Candidacidal effect of fluconazole and chlorhexidine released from acrylic polymer

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Received 14 August 2012; returned 18 September 2012; revised 27 September 2012; accepted 16 October 2012

Objectives: To investigate the efficacy and rate of killing of a fluconazole- or chlorhexidine-impregnated polymeric delivery system against fluconazole-susceptible and -resistant Candida albicans and fluconazole-resistant Candida glabrata.

Methods: Poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) discs impregnated with chlorhexidine, pure fluconazole (FLCp) or fluconazole from capsules (FLCc) were prepared by substituting a portion of PEM powder with an equivalent amount of each drug. Discs were incubated in sterile water for 1, 3, 7, 14, 21 and 28 days. The amounts of drugs in the leachates were measured spectrophotometrically and their antifungal activity against fluconazole-susceptible (n = 1) and fluconazole-resistant (n = 2) candidal isolates was determined using a time–kill method and by comparing the released concentrations with the corresponding MICs.

Results: Fluconazole and chlorhexidine leached from PEM/THFM polymer for up to 28 days and the released concentrations were fungicidal against all three Candida isolates for at least the first 7 days. Chlorhexidine leachates killed all Candida isolates more rapidly than the two fluconazole formulation leachates throughout the study period. FLCc leachates required longer incubation for 100% killing than FLCp leachates. The proportion of viable C. glabrata dropped more slowly than that of C. albicans with the same MIC.

Conclusions: The concentrations of chlorhexidine and fluconazole leached from the PEM/THFM polymer were fungicidal against all Candida isolates, including those resistant to fluconazole, for the first 7 days. Chlorhexidine leachates showed a rapid fungicidal activity for up to 4 weeks, which can be of use in cases with poor response to conventional antifungals.

Keywords: Candida, leachates, biomaterial infection, antimicrobials, poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate

Introduction

Oral candidosis is a prevalent problem among the elderly and medically compromised patients.1,2 It is a complex inflammatory process involving formation of candidal–bacterial biofilms on the various natural and artificial surfaces present in the oral cavity.3 Candida species are common oral colonizers and they readily form mixed-species biofilms on non-renewing surfaces such as teeth, dental fillings, intubation tubes and dentures. These biofilms form a potential source of systemic infection, especially as they are inherently resistant to antimicrobial treatment.4 They are also associated with a high level of mucosal inflammation locally.2–4

Peroral fluconazole is commonly used for treatment, as it is well tolerated with few side effects.4 However, microbiological and clinical resistance is a major clinical concern. This has been associated with the selection and emergence of fluconazole-resistant Candida species such as Candida glabrata, as well as poor efficacy against candidal–bacterial biofilms.4–6 Moreover, in patients with reduced saliva production, therapeutic drug levels are difficult to achieve in the oral cavity with systemic therapy.7 Interestingly, alternative topical agents such as chlorhexidine have shown excellent efficacy against Candida species.4,6,9 In addition, chlorhexidine has anti-biofilm activity with low MIC values.8,10
One of the main challenges in topical treatment is the rapid clearance of an administered drug from the site of infection by saliva.\textsuperscript{11} Moreover, rigid patient compliance with frequent drug administration is paramount for an optimal outcome.\textsuperscript{11} Therefore, alternative approaches are needed. Local self-release delivery systems have been suggested as an alternative approach for achieving and maintaining therapeutic drug levels at the site of infection.\textsuperscript{12–15} Schneider\textsuperscript{16} showed that leachates of discs made from soft acrylic lining material impregnated with chlorhexidine, fluconazole, nystatin or clotrimazole inhibited candidal growth. It has also been demonstrated that chlorhexidine and fluconazole are steadily released in anti-candidal concentrations from a hard poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) acrylic for up to 4 weeks.\textsuperscript{17} However, the fungicidal potential and the rate of killing of such leachates have not been studied previously.

The aim of the present study was to investigate the efficacy of a fluconazole- or chlorhexidine-impregnated PEM/THFM delivery system against Candida albicans and fluconazole-resistant C. glabrata using a time–kill approach. We also wanted to compare the rate of killing of the two agents and the impact of the fluconazole formulations. Fluconazole powder is available in two formulations: pure powder and capsules, which contains both active compound and excipients.

Materials and methods

Study design

Acrylic discs were prepared using PEM/THFM impregnated with chlorhexidine, pure fluconazole (FLCp) or fluconazole from capsules (FLCc) by substituting a portion of the PEM powder with an equivalent amount of each drug. The drug-free control discs were prepared following the manufacturer's instructions. All discs were incubated in sterile distilled water at 37°C for 28 days. The leachates were collected and water was replaced at 1, 3, 7, 14, 21 and 28 days. The amounts of released drugs were measured using a spectrophotometer and the antifungal activity of the leachates was determined using a time–kill method. The MIC of each drug for the Candida isolates was determined and compared with the released concentrations. The experiment was performed in quintuplicate and all leachates were analysed in parallel.

