An Acad Bras Cienc (2022) 94(2): e20211021 DOI 10.1590/0001-3765202220211021
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Synergistic combination of duloxetine hydrochloride and fluconazole reduces the cell growth and capsule size of *Cryptococcus neoformans*

RAQUEL T. MENEZES, THAÍS C. PEREIRA, JULIANA C. JUNQUEIRA, LUCIANE D. OLIVEIRA & LILIANA SCORZONI

Abstract: This study aimed to evaluate the effect of duloxetine hydrochloride (DH) on *Cryptococcus neoformans*. DH minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were 18.5 µg/mL, and the combination with fluconazole (FLZ) reduced the MIC value by 16-and 4-fold for DH and FLZ, respectively. The capsule size decreased by 67% and 16% when treated with DH and DH with FLZ, respectively. Therefore, this study showed that DH is active against *C. neoformans* alone and in combination with FLZ, leading to the reduction of the capsule size of this yeast.

Key words: *Cryptococcus neoformans*, capsule, fluconazole, duloxetine hydrochloride, synergistic combination.

INTRODUCTION

Cryptococcus neoformans and *Cryptococcus gattii* are the mains etiologic agents of cryptococcosis, an infection that occurs by inhalation of yeasts or spores than causes pulmonary infection and reaches the central nervous system (Squizani et al. 2018, Dubot-Peres et al. 2019). *C. neoformans* and *C. gattii* have a polysaccharide capsule, composed mainly by glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), this structure has multiple functions as provide resistance against the host immunological defenses and to antifungal agents (Casadevall et al. 2019, Zaragoza 2019, Nichols 2021).

The recommended therapeutic regimen for cryptococcosis is performed with a combination of amphotericin B (AMB) and 5-fluorocytosine (5-FC) followed by maintenance treatment with FLZ (Perfect et al. 2010). However, toxicity, antifungal resistance development, and cost are limitations of the available therapy (Bosco-Borgeat et al. 2016, Elsegeiny et al. 2018, Spadari et al. 2020).

Faced with these challenges, drug repositioning has emerged as a viable approach, as it allows the reuse of molecules for economic and rapid identification of new pharmacological effects. Numerous studies have addressed the repositioning of drugs to identify compounds that can act synergistically with traditional antifungals, thus generating a potent therapeutic alternative against pathogenic fungi (Rossato et al. 2016, Oliveira et al. 2018). Antidepressants from the selective serotonin reuptake inhibitors (SSRIs) class have demonstrated antifungal activity and synergy with antifungal agents (Lass-Flörl et al. 2001, Serafin et al. 2019, Rosa et al. 2020, Pereira et al. 2021). Belonging to serotonin and norepinephrine reuptake inhibitor (SNRIs) class, duloxetine hydrochloride (DH) is also an antidepressant which, to our knowledge,

the antifungal and synergistic activity were not previously investigated. Because of the promising antifungal activity demonstrated by antidepressants, could be an interesting molecule to be investigated.

Therefore, because of the previously discussed problems related to antifungal therapy, the antifungal activity and synergy with fluconazole of the antidepressant DH, could be an option to be studied in the treatment of cryptococcosis.

MATERIALS AND METHODS

Microorganisms and drugs

C. neoformans H99 (ATCC 208821) was used for the experiments. *Candida krusei* ATCC 6258 was used as a quality control strain for the broth microdilution assay. Duloxetine hydrochloride (DH) (Galena, Campinas, Brazil), a selective serotonin and norepinephrine reuptake inhibitor class, and the antifungal fluconazole (FLZ) (Sigma-Aldrich, St Louis, MO) were used to assess susceptibility and synergistic effects in *C. neoformans*.

Evaluation of antifungal activity and determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of DH

To determine the susceptibility of DH, the microdilution technique was performed according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2017). FLZ was used as a test control. DH was diluted in dimethyl sulfoxide (DMSO) and diluted in RPMI at a concentration of 1,000 µM. Serial dilutions of DH compound were performed in a 96-well plate in the concentrations range of 148.5 to 0.29 µg/ml. The concentration of the fungal suspension was 2.5×10^5 cells/mL. The MIC was considered the lowest concentration at which no turbidity of the culture medium was observed, indicating growth inhibition, with 90–100% inhibition. For the MFC assay, an aliquot from each of the 96 wells of the microdilution plate was removed with the aid of a sterile wooden toothpick and placed carefully on Sabouraud agar. After 48 h of incubation at 37°C, the MFC was determined as the lowest concentration at which yeast growth was not observed.

