Protective Effect of Low Concentrated Boric Acid in Oral Candidiasis: An 
In Vitro Study

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Received: January 29, 2018; Published: March 05, 2018

Abstract

Candida albicans (C. albicans) is the most common human commensal fungi found adhered to the mucosal membranes of the oral cavity. Under certain conditions, previously asymptomatic colonization can become virulent, resulting in oral candidiasis (OC). Acrylic denture bases are porous and can potentially harbor infection in the denture, the denture cup and the soft tissues of the mouth, promoting C. albicans overgrowth. Patients with OC who wear removable dentures are not only susceptible to Candida infection, but also to reinfection. The present study aimed to test the efficiency of using low concentrations of boric acid (BA) solution for the topical treatment of OC, and to prevent reinfection. Overall, our findings support the potential use of low concentrated BA solutions for the topical treatment of OC, disinfection of dentures, and prevention of reinfection.

Keywords: Boric Acid; Candida albicans; Oral Candidiasis; Oral Fibroblasts; Denture-Resin Acrylic Material

Abbreviations

BA: Boric Acid; OC: Oral Candidiasis; IC: Invasive Candidiasis; VC: Vaginal Candidiasis; HGF-1: Human Gingival Fibroblasts-1; YPD: Yeast Extract Peptone Dextrose

Introduction

The polymorphic fungus Candida albicans is a common human commensal which can be found adhered to the skin and mucous membranes of the oral cavity [1]. While C. albicans colonization is typically harmless in healthy individuals, it may become virulent for patients with compromised immune system, poor diet, stress, chronic diseases, or those undergoing long-term antibiotic therapy [2-6]. This change in virulence is driven by C. albicans’ ability to switch from a commensal yeast morphology to the highly polarized invasive hyphal morphology [7]. C. albicans is also the most common pathogen associated with invasive candidiasis (IC) and is a leading cause of mycosis-associated mortality in the United States [8].

Of relevance and interest to dental professionals, oral candidiasis (OC) is also an issue in patients using removable acrylic appliances (especially complete dentures), where the porous and microaerobic environment can promote Candida biofilm formation, particularly for patients who fail to remove their dentures at night [9]. Approximately 60-100% of denture wearers express varying clinical manifes-
tions of appliance-induced OC [10]. Current treatment of *C. albicans* infected appliances centers around either mechanical removal of the affected acrylic surfaces or treating the mucosal surface. Elimination of the affected surface eliminates the embedded fungal biofilm, but negatively affects the denture's retention and resistance to fracture. Additionally, roughening of the polished acrylic surface with an acrylic bur or another abrasive instrument is likely to increase the area and porosity and promote future fungal growth. Because acrylic appliances contain methyl methacrylate (a substrate for carbon), invasion can extend deep into the denture material, making complete removal of the fungal infection problematic [11]. As with any fungal infection, complete treatment of all surfaces involved is imperative to preventing reinfection. Even after successful treatment, recurrent Candida infections are common [12]. Patients wearing acrylic appliances would greatly benefit from a treatment option that prevents invasive growth into the denture and surrounding oral tissue, rather than treating the overgrowth after it has established. This treatment would also have the benefit of preserving the structural integrity of the prosthesis, thus reducing the propensity of future fungal infections.

Boric acid (BA) is a colorless and odorless compound acid of boron, traditionally used as an antiseptic and pesticides. In small quantities, BA is as also used as an active component in a wide variety of household products, such as cleaners, cosmetics, eye washes, and toothpaste [13]. However, at high concentrations, BA can be toxic. According to the Environmental Protection Agency (EPA), a single dose of 15 - 20g of BA is sufficient to cause acute toxicity in human adults (United States Environmental Protection Agency (EPA). Toxicological Review of Boron and Compounds. 2004 Sep. Report No. EPA 635/04/052). Despite its potential toxicity at high concentrations, BA is a well-known therapeutic compound which is successfully used to treat vaginal candidiasis (VC) [14]. However, its efficacy to treat OC remains to be shown. The present study has been designed to bridge our gap in knowledge on BA's effectiveness to treat OC and shed light on the potential mechanism underlying its anti-fungal activity. Overall, our results provide evidence supporting the benefits of low concentrated BA solutions to treat and prevent OC, rather than conventional techniques including denture cleaners (i.e. Efferdent, Polident) or in severe cases solutions of household bleach and water.

