved medicine efficiency in the target area.

Comparing the findings reported here with others previously published, discrepant results are noted. Thus, when FLZ was individually carried by IONPs-CS, the effects of the mono nanocarrier on single *Candida* biofilms did not differ from those found for the antifungal applied alone [37]. The same trend was found for a mono nanocarrier of CHX applied to *S. mutans* biofilms [35]. For *C. albicans*, however, IONPs-CS-CHX outperformed the antibiofilm effects of CHX alone, depending on the parameter analyzed [35]. Therefore, the construction of a dual nanocarrier containing CHX and FLZ is fundamental to improve the effects on microorganisms within polymicrobial and inter-kingdom consortia by involving medicines that act on different targets.

The results of CFUs and cell viability by confocal microscopy are also associated with the type of interaction established between bacteria and fungi within inter-kingdom biofilms. In this sense, *C. albicans* hyphae are essential to increase and maintain biofilm architecture's complexity and serve as attachment points for other yeasts, hyphae, and bacteria [8]. Consequently, the complete eradication of *C. albicans* seen in the present study after biofilm treatment with IONPs-CS-CHX156-FLZ624 may have contributed to the reduction in CFU numbers of total anaerobes and aerobes, *S. mutans*, *Lactobacillus* spp., and *C. glabrata*, considering that these species lost their Anchorage [66].

Another interesting finding of the present study was that the dual nanocarrier and the combination of CHX with FLZ in the free form significantly reduced lactic acid production compared to CN (Fig. 3). This result may be attributed mainly to the cell death of *Lactobacillus* spp. and *S. mutans* after exposure to these compounds, as these microorganisms are closely associated with acid production in oral biofilms. Although *Candida* species are not protagonists in the metabolism of sucrose and other sugars into lactic acid, when present in inter-kingdom biofilms with acidogenic microorganisms, they do not prevent the production of this type of acid by bacteria [67]. Thus, reductions in acid production have major clinical implications, as they could minimize both the emergence of carious lesions and the activation of acidic proteolytic exoenzymes produced by *Candida* species, which damage mucosal tissues in cases of oral candidiasis. It should also be noted that, although IONPs and CS alone had a not very expressive reducing effect on the viability and number of microcosm biofilm cells, these compounds significantly reduced lactic acid production, behaving similarly to the other compounds. The mechanisms that led to these effects remain unclear and should be explored in the future.

The dual nanocarrier tested in the present study also showed the potential to affect the extracellular matrix of the microcosm biofilm, significantly reducing the concentrations of carbohydrates and nucleic acids (Table 2). These effects contribute to disorganizing the biofilm architecture, since carbohydrates represent one of the main constituents of the extracellular matrix,

while nucleic acids play an important role in the structural and protective properties of the matrix [8]. Consequently, a less compact polymicrobial community and less resistance to future antimicrobial challenges may have been obtained after exposure to the dual nanocarrier. The results of extracellular matrix composition are also related to those found for CFU quantification and cell viability by confocal microscopy, considering that the matrix degradation may have enabled the penetration of the nanostructure into the various layers of the biofilm, increasing CHX and FLZ antimicrobial efficacies.

Regarding cytotoxicity, the viability of oral keratinocytes was dependent on the concentration of the dual nanocarrier, regardless of the exposure period tested (24 or 48 h; Fig. 5). Furthermore, in general, IONPs-CS-CHX-FLZ and CHX-FLZ significantly reduced keratinocyte viability at CHX and FLZ concentrations equal to or greater than 7.8 and 31.25 μg/mL, respectively. Taking into account that IONPs and CS reduced cell viability at concentrations equal to or greater than 700 and 87.5 μg/mL, respectively, it is plausible to assume that CHX and FLZ are primarily responsible for the cytotoxic effect of the dual nanocarrier, with less interference from the core-shell carrier structure. Similar results were previously reported when murine fibroblasts were exposed to mono nanocarriers of CHZ, FLZ, miconazole, and cetylpyridinium chloride [36,39,68]. The cytotoxic mechanisms of action of CHX and FLZ are varied and cell type dependent. For example, CHX may induce DNA damage in oral mucosal cells [69], lead to apoptotic and autophagic/necrotic death in osteoblasts [70], and cause an increase in oxidative stress and necrosis in human gingival fibroblasts [71]. In turn, FLZ may lead to DNA damage and mutagenicity in blood cells, in addition to increasing levels of TNF-α and reducing those of IL-6 and IL-10 [72].

