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Anticandidal Activity and Biocompatibility of a Rechargeable Antifungal Denture Material

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Abstract

Objectives—*Candida*-associated denture stomatitis is a recurrent and debilitating oral mucosal disease. Development of anticandidal denture materials represents a promising strategy to manage this condition. We have previously shown that miconazole incorporated in methacrylic acid (MAA) copolymerized diurethane dimethacrylate (UDMA) denture materials has long-term anticandidal activity. In this study, we examined the ability of culture medium conditioned with drug-free- or miconazole-MAA-UDMA discs to prevent *Candida* infection in an *in vitro* oral epithelial cell/*Candida albicans* co-culture system.

Material and Methods—*Candida albicans* (*C. albicans*) induced OKF6/TERT-2 cell damage was quantified by the release of lactate dehydrogenase from epithelial cells, cytokine production was quantified using protein cytokine arrays, and the expression of *C. albicans* genes was measured by RT-qPCR.

Results—*C. albicans* had limited growth with altered expression levels of secreted aspartyl proteinase-2 and -5 in culture medium conditioned by miconazole-MAA-UDMA discs. Significantly, the ability of *C. albicans* to induce oral epithelial cell damage and trigger epithelial proinflammatory cytokine production was also inhibited by miconazole disc conditioned media.

Conclusion—Miconazole released from MAA-UDMA denture materials effectively prevents the development of candidal infection in an *in vitro* oral epithelial system. Further characterization of this drug-rechargeable denture material is warranted.

Keywords

Anticandidal; Rechargeable; Denture Material; Denture Stomatitis; *Candida albicans*; Epithelial cells

Introduction

Dentures are invaluable to the nutritional status, speech, appearance, and quality of life of patients missing multiple or all teeth. Unfortunately, due to candidal colonization and subsequent formation of biofilms on denture surfaces, the use of these prostheses is often associated with the development of *Candida*-associated denture stomatitis (CADS), a common and recurring disease among denture wearers (Radford *et al.*, 1999; Ikebe *et al.*, 2006). CADS can lead to oral mucosal discomfort, altered taste sensation and dysphagia, resulting in poor nutritional intake, and therefore compromising the quality of life of affected patients. Moreover, CADS can lead to life-threatening complications, especially among immunosuppressed and immunocompromised populations (Bergmann, 1988; Radford *et al.*, 1999; Ikebe *et al.*, 2006).

Current management strategies for CADS include denture cleaning and disinfection, proper denture wearing habits, use of tissue conditioners/liners, modification of denture surface energy, and administration of topical or systemic antifungals (Arendorf and Walker, 1987; Merz, 1990; Perezous *et al.*, 2005; ten Cate *et al.*, 2009). However none of these strategies completely prevent *Candida* colonization/biofilm formation on denture surfaces, and the reinfection rate is high. Anticandidal dentures represent another approach to treat CADS. These dentures are fabricated by impregnating drugs into denture liners or base materials that deliver antifungal agents directly onto infection sites (Quinn, 1985; Schneid, 1992; Truhlar *et al.*, 1994; Matsuura *et al.*, 1997; Chow *et al.*, 1999; Lefebvre *et al.*, 2001; Kalachandra *et al.*, 2002; Lin *et al.*, 2003; Abe *et al.*, 2004; Raj and Dentino, 2005; Yoshinari *et al.*, 2006; Pesci-Bardon *et al.*, 2006; Redding *et al.*, 2009). The efficacy of this approach in short-term applications (e.g., days to weeks) has been well demonstrated in a number of studies (Quinn, 1985; Schneid, 1992; Chow *et al.*, 1999; Lin *et al.*, 2003; Abe *et al.*, 2004). However, currently available anticandidal dentures fail to provide long-term (e.g., months to years) protection against CADS. The lack of long-term efficacy of these devices is primarily due to their inability to incorporate enough drugs for extended use. Also, existing anticandidal denture materials release the impregnated drugs in an uncontrolled manner. More specifically, these antimicrobial dentures initially release high concentrations of antifungal drugs regardless of whether active infection is present, and this is followed by an exponential decrease in drug release. When the minimal inhibitory concentration is no longer achieved, the anticandidal activity of these appliances is lost.

