Glass fibre-reinforced composite laced with chlorhexidine digluconate and yeast adhesion

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The aim of this study was to lace dental glass fibre reinforced composite (FRC) prepreg with chlorhexidine digluconate and to examine the adherence of common oral fungal pathogen *Candida albicans* to FRC made of the prepreg. Four different test and control material groups each comprising 16 test specimens ($(5.0 \times 5.0 \times 0.8)\ mm^3$) each were used as substrates for *C. albicans* adherence. A porous polymer pre-impregnated woven glass fibre prepreg was laced with solution of chlorhexidine gluconate and it was used with autopolymerized denture base polymer to fabricate FRC test specimens. Control group (Group 1) consisted of FRC test specimens stored in water. In Group 2, the test specimens were stored in 10% chlorhexidine digluconate solution for 24 h. Group 3 consisted of specimens fabricated using such fibre reinforcements which were pre-soaked in 20% chlorhexidine digluconate and dried before preparation with denture base resin, and followed by storage of the specimens in water. Group 4 was similar to Group 3 but instead of water storage the specimens were immersed in 10% chlorhexidine digluconate for 24 h. For the candidal adhesion assay the test and control specimens were incubated in standardized suspensions of four different strains of *C. albicans*, rinsed and prepared for light-microscopy. The mean number of adherent cells in each group was counted microscopically and analysed statistically. There were significantly ($P < 0.05$) more adherent *C. albicans* cells found in Group 1 than in the other three groups which did not differ significantly from each other. The lowest numbers of adherent cells were found in Group 3. Pretreating the porous polymer pre-impregnated glass fibre reinforcement with chlorhexidine digluconate result in reduction in the number of adherent yeast cells on the surface FRC material.

1. Introduction

The use of fibre-reinforced composites (FRC) in dentistry has rapidly increased during last years. FRCs are used in denture bases, permanent and temporary fixed partial dentures, periodontal splints, and orthodontic appliances [1–5]. In these applications, the biomechanical and adhesive properties of FRCs have justified their use. Discussion has risen about using FRCs as vehicle material for some antimicrobial agents to the oral cavity. In FRC appliances having a limited usage time, e.g. temporary bridges or temporary dentures, it could be hypothesized that fibre reinforcement can be a reservoir for some antimicrobial agents. This may help to reduce plaque accumulation dental appliance and reduce local tissue infections.

Earlier studies have shown that incubation of denture base polymer in chlorhexidine digluconate solution decreases the number of adherent of yeasts *in vitro* [6, 7]. This effect appears to be based on the perfusion of chlorhexidine into the denture base polymer and its subsequent diffusion from the polymer. Therefore, it seems that even a relatively small quantity of chlorhexidine on the denture surface may affect initial yeast adherence. The successful combination of fibre reinforcement with highly viscous denture base resins requires polymer pre-impregnation of the glass fibres as recently introduced by Vallittu [8].

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Oral *Candida* species, especially *C. albicans*, are often associated with denture stomatitis [9–11]. These yeasts together with bacterial flora of denture plaque, adhere to and accumulate on the denture surface which acts as a reservoir of microorganisms being a chronic source of mucosal irritation [12]. The ability of yeasts to adhere to polymeric surfaces is due to attractive London–van der Waals forces (hydrophobic forces) and electrostatic forces [12–14]. These forces, which initiate adherence of yeasts and bacteria also offer an opportunity for further microbial bonding and denture plaque formation. Factors such as saliva, serum and other microorganisms and differences in surface topography and chemistry may affect this complicated process [6, 15–17].

The aim of this *in vitro* study was to examine the adherence of *C. albicans* to denture base polymer reinforced with E-glass fibres enriched with chlorhexidine digluconate.

