Ions released from a S-PRG filler induces oxidative stress in *Candida albicans* inhibiting its growth and pathogenicity

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Received: 7 March 2018 / Revised: 10 May 2018 / Accepted: 30 May 2018 / Published online: 6 June 2018
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Abstract

Candida albicans causes opportunistic fungal infections usually hidden among more dominant bacteria and does not exhibit high pathogenicity in vivo. Among the elderly, due to reduced host resistance to pathogens attributable to immunosenescence, oral candidiasis is more likely to develop often leading to systemic candidiasis. Surface pre-reacted glass ionomer filler (S-PRG filler) is an ion-releasing functional bioactive glass that can release and recharge six ions which in turn strengthens tooth structure, inhibits demineralization arising from dental caries, and suppresses dental plaque accumulation. However, its effects on *C. albicans* have never been elucidated. Here, we evaluated the effects of ion released from S-PRG filler on *C. albicans*. Results show that extraction liquids containing released ions (ELIS) decreased the amount of hydrogen peroxide and catalase activity in *C. albicans*. Moreover, ELIS presence was found to affect *C. albicans*: (1) suppression of fungal growth and biofilm formation, (2) prevent adherence to denture base resin, (3) inhibit dimorphism conversion, and (4) hinder the capability to produce secreted aspartyl proteinase. Taken together, our findings suggest that ELIS induces oxidative stress in *C. albicans* and suppresses its growth and pathogenicity. In this regard, we propose that ELIS has the potential to be clinically used to help prevent the onset and inhibition of oral candidiasis among the elderly population.

Keywords *Candida albicans* · Oxidative stress · S-PRG filler · Pathogenicity

Introduction

Candida albicans is a fungus most frequently detected as part of the indigenous microbial flora found in the oral cavity (Cannon and Chaffin 1999). Moreover, opportunistic infections and systemic diseases associated to this fungus include: pulmonary mycosis, gastrointestinal mycosis, mycosis due to compromised immune function, and superinfection following prolonged use of antimicrobial agents (Alasio et al. 2003; Clemons and Stevens 2001; Pace and McCullough 2010; Zupanić-Krmek and Nemet 2004). In the elderly oral cavity, *C. albicans* easily thrive attributable to the high rate of denture use within the elderly population (Salerno et al. 2011; Yoshida et al. 2001) and, likewise, *C. albicans* has several known

pathogenic factors that greatly contribute to fungal pathogenicity (Kim and Sudbery 2011; Mayer et al. 2013). Both *C. albicans* growth and pathogenicity contribute to oral candidiasis and other related infections within the elderly population which would highlight the growing importance of developing oral antifungal therapies (Salerno et al. 2011; Yoshida et al. 2001; Kim and Sudbery 2011; Mayer et al. 2013). Additionally, an ideal strategy to cater to oral candidiasis infection would require a holistic approach, wherein, both *C. albicans* growth and pathogenicity would be inhibited.

Surface pre-reacted glass ionomer (S-PRG) filler is an ion-releasing functional bioactive glass where it releases six ions (fluoride, strontium, sodium, aluminum, boric acid, and silicic acid) (Fujimoto et al. 2010). During high ion concentrations, ions are recharged and re-released from the S-PRG filler which in turn have various effects: (1) strengthening of tooth structure, (2) inhibition of tooth demineralization, (3) buffering capacity against intraoral acids, and (4) inhibition of dental plaque adhesion on the resin surface (Kaga et al. 2014; Kotaku et al. 2014; Hotta et al. 2014). At present, S-PRG filler has already been used clinically by being incorporated into filling materials, bonding systems, temporary cements, and

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orthodontic resins (Nakamura et al. 2009). However, its potential application during fungal infection particularly among the elderly has not been elucidated.