**Materials and Methods**

**Chemicals and Reagents:** Reagents boric acid (BP168), dextrose (D16), DMEM (SH3002101), DMSO (D128), peptone (BP1420), paraformaldehyde (T353), phalloidin (A12380), SYBR green (4309155) and yeast extract (BP1422) were purchased from ThermoFisher Scientific (Waltham, USA). Calcofluor (18909), MTT (M2128) and Triton X (T8787) were purchased from SigmaAldrich (St. Louis, USA). Human Gingival Fibroblasts, HGF-1 (CRL-2014) cells were obtained from ATCC (Manassas, USA). RNasey kit (74104) was from (Qiagen, Hilden, Germany).

**Cell culture and *C. albicans* growth conditions:** HGF-1 cells were cultured in DMEM, supplemented with 10% FBS. HGF-1 cells were maintained at 37°C, 5% CO₂ and used between 2nd and 4th passage. *C. albicans* was grown in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% dextrose) or YPD agar plates, and incubated at 30°C in an orbital shaker at 250 rpm.

**Cytotoxicity assay:** HGF-1 cells were seeded in triplicate at 10,000 cells/well in a 96-well plate and incubated in media containing different concentrations of BA (2 mg/mL, 4 mg/mL, and 8 mg/mL). After 24h, the cell-culture media was replaced with fresh media containing thiazolyl blue tetrazolium bromide (MTT) reagent and incubated at 37°C in 5% CO₂ for 4h. Thereafter, supernatant was removed and the formazan product was dissolved in DMSO. Absorbance was measured at 570 nm. Untreated HGF-1 cells were taken as controls. Triplicate wells were used for each concentration of BA and untreated control. Percentage cytotoxicity was calculated by substituting the corresponding mean absorbance values in the following equation:

% Cytotoxicity = ([Untreated cells] - (BA-treated cells)/(Untreated cells)]) x 100  

The graphs were plotted using % Viability, calculated as: % Viability = 100 - % Cytotoxicity.

**In vitro Infection assay:** HGF-1 cells cultured in DMEM were treated with 4 mg/mL BA for 1h. The control consisted of HGF-1 cells treated with equal volume of distilled water. *C. albicans* inoculum was prepared in the meantime. Overnight grown *C. albicans* was quantified by measuring the absorbance at 600 nM (NanoDrop ND-1000). *C. albicans* was adjusted to 10⁷ cfu/mL, optimal for *in vitro* infection (OD1.0 ≡ 10⁴ cfu) [15]. Following 1h of BA pretreatment, HGF-1 cells were washed and incubated in 490 µl of fresh DMEM media. After that, HGF-1 cells were infected (wherever applicable) with 10 µl of 10⁷ cfu/mL *C. albicans*, and incubated at 37°C, 5% CO₂ for 1.5h (immuno-fluorescence labeling and lysis assay).

Immunofluorescence labeling: Untreated Candida-infected HGF-1 cells and BA-treated Candida-infected HGF-1 cells were grown on glass coverslips and fixed in 4% PFA for 20 minutes at room temperature. After that, 10 µg/mL calcofluor white was added for 20 minutes at 37°C. Coverslips were washed three times with PBS and incubated in 1:200 phalloidin Alexa-568 for 1h at 37°C. Non-specific binding was removed by washing the coverslips with PBS three times followed by mounting in glycerol. The images were captured using Nikon Eclipse 80i.

HGF-1 cell lysis assay: 0.5% Triton X was prepared in PBS and added to untreated and BA-treated HGF-1 cells infected with C. albicans. The lysate was prepared by pipetting up and down several times, and the lysate was further spread on YPD agar plate and incubated at 30°C overnight. The number of colonies in Untreated Candida-infected HGF-1 cells and BA-treated Candida-infected HGF-1 cells were photographed.