Analyzing the cytotoxicity results together with the microbiological findings, it is evident that the MIC values of the dual nanocarrier were not cytotoxic to oral keratinocytes. Otherwise, a contrary finding was observed for nanocarrier concentrations that were effective against microcosm biofilms. Given this scenario, the use of IONPs-CS carrying lower concentrations of CHX and FLZ

would represent a safer alternative for clinical use and with great potential to prevent the emergence of oral candidiasis, considering that the nanostructure could hinder biofilm formation due to the reduction of *Candida* planktonic cells. However, in clinical situations where oral candidiasis is already installed and associated with biofilm formation, the use of dual nanocarriers at higher concentrations would be necessary. Thus, further investigations exploring the effects of the nanocarrier on more complex models of oral epithelium, on the composition of the microcosm biofilm by next-generation sequencing, and the analysis of the CHX and FLZ release mode from the nanocarrier will provide guiding answers on the best administration form of dual nanocarrier-containing formulations.

Conclusions

CHX and FLZ were conjugated to CS-coated IONPs, forming a dual nanocarrier with dimensions around 6 nm. This nanocarrier showed a synergistic antimicrobial effect on planktonic cells of *C. albicans* and *C. glabrata*, as well as on cultivable cells of polymicrobial biofilms simulating a microcosm of oral candidiasis. The dual nanocarrier also reduced lactic acid production by biofilm cells and levels of carbohydrates and nucleic acids in the extracellular matrix. For some of the parameters examined, the antibiofilm effects of the dual nanocarrier exceeded those promoted by the combination of CHX with FLZ in the free form. Furthermore, a dose-dependent cytotoxic effect against oral keratinocytes was observed for the dual nanocarrier. Finally, the nanotherapy tested in the current study is promising and constitutes a major advance in alternative methods to traditional antimicrobials for oral candidiasis control.

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Table 1. Susceptibility of *Candida albicans* and *Candida glabrata* planktonic cells to iron oxide nanoparticles (IONPs), chitosan (CS), chlorhexidine (CHX), and fluconazole (FLZ) in their free forms or as a dual nanocarrier. Susceptibility was determined by the minimal inhibitory concentration (MIC) values.

Species	MIC (µg/mL)								FICI	Classification
	Alone				Forming the nanocarrier					
	IONPs	CS	CHX	FLZ	IONPs	CS	CHX	FLZ		
Candida albicans	> 500	> 140	3.12	> 12.5	0.27-0.54	0.27-0.54	0.19- 0.39	0.78- 1.56	< 0.25	Synergism
Candida glabrata	> 500	> 140	3.12	> 50	0.54-1.09	0.54-1.09	0.39- 0.78	1.56- 3.12	< 0.32	Synergism

Note: FICI, fractional inhibitory concentration index.

Table 2. Mean concentrations (standard deviation) of proteins, carbohydrates, and nucleic acids from the extracellular matrix of microcosm biofilms after exposure for 24 h to different compounds. Biofilms were exposed to 218.5 µg/mL iron oxide nanoparticles (IONPs), 218.5 µg/mL chitosan (CS), chlorhexidine (CHX) at 156 µg/mL + fluconazole (FLZ) at 624 µg/mL (CHX156-FLZ624), and IONPs-CS carrying 39 µg/mL CHX + 156 µg/mL FLZ (IONPs-CS-CHX39-FLZ156), 78 µg/mL CHX + 312 µg/mL FLZ (IONPs-CS-CHX78-FLZ312), and 156 µg/mL CHX + 624 µg/mL FLZ (IONPs-CS-CHX156-FLZ624). The exposure of biofilms for 24 h to pure McBain medium corresponds to the negative control (NC).