Here, we evaluated the effects of extraction liquid containing the ions released from the S-PRG filler (S-PRG ion extraction liquid: ELIS) on *C. albicans* oxidative stress induction. Moreover, we demonstrated the effects of ELIS on *C. albicans* growth, biofilm formation, and pathogenicity.

Materials and methods

Fungal cells and materials

C. albicans strain tested was NUD202 (a clinical isolate). S-PRG filler, produced according to a previously published method, was obtained from SHOFU Inc. (Kyoto, Japan). S-PRG filler was mixed with an equal amount of distilled water under constant stirring for 24 h. Subsequently, we centrifuged and filtered the solution to separate the powder and liquid components. Recovered filtrate (ELIS) served as the test sample used throughout this study (Fujimoto et al. 2010). Element analysis of the ELIS components was conducted using both an inductively coupled plasma atomic emission spectroscopy (ICP-AES; ICPS-8000, Shimadzu Corp., Kyoto, Japan) and a fluoride ion electrode method (fluoride electrode, model 9609BN; pH/ion meter, model 720A; Orion Research Inc., Beverly, MA, USA).

Hydrogen peroxide and catalase measurement

Fungus was inoculated into Sabouraud glucose (SG) broth containing ELIS at varying dilutions (1:8, 1:16, 1:32, and 1:128). Subsequently, inoculated samples were incubated aerobically at 37 °C for 24 h. Fungus was harvested through centrifugation at 5000×g for 10 min under 4 °C chamber conditions and washed twice with PBS. Cells, approximately 1.0×10^8 , were suspended in 150 µL solution containing 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, and 1% SDS. Subsequently, the suspension was placed in Zircoprep Mini (Nippon Genetics Co. Ltd., Tokyo, Japan) and violently agitated for 10 min in a Disruptor Genie cell disruptor (Scientific Industries Inc., NY, USA). After sample centrifugation, supernatant was used.

Red hydrogen Peroxide Assay Kit (Enzo Life Sciences Inc., CA, USA) was used to establish bacterial hydrogen peroxide amounts following our earlier work (Cueno et al. 2014; Ohya et al. 2016), whereas, the Catalase Assay Kit (Cayman Chemical Company Inc., MI, USA) was used to establish catalase amounts in *C. albicans*. Both kits were performed according to manufacturer's recommendation.

Fungal growth evaluation

ELIS at the same varying dilutions previously described above was mixed with SG broth. Each mixture was added in a 96-well plate, test strains were inoculated (pre-incubated with SG broth at 37 °C for 18 h) to a final OD600 = 0.01 and aerobically incubated at 37 °C for 18 h. Turbidity was measured at OD600 nm using a colorimeter (TriStar LB 941, Berthold Technologies, Bad Wildbad, Germany) to compare fungal growth among the test media.

Fungal form visualization

Test fungus was incubated in an ELIS-containing medium similar to earlier dilutions with 5% fetal calf serum (FCS) at 37 °C for 18 h and, likewise, was smeared onto a glass slide, fixed, and stained with phenol crystal violet. Morphological observation was observed under a light microscope (BH2, Olympus Corp., Tokyo, Japan) in order to establish and compare fungal cell formation. The number of cells in 10 fields (10×) of view was counted to calculate the ratio of hyphae cells relative to the total number of fungi.

Effects on fungal adhesion

PMMA-based resin pieces (10-mm diameter × 1-mm thickness, SHOFU Inc., Kyoto, Japan) were used to assess fungal adhesion. Resin pieces were placed in a 24-well plate. Whole saliva was filtered and treated at 56 °C for 1 h and added to each well (2 mL each). Each resin piece was incubated at 37 °C for 1 h to allow saliva film envelopment. Subsequently, resin pieces were washed with phosphate-buffered saline (PBS), combined with a test fungus suspension (1.0×10^7 cells/2 mL) containing ELIS dissolved at each PBS concentration, and incubated at 37 °C for 1 h. Fungus non-adhering to the resin pieces were removed by PBS washing and, afterwards, the resin pieces were exposed to ultrasound in 2 mL PBS (20 W, 90 s on ice; Handy Sonic UR-21P, Tomy Seiko Co., Ltd., Tokyo, Japan) in order to release and harvest the adherent fungus. Subsequently, fungal suspensions were then diluted, applied to SG agar medium and incubated. Similarly, CFU counting was performed.