The major challenge related to the development of antifungal dentures designed for long-term use is the lack of strategies to incorporate enough antifungal drugs into the prostheses and control antifungal drug release rate. Our research group has developed a rechargeable, “click-on/click-off” antifungal delivery system technology to extend anticandidal duration and control drug release rates based on whether candidal infection is present (Cao *et al.*, 2010). Methacrylic acid (MAA) is a biocompatible monomer that is widely used in glass-ionomer cements (Fruits *et al.*, 1996; Mount, 1999; De Bruyne and De Moor, 2004). MAA has also been proposed for use in controlled drug delivery systems (Saito and Isogai, 2004; Dehousse *et al.*, 2010). We have shown that MAA (10% wt) can be copolymerized with denture base resin monomer, urethane dimethacrylate (UDMA), in the curing step, without negatively affecting the physical/mechanical properties of the resulting resins (Cao *et al.*, 2011). The anionic MAA moieties in the denture act as a “rechargeable battery” to bind and then slowly release cationic antifungal drugs for weeks to months. Along with this line, we have demonstrated that MAA-containing UDMA resins bind miconazole and chlorhexidine gluconate through ionic interactions and provide potent antifungal effects against *Candida albicans* (*C. albicans*) (Cao *et al.*, 2010). Analysis of drug release kinetics demonstrated a sustained release of antifungal agents from MAA-UDMA loaded resin discs. After 60 days of releasing activity *in vitro* at neutral pH, MAA-UDMA resin discs still contained the

minimal inhibitory concentration of miconazole [$18.22 \pm 1.2 \mu\text{g}/\text{cm}^2$ (mean $\pm$ SD)]. The antifungal drugs could be removed from the discs by immersion in EDTA solution and recharged to the quenched denture materials to regenerate their anticandidal properties (Cao *et al.*, 2010). Allowing click on/click off and the interchange of anticandidal drugs on MAA-containing denture materials is essential for avoiding the development of drug resistant *Candida* spp. MAA-containing dentures can be worn as conventional dentures. However if CADS occurs, these dentures can be charged with antifungal drugs. Once CADS is cleared, the antifungal drugs are washed off (quenched) the MAA-containing dentures, so these prosthetic appliances no longer release antifungal drugs. If CADS recurs or drug resistance is suspected, MAA-containing dentures can be recharged with drugs with different anticandidal mechanisms.

In previous studies, we have only demonstrated the anticandidal activity and cytotoxicity of MAA-containing denture materials using a conventional microbiological assay (i.e., zone inhibition on agar) (Cao *et al.*, 2010) and fibroblasts (Cao *et al.*, 2011). To further validate the potential therapeutic use of MAA copolymerized UDMA resin materials containing antifungal drugs in oral conditions, here we tested the biocompatibility and anticandidal activity of miconazole released from MAA-UDMA resin discs using an oral epithelial cell/*C. albicans* co-culture system.

Materials and Methods

Organisms

C. albicans strain SC5314, originally isolated from a patient with disseminated candidiasis, was obtained from the American Type Culture Collection (ATCC; Rockville, MD). The organisms were routinely propagated using yeast–peptone–dextrose (YPD) agar (Difco Laboratories, Detroit, MI) at 25°C.

Cell Culture

Human oral keratinocyte cell line OKF6/TERT-2 was used in this study, as oral epithelial cells are the first cells to interact with *C. albicans* during the establishment of CADS. The OKF6/TERT-2 cells are normal human oral mucosal epithelial cells that have been immortalized by forced expression of telomerase 2 via retroviral transduction and deletion of p16INK4a regulatory protein (Dickson *et al.*, 2000). Cells were maintained in keratinocyte serum-free medium (KSFM) (Invitrogen, Carlsbad, CA) supplemented with 0.1 ng/ml epidermal growth factor (Invitrogen, Carlsbad, CA), 50 $\mu\text{g}/\text{ml}$ pituitary bovine extract (Invitrogen, Carlsbad, CA), 0.4 mM CaCl_2 and antibiotics (100 U/ml penicillin; 100 $\mu\text{g}/\text{ml}$ streptomycin). This study was approved by the Institutional Review Board of the University of Texas Health Science Center at San Antonio.

Miconazole Loaded Denture Materials

Rechargeable denture materials were prepared as described previously with minor modifications (Cao *et al.*, 2010). In brief, denture discs ($10 \times 10 \times 2\text{mm}$) (L $\times$ W $\times$ H) were synthesized by co-polymerization of methacrylic acid (MAA) (Sigma-Aldrich, St. Louis, MO) with diurethane dimethacrylate (UDMA) (Sigma-Aldrich, St. Louis, MO) at 1:10 ratio (wt/wt) in the presence of 1% initiator azobisisobutyronitrile (Sigma-Aldrich, St. Louis, MO) (wt/wt) in aluminum molds. Polymerization was carried out at 70°C for 3 h. Specimens were visually inspected to ensure they were free of voids. A series of MAA-UDMA discs were then immersed in 5% miconazole ethanol solution for 24 h at room temperature under constant shaking. Next, the discs were rinsed individually with water (3 $\times$, 10 ml and 1 min for each rinse), air-dried, and stored in a desiccator. MAA-UDMA discs absorbed $59.8 \pm 2.5 \mu\text{g}/\text{cm}^2$ of miconazole (Cao *et al.*, 2010).