	Compounds						
Matrix components (mg/g of biofilm dry weight)	**Negative control**	**Iron oxide nanoparticles (IONPs)**	**Chitosan (CS)**	**Chlorehxidine-Fluconazole (CHX156-FLZ624)**	**IONPs-CS-CHX39-FLZ156**	**IONPs-CS-CHX78-FLZ312**	**IONPs-CS-CHX156-FLZ624**
Proteins	3.41 (1.31)^{ab}	6.50 (0.58)^{a}	5.14 (3.55)^{ab}	12.38 (2.64)^{c}	1.59 (0.30)^{b}	2.45 (0.33)^{b}	1.34 (1.10)^{b}
Carbohydrates	256.70 (39.62)^{a}	183.26 (6.32)^{b}	111.22 (72.31)^{c}	519.0 (29.90)^{d}	75.18 (18.57)^{c}	55.43 (29.62)^{c}	57.94 (5.85)^{c}
Nucleic acids	8.28 (1.02)^{a}	9.94 (1.62)^{a}	5.95 (0.94)^{b}	8.44 (0.96)^{a}	8.10 (1.83)^{a}	5.15 (0.93)^{bc}	3.25 (0.61)^{c}

Note: for proteins, carbohydrates, and nucleic acids, separately, different lowercase letters symbolize significant differences among compounds (one-way ANOVA and Fisher LSD's post hoc test; $p < 0.05$).