Evaluation of synergistic activity between DH and FLZ

The synergistic activity of FLZ and DH was assessed using the "chessboard" technique. This technique is based on the broth microdilution test recommended by EUCAST (EUCAST 2017). The concentration of the fungal suspension was 2.5×10^5 cells/mL. To calculate synergistic activity, the fractional inhibitory concentration index (FICI) was calculated using the equation: ΣFIC = FICA + FICB, where the FIC is the ratio of the MIC of the drug in combination with the MIC alone (Odds 2003). A combination was considered synergistic at an FICI ≤ 0.5, regardless of FICI > 1 and ≤ 4 and antagonist at FICI > 4.0.

Evaluation of the effect of DH in subinhibitory concentration and in synergistic combinations with FLZ on the size of the *C. neoformans* capsule

C. neoformans underwent capsule induction according to the protocol described by Zaragoza & Casadevall (2004). Subsequently, cells with induced capsules were treated with a subinhibitory concentration (Sub-MIC) related to isolated duloxetine hydrochloride (DH) treatment (9.28 µg/mL) and with a synergistic concentration (DH, 1.16 µg/mL; FLZ, 1 µg/mL). As a control, cells with untreated induced capsules and cells without capsule induction were used. The treatment was carried out for 24 h at 37°C, after which the cells were stained with Chinese

ink and analyzed using an optical microscope (Zeiss, Germany) with a 400-fold magnification. The capsule size was calculated using ImageJ software (Rueden et al. 2017) and for each experimental group fifty cell were measured. For the calculation, the cell body value was subtracted from the cell body value plus capsule, resulting in the value of the capsule size.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). The results obtained by the effect of the drug alone and the synergistic combinations on the size of the capsule were analyzed by one-way ANOVA (Tukey's test) and t-test (Mann-Whitney test) with a significance level of $p < 0.05$.

RESULTS

Assessment of the susceptibility of *C. neoformans* to DH and to synergistic combinations with FLZ

DH was evaluated against *C. neoformans*, and this compound was active with an MIC and MFC of 18.5 µg/mL. In addition, the synergistic combination was able to decrease the MIC and MFC values by 16 times for DH and 4 times for FLZ. The FICI values were considered promising, showing a synergistic effect (Table I).

Evaluation of the effect of the treatment of DH at subinhibitory concentration and in synergistic combinations in the size of the capsule of *C. neoformans*

DH, at a subinhibitory concentration (9.28 µg/mL), reduced the capsule size of *C. neoformans* by 67% ($p < 0.0001$) compared to the untreated control (Figure 1a). Regarding the synergistic concentration, the combination of 1.16 µg/mL DH and 1 µg/mL FLZ was responsible for reducing the *C. neoformans* capsule by 16% ($p = 0.0019$) in relation to the control (Figure 1b). In addition, the isolated treatment with DH (1.16 µg/mL) reduced the capsule size by 23% ($p < 0.0001$) compared to the control, and 1 µg/mL FLZ was responsible for reducing the capsule size by 3% ($p = 0.2931$). When comparing the isolated treatment of DH (1.16 µg/mL) with the combination of DH (1.16 µg/mL) and FLZ (1 µg/mL), there was a significant difference ($p = 0.0312$). However, the isolated FLZ (1 µg/mL) did not show a statistical difference in relation to the combination of DH (1.16 µg/mL) and FLZ (1 µg/mL) (Figure 1b). The capsule cell reduction is shown in Figure 2.

DISCUSSION AND CONCLUSIONS

In the present study, the antifungal activity of the antidepressant form SNRIs class, DH, was verified, with MIC and MFC values of 18.5 µg/mL. After confirming the antifungal activity of

Table I. Susceptibility assay duloxetine hydrochloride (DH) alone or in combination with fluconazole against *C. neoformans*.