To test the biocompatibility and the anticandidal effect of miconazole- and drug-free-MAA-UDMA discs, 1cm² discs were immersed in complete KSFM at ratio of 14ml/cm² with gentle shaking (60 rpm) for 48 h at 37°C. MAA-UDMA disc conditioned medium was then filtered through a 0.22 µm filter unit (Nalge Rochester NY) and used for testing immediately. The residual miconazole on the discs was extracted with deionized water using an automatic soxhlet extraction apparatus for 24 h and measured at λ=220 (residual drug). Concentration of miconazole in the conditioned medium was calculated by subtracting the residual drug from the original absorbed concentration.

OKF6/TERT-2/*C. albicans* co-culture system

OKF6/TERT-2 cell and *C. albicans* co-culture system has been established for examining oral epithelial cell responses to *Candida* infection (Dongari-Bagtzoglou and Kashleva, 2003a; Dongari-Bagtzoglou *et al.*, 2004; Li *et al.*, 2007). Stationary-phase *C. albicans* SC5314 yeast cells were prepared by growth for 18 h at room temperature in YPD agar broth (Difco Laboratories, Detroit, MI). The fungal cells were centrifuged, washed with phosphate-buffered saline (PBS), and counted with hemacytometer. OKF6/TERT-2 cells were seeded at density of 4×10^5 cells/well in 2 ml of complete KSFM in 6-well plates (Corning Incorporation, Corning, NY) and cultured overnight at 37°C and 5% CO₂ atmosphere. The following day, epithelial cells were challenged with suspensions of stationary-phase viable *C. albicans* at epithelial cell to fungal cell ratio of 1:0.1 (8×10^4 fungal cells/well) or 1:1 (8×10^5 fungal cells/well) in conditioned medium or complete KSFM for up to 18 h. The resulting supernatants were collected and stored at -80°C prior to use in cytokine and cell damage assays. OKF6/TERT-2/*C. albicans* co-cultures were processed for RNA isolation. Negative controls for these experiments included uninfected OKF6/TERT-2 cultures and *C. albicans* alone.

Cell damage assay

The potential cytotoxic effect of substances released from drug free- and miconazole-MAA-UDMA discs on oral epithelial cells was assessed using the CytoTox-96 Non-Radioactive Cytotoxicity assay (Promega, Madison, WI) which measures the release of lactate dehydrogenase (LDH) from damaged and dying cells (Villar and Zhao, 2010). The same assay was also used to examine cell damage in oral epithelial cells infected with *C. albicans* in the presence or in the absence of miconazole released from miconazole-MAA-UDMA discs. In these experiments, OKF6/TERT-2 epithelial cells were co-cultured with *C. albicans* strain SC5314 for 18 h, and the LDH released from the culture system into the culture medium was quantified based on the enzymatic conversion of the tetrazolium salt into a red formazan product. The amount of LDH in medium was measured spectrophotometrically at 490nm according to the manufacturer's instructions. Uninfected oral epithelial cultures (epithelial control) and *C. albicans* alone (*C. albicans* control) incubated under the identical conditions (e.g. KSFM or conditioned media) were included as negative controls. The total amount of LDH was estimated by treating uninfected OKF6/TERT-2 cells with 9% Triton X-100 for 1h. The LDH release in the presence of *C. albicans* was quantified by using the following formula [(experimental – epithelial control – *C. albicans* control)/(total – epithelial control)] × 100. The values were expressed as percentage of the total amount of LDH release.