Preparation of PEM/THFM discs

For the control discs, 1 g of PEM (Lucite International, Durham, UK) and 0.6 mL of THFM (Sigma–Aldrich, Dorset, UK) were mixed. For the impregnated discs, 10% (w/w) of the PEM was replaced with HPLC grade 98% pure chlorhexidine diacetate powder (Sigma–Aldrich), 10% (w/w) with FLCp (Pfizer, Kent, UK) or 25% (w/w) with FLCC (Pfizer), resulting in 100 mg of active drug per disc for all. Powder from fluconazole capsules contains a large amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25% of it is equivalent to 10% of FLCp. The drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed. All mixtures were packed into disc-shaped steel moulds (40 mm diameter and 0.5 mm height) and allowed to cure for 15 min.

Disc incubation

Each disc was soaked individually in 20 mL of sterile distilled water at 37°C in tightly sealed plastic flasks. The flasks were gently shaken by hand three times daily throughout the experiment to simulate the oral environment. The leachates of the drug-free control discs were used as negative controls.

Drug concentration measurement

The amount of drug released was quantified using a UV spectrophotometer at 220 nm (Shimadzu, Kyoto, Japan).\textsuperscript{18} Triplicate 10-fold dilution series of the leachates were analysed at 1, 3, 7, 14, 21 and 28 days. The absorbance readings of the drug-free control leachates were used as a reference. The unknown concentrations of drug in each leachate were calculated using standard curves of known concentrations of chlorhexidine or fluconazole.

Isolates and inoculum preparation

One Candida strain susceptible to fluconazole and two isolates resistant to fluconazole were used: C. albicans ATCC 90028 (MIC 0.25–1 mg/L), and C. albicans F/2511 and C. glabrata FA023 (MIC >64 mg/L reported for both). The two latter isolates were obtained from the culture collection of the Mycology Reference Centre (Manchester, UK). Isolates were grown on Sabouraud agar for 48 h and then a couple of colonies were inoculated into 10 mL of distilled water and the cell density was adjusted spectrophotometrically (BMG Labtech, Aylesbury, UK) to 1.0 × 10^8 cells/mL and further diluted to a final cell density of 1.0 × 10^5 cells/mL (working dilution).

MIC determination

MICs were determined for FLCc, FLCp and chlorhexidine using CLSI M27-A3 microdilution methodology.\textsuperscript{19} Briefly, 2-fold dilution series of FLCp and FLCC (0.125–2048 mg/L) and chlorhexidine (0.1–50 mg/L) were prepared in sterile distilled water and an inoculum of 1 × 10^3 organisms/mL was used. After 48 h of incubation at 37°C, the growth in each well was measured by spectrophotometry (BMG Labtech) at 490 nm. For fluconazole, the MIC was the lowest drug concentration that reduced the OD_{490} by 50% compared with the drug-free control. The CLSI standard breakpoints for fluconazole were used for susceptibility interpretation.\textsuperscript{20} For chlorhexidine, the MIC was the lowest drug concentration that reduced the OD_{490} by 80% compared with the drug-free control.\textsuperscript{20}

Time–kill studies

Time–kill studies were carried out following standard protocols.\textsuperscript{21} To detect the fungicidal activity of the leachates, 100 μL of the working dilution was transferred to sterile tubes containing 900 μL of leachate of chlorhexidine, FLCp, FLCC or the drug-free control discs. The tubes were incubated at 37°C and aliquots were removed at 1, 2, 4, 6 and 24 h post-incubation. Ten microlitre aliquots of the neat sample and two 10-fold serial dilutions were plated on Sabouraud agar (Oxoid, Basingstoke, UK) and were incubated for 48 h at 37°C to test for viability. This was repeated for all leachates collected at 3, 7, 14, 21 and 28 days and against the three isolates tested. Viability counts of the working dilutions were used as controls. A fungicidal effect was defined as 100% kill. Three hundred data points were collected for each of the six time intervals of leaching: four disc types, five replicates, five time–kill measurements and three isolates.

Statistical analyses

One-way ANOVA was used to analyse all data and the significance level was set at P ≤ 0.05. All data were tested using Levene’s test for homogeneity of variance (P ≤ 0.05), following the assumption of equal variances. Equal variances were confirmed (P > 0.05), hence the Bonferroni post
hoc test was used to determine the differences in the leached amounts of the impregnated drugs at each time interval.