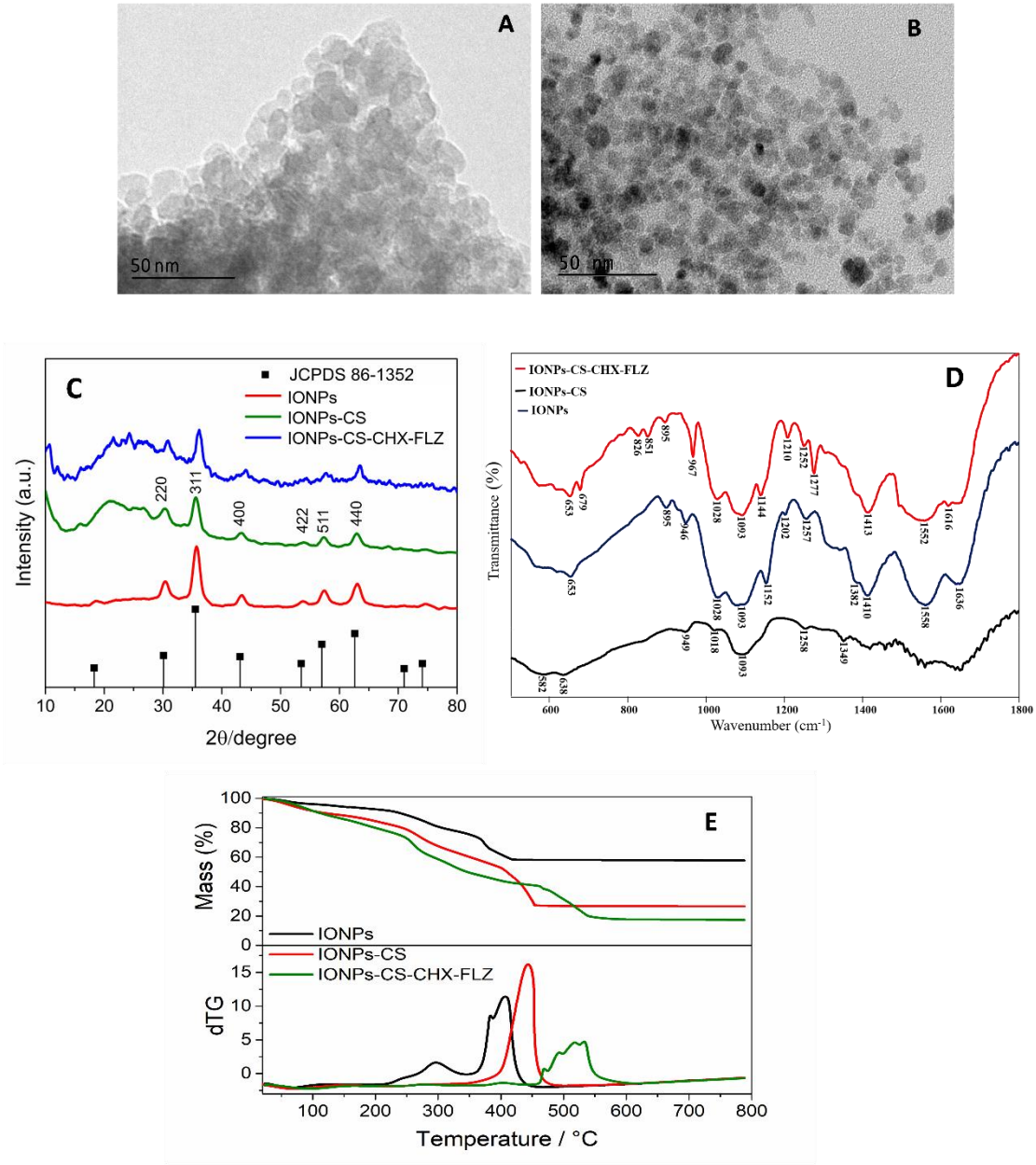

Figure 1. Transmission electron microscopy of iron oxide nanoparticles (IONPs; image A) and chitosan (CS)-coated IONPs carrying chlorhexidine (CHX) and fluconazole (FLZ), forming the dual nanocarrier (image B). X-ray diffraction patterns (image C), Fourier transform infrared spectroscopy (image D), and thermogravimetric curves (image E) for IONPs, IONPs-CS, and IONPs-CS-CHX-FLZ.

