Archives of Oral Biology 60 (2015) 1565–1570

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Synergistic effects of tea catechin epigallocatechin gallate and antimycotics against oral *Candida* species

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ARTICLE INFO

Article history:
Received 1 November 2014
Received in revised form 25 June 2015
Accepted 4 July 2015

Keywords:
Epigallocatechin gallate
Antifungal agents
Candida species
Synergism

ABSTRACT

Objective: Epigallocatechin gallate (EGCG), the major antimicrobial tea polyphenol, has been reported to inhibit the growth of *Candida albicans* planktonic cells and enhance the antifungal activity of antimycotics. We hypothesised that synergism exists between EGCG and conventional antimycotics against biofilms of *Candida* species.
Design: The minimal inhibitory concentrations (MIC) of EGCG, miconazole, fluconazole and amphotericin B against planktonic cells and the sessile MIC (SMIC) against biofilms of *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida kefyr* and *Candida krusei* were determined by a microdilution method. For assessment of biofilm metabolic activity, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay was used. The interactions between EGCG and antimycotics were evaluated by checkerboard microdilution assay and determined by fractional inhibitory concentration index (FIC).
Results: Synergism was observed between EGCG and miconazole, fluconazole or amphotericin B against most test planktonic and biofilm cells of *Candida* species (FIC $\leq$ 0.5). All biofilm cells were significantly more resistant to EGCG and antimycotics (20–3200 times higher) compared with their planktonic counterparts.
Conclusions: We conclude that EGCG enhances the antifungal effects of miconazole, fluconazole and amphotericin B. Combined treatment with EGCG may lower the dosages of antimycotics, thus preventing adverse effects and the emergence of drug-resistant oral *Candida* species.
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1. Introduction

In recent years, the incidence of fungal infections has increased significantly due to increased usage of broad-spectrum antibiotics, immunosuppressive therapy, organ and bone marrow transplantation and the continued spread of HIV infection (Ramos-E-Silva, Lima, Schechtman, Trope, & Carneiro, 2010; Sardi, Scorzoni, Bernardi, Fusco-Almeida, & Mendes Giannini, 2013). Candida species, the most common human fungal pathogens, are associated with superficial infections of the skin, hair, oral or vaginal tract and with life-threatening systemic infections (Luo, Skerka, Kurzai, & Zipfel, 2013; Paramythiotou, Frantzeskaki, Flevari, Armaganidis, & Dimopoulos, 2014). Among these *Candida* species, *Candida albicans* has been considered the most virulent and frequently isolated species, especially in oral candidiasis (Williams, Kuriyama, Silva, Malic, & Lewis, 2011). The non-albicans *Candida* species, such as *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, *Candida kefyr* and *Candida krusei*, which have also been frequently detected in medically compromised patients with a history of multiple courses of azole antifungals, are related to a series of invasive infections including pyelonephritis, endocarditis and meningitis (Falagas, Roussos, & Vardakas, 2010; Miceli, Díaz, & Lee, 2011).

Recent evidence has demonstrated that the majority of fungal infections are associated with biofilm growth (Ramage, Saville, Thomas, & López-Ribot, 2005; Ramage, Rajendran, Sherry, & Williams, 2012). Biofilms are three-dimensional structures consisting of densely packed microorganisms that attach to a surface and to each other, and are enclosed in an extracellular polymeric matrix (Ramage et al., 2005). Biofilms are the most common form of microorganisms found in nature and cause a majority of human infections (Douglas, 2003). The ability of *Candida* species to form biofilms is an important virulence factor in their pathogenicity. The adherent sessile cells within a biofilm are highly resistant to

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http://dx.doi.org/10.1016/j.archoralbio.2015.07.001
0003-9969/Published by Elsevier Ltd.

antimicrobials and host defence compared with the planktonic cells (Douglas, 2003; Ramage et al., 2012). Biofilm provides microbial pathogens a form of protection and allows them to survive in hostile environments (Ning et al., 2013). Therefore, the elimination of pathogenic biofilms remains a major challenge in the control of fungal infections.

To date, two major problems exist in antifungal therapy, i.e., the adverse effects of selected antimycotics and the increase in drug-resistant fungal pathogens (Ashbee et al., 2014; Douglas, 2003). Because of the intrinsic limitations of conventional antimycotics (such as gastrointestinal toxicity, nephrotoxicity, hepatotoxicity and neurologic toxicity), much attention has been focused on the development of alternative antifungal strategies that are effective but less toxic (Andes, 2013; Hamill & Amphotericin, 2013). Higher plants have traditionally served as a rich source for antimicrobial compounds. Many plant-derived natural products have been shown to possess antifungal properties (Feás & Estevinho, 2011; Sardi et al., 2013; Estevinho, Afonso, & Feás, 2011; Bisignano, Filocamo, Faulks, & Mandalari, 2013). Studies have shown that some of these compounds enhance the bioactivity of antimycotics, thus lowering drug dosage and reducing toxic side-effects (Han, 2007; Wei, Xu, & Wu, 2011). We have previously reported that berberine, an alkaloid isolated from *Hydrastis canadensis* and other medicinal plants, demonstrated a synergetic effect with miconazole against planktonic and biofilm cells of *Candida* species (Wei et al., 2011). Epigallocatechin gallate (EGCG), an antimicrobial polyphenol (chemical structure shown in Fig. 1) found in many plants, especially tea leaves (*Camellia sinensis*), has been found to inhibit the growth and hyphal formation of *Candida* species (Han, 2007; Hirasawa & Takada, 2004; Daglia, 2012; Chen, Zhai, & Arendrup, 2015; Sitheeque et al., 2009). A study in a murine model of disseminated candidiasis indicated that EGCG demonstrated a synergetic antifungal effect with amphotericin B (Han, 2007). It also enhanced the antifungal activity of fluconazole, amphotericin B and ketoconazole against *C. albicans* planktonic cells (Hirasawa & Takada, 2004; Navarro-Martínez, García-Cánovas, Rodríguez-López, 2006). However, whether such synergy exists against the more resistant *Candida* biofilms is unclear. We hypothesise that EGCG, when combined with antifungal drugs, exerts synergistic growth inhibition against oral *Candida* biofilms. In this study, we examined the antifungal activity of EGCG alone and in combination with miconazole, fluconazole or amphotericin B against biofilm cells of *Candida albicans* and the non-*albicans Candida* species. Similar drug combinations against planktonic cells were also determined as a comparison with the biofilm cells.