2. Material and methods

2.1. Test specimens

The denture base resin used in this study was autopolymerized denture base material (Palapress®, Heraeus Kulzer, Wehrheim, Germany; powder lot 012151, liquid lot 010984) reinforced with a commercially available Stick Net™ (Stick Tech Ltd., Turku, Finland; lot 1990906-W-0037) woven E-glass fibre reinforcement pre-impregrnated with porous polymethyl methacrylate (PMMA, Mw 220.000) [8]. The pre-impregnation polymer allowed the formation of a well impregnated FRC material with highly viscous mixture of denture base resin powder and monomer liquid. The Palapress® resin contained methylmethacrylate (MMA) and butanedioldimethacrylate (BDMA) monomers with a barbituric acid–copper ion initiator system. The pre-impregnated glass fibre reinforcements were further impregnated with a mixture of polymer powder and monomer liquid of Palapress® resin, after which the reinforcements were embedded into the resin mixture. The powder/liquid ratio of the resin was 1.90/1.00 by weight and polymerization of the resin was carried out in water at (55 ± 1) °C under air pressure of 300 kPa for 15 min. Thickness of the glass fibre reinforcement was 0.06 mm and one layer of reinforcement was incorporated to the rhombic test specimens of a size $(5.0 \times 5.0 \times 0.8)\,mm^3$.

Four different material groups, each with 16 individual test specimens were tested. Group 1 consisted of FRC prepared as above and stored in sterile tap water for 24 h in order to remove majority of the residual MMA monomers and to be water saturated [18]. Water immersion time of 24 h was selected after storing six test specimens in water for 50 h and measuring the weight increase of the specimens by water sorption (Fig. 1). Group 2 was similar to Group 1 but instead of water storage the specimens were stored in 10% chlorhexidine digluconate solution (medical grade, lot ya 9901028, University Pharmacy, Turku, Finland) for 24 h. The test specimens of Group 3 were fabricated from the polymer pre-impregnated fibre reinforcements which were immersed in 20% chlorhexidine digluconate solution for 1 min and dried before preparation of the test specimens. During the 1 min immersion time the porous prepolymer matrix of the reinforcement absorpted chlorhexidine digluconate which was ensured by measuring the weight increase of the fibre reinforcement (Fig. 2). The dried reinforcements were used in fabrication of test specimens as described above and the test specimens were then stored in water for 24 h. Group 4 was similar to group 3 but instead of water storage the specimens were immersed in 10% chlorhexidine digluconate solution for 24 h before yeast adhesion assay.

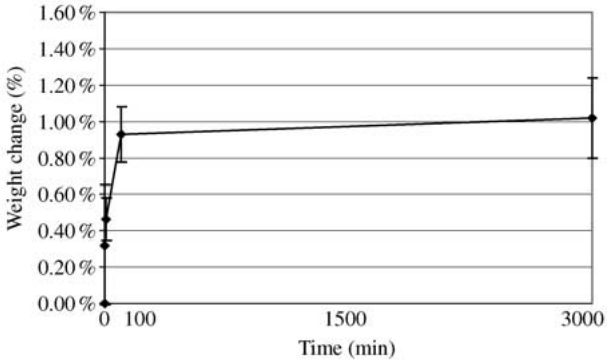

Figure 1 Weight increase (%mean ± SD) of the FRC test specimens in water showing water saturation of the test specimens ($n = 6$).

2.2. Microbiological procedures

2.2.1. Organisms and growth conditions

Candida albicans was selected as testing microbe because well established methodology in determining its adherence on dental materials. Strains of *Candida albicans* used were Ca1 (ATCC 90028) and three oral isolates, Ca2–Ca4. The organisms were identified by a commercially available API 20C Aux identification kit (Analytical Profile Index; Bio Merieux SA, France). Stock cultures were maintained at 4 °C. After recovery these were maintained on Sabouraud dextrose agar (Oxoid Ltd, Basingstoke, Hampshire, England), stored at 4–6 °C during the experimental period. Purity of cultures was ensured by regular random identification of isolates by techniques described above. A loopful of stock culture was incubated on a Sabouraud-agar plate in air at 37 °C for 18 h. Four loopfuls of this culture were transferred to Sabouraud dextrose broth (Oxoid Ltd,

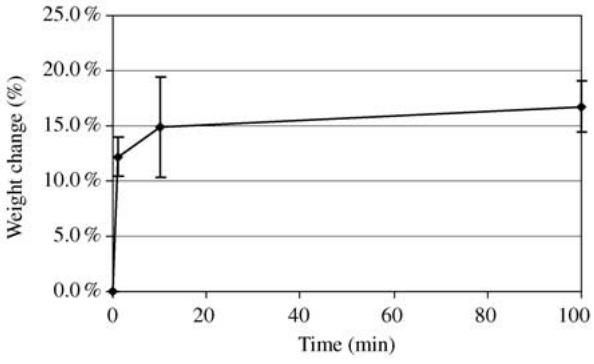

Figure 2 Weight increase (%mean ± SD) of the woven polymer pre-impregnated glass fiber reinforcement incubated in chlorhexidine digluconate solution ($n = 6$).