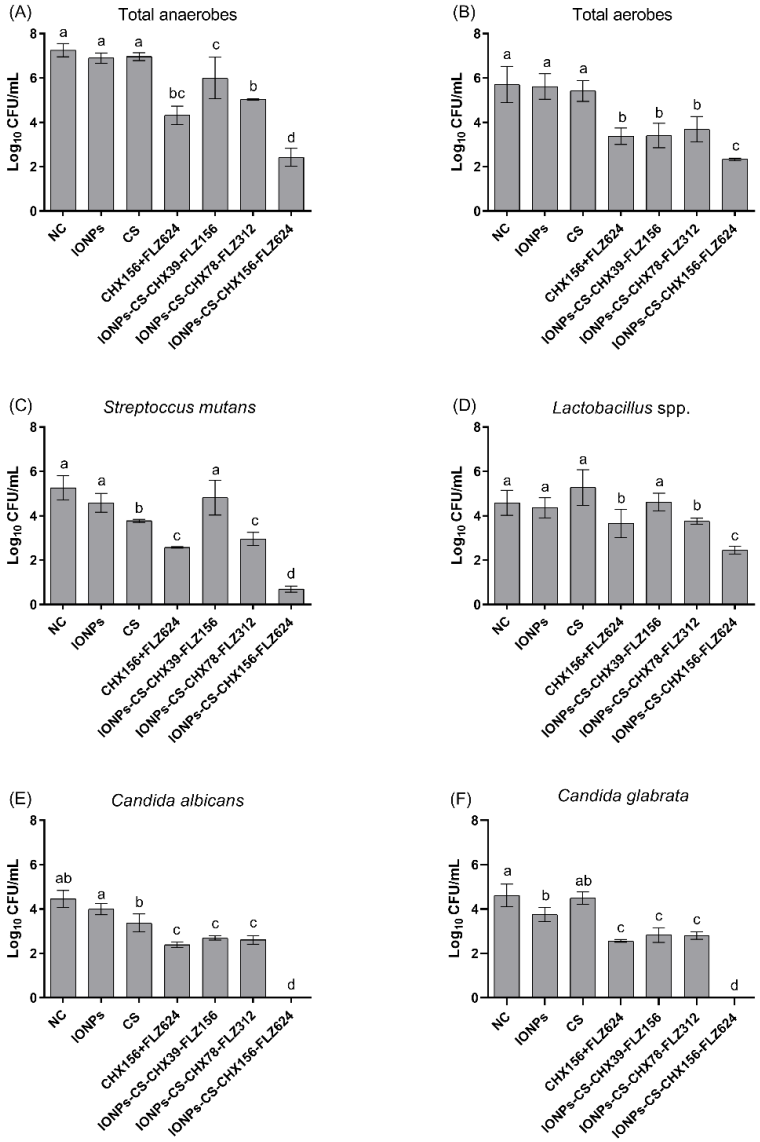

Figure 2. Effects of dual nanocarriers and controls on the quantification of colony-forming units (Log_{10} CFU/mL) of total anaerobes (A), total aerobes (B), *Streptococcus mutans* (C), *Lactobacillus* spp. (D), *Candida albicans* (E), and *Candida glabrata* (F) from salivary microcosm biofilms. Biofilms were developed during 72 h and treated for 24 h with the following compounds: 218.5 µg/mL iron oxide nanoparticles (IONPs), 218.5 µg/mL chitosan (CS), chlorhexidine (CHX) at 156 µg/mL combined with fluconazole (FLZ) at 624 µg/mL (CHX156-FLZ624), and IONPs-CS carrying 39 µg/mL CHX + 156 µg/mL FLZ (IONPs-CS-CHX39-FLZ156), 78 µg/mL CHX + 312 µg/mL FLZ (IONPs-CS-CHX78-FLZ312), and 156 µg/mL CHX + 624 µg/mL FLZ (IONPs-CS-CHX156-FLZ624). Biofilm exposure for 24 h to pure McBain medium corresponds to the negative control (NC). The error bars depict the standard deviations of the means. For each microorganism evaluated, different lowercase letters show significant differences among compounds (one-way ANOVA and Fisher LSD's test; $p < 0.05$).

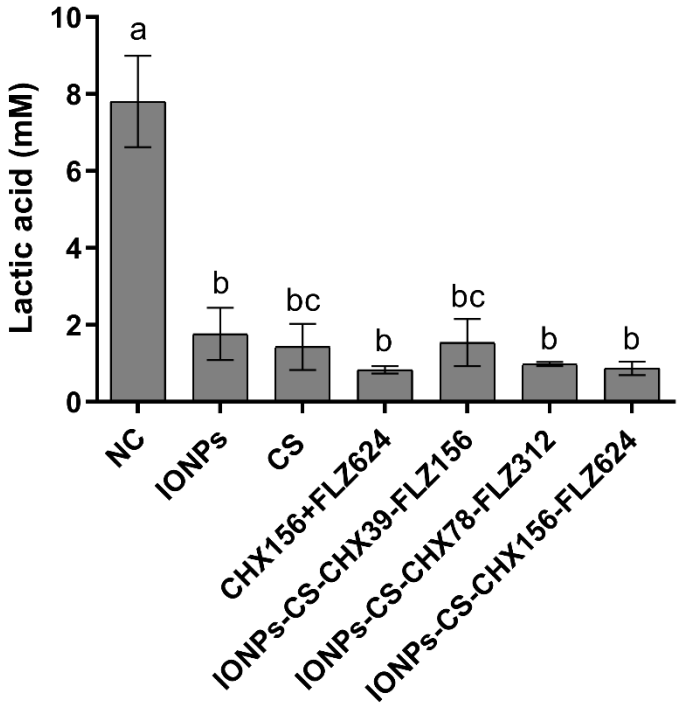

Figure 3. Mean concentrations of lactic acid (mM) produced by microcosm biofilms after treatment with different compounds. Biofilms were developed during 72 h and treated for 24 h with the following compounds: 218.5 µg/mL iron oxide nanoparticles (IONPs), 218.5 µg/mL chitosan (CS), chlorhexidine (CHX) at 156 µg/mL combined with fluconazole (FLZ) at 624 µg/mL (CHX156-FLZ624), and IONPs-CS carrying 39 µg/mL CHX + 156 µg/mL FLZ (IONPs-CS-CHX39-FLZ156), 78 µg/mL CHX + 312 µg/mL FLZ (IONPs-CS-CHX78-FLZ312), and 156 µg/mL CHX + 624 µg/mL FLZ (IONPs-CS-CHX156-FLZ624). Biofilm exposure for 24 h to pure McBain medium corresponds to the negative control (NC). The error bars depict the standard deviations of the means. Different lowercase letters show significant differences among compounds (one-way ANOVA and Fisher LSD's test; $p < 0.05$).