Cytokine detection

Multiple cytokines were simultaneously detected in the supernatants using the RayBio enzyme-linked immunosorbent assay (ELISA) cytokine array (RayBiotech, Norcross, GA). The array consists of 23 pairs of cytokine monoclonal antibodies spotted onto a nitrocellulose membrane and the assay was performed according to the manufacturer's recommendation. In brief, after membrane blocking, 1 ml of supernatants were added and

incubated for 2 h at room temperature, and this was followed by addition of a cocktail of biotinylated detection antibodies (1:1000 dilution) for 2 h, and subsequent addition of horseradish peroxidase-conjugated streptavidin for 2 h. The membranes were then developed by addition of an enhanced chemiluminescence-type solution and exposed to X-ray film (Kodak BioMax film; Kodak, Rochester, NY). The signals on films were then scanned and stored as TIF images and their intensities were determined by densitometry analysis using the ImageJ software (U. S. National Institutes of Health, Bethesda, MD). Positive control signals (provided by the manufacturer) on each membrane were used to normalize cytokine signal intensities.

Reverse transcriptase – quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was used to examine the expression levels of *C. albicans* virulent factors secreted aspartyl proteinases (SAP) –2 and –5. Total mRNA was isolated from co-cultures using TRI Reagent (Molecular Research Center, Cincinnati, OH), following the manufacturer's instructions, and the samples were processed using minibead beater (BioSpec Products, Bartlesville, OK) or Maxwell 16 System Personal Automation™ (Promega, Madison, WI). RNA samples were treated with RNase-free DNase I using a kit from Applied Biosystems (Foster City, CA). Complementary DNA (cDNA) synthesis was carried out by reverse transcription of the DNase free RNA samples following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Quantitative RT-PCR assay was then performed on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) and subjected to RT-PCR and DynAmo™ SYBR® Green qPCR (Applied Biosystems, Foster City, CA) using published oligonucleotide primers (Naglik *et al.*, 2008), i.e., *C. albicans* SAP2: forward 5' - TCCTGATGTTAATGTTGATTGTCAAG-3' and reverse 5' - TGGATCATATGTCCCCTTTTGTT-3', *C. albicans* SAP5 pairs: forward 5' - CATTGTGCAAAGTAACTGCAACAG-3', and reverse 5' - TTACGCAAAAGGTAAGTGTATCAAGA-3', *C. albicans* ACT1 pairs: forward 5' - GACAATTTCTCTTTCAGCACTAGTAGTGA-3' and reverse 5' - GCTGGTAGAGACTTGACCAACCA-3', human β -actin pairs: forward 5' - TCGACAACGGCTCCGGCAT-3', and reverse 5' - AAGGTGTGGTGCCAGATTTTC-3' (Milam *et al.*, 1991). The expression of SAP2 and SAP5 mRNA transcripts was normalized to the relative levels of *C. albicans* housekeeping gene ACT1. The expression levels of *C. albicans* SAP2 and SAP5 in co-cultures maintained in the presence of miconazole-MAA-UDMA conditioned medium was expressed as fold changes over expression levels in control cultures maintained in drug-free-MAA-UDMA, at both high (1:1) and low (1:0.1) infection ratios.

Statistical analyses

Statistically significant differences were determined by analysis of variance (ANOVA) followed by Dunnett's test or by two-tailed *t*-test. Differences were considered statistically significant at a p-value <0.05. The values were expressed as means $\pm$ standard deviation (SD) or standard error (SE).

RESULTS

Miconazole concentration in the conditioned medium

The amount of miconazole released from drug charged MAA-UDMA discs into the conditioned medium was first determined. Previous studies estimated that MAA-UDMA-discs absorbed on average 59.8 ± 2.5 (Mean $\pm$ SD) $\mu\text{g}/\text{cm}^2$ of miconazole (Cao *et al.*, 2010). In the current study, miconazole-MAA-UDMA discs still contained 33.16 ± 5.07 $\mu\text{g}/\text{cm}^2$ of miconazole following 48 h of immersion in KSFM. Therefore, the concentration of

miconazole released from miconazole-MAA-UDMA discs into the culture medium was $1.90 \pm 0.34 \mu\text{g/ml}$ ($n=6$) (miconazole-MAA-UDMA conditioned medium).

Microscopic assessment of the co-cultures

The co-culture system used in this study is shown in Figure 1A. OKF6/TERT-2 formed confluent cell monolayers in the absence of *C. albicans*. Of interest, uninfected OKF6/TERT-2 cells maintained their original characteristics when incubated in medium conditioned by drug-free- or miconazole-MAA-UDMA discs (Figure 1A and 1B). At a low infection ratio (1:0.1 epithelial to yeast cell ratio), yeast and hyphal forms of *C. albicans* adhere to and partially covered OKF6/TERT-2 monolayers maintained in KSFM media (data not shown). Nonetheless, under these experimental conditions, some epithelial cells were not covered by *C. albicans* cells and were still clearly visible under the microscope (not shown). At a high infectivity ratio (1:1 epithelial to yeast cell ratio), OKF6/TERT-2 cells incubated in KSFM were virtually completely covered by *C. albicans* yeast cells and hyphal forms (not shown). Similar findings were observed when co-cultures were maintained in medium conditioned by drug-free-MAA-UDMA discs (Figure 1A). In sharp contrast, *C. albicans* failed to cover epithelial cells maintained in medium conditioned by miconazole-MAA-UDMA discs, regardless the level of infection (Figure 1B). Under this experimental condition, *C. albicans* formed floating aggregates, failed to form hyphae and adhere to OKF6/TERT-2 cells (Figure 1B).