Effects on biofilm formation

Protocols for biofilm formation followed a modified version of an earlier work (Li et al. 2003). Briefly, NUD202 was inoculated into 0.2 mL SG broth supplemented with or without 5% FCS with ELIS at varying dilutions similar to earlier in individual wells that were treated with heat-inactivated whole saliva in polystyrene 96-well plates (flat bottom, Nunc, Thermo Fisher Scientific, Japan). The plate was incubated at 37 °C for 48 h. After biofilm formation and growth, planktonic cells

were discarded through two rounds of washing with PBS. In the method involving crystal violet (CV) staining, 0.1 mL 0.1% CV was added to each well and incubated for 20 min at 37 °C. After incubation, samples were washed with PBS and 0.1 mL 95% ethanol was added to dissolve the dyed biofilm cells and the absorbance for each well was determined using a microplate reader at 570 nm (TriStar LB 941, Berthold Technologies, Bad Wildbad, Germany).

Proteinase activity production

Test fungus was inoculated into a medium supplemented with both FCS and varying ELIS concentrations. Samples were aerobically incubated at 37 °C for 18 h. Supernatant was harvested by centrifugation at 5000×g for 10 min under 4 °C chamber conditions. Samples were concentrated 25-fold using a concentration unit (Ultracel®-10K Centrifugal Filter Unit, Millipore, Darmstadt, Germany). Secreted aspartyl proteinase (SAP) activity was quantified following a previously published work (Carvalho et al. 2010) with some modifications. Briefly, concentrated sample (0.3 mL) was combined with 0.5 mL 1% azocasein in Tris-HCl buffer (pH 4.8) and treated at 37 °C for 1 h. Reaction was stopped with the addition of 0.8 mL 10% trichloroacetic acid and, subsequently, 0.5 mL of supernatant harvested by centrifugation (at 10,000×g for 10 min under room temperature conditions) was combined with an equal volume of 0.5 M NaOH. OD440 nm value was determined using a colorimeter (TriStar LB 941) after 15 min. Standard curves of enzyme activity were generated using pepsin.

Statistical analyses

Statistical significance of differences between samples was determined by one-way ANOVA with Scheffe's test. A significance level of 95% ($p < 0.05$) was considered statistically significant.

Results

Confirmation of ions in ELIS components

We found that ELIS contained ions at the following concentrations: 57.6 ppm [Al^{3+}], 8719.2 ppm [BO_3^{3-}], 534.1 ppm [Na^+], 52.3 ppm [SiO_3^{2-}], 223.9 ppm [Sr^{2+}], and 130.0 ppm [F^-]. Among these ions, Al^{3+} and BO_3^{3-} manifest an enzyme-inhibiting effect while high concentration of Na^+ and SiO_3^{2-} inhibits the mycelial growth of fungus. Moreover, Sr^{2+} and F^- have been reported to inhibit dental demineralization and F^- has an antibacterial effect (Bekker et al. 2006; Cho and Joshi 1989; Ma et al. 2014; McMillen et al. 1998; Smoum et al. 2012; Thuy et al. 2008). Considering all the ions released

and found in ELIS, this would imply that the ELIS components would have beneficial dental applications. It is worth mentioning that we also mixed S-PRG filler with an equal amount of heat-treated and filtered whole saliva under constant stirring for 24 h. Ion concentration difference between ions released into saliva and distilled water was not observed (data not shown). In this regard, the ion concentration used in this study is presumed to be sufficiently obtained even in the oral cavity. It is worth mentioning that the ion concentration in water is higher than previous clinical work (Kaga et al. 2011). However, that current study was performed using in vitro conditions and serves as a guide towards possible clinical applications.