TABLE I Mean adherence ± SD of four strains of *C. albicans* (Ca1–Ca4) to different groups of test specimens (adherent cells per microscope field ($\sim 1.6\,mm^2$))

Group	Isolate	Mean[1]	SD	Mean of group	SD of group
1	Ca1	51.25	7.41		
	Ca2	63.00	15.98		
	Ca3	59.25	22.95		
	Ca4	65.75	15.02	59.81	6.30
2	Ca1	51.25	13.72		
	Ca2	41.75	18.55		
	Ca3	39.75	19.60		
	Ca4	43.75	20.84	44.13	5.02
3	Ca1	36.50	9.61		
	Ca2	34.75	10.14		
	Ca3	42.50	17.33		
	Ca4	41.00	14.21	38.69	3.66
4	Ca1	41.00	6.16		
	Ca2	47.25	20.76		
	Ca3	51.25	13.25		
	Ca4	35.75	16.68	43.81	6.83

[1] $n = 4$.

Basingstoke, Hampshire, England) enriched with sucrose ($500\,mmol\,l^{-1}$) (KEBO Lab, Oslo, Norway) and incubated at 37 °C for 24 h. The culture was centrifuged at 1500 *g* for 10 min and the deposit washed twice with PBS. A final, spectrophotometrically standardized, yeast suspension of approximately $\sim 1 \times 10^7\,cfu\,ml^{-1}$ was prepared in PBS.

2.2.2. Adhesion assay

In brief, the candidal adhesion assay was performed according to Samaranayake and MacFarlane [6]. The prepared test specimens were placed vertically in the wells of a sterile serology plate (Corning Glass Works, Corning, NY, USA), each well measuring 5 mm in diameter. Approximately 0.4 ml of the yeast suspension was added to each well and incubated with gentle agitation at 75 rpm at 37 °C for 1 h. The test specimens were then removed from the wells, washed in sterile distilled water, air dried, and stained using a modified Gram-stain, without counter staining. Thereafter, the test specimen were dried at room temperature and mounted on glass slides with Permount®-glue (Fisher Scientific, Fair Lawn, NJ, USA). Randomly selected 20 fields were counted for each sample at 400× magnification using light microscopy. The mean number of adherent yeast cells per field was expressed as cells per microscope field ($\sim 1.6\,mm^2$). Each experiment with each isolate/test specimen combination was performed on two separate occasions, with two strips on each occasion.

2.2.3. Statistics

The statistical differences between the drug-free control and drug exposed test groups were analysed by the nonparametric Mann–Whitney U test, using the GraphPad Instat Program (GraphPad Software Corp, San Diego, USA). P value of < 0.05 was considered statistically significant.

3. Results

3.1. Adherence of yeasts to test and control samples

When the mean adhesion levels of the yeasts to four different test specimens were compared the lowest numbers of adherent cells were found in Group 3. This group with chlorhexidine laced polymer and immersed in water had a mean of 38.69 yeasts per unit area compared with 59.81, 44.13 and 43.81 yeasts per unit area in Groups 1, 2 and 4, respectively (Table I). Thus, there were significantly more adherent *C. albicans* cells found in Group 1 without chlorhexidine than in the other three groups which either contained chlorhexidine-laced polymer (Groups 3 and 4) or normal polymer immersed in chlorhexidine (Group 2) ($P < 0.05$). There was no significant difference in yeast adherence to the acrylic specimens in the latter three groups (Groups 2, 3 and 4), which were exposed to chlorhexidine in one way or another ($P > 0.05$).

3.2. Intra-species variation in yeast adhesion

On analysis of the intra-species variation between the four *C. albicans* isolates tested no significant difference could be noted in their adhesion ($P > 0.05$).