Evaluation of BA’s antifungal properties: To evaluate the potential for using the BA-based solution as a disinfection method for acrylic-based dentures, C. albicans at 10^5 cfu/mL in YPD media was used as an inoculum. Identical acrylic samples (10 x 10 x 5 mm) were disinfected, sterilized, and pre-soaked in 4 mg/mL BA solution for 6h or 12h. Controls consisted of samples pre-soaked in distilled water. Next, each denture sample was incubated for 12h in 6 mL of inoculum. On the following day, denture resins were transferred to fresh, C. albicans-free YPD media. Following overnight incubation, the concentration of C. albicans in the media was quantified using a nanodrop. The growth of C. albicans in BA samples was calculated by substituting the corresponding mean absorbance values in the following equation:

% Growth rate = [(Control) − (BA-treated)/(Control)] x 100

Statistical Analyses: All experiments were performed in triplicates (n = 3) and repeated three times. The effect of BA on growth of C. albicans or HGF-1 survival was analyzed by measuring absorbance (outcome variable). To test inter-group differences and determine statistical significance, one-way ANOVA was performed. Post-hoc analyses between groups was done using t-test (Two-Sample Assuming Equal Variances). To account for multiple testing of groups Bonferroni correction was done.

Results

In vitro BA effect in C. albicans: To assess BA’s impact in Candida growth, C. albicans was grown in YPD broth containing varied BAs concentrations (2 mg/mL, 4 mg/mL and 8 mg/mL) for 24h. Overall, a dose dependency on Candida’s growth inhibition was observed. Nevertheless, only a marginal, statistically insignificant (p value = 0.34) increase in cell death was observed for BA concentrations above 4 mg/mL (Figure 1A). Therefore, all subsequent experiments were performed with 4 mg/mL BA. To investigate the effect of incubation time on BA’s Candida growth inhibition, C. albicans was cultured with 4 mg/mL BA for 8h, 16h, and 24h. While untreated (control) Candida cultures showed logarithmic growth over time, Candida growth was significantly inhibited in the presence of BA (Figure 1B).

Figure 1: Effect BA treatment on C. albicans growth (A) Strong growth inhibition was observed in C. albicans colonies grown in media containing different concentrations of BA (2 mg/mL, 4 mg/mL and 8 mg/mL). Statistically significant result was observed between control and three BA samples. No statistically significant difference was observed between BA concentrations 4 mg/mL and 8 mg/mL (p value = 0.34) (B) Contrasting with the normal logarithmic growth observed in control colonies, a near complete growth inhibition was observed in C. albicans treated with 4 mg/mL BA for various time intervals (8h, 16h and 24h). Error bars represent Standard Error (SE).
BA inhibits \textit{C. albicans} infection of HGF-1 oral fibroblasts: Earlier studies have shown oral fibroblasts to be a major player in orchestrating an anti-microbial immune response against \textit{Candida} infection [16,17]. Next, to assess BA's potential cytotoxicity to oral fibroblasts, HGF-1 cells were seeded into the microplates and incubated with various concentrations of BA for 24h. At the concentrations tested, BA exhibit no or low cytotoxic effects (Figure 2A). These results suggested that although BA is highly effective in killing \textit{C. albicans}, it posed no major harm to the resident oral fibroblasts. Interestingly, pretreatment of HGF-1 cells with BA followed by infection with \textit{C. albicans} led to the formation of unusual blob-like aggregates in the cell-culture supernatant (Figure 2F' insert). This phenomenon, previously observed elsewhere [18], is attributed to the hydrophobic nature of sialic acid residues on \textit{C. albicans}' surface, which leads to the formation of the 'self-aggregates' [18]. Of note, \textit{C. albicans}' aggregation property was markedly enhanced upon treatment with BA in cell culture media (Figure 2F' insert). Consistent with the enhanced response, large cell aggregates were visualized in BA-treated HGF-1 cells (Figure 2F' insert) compared to untreated controls (Figure 2F insert). Although \textit{C. albicans} self-aggregating properties have been previously reported [18], this is the first-time BA is shown to enhance that property. To further assess the interaction of \textit{C. albicans} with untreated- and BA-treated, infected HGF-1 cells were observed by light microscopy (Figure 2B-D). BA-treated HGF-1 cells showed a reduction in \textit{C. albicans} infection (Figure 2F', C') compared to untreated controls (Figure 2F, C). Morphological analyses of untreated \textit{C. albicans} indicated a switch from fungal to hyphal form, which is critical for infection under \textit{in vivo} conditions (Figure 2D). BA's effect on \textit{C. albicans}' cytoskeleton has previously been shown to be an important mechanism of its activity [19]. While untreated HGF-1 cells displayed massive \textit{C. albicans} filamentous growth after 24h of incubation (Figure 2D), BA treatment visibly inhibited fungal growth in \textit{C. albicans} infected cells