Effect of medium conditioned by miconazole-MAA-UDMA discs on epithelial cell damage in response to candidal infection

We next compared the levels of epithelial cell injury by measuring the amount of LDH released from OKF6/TERT-2 cells cultured with *C. albicans* suspended in medium conditioned by miconazole- or drug-free- MAA-UDMA discs (experimental and disc control media, respectively) or in complete KSFM (control). At a high infectivity ratio (1:1 epithelial to yeast cell ratio), SC5314 suspended in complete KSFM or drug-free-MAA-UDMA conditioned medium promoted the injury of 67% and 54% of OKF6/TERT-2 cells, respectively (Figure 2). While, at a low infectivity ratio (1:0.1 epithelial to yeast cell ratio), SC5314 suspended in complete KSFM or drug-free-MAA-UDMA conditioned medium injured 50% and 45% of OKF6/TERT-2 cells, respectively (Figure 2). In sharp contrast, SC5314 did not inflict any considerable damage to OKF6/TERT-2 cells when suspended in medium conditioned by miconazole-MAA-UDMA discs (Figure 2). This was true for both infectivity ratios tested (Figure 2). Finally, treatment of uninfected OKF6/TERT-2 cells with drug-free- or miconazole-conditioned medium had no effect on the integrity of these cells (data not shown). Altogether, this indicates that the miconazole released from MAA-UDMA discs curtailed the ability of *C. albicans* to induce oral epithelial cells damage and had no apparent cytotoxicity to epithelial cells.

Effect of medium conditioned by miconazole-MAA-UDMA discs on the proinflammatory cytokine response to infection

Using antibody based-arrays, we found that OKF6/TERT-2 cells maintained in complete KSFM did not produce IL-1 at detectable levels, but produced relatively high constitutive levels of IL-8 and IL-6 as compared to other measured cytokines (Figure 3). OKF6/TERT-2 responded to medium conditioned by drug-free- or miconazole-MAA-UDMA discs with no changes on the cytokine profile (Figure 3). At high infectivity ratio, *C. albicans* induced OKF6/TERT-2 cells to secrete increased amounts of IL-1 ($p < 0.001$), and IL-1 ($p < 0.001$) (Figure 3). In contrast, production of IL-4, IL-6, IL-8, IL-10 and GM-CSF was not significantly affected by *C. albicans* infection (Figure 3). Quantitative differences were found in the overall inflammatory responses of OKF6/TERT-2 cells to *C. albicans* when infection was carried in miconazole-MAA-UDMA discs conditioned medium. More

specifically, *C. albicans*-induced epithelial inflammatory responses (e.g., IL-1 and IL-1) were significantly inhibited when infection was carried out in medium conditioned by miconazole-MAA-UDMA discs (Figure 3). Overall, *C. albicans*-induced epithelial inflammatory responses were not affected by medium conditioned by drug-free-MAA-UDMA discs. The exception was an increase in IL-6 secretory levels when infection was maintained in medium conditioned by drug-free-MAA-UDMA discs ($p<0.05$) (Figure 3).

Effect of medium conditioned by miconazole-MAA-UDMA discs on SAP2 and SAP5 expression levels during infection

Expression level of candidal genes was evaluated using RT-qPCR. Total RNAs extracted from the co-cultures indicated the expression of both human and candidal genes in this *in vitro* system. As expected, candidal genes were not expressed in uninfected OKF6/TERT-2 cells. Moreover, *C. albicans ACT1* was expressed at significantly lower levels in cultures infected at a low infectivity ratio, as compared to the levels measured in cultures infected at a high infectivity ratio. Noticeably, *C. albicans ACT1* expression was significantly reduced when infection was maintained in medium conditioned by miconazole-MAA-UDMA discs, regardless of the infectivity ratio (Table 1).