ELIS induces oxidative stress

To measure ELIS-related oxidative stress induction, we measured the amount of hydrogen peroxide and catalase levels in *C. albicans*. We found that the hydrogen peroxide level had no effect up to the 16-fold dilution concentration. However, significant suppression was seen from the 8-fold (Fig. 1a). It is worth mentioning that although the amount of catalase increased at the 128-fold dilution concentration, it decreased thereafter in a concentration-dependent manner and was almost undetectable at the 8-fold dilution concentration (Fig. 1b). Nevertheless, we believe that the variations in catalase amounts at varying ELIS dilution concentration would suggest that oxidative stress induction differs.

ELIS inhibits fungal growth

To determine the effect of ELIS on fungal growth, we measured optical density after incubation of the test strain in ELIS-containing media. Figure 1c shows post incubation turbidity in media containing various ELISA concentrations after 18-h incubation. ELIS with a $\leq 1:16$ dilution ratio inhibited growth in both test fungus strains while growth was almost inhibited at 1:8 dilution ratio. This demonstrates that ELIS has the potential to inhibit *C. albicans* growth.

ELIS inhibits fungal dimorphism

Regarding the effect of ELIS on dimorphism of *C. albicans*, it was cultured in an ELIS-containing medium at varying dilutions, stained, and observed under a light microscope. In FCS presence, most cells showed hyphae form (Fig. 2). Moreover, ELIS inhibited dimorphism conversion, a trait closely associated with pathogenesis, at a concentration-dependent manner and little mycelial form was seen at the 8-fold dilution which we associated to fungal dimorphism inhibition.

Fig. 1 ELIS induces oxidative stress and inhibits fungal growth. **a** Amount of hydrogen peroxide compared with a control. **b** Amount of catalase same as above. **c** *Candida albicans* growth. Strains growth measured at OD600 are indicated. Results shown correspond to $n = 6$ independent samples. Each sample indicated the statistical significance of differences between control and treated samples, determined using one-way ANOVA with Scheffe's test (** $p < 0.01$)

ELIS is detrimental to major pathogenic factors

Major pathogenic factors of *C. albicans* reported include: adhesion, biofilm formation, and ability to produce proteinase (Kim and Sudbery 2011; Mayer et al. 2013). This would imply that an effective antifungal strategy that follows a holistic approach would also require the inhibition of these pathogenic factors.

Fig. 2 ELIS inhibits fungal dimorphism. **a** Dimorphic forms of *Candida albicans* in the pictures. Upper left sample representing hyphal form without ELIS and treated samples (upper right, lower left and right panels) representing yeast form follow in a concentration-dependent manner are shown. **b** The ratio of hyphae form to the total cell number counted under a microscope compared to control

To evaluate *C. albicans* adhesion ability, we exposed resin pieces to the fungal suspension and treated it with ELIS. As shown in Fig. 3a, we found that at lower ELIS concentrations (1:32 dilution ratio), no significant difference was noted between the experimental and control groups. However, a significant reduction of fungus adhesion to the resin piece relative to the control group was observed at $\leq 1:16$ ELIS dilution. This would suggest that ELIS has the potential to inhibit fungal adhesion.

To determine the effect of ELIS on biofilm formation, we measured optical density after staining of biofilms formed in the bottom of microplate. Figure 3b shows the inhibition on biofilm formation rate of ELIS. Biofilm formation ability of NUD202 was suppressed in a concentration-dependent manner by ELIS. This suppression pattern was different from the results of the culture experiment.