Susceptibility test (µg/mL)			Chequerboard assay		MIC reduction	
Drug	MIC	MFC	MIC^{co} (CD and FLZ)	FICI (effect)	CD	FLZ
CD	18.5	18.5	1.1 and 1	0.4891 (Syn)	16 x	4 x
FLZ	4	4				

CD, Duloxetine hydrochloride; FLZ, fluconazole; MIC, minimum inhibitory concentration; MFC, minimum fungicial concentration; FICI, fractional inhibitory concentration; Syn, Synergistic; MIC^{co}, minimum inhibitory concentration in the combination.

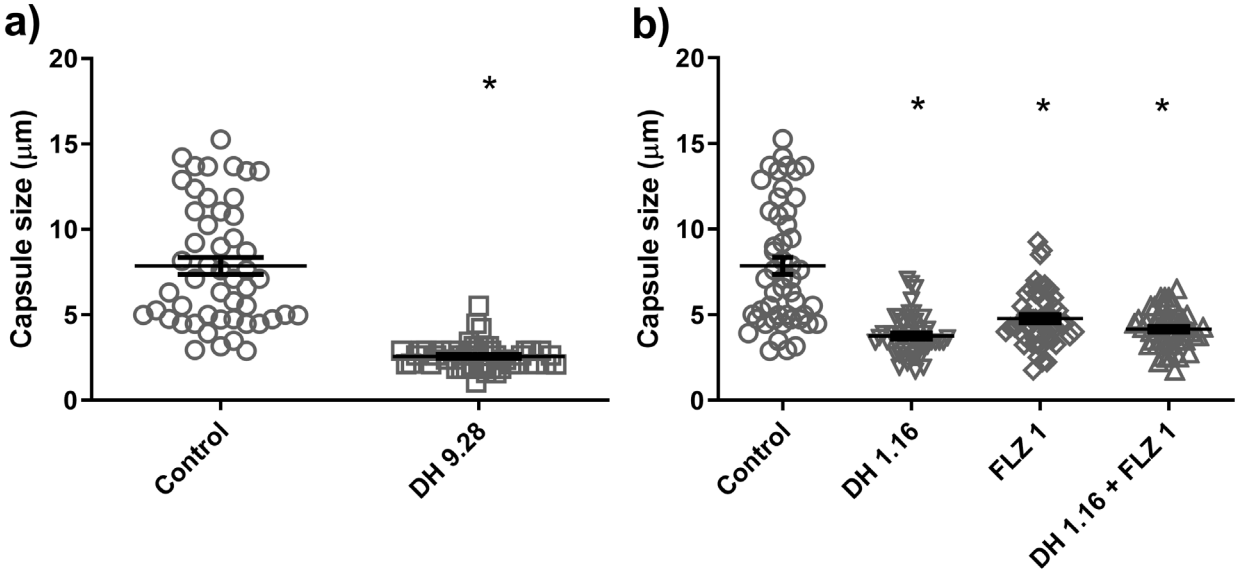

Figure 1. Effect of treatment on capsule size of *C. neoformans*. a) DH at the minimal subinhibitory concentration (Sub-MIC) of 9.28 µg/mL. **b)** DH in concentrations equivalent to the synergistic value (treatments alone and in combination). Fifty cells were measured for each group. DH: duloxetine hydrochloride; FLZ: fluconazole; Control: *C. neoformans* ATCC H99 5 × 10^6 cells /mL. (*): represents statistical difference in relation to the control ($p < 0.05$).