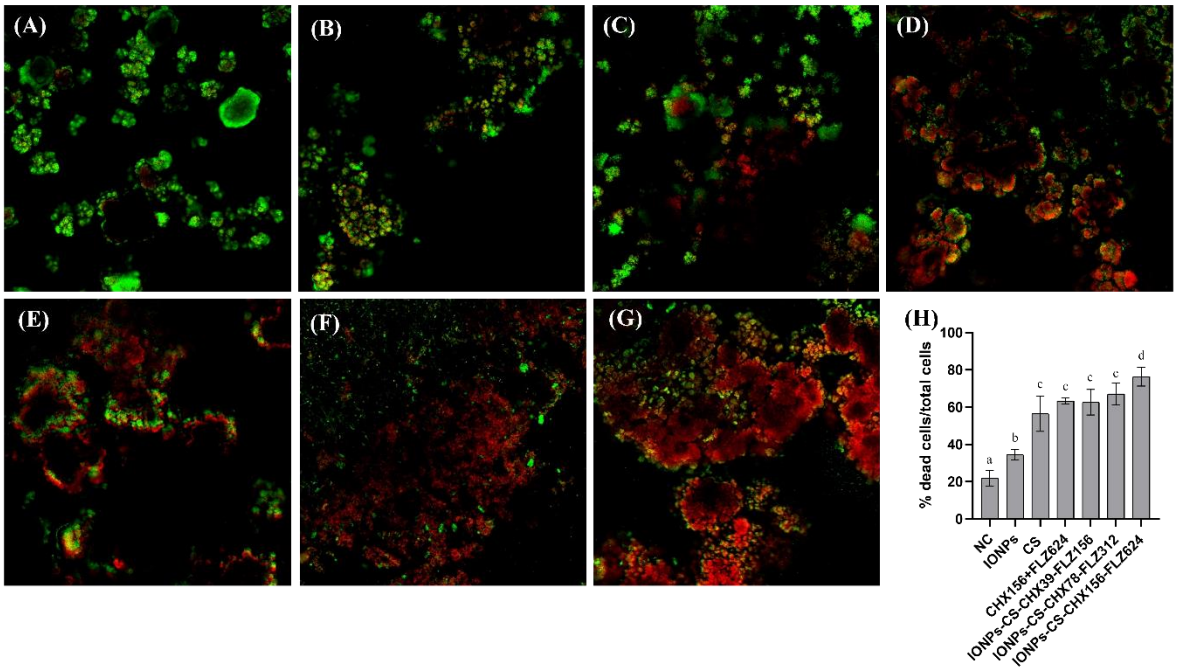

Figure 4. Structural and viability analyses of the microcosm biofilm by confocal laser scanning microscopy. Biofilms were developed during 72 h and treated for 24 h with the following compounds: 218.5 µg/mL iron oxide nanoparticles (IONPs; image B), 218.5 µg/mL chitosan (CS; image C), chlorhexidine (CHX) at 156 µg/mL combined with fluconazole (FLZ) at 624 µg/mL (CHX156-FLZ624; image D), and IONPs-CS carrying 39 µg/mL CHX + 156 µg/mL FLZ (IONPs-CS-CHX39-FLZ156; image E), 78 µg/mL CHX + 312 µg/mL FLZ (IONPs-CS-CHX78-FLZ312; image F), and 156 µg/mL CHX + 624 µg/mL FLZ (IONPs-CS-CHX156-FLZ624; image G). Biofilm exposure for 24 h to pure McBain medium corresponds to the negative control (NC; image A). Live and dead cells emit green and red fluorescences, respectively. Magnification: 20×. The quantitative analysis of cell viability (% of dead cells/total cells) is represented in image H, and different lowercase letters show significant differences among compounds (one-way ANOVA and Fisher LSD's test; $p < 0.05$).