**Results**

The impregnated drugs were released from the PEM/THFM discs over the 28 day period with a high rate of initial leaching followed by controlled slow release (see key in Figures 1–3). At all timepoints, the rate of release of chlorhexidine and FLCc was significantly higher compared with FLCp ($P \leq 0.05$). The MIC of chlorhexidine for all three isolates was 6.25 mg/L. The MIC of FLCp and FLCc for *C. albicans* ATCC 90028 was 0.25 mg/L and was 128 mg/L for both *C. albicans* F/2511 and *C. glabrata* F/4023.

For chlorhexidine and FLCp, all leachates with concentrations above the MIC (range 173–1019 mg/L, and 68–195 mg/L, respectively) led to 100% killing of all three isolates during 24 h of incubation. No significant killing was detected for leachates with concentrations below the MIC (Figures 1 and 2). All FLCc leachates (concentration range 116–834 mg/L) resulted in 100% killing of the fluconazole-susceptible strain. Of the two fluconazole-resistant isolates, 100% killing was detected with leachates collected during the first 7 days (concentration range 262–834 mg/L; Figure 3). More than 2 times the MIC was required for 100% killing with the FLCc compared with 1.2 times the MIC with FLCp. A concentration of 1.95 times the MIC of FLCc resulted in 60% killing of the resistant isolates in 24 h of incubation.

Leachates from chlorhexidine-impregnated discs collected at all time intervals resulted in 100% killing of all three *Candida* isolates during 60 min of incubation (Figure 1). All FLCp leachates led to 100% killing of the fluconazole-susceptible *C. albicans*.

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**Figure 1.** Representative time–kill curve plot for *C. albicans* ATCC 90028, *C. albicans* F/2511 and *C. glabrata* F/4023 of leachates of different time intervals of chlorhexidine-impregnated discs. The MIC of chlorhexidine for all isolates was 6.25 mg/L. Numbers after key symbols represent the concentrations measured in each leachate. CTR, control; D, days leachates collected.

**Figure 2.** Representative time–kill curve plots for (a) *C. albicans* ATCC 90028, (b) *C. albicans* F/2511 and (c) *C. glabrata* F/4023 of leachates of different time intervals of FLCp-impregnated discs. The MIC of FLCp for *C. albicans* ATCC 90028 was 0.25 mg/L, while the MIC of FLCp for *C. albicans* F/2511 and *C. glabrata* F/4023 was 128 mg/L. Numbers after key symbols represent the concentrations measured in each leachate. CTR, control; D, days leachates collected.

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**Figure 3.**
ATCC 90028 within 60 min (Figure 2a). For the fluconazole-resistant C. albicans F/2511, 100% killing was achieved within 60 min with FLCp leachates collected during the first 7 days (Figure 2b). For C. glabrata F/4023, 100% killing was seen at the 2, 4 and 24 h timepoints with leachates collected on days 1, 3 and 7, respectively (Figure 2c). The killing efficiency of FLCp leachates collected after 7 days was insignificant against both C. albicans F/2511 and C. glabrata F/4023 (Figure 2b and c). All FLCc leachates led to 100% killing of C. albicans ATCC 90028 within 24 h of incubation (Figure 3a). For C. albicans F/2511, 100% killing was seen in 4 h of incubation with leachates collected on day 1 and in 24 h of incubation with leachates collected on days 3–7 (Figure 3b). For C. glabrata F/4023, leachates collected during the first 7 days resulted in 100% of killing during 24 h of incubation. FLCc leachates collected between days 7 and 14 resulted in 60% killing during 24 h of incubation compared with 20% for equivalent FLCp leachates of both C. albicans F/2511 and C. glabrata F/4023 (Figure 3b and c).