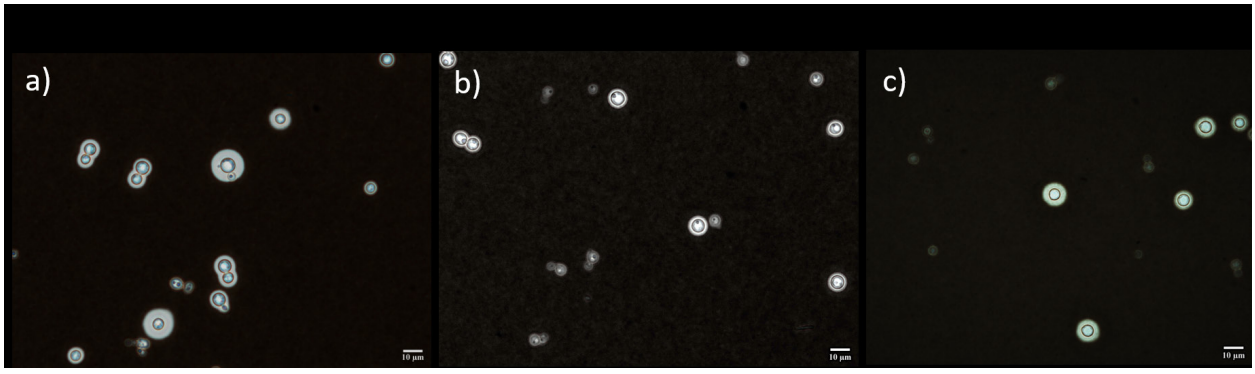

Figure 2. Effect of DH on the polysaccharide capsule size of *C. neoformans*. *C. neoformans* cells were stained with Chinese ink and analyzed with a magnification of 400x. a) Control. **b)** DH at the minimal subinhibitory concentration (Sub-MIC) of 9.28 µg / mL. **c)** DH in synergistic concentrations (1.16 µg/mL and FLZ 1 µg /mL). DH: duloxetine hydrochloride; FLZ: fluconazole; Control: *C. neoformans* ATCC H99 5x10^6 cells /mL.

the isolated drug, it was verified whether this compound had synergistic activity with the FLZ antifungal. A decrease in MIC value was observed by 16-fold for DH and 4-fold for FLZ when used in combination.

The main challenge in the in cryptococcosis therapy with fluconazole is related fungal resistance and high toxicity (Bongomin et al. 2018). In this sense, the combination of compounds could be one interesting alternative since synergistic combination permits to reduce the concentrations of both drugs and consequently reduce drugs toxicity and microorganism resistance (Rohilla et al. 2019, Gushiken et al. 2021, Chen et al. 2014).

Zhai et al. (2012) demonstrated that the antidepressant sertraline, has antifungal activity against *C. neoformans* and interacts synergistically or additively with FLZ. Moreover, assays of systemic cryptococcosis in a mouse

model demonstrated that sertraline alone reduces the brain fungal burden with an efficacy comparable to that of FLZ. Pereira et al. (2021) demonstrated the anti-*C. neoformans* effect of the fluoxetine hydrochloride (FLH) and paroxetine hydrochloride (PAH), alone and in combination with amphotericin B (AmB). Both compounds had antifungal effect and in combination with AmB reducing the AmB MIC up to 8-fold. Furthermore, FLH and PAH alone or in combination with AmB significantly reduced *C. neoformans* capsules size.

Fluconazole mechanism of action is the inhibition of 14α-lanosterol demethylase, one of the enzymes responsible for ergosterol biosynthesis (Maertens 2004). It has been demonstrated that sertraline acts in the intracellular membrane organization, translation and vesicle transport (Zhai et al. 2012). We believe that DH could act similarly, since both drugs are antidepressants with similar mechanism of action. Despite reducing doses and toxicity, one important advantage of drug combination is the possibility of multiple mechanisms of action in the yeast cell, fact that can became the treatment more efficient (Chen et al. 2014).

One of the main components of the *Cryptococcus* spp. is glucuronoxylomannan (GXM), and its release for the formation of the capsule depends on extracellular vesicles (Zaragoza et al. 2009, Guess et al. 2018). Taking into account that SIRS affects intracellular membrane organization, translation and vesicle transport (Zhai et al. 2012), and those vesicles are important for the transport of GXM to extracellular compartment, we hypnotize that the action of DH could be related to inhibition of vesicle transport, however, further studies are required.

In addition, *C. neoformans* capsule are responsible for masking pathogen-associated molecular patterns (PAMPs) such as mannan and 1-3 β glucan, thus preventing phagocytosis and recognition by the immune system (Hatinguais et al. 2020). Thus, the use of compounds with action in the capsule can contribute to the recognition of this yeast by the immune system, and consequently improve its elimination (Kuttel et al. 2020). In this study, DH at sub-inhibitory and synergistic concentrations (isolated and combined) decreased the yeast capsular size from 11% to 67%.

Although DH showed promising results against *C. neoformans,* only laboratory reference strains were tested in the present study. Considering the diversity of *Cryptococcus* species, our findings need to be extended to other species and clinical isolates. The inclusion of clinical strains in future studies is essential as these strains differ genotypically and phenotypically from the reference strains. Within the limitations of this study, it was concluded that DH had antifungal activity against *C. neoformans* and synergistic antifungal activity with FLZ. Furthermore, DH alone, as well as combined with FLZ, demonstrated a potent effect in reducing the capsule of *C. neoformans*

Acknowledgments

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Brazil).