Relative expression of *C. albicans* virulent factors *SAP2* and *SAP5* was examined in cultures maintained in complete KSFM or medium conditioned by drug-free- or miconazole-MAA-UDMA discs. Expression levels of *SAP2* and *SAP5* were not affected when infection was carried out in the presence of medium conditioned by drug-free-MAA-UDMA discs (Figure 4). In contrast, *SAP2* and *SAP5* expression levels were altered when infection was maintained in medium conditioned by miconazole-MAA-UDMA discs. Interestingly, in the presence of medium conditioned by miconazole-MAA-UDMA discs, *SAP2* expression was up-regulated by 2.3 and 3.5 folds at the lower and higher infectivity ratios, respectively. In contrast, under the same experimental conditions, *SAP5* expression was reduced to 42% and 36% of the original levels, respectively. These results suggest that miconazole released from MAA-UDMA discs reduces *C. albicans* counts and affects the expression of virulence factors on this fungal organism.

Discussion

We have previously shown that miconazole charged MAA-UDMA discs have prolonged antifungal activities against *C. albicans* (Cao *et al.*, 2010). In the current study, we have demonstrated that miconazole is released from MAA-UDMA discs and possesses antifungal activities in an *in vitro* model of oropharyngeal candidiasis. More specifically, we have demonstrated for the first time that medium conditioned by miconazole-MAA-UDMA discs prevented the ability of *C. albicans* to form hyphae, cover and damage oral epithelial cells, and trigger a strong epithelial proinflammatory response. Moreover, we have shown that *C. albicans* gene expression was affected when this fungal organism was incubated in medium conditioned by miconazole-MAA-UDMA discs.

Oral epithelial cell-*C. albicans* co-culture systems have been used as *in vitro* study models to examine the interactions between oral mucosal cells and *C. albicans* (Dongari-Bagtzoglou and Fidel, Jr., 2005). OKF6/TERT-2 cells originate from the epithelium of the floor of mouth and are considered a normal epithelial cell line based on growth rate and differentiation patterns (Dickson *et al.*, 2000). Of interest, OKF6/TERT-2 cells respond to *Candida glabrata* by secreting a set of proinflammatory cytokines that are similar in composition and magnitude to the one produced by infected primary oral epithelial cells (Li and Dongari-Bagtzoglou, 2007). Here we report that *C. albicans* strain SC5314 adhered to and covered OKF6/TERT-2 monolayers. However, in the presence of medium conditioned by miconazole-MAA-UDMA discs *C. albicans* formed aggregates, and failed to filament,

adhere to and damage OKF6/TERT-2 cells, demonstrating the efficacy of miconazole released from MAA-UDMA discs against *C. albicans*. These results were expected, as the amount of miconazole released from MAA-UDMA discs exceeded the reported Minimal Inhibitory of Concentration (MIC) of miconazole (0.06–1.00 µg/ml) against *C. albicans* (Yoshida *et al.*, 2006; Ghannoum *et al.*, 2011). Azoles are the most widely used antifungal drugs. They exert fungistatic activity by inhibiting fungal cytochrome P450-dependent enzyme 14 α -sterol-demethylase, an essential enzyme involved in the oxidative removal of the 14 α -methyl group of lanosterol. The inhibition of 14 α -sterol demethylase leads to depletion of sterol biosynthesis and subsequent structural changes in the fungal lipid membrane. Unlike other azoles, miconazole is also thought to have fungicidal activity by promoting accumulation of reactive oxygen species (ROS) in yeast cells (Kobayashi *et al.*, 2002). Along with this, miconazole released from MAA-UDMA discs drastically reduced the number of *C. albicans* cells.

While *C. albicans* suspended in KSFM or drug-free conditioned medium injured more than 50% of OKF6/TERT-2 cells, this fungal organism did not inflict any considerable damage to oral epithelial cells when suspended in medium conditioned by miconazole-MAA-UDMA discs. These results demonstrated the efficacy of miconazole in inhibiting the ability of *C. albicans* to infect and damage oral epithelial cells. OKF6/TERT-2 cells incubated alone in the presence of medium conditioned by drug-free-MAA-UDMA discs showed negligible levels of cell damage, demonstrating the safety of the proposed long-term anticandidal denture resin materials. UDMA and MAA are commonly used in clinical dentistry (De Bruyne and De Moor, 2004; Ahmad *et al.*, 2009). Our results are consistent with previous findings that UDMA and MAA are biocompatible (Fruits *et al.*, 1996; Mount, 1999; De Bruyne and De Moor, 2004). Finally, these results also agree with results from previous studies showing that miconazole is an effective antifungal drug of low toxicity (Sawyer *et al.*, 1975; Cope, 1980; Vandenbosch *et al.*, 2010).