Discussion

The present study shows that fluconazole and chlorhexidine become readily leached from the PEM/THFM polymeric system for up to 28 days and that the released concentrations were fungicidal against the three Candida isolates, including the two isolates resistant to fluconazole, for at least the first 7 days. Significant differences between the rates of killing of the released agents were detected. Chlorhexidine leachates killed all Candida isolates more rapidly than either of the two fluconazole formulation leachates. Interestingly, FLCc leachates required longer incubation for 100% killing than FLCp leachates, although higher concentrations of active drug were measured in the leachates at all timepoints and the MIC of both fluconazole compounds was the same for the isolates. In addition, marked differences in the rate of killing between the two fluconazole-resistant isolates were detected with both fluconazole formulations: the proportion of viable C. glabrata F/4023 decreased more slowly than that of C. albicans F/2511 with the same MIC. A PEM/THFM polymer impregnated with either fluconazole or chlorhexidine has previously been shown to have antifungal activity, but the rate of killing or the impact of the formulation used has not been studied before.14,17 All chlorhexidine leachates were equally effective and rapid in killing fluconazole-resistant and -susceptible isolates for up to 28 days. This can be explained by the high concentrations of chlorhexidine released exceeding the MIC values (6.25 mg/L) for all isolates at all time intervals. This can also be further explained by the rapid uptake of chlorhexidine by C. albicans and C. glabrata, which has been reported previously.22 The present work extends the findings of previous studies where a
similar polymeric system impregnated with chlorhexidine or fluconazole was used and efficacy against fluconazole-resistant *C. albicans* was seen,13,23 However, in these previous studies the kinetics of killing and the impact of different fluconazole formulations were not defined. The present study shows that chlorhexidine leachates killed all *Candida* isolates in 1 h compared with the two fluconazole formulation leachates, where up to 24 h was required.

Significantly higher concentrations of fluconazole were detected in leachates from discs impregnated with FLCc compared with those impregnated with FLCp, as reported previously using a different detection method.17 However, despite a greater than four times higher concentration of active drug for day 1 leachates of FLCc, 4–24 h was required for 100% killing in contrast to 1–2 h for the FLCp leachates. Both of the fluconazole-resistant isolates were killed with the FLCp leachates when the concentration was higher than the MIC but twice the MIC was required for this with the FLCc leachates. When killing of the fluconazole-resistant isolates was seen, it was slower than that of the susceptible isolate. In addition, the killing of *C. glabrata* was slower than that of the fluconazole-resistant *C. albicans*, which is in agreement with previous reports.24 These unexpected differences between the two formulations may be due to the excipients in FLCc, which may interfere with drug uptake by *Candida* and modify the action of the drug. A slow rate of killing has significant consequences, as it may lead to the selection and emergence of resistance.4

The findings of the current study clearly demonstrate that both chlorhexidine and fluconazole are readily leached from the polymer and the antifungal activity of the impregnated agents was not affected by acrylic polymerization even after prolonged incubation. A time–kill approach was used in this study to show precisely how quickly the agents act against the tested isolates, which was a limitation of previously used methods such as cfu counts and well diffusion tests.14,15 Although a limited number of isolates were studied, the two species included in this study, *C. albicans* and *C. glabrata*, represent those most commonly associated with denture stomatitis.2 Importantly, the activity of the leachates against the three indicator isolates corresponded with the susceptibility of the isolates to the pure compounds. Based on our findings, the released amounts of the antimicrobial agents were the highest during the first day (20 mg of chlorhexidine and 17 mg of FLCp). For an average full denture, this would be equivalent to 37 mg of chlorhexidine and 30 mg of fluconazole.25 These amounts are safe and within the recommended doses.26,27 Impregnation with antimicrobials presents a challenge to the physical and mechanical properties of a polymer. This is also the case with the PEM/THFM polymer used in the present study. However, despite a reduction in all tested physicomechanical properties (degree of conversion, shear strength, water sorption and colour stability), all values are still within acceptable ranges and similar to those reported for some other acrylic liners when tested without impregnation.28,29 As this is an *in vitro* study, further clinical studies are warranted to determine its clinical applicability.

In conclusion, a polymer containing either chlorhexidine or fluconazole is a promising treatment modality and effective amounts of drugs could be released at the site of pathology. The rapid kill rate of chlorhexidine compared with fluconazole is encouraging and may prove to be an effective option, e.g. when azole antifungal treatment fails, in immunocompromised patients with a need for long-term treatment or when fungal biofilms are suspected. Chlorhexidine impregnation has also been shown to effectively prevent biofilm formation on acrylic discs for up to 28 days.30 Using impregnated lining materials resistant to reinfection and leaching fungicidal amounts of drug capable of killing adjacent mucosa-bound *Candida* could be an efficient approach to the treatment of foreign body-associated infections. Moreover, the common side effects experienced with chlorhexidine mouth rinse, including superficial staining of the teeth and oral mucosa and gustatory dysfunction, can be minimized by the targeted release of the delivery device. On the other hand, the well-tolerated fluconazole appears to have potential for the treatment of uncomplicated infections in otherwise healthy patients where the risk for development of resistance is limited. Combining chlorhexidine and fluconazole will need further studies to investigate any possible synergistic effect, but this could be a promising therapy.

**Funding**

This work was supported in part by the University of Jordan (to N. Salim), The University of Manchester Research Funds (to all) and the NHS National Commissioning Group Chronic Pulmonary Aspergillosis National Service, UK (to R. R.).

**Transparency declarations**

None to declare.

**References**