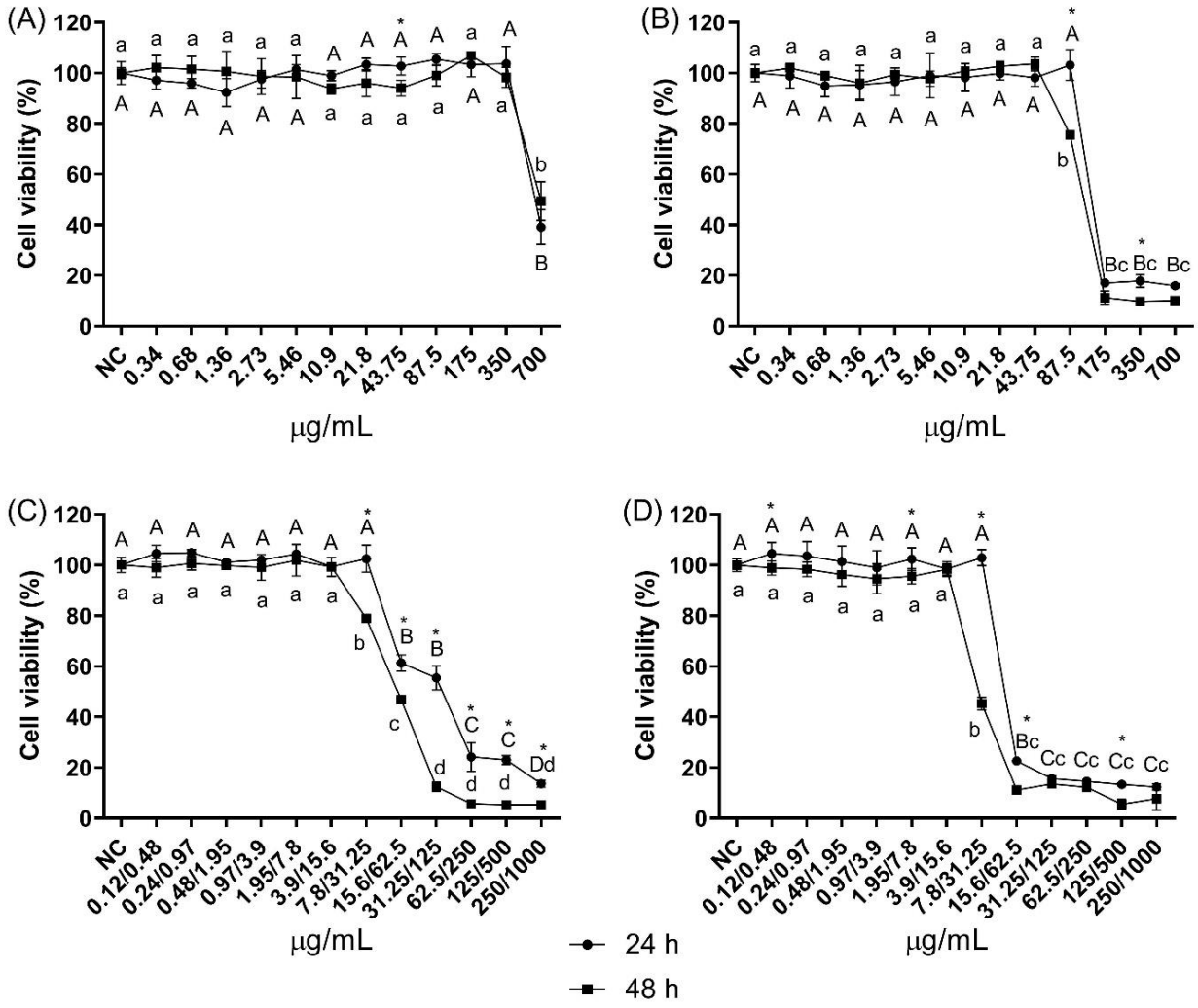

Figure 5. Percentages of viable human oral keratinocytes (NOKsi lineage) determined by the MTT reduction assay after 24 or 48 h of exposure to the following compounds: iron oxide nanoparticles (IONPs; image A), chitosan (CS; image B), chlorhexidine (CHX; 0.12-250 μg/mL) combined with fluconazole (FLZ; 0.48-1000 μg/mL; image C), and CS-coated IONPs carrying CHX/FLZ (0.12/0.48 to 250/1000 μg/mL; image D). Keratinocytes exposed to pure DMEM medium were considered as negative control (NC). Different uppercase and lowercase letters show significant differences among the concentrations of the compounds, respectively for 24 and 48 h of exposure. Asterisks (*) depict significant differences between 24 and 48 h of exposure, for each concentration of the compounds (two-way ANOVA or Kruskal-Wallis test followed by Tukey's test; $p < 0.05$).