Fig. 3 ELIS is detrimental to major pathogenic factors. **a** *Candida albicans* adhesion in ELIS solution. Colony number with regards to ELIS dilution ratio are shown. **b** ELIS inhibited biofilm formation in a concentration-dependent manner. **c** *C. albicans* proteinase activity. SAP activity with regards to ELIS dilution ratio is indicated. Each sample indicated the statistical significance of difference between control and treated samples, determined using one-way ANOVA with Scheffe's test (** $p < 0.01$)

To determine the effects of ELIS on proteinase production, we measured *C. albicans* proteinase production. Figure 3c shows enzymatic activity values in supernatants of the fungal culture media after incubation in varying ELIS concentrations. SAP, known to be the main proteinase produced by *C. albicans*, accounts for a majority of the proteinases contained

in the fungal culture supernatant. There was no effect at a dilution of 1:128, but SAP activity was markedly reduced in an ELIS concentration-dependent manner at a dilution of 1:32–1:8 (Fig. 3c). As proteinase activity is involved in intracellular invasion and nutrient acquisition of this fungus (Naglik et al. 2008), this would imply that ELIS has the potential to inhibit these *C. albicans* actions by possibly affecting SAP activity.

Discussion

C. albicans is involved in both oral and systemic disease induction (Cannon and Chaffin 1999). Moreover, denture-wearing elderly people with compromised immune function are more likely to develop oral candidiasis as a result of *C. albicans* growth (Zupanić-Krmek and Nemet 2004). Various antifungal agents have been developed to treat oral candidiasis, however, while the expected antifungal effect of the agents is being manifested, prolonged use of such drugs can result in the sudden emergence of drug-resistant strains (Clemons and Stevens 2001). Through this study, we demonstrated ELIS-released oxidative stress induction in *C. albicans*. Moreover, we showed it effects a fungal proliferation and pathogenicity.

Experiments on *C. albicans* oxidative stress have been reported since oxidative stress shows various adverse effects on living organisms (Dantas et al. 2015). In particular, an imbalance in intracellular ROS has shown to be one of the steps leading to apoptosis (Benaroudj et al. 2001). For oxidative stress experimentation, there are several evaluations, but the most basic one is the measurement of a pro-oxidant (hydrogen peroxide) and anti-oxidant (catalase) amounts. Thus, ELIS effects on these biochemical components were measured.

Although the amount of hydrogen peroxide was constant up until 16-fold of ELIS concentration, hydrogen peroxide was thought to be functioning because it is also related to intracellular signals in addition to oxidative stress (da Silva et al. 2010). In this regard and for this study, we presumed that hydrogen peroxide suppression is related to toxicity (da Silva et al. 2010; Gough and Cotter 2011). Most especially, it showed a significant decrease at the 8-fold dilution concentration which we hypothesize to be attributed to toxicity leading to cell death.

From our results, induction of oxidative stress in *C. albicans* was considered as one of the antifungal mechanisms of ELIS. It has been reported that PKA pathway-related hyphae formation is weak against oxidative stress (Wilson et al. 2007), it was inferred that this oxidative stress works to suppress the adhesion, hyphal transformation and protease production.

C. albicans is detected at a high ratio in the oral cavity of the elderly population because elderly individuals have

decreased immunity owing to immunosenesence and a high denture wearing rate (Yoshida et al. 2001). An immediate requirement for any antifungal therapy is fungal growth inhibition. Some of the ELIS components are known to have antifungal activity which would imply that ELIS can be directly used to inhibit *C. albicans* growth.

Although fungal growth is the immediate concern during fungal infection, for long-term treatment, *C. albicans* pathogenic factors have to be likewise targeted. *C. albicans* is known to have a high capacity to adhere to denture base resin attributable to two adhesion mechanisms (ionic charge and glycoproteins) and, likewise, is the main microorganism involved in the formation of denture plaques (Salerno et al. 2011). In the adhesion experiment, even if ELIS was present in the environment or S-PRG filler was added to the resin of the denture (data not shown), adhesion was suppressed in a concentration-dependent manner. It was suggested that addition of S-PRG filler to denture resin could exert the preventive effect of oral candidiasis by suppressing adherence of *Candida* even in clinical practice.