OKF6/TERT-2 has been used to characterize the profile of proinflammatory responses of oral epithelial cells to *C. glabrata* (Li and Dongari-Bagtzoglou, 2007). To our knowledge, the cytokine profiles of OKF6/TERT-2 cells in response to *C. albicans* infection have not been previously documented. The current study showed that uninfected OKF6/TERT-2 cells did not produce IL-1 at detectable levels, but constitutively produced other inflammatory cytokines at low (e.g. IL-1 β , IL-4, IL-10 and GM-CSF) and high (e.g. IL-8 and IL-6) levels. Like in other co-culture systems, production of pro-inflammatory cytokines IL-1 β and IL-1 α by OKF6/TERT-2 cells was remarkably increased in response to *C. albicans* (Dongari-Bagtzoglou and Kashleva, 2003a; Dongari-Bagtzoglou and Fidel, Jr., 2005; Jayatilake *et al.*, 2007). In the current studies, we were unable to demonstrate a statistical significant increase in IL-8 secretion by OKF6/TERT-2 in response to *C. albicans*, as has been previously reported for other epithelial cell lines (Dongari-Bagtzoglou and Kashleva, 2003b; Villar *et al.*, 2004; Villar *et al.*, 2005). However, it is important to note that compared to other oral epithelial cell lines (e.g., SCC4 and SCC15), OKF6/TERT-2 secretes low amounts of IL-8 in response to *C. albicans* (Dongari-Bagtzoglou and Kashleva, 2003a). This discrepancy in findings obtained with different cell types sheds some light on the importance of choosing a specific cell line when studying host/microbial interactions.

Epithelial cell/*C. albicans* co-cultures maintained in drug-free-MAA-UDMA conditioned medium showed a similar pattern of cytokine activation as infected co-cultures maintained in KSFM. Nonetheless, IL-6 levels were increased in infected epithelial cultures maintained in drug-free-MAA-UDMA conditioned medium as compared to the levels measured in infected cultures maintained in unconditioned KSFM. The clinical and biological significance of the increased IL-6 response requires further investigation of MAA-UDMA discs in *in vivo* model. In sharp contrast, medium conditioned by miconazole-MAA-UDMA

discs effectively limited *C. albicans*-induced inflammatory cytokine responses (e.g., increase in IL-1 β and IL-17 levels). Altogether, these results suggest that miconazole released from MAA-UDMA discs can inhibit *C. albicans* infection and reduce oral mucosal inflammatory responses.

The effect of conditioned medium on the expression of *C. albicans* housekeeping gene *ACT1* and virulence factors *SAP2* and *SAP5* was also evaluated. The relative expression of candidal housekeeping gene *ACT1* over human epithelial housekeeping gene β -actin, was drastically reduced in cultures maintained in miconazole-MAA-UDMA conditioned medium. This indicates a decrease in *C. albicans* cell counts in the presence of miconazole, and further confirms the fungistatic effect of miconazole released from MAA-UDMA discs in our oral mucosal system. Expression of *C. albicans* SAP gene family may affect the ability of *C. albicans* to adhere to, invade and damage oral mucosal surfaces and has been associated with *C. albicans* virulence and pathogenesis of oropharyngeal candidiasis (Naglik *et al.*, 2003). In this study, *SAP2* and *SAP5* were expressed in co-cultures maintained in drug-free-MAA-UDMA conditioned medium at levels similar to the ones found in co-cultures maintained in complete KSM. This observation is consistent with previous studies showing that both *SAP2* and *SAP5* are expressed during oropharyngeal candidiasis (Naglik *et al.*, 2008; Lermann and Morschhauser, 2008). Interestingly, the few *C. albicans* cells that survived in co-cultures maintained in miconazole-MAA-UDMA conditioned medium showed increased *SAP2* and decreased *SAP5* expressions. Previous studies have also demonstrated that *C. albicans* *SAP2* expression and activity levels are increased in response to azole exposures (Copping *et al.*, 2005; Barelle *et al.*, 2008; Costa *et al.*, 2010). Nonetheless, the clinical significance of the induction of *SAP2* expression by miconazole still requires further investigation. *SAP5* expression has been associated with hyphal and pseudohyphal transformation and has been detected in clinical cases of oropharyngeal candidiasis (Naglik *et al.*, 2003; Naglik *et al.*, 2008). Down-regulation of *SAP5* expression in co-cultures maintained in miconazole-MAA-UDMA disc conditioned medium may reflect the lack of hyphal formation under these conditions.