Anexos

ANEXO A

*Normas da revista na qual será submetido o artigo desta tese (Colloids and Surfaces B: Biointerfaces).

Author Guidelines (Colloids and Surfaces B: Biointerfaces)

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Introduction

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ANEXO B

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeitos antibiofilme e citotóxico de um novo nanosistema carreador de clorexidina e fluconazol

Pesquisador: Heitor Ceolin Araujo

Área Temática:

Versão: 2

CAAE: 10330919.4.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: UNIVERSIDADE ESTADUAL PAULISTA JULIO DE MESQUITA FILHO

DADOS DO PARECER

Número do Parecer: 3.326.480

Apresentação do Projeto:

O objetivo deste projeto é sintetizar um novo nanocarreador dual de clorexidina (CLX) e fluconazol (FLZ), com base em nanopartículas magnéticas de óxido de ferro (NPMs) e quitosana (QTS), e avaliar seus efeitos antibiofilme e citotóxico. O nanocarreador será preparado através do carregamento de CLX e FLZ sobre NPMs revestidas com QTS, e caracterizados por difratometria de raios-X, espectroscopia na região do infravermelho, análise termogravimétrica e microscopia eletrônica de transmissão. Saliva proveniente de um doador sadio será usada como inóculo para a formação de biofilmes microcosmos, os quais serão formados em discos de vidro no modelo de Adesão Ativa de Amsterdam. Os biofilmes serão formados por 24, 48, 72 e 96 horas. Após cada período de formação de biofilmes, os mesmos serão tratados durante 24 horas com diferentes concentrações do nanocarreador CLX-FLZ-QTS-NPM. O efeito antibiofilme será avaliado por meio da contagem do número de células cultiváveis, composição da matriz extracelular (proteínas, carboidratos e ácidos nucleicos), produção de ácido e análise da estrutura dos biofilmes. O efeito citotóxico do nanocarreador frente a células de fibroblastos de rato será determinado através do ensaio de MTT. Controles apropriados serão

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incluídos em todas as análises. Os dados serão submetidos ao teste de normalidade e, a seguir, será aplicada análise estatística adequada para
cada resultado, com nível de significância de 5%.

Objetivo da Pesquisa:

Objetivo Primário:

Portanto, o objetivo deste projeto de pesquisa é sintetizar um novo nanocarreador dual de CLX e FLZ, com base em NPMs de óxido de ferro e QTS,
avaliar seu efeito sobre biofilmes microcosmos e sua citotoxicidade frente a células de fibroblastos de rato.

Avaliação dos Riscos e Benefícios:

Riscos:

A coleta de saliva será feita por um método indolor, que causa risco mínimo, utilizando tubos de polipropileno, pelo método da expectoração. Os
riscos são mínimos, sendo os mesmos que ocorrem em exame de rotina da boca.

Benefícios:

O benefício será para o desenvolvimento de um nanosistema que poderá auxiliar no tratamento de infecções virais e fúngicas

Comentários e Considerações sobre a Pesquisa:

Pesquisa apresenta-se apta para a sua realização.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram adicionados de acordo com a resolução 466/12 do Conselho Nacional de saúde CNS.

Recomendações:

não há

Conclusões ou Pendências e Lista de Inadequações:

Projeto aprovado para seu início.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/11/2019.

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Continuação do Parecer: 3.326.480

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1319906.pdf	18/04/2019 15:27:29		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Doutorado_CEP.pdf	18/04/2019 15:27:12	Heitor Ceolin Araujo	Aceito
Folha de Rosto	Folha_de_rosto.pdf	27/03/2019 09:38:12	Heitor Ceolin Araujo	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	25/03/2019 16:01:54	Heitor Ceolin Araujo	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARACATUBA, 15 de Maio de 2019

Assinado por:
Aldiéris Alves Pesqueira
(Coordenador(a))

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