REFERENCES

BONGOMIN F, OLADELE RO, GAGO S, MOORE CB & RICHARDSON MD. 2018. A systematic review of fluconazole resistance in clinical isolates of Cryptococcus species. Mycoses 5: 290-297.

BOSCO-BORGEAT ME, MAZZA M, TAVERNA CG, CÓRDOBA S, MURISENGO OA, VIVOT W & DAVEL G. 2016. Amino acid substitution in *Cryptococcus neoformans* lanosterol 14-α-demethylase involved in fluconazole resistance in clinical isolates. Rev Argent Microbiol 48: 137-142.

CASADEVALL A, COELHO C, CORDERO RJ, DRAGOTAKES Q, JUNG E, VIJ R & WEAR MP. 2019. The capsule of *Cryptococcus neoformans*. Virulence 1: 822-831.

CHEN X, REN B, CHEN M, LIU MX, REN W, WANG QX & YAN GY. 2014. ASDCD: antifungal synergistic drug combination database. PLoS ONE 1: e86499.

DUBOT-PERES A, MAYXAY M, PHETSOUVANH R, LEE SJ, RATTANAVONG S, VONGSOUVATH M, DAVONG V, CHANSAMOUTH V, PHOMMASONE K & MOORE C. 2019. Management of Central Nervous System Infections, Vientiane, Laos, 2003-2011. Emerg Infect Dis 25: 898-910.

ELSEGEINY W, MARR KA & WILLIAMSON PR. 2018. Immunology of Cryptococcal Infections: Developing a Rational Approach to Patient Therapy. Front Immunol 9: 651.

EUCAST. 2017. Definitive document E. DEF7.3.1. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. European Committee on Antimicrobial Susceptibility Testing.

GUESS T, LAI H, SMITH SE, SIRCY L, CUNNINGHAM K, NELSON DE & MCCLELLAND EE. 2018. Size Matters: Measurement of Capsule Diameter in *Cryptococcus neoformans*. J Vis 132: e57171.

GUSHIKEN AC, SAHARIA KK & BADDLEY JW. 2021. Cryptococcosis. Infect Dis Clin North Am 2: 493-514.

HATINGUAIS R, WILLMENT JA & BROWN GD. 2020. PAMPs of the Fungal Cell Wall and Mammalian PRRs. Curr Top Microbiol Immunol 425: 187-223.

KUTTEL MM, CASADEVALL A & OSCARSON S. 2020. *Cryptococcus neoformans* capsular GXM conformation and epitope presentation: A molecular modelling study. Molecules 11: 2651.

LASS-FLÖRL C, DIERICH MP, FUCHS D, SEMENITZ E & LEDOCHOWSKI M. 2001. Antifungal activity against Candida species of the selective serotonin-reuptake inhibitor, sertraline. Clin Infect Dis 12: e135-e136.

MAERTENS JA. 2004. History of the development of azole derivatives. Clin Microbiol Infect 1: 1-10.

NICHOLS CB. 2021. Visualization and Documentation of Capsule and Melanin Production in *Cryptococcus neoformans*. Curr Protoc 1: e27.

ODDS FC. 2003. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 52: 1.

OLIVEIRA AS, MARTINEZ-DE-OLIVEIRA J, DONDERS GGG, PALMEIRA-DE-OLIVEIRA R & PALMEIRA-DE-OLIVEIRA A. 2018. Anti-*Candida* activity of antidepressants sertraline and fluoxetine: effect upon pre-formed biofilms. Med Microbiol Immunol 207: 195-200.

PEREIRA TC, MENEZES RT, OLIVEIRA HC, OLIVEIRA LD & SCORZONI L. 2021. In vitro synergistic effects of fluoxetine and paroxetine in combination with amphotericin B against *Cryptococcus neoformans*. Pathog Dis 2: ftab001.

PERFECT JR, DISMUKES WE, DROMER F, GOLDMAN DL, GRAYBILL JR, HAMILL RJ, HARRISON TS, LARSEN RA, LORTHOLARY O & NGUYEN MH. 2010. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of america. Clin Infect Dis 50: 291-322.