One of the *C. albicans* adhesion mechanisms involve cell surface glycoproteins (Hoyer 2001), and these genes encoded proteins that are related to host tissue adhesion, tissue invasion activity, and fungal morphological changes (Ene and Bennett 2009; Hoyer 2001; Ueno et al. 2004). Similarly, gene family that encoded *C. albicans* SAP activity is important for digesting host protein, useful for tissue invasion, avoiding host immune mechanisms, and exhibit increased expression during the hyphal stage which suggests a role in hyphal formation (Chen et al. 2002). This would insinuate that both adhesion and proteinase production related genes can affect *C. albicans* dimorphism and, likewise, may influence what fungal form (hyphal or yeast) *C. albicans* would exist. Moreover, it was previously suggested that these genes are in so many ways parallel to one another (Hoyer 2001). Thus, we postulate that ELIS can hamper SAP gene expression which in turn would suggest that fungal adhesion and hyphal formation are both deferred.

Between the two *C. albicans* fungal forms, pathogenicity is higher in the hyphal form compared to the yeast form. It has been known that antiphagocytic and proteinase activities are enhanced during the hyphal form (Ueno et al. 2004) which is why the dimorphism property is one of the therapeutic targets of candidiasis (Jacobsen et al. 2012). *C. albicans* is a dimorphic fungus which undergoes morphological change depending on surrounding environmental conditions (Mitchell 1998). When adhering to human tissues, *C. albicans* assumes the yeast form and, after contacting the superficial layer of the tissue, it converts to the hyphal form under the influence of several environmental factors such as serum, temperature, pH, and nutrients (carbon, nitrogen, and amino acid source) (Sato et al. 2004). *C. albicans* yeast form as compared to hyphal form has a higher level of cellular invasiveness exhibiting anti-phagocytic activity resulting to pathogenicity (Hoyer 2001).

In this regard, this would emphasize the importance of inhibiting both fungal forms. Moreover, our results highlight the significance of ELIS action against these two fungal forms. Similarly, we examined the influence of ELIS on the formation of *C. albicans* biofilm. While biofilm formation by human pathogenic yeasts has been recognized as a potentially important medical problem (Chandra et al. 2001), relatively little is known about the variation and evolution of biofilm formation within populations of these yeasts. Thus, inhibition of *Candida* biofilm formation ability is one way to prevent candidiasis. Biofilm formation was inhibited in a concentration-dependent manner, specifically, its inhibition rate was higher than the result of growth inhibitory effect. From these results, it seems ELIS can be useful in the prevention of candidiasis.

Finally, the effect on proteolytic enzyme activity was investigated. Proteolytic enzymes are very important for the localization of this fungus. It is also deeply involved in physiological activity. ELIS suppressed proteolytic enzyme activity in a concentration-dependent manner and was hardly observed at 8-fold dilution concentration. Thus, it was confirmed that ELIS is useful for suppressing the pathogenicity of *C. albicans*.

In summary, we shared that ELIS induces oxidative stress in *C. albicans* and demonstrated ELIS-related inhibition on the following fungal factors: (1) growth and biofilm formation, (2) fungal adhesion capability, (3) conversion from the yeast form to the hyphal form, and (4) proteinase production. In this regard, these results indicate that the ions released from S-PRG filler effectively inhibit both the growth and pathogenic factors of *C. albicans* which in turn we propose may have possible clinical applications in preventing the onset of candidiasis especially among the elderly including denture wearers.

Acknowledgements We thank to Dr. Toshiyuki Nakatsuka (Shofu Inc.) for providing the S-PRG filler. This research was supported in part by funding from the Satoh Research Fund, a grant from the Dental Research Center of Nihon University School of Dentistry, KAKENHI (Japan Society for the Promotion of Science, C: 24593168 and 15K11456, Japan).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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