4. Discussion

The adherence of *C. albicans* to denture base polymer reinforced with E-glass fibres enriched with chlorhexidine digluconate was studied. The method used was a standard technique [6] that is widely used to study candidal adhesion to prosthetic substrates [19]. The very low standard deviation of the emanating data (ranging from 3.66 to 6.83 yeasts per unit area) indicates the sensitivity of the method and validates its use in the present study.

We employed four strains of *C. albicans* in this study as previous workers have shown significant intra-species variation in candidal adhesion [16]. However, we were unable to show significant intra-species variation in the

four *Candida* strains used, irrespective of the varying experimental conditions and this lends further credence to the data obtained.

To our knowledge, this is the first study to investigate the effect of chlorhexidine incorporation in fibre reinforced material on *C. albicans* adherence. We were particularly interested in administration of chlorhexidine digluconate in porous polymer pre-impregnated glass fibre reinforcement as a reservoir source. This was controlled with a nonchlorhexidine group (Group 1) and two groups of FRC specimens with and without chlorhexidine pretreated fibres (Groups 2 and 4). A well-established methodology was chosen to study the adherence of yeasts to obtain data comparable with previous studies. The water absorption of the specimens was controlled in order to remove residual monomers [18, 20–22], which may affect the adherence of yeasts as suggested recently [23].

Earlier studies have shown that chlorhexidine incubated either together with yeast cells or test specimens decreases the adherence of *C. albicans* to acrylic surfaces [15, 24]. This seems to be due to the ability of chlorhexidine to affect cell membrane and cell wall characteristics [25]. The results of the present study are in accordance with these previous studies showing decreased candidal adherence in all the three groups with chlorhexidine exposure in comparison to the chlorhexidine-free control. In the present study, chlorhexidine caused approximately one-third reduction in the number of adherent cells. Samaranayake *et al*. [24] have shown a dose-response between yeast adherence and chlorhexidine, i.e. higher concentration of chlorhexidine in the immersion liquid of the test specimens caused stronger inhibition of adherence. Further studies are needed to clarify whether this dose-dependency can be achieved also using the reinforcement as medicament vehicle. In the present study, both groups of specimens incubated in chlorhexidine (Groups 2 and 4) had identical numbers of adherent yeast cells. However, Group 3 with chlorhexidine-laced reinforcement with water-storage showed even lower number of cells than Group 4 which was similar in all aspects except that it was incubated in chlorhexidine solution. However, this difference was not statistically significant.

From a clinical perspective, the use of chlorhexidine pretreated glass fibre reinforcement in denture bases could be beneficial for the management of denture-induced stomatitis. The current concept of using FRCs in denture base polymers is based on the use of partial fibre reinforcements where a relatively small quantity of fibres is polymerized onto the fracture initiation region of the denture [2, 4]. This approach to use fibre reinforcement may not be ideal for the use of reinforcement as a reservoir for chlorhexidine. The use of total fibre reinforcement could be more beneficial as a delivery mode of chlorhexidine. The total fibre reinforcement entails including woven or chopped fibres into the entire denture base plate as described by Ladizesky *et al*. [1] and Braden *et al*. [5]. By this, the distribution of the chlorhexidine enriched fibre reinforcement would be more uniformly distributed over the palatal mucosa. The approach can also be used as a part rebasing of dentures by autopolymerizing denture base resins and chlorhexidine enriched fibre reinforcement. Other possible applications of chlorhexidine enriched fibre reinforcement could be in provisional fixed partial dentures or periodontal splints that are reinforced with glass fibres [3, 26].

Clinical importance of *in vitro* studies can always be questioned. However, this type of development of materials with properties that inhibit microbial colonization is of particular interest in both medicine and dentistry, particularly when dealing with medically compromised patient groups that are highly susceptible to infections with opportunistic pathogens. However, further studies are needed to evaluate the possible adverse effects of the chlorhexidine pretreated fibre reinforcements with regard to mechanical and biological properties of these materials. Furthermore, more information is needed on the long-term influence of the chlorhexidine treatment on the microbial adhesion, possible emergence of resistance strains as well as of its effects on host tissues during prolonged contact.

5. Conclusions

Pretreating the porous polymer pre-impregnated glass fibre reinforcement with chlorhexidine digluconate result in a reduction in the number of adherent yeast cells on the surface FRC material.

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