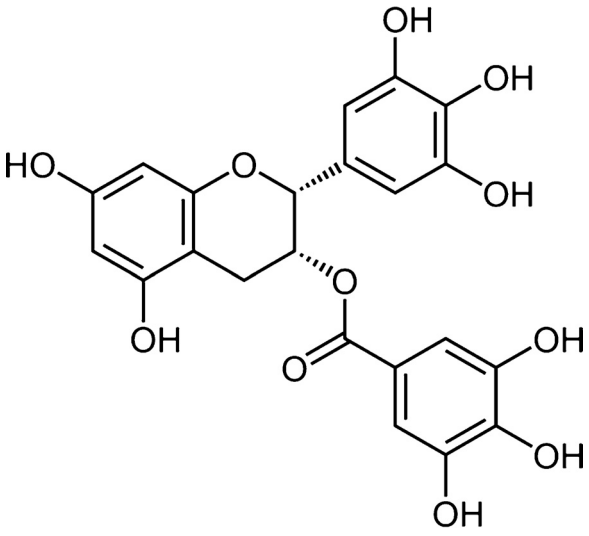

Fig. 1. Chemical structure of epigallocatechin gallate (EGCG).

2. Materials and methods

2.1. Test Candida species and antifungal agents

C. albicans SC5314, *C. albicans* American Type Culture Collection (ATCC) 10231, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 13803, *C. glabrata* ATCC 66032, *C. kefyr* ATCC 46764 and *C. krusei* ATCC 14243 were used in this study. All *Candida* species were grown in Sabouraud's dextrose agar (SDA) at 37 °C for 48 h, isolated colonies were subcultured in Sabouraud's dextrose broth (SDB) to late-exponential growth phase, and cells were collected and resuspended in RPMI 1640 to the concentrations desired for further use. All test agents—including miconazole (MCZ), fluconazole (FLC), amphotericin B (AmB), EGCG, 2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and menadione—were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). EGCG solution was freshly prepared in RPMI 1640 before assays. Stock solutions of MCZ and AmB were dissolved in dimethyl sulfoxide (DMSO) at high concentration and further diluted in RPMI for use. FLC was dissolved in RPMI. To minimise the oxidation of EGCG, all plates were incubated in an anaerobic growth chamber (85% N_2, 10% H_2, and 5% CO_2; Forma Scientific, Marietta, OH, USA).

2.2. Effects of test antimycotics against Candida planktonic cells

The minimum inhibitory concentrations (MICs) of EGCG, MCZ, FLC and AmB against planktonic *Candida* cells were determined by a microdilution method in 96-well cell plates according to the protocol of the CLSI (M27-A3) with some modifications (Fothergill, 2008). Test agents were added to wells and serially diluted to final concentrations ranging from 93.8 μg/mL to 6000 μg/mL for EGCG; from 0.125 μg/mL to 4 μg/mL for MCZ; from 0.125 μg/mL to 4 μg/mL for FLC; and from 0.031 μg/mL to 1 μg/mL for AmB. *Candida* cells were subsequently added to wells (final concentration, 2.5×10^3 CFU/mL). The control wells included RPMI 1640 and *Candida* species without test agents. Plates were anaerobically cultured for 24 h at 37 °C, and growth was spectrophotometrically determined at 530 nm in a plate reader (Victor X5 Multilabel, PerkinElmer, Waltham, MA, USA). The MIC was defined as the lowest concentration of test agent that inhibited more than 90% growth compared with that of the agent-free control. All assays were performed in triplicate.

2.3. Effects of test antimycotics against Candida biofilms

The effects of EGCG, MCZ, FLC and AmB against *Candida* biofilm cells were examined in 96-well plates according to the method described by Pierce et al. (2008) with some modifications. Each well contained 200 μL *Candida* suspension (1×10^6 CFU/mL), and was anaerobically incubated in RPMI at 37 °C for biofilm formation. After 48 h, wells were washed twice with phosphate-buffered saline (PBS, pH 7.0) to remove non-adherent cells. EGCG, MCZ, FLC and AmB were subsequently added to the plates and serially diluted. The final concentrations of test agents ranged from 750 μg/mL to 48 000 μg/mL for EGCG; from 6.25 μg/mL to 12 800 μg/mL for MCZ; from 156 μg/mL to 25,600 μg/mL for FLC; and from 0.15 μg/mL to 10 μg/mL for AmB. After further incubation for 24 h, the biofilms were washed twice with PBS, and the metabolic activity of the biofilms was examined by the XTT reduction assay (Kuhn, Balkis, Chandra, Mukherjee, & Ghannoum, 2003). For this, 158 μL PBS, 40 μL XTT (1 mg/mL) and 2 μL menadione (0.4 mM) were add to each well and incubated in the dark for 3 h at 37 °C. The absorbance of the supernatant was measured at 492 nm by means