ROHILLA R, MEENA S, KAISTHA N, KRISHNA RAJ A & GUPTA P. 2019. Disseminated cryptococcosis and hepatitis C virus infection: A fatal co-infection. Curr Med Mycol 4: 50-53.

ROSA TF, SOUZA MC, SERAFIN MB, BOTTEGA A, FOLETTO VS, COELHO SS & HÖRNER R. 2020. Repositioning or redirection of antidepressant drugs in the treatment of bacterial and fungal infections. Am J Ther 5: e528-e532.

ROSSATO L, LORETO ES, ZANETTE RA, CHASSOT F, SANTURIO JM & ALVES SH. 2016. In vitro synergistic effects of chlorpromazine and sertraline in combination with amphotericin B against *Cryptococcus neoformans* var. grubii. Folia Microbiol 61: 399-403.

RUEDEN CT, SCHINDELIN J, HINER MC, DEZONIA BE, WALTER AE, ARENA ET & ELICEIRI KW. 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics 18: 529.

SERAFIN MB, BOTTEGA A, FOLETTO VS, DA ROSA TF, MACHADO CS, COELHO SS & HÖRNER R. 2019. Drug Repurposing Identifies New Promising Treatment Options for Invasive Fungal Diseases. Clin Ther 11: 2454-2459.

SPADARI CDC, WIRTH F, LOPES LB & ISHIDA K. 2020. New approaches for cryptococcosis treatment. Microorganisms 4: 613.

SQUIZANI ED, OLIVEIRA NK, REUWSAAT JCV, MARQUES BM, LOPES W, GERBER AL, DE VASCONCELOS ATR, LEV S, DJORDJEVIC JT & SCHRANK A. 2018. Cryptococcal dissemination to the central nervous system requires the vacuolar calcium transporter Pmc1. Cellular Microbiology 20: e12803.

ZARAGOZA O. 2019. Basic principles of the virulence of Cryptococcus. Virulence 10: 490-501.

ZARAGOZA O & CASADEVALL A. 2004. Experimental modulation of capsule size in *Cryptococcus neoformans*. Biol Proced Online 6: 10-15.

ZARAGOZA O, RODRIGUES ML, DE JESUS M, FRASES S, DADACHOVA E & CASADEVALL A. 2009. The capsule of the fungal

pathogen *Cryptococcus neoformans*. Adv Appl Microbiol 68: 133-216.

ZHAI B, WU C, WANG L, SACHS MS & LIN X. 2012. The antidepressant sertraline provides a promising therapeutic option for neurotropic cryptococcal infections. Antimicrob Agents Chemother 56: 3758-3766.

How to cite

MENEZES RT, PEREIRA TC, JUNQUEIRA JC, OLIVEIRA LD & SCORZONI L. 2022. Synergistic combination of duloxetine hydrochloride and fluconazole reduces the cell growth and capsule size of *Cryptococcus neoformans*. An Acad Bras Cienc 94: e20211021. DOI 10.1590/0001-3765202220211021.

Manuscript received on August 9, 2021;
accepted for publication on December 13, 2021

RAQUEL T. MENEZES[1]
https://orcid.org/0000-0003-4250-5528

THAÍS C. PEREIRA[1]
https://orcid.org/0000-0002-7206-3348

JULIANA C. JUNQUEIRA[1]
https://orcid.org/0000-0001-6646-6856

LUCIANE D. OLIVEIRA[1]
https://orcid.org/0000-0002-5465-9551

LILIANA SCORZONI[1,2]
https://orcid.org/0000-0002-0178-6653

[1]Universidade Estadual Paulista (UNESP), Instituto de Ciência e Tecnologia, Departamento de Biociências e Diagnóstico Oral, Av. Engenheiro Francisco José Longo, 777, 12245-000 São José dos Campos, SP, Brazil

[2]Pós-Graduação em Enfermagem, Universidade de Guarulhos (UNG), Rua Engenheiro Prestes Maia, 88, 07023-070 Guarulhos, SP, Brazil

Correspondence to: **Liliana Scorzoni**
E-mail: liliscorzoni@yahoo.com.br

Author contributions

Conceived and designed the experiments: RTM, TCP, LS. Performed the experiments: RTM, TCP. Analyzed the data: RTM, TCP, LS; Wrote the paper: RTM, TCP, JCJ, LDO, LS; Revised the paper: JCJ, LDO, LS.