In summary, we have expanded our previous characterization of the proposed MAA-UDMA denture material charged with miconazole and demonstrated that the miconazole released from MAA-UDMA discs inhibited the development of candidal infection in an *in vitro* model of oropharyngeal candidiasis without promoting toxic effects on human oral epithelial cells. Future studies should focus on the development, characterization and applications of long-term anticandidal MAA-UDMA dentures in animal models of CADS.

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Figure 1.

Effects of medium conditioned by miconazole-MAA-UDMA discs on OKF6/TERT-2/*C. albicans* co-cultures. OKF6/TERT-2 cells were co-cultured with *C. albicans* SC5314 suspended in drug-free or miconazole-conditioned media or complete KSFM at various epithelial cell (E) to fungal cell (C) ratios (E/C) (1:0, 1:0.1 and 1:1), for 18 h. Representative microscopic results of one of three independent experiments show (A) co-cultures maintained in medium conditioned by drug-free-MAA-UDMA resin discs, and (B) co-cultures maintained in medium conditioned by miconazole-MAA-UDMA resin discs.

Figure 2.

Effects of medium conditioned by miconazole-MAA-UDMA discs on *C. albicans*-induced OKF6/TERT-2 cell damage. OKF6/TERT-2 cells were co-cultured with *C. albicans* SC5314 suspended in conditioned media or complete KSFM at increasing fungal cell to epithelial cell ratios, for 18 h. The release of lactate dehydrogenase (LDH) into the culture supernatants was quantified using CytoTox96® non-radioactive cytotoxicity assay. Data are shown as mean percentage of damaged/dead cells obtained by analysis of quadruplicate wells in at least two separate experiments; Bar graphs represent means $\pm$ SD. * indicates statistically significant differences ($p < 0.05$) for a comparison with OKF6/TERT2 cells co-cultured with *C. albicans* SC5314 suspended in complete KSFM.

Figure 3.

Effects of medium conditioned by miconazole-MAA-UDMA discs on OKF6/TERT-2 cytokine responses to *C. albicans*. OKF6/TERT-2 cell cultures were cultured in control KSFM, drug-free or miconazole-disc conditioned medium with or without *C. albicans* (at an infectivity ratio of 1:1). Cytokine release in culture medium was evaluated using RayBio Cytokine protein array. The density of individual cytokine signals was quantified by densitometry as described in Materials and Methods. Data are shown as means $\pm$ SE of the optical density (O. D) of four independent experiments. * indicates that the p-value is <0.05 for a comparison with untreated OKF6/TERT2 cells; and # indicates that the p-value is <0.01 for a comparison with OKF6/TERT2 cells co-cultured with *C. albicans* SC5314 in KSFM.

Figure 4.

Effects of medium conditioned by miconazole-MAA-UDMA discs on the expression of candidal genes. Total RNAs were extracted as described in Materials and Methods and subjected to RT-PCR and DynAmoTM SYBR® Green qPCR using published primers. All primers resulted in amplification efficiencies of at least 95%. Relative *SAP2* and *SAP5* expression levels were calculated using the 2^{-C_t} method. Expression levels of *C. albicans* *SAP2* and *SAP5* in co-cultures maintained in the presence of miconazole-MAA-UDMA conditioned medium was expressed as fold changes over expression levels in control cultures maintained in drug-free-MAA-UDMA, at both high (1:1) and low (1:0.1) infection ratios. Bar graphs represent means $\pm$ SE of eight independent experiments. * indicates that the p-value is <0.02 for a comparison with drug-free disc conditioned media.

Table 1

Analysis of expression of *C. albicans* ACT1 in the co-culture system.

Medium	Drug-free-MAA-UDMA disc conditioned		Miconazole-MAA-UDMA disc conditioned	
OKF6/TERT-2/SC531	1: 1	1: 0.1	1: 1	1: 0.1
<i>C. albicans</i> ACT1/Human α -actin	1	$0.560 \pm 0.103^*$	$0.031 \pm 0.012^{**}$	$0.018 \pm 0.006^{**}$

The transcription level of *C. albicans* ACT1 relative to the expression of human α -actin was measured at low and high epithelial cell to fungal cell ratios (1:0.1 and 1:1, respectively). The oral epithelial cell/*C. albicans* infection ratio 1:1 was used as control (calibrator) for other cultures in 4 independent measurements (means $\pm$ SE).

*
p<0.02,

**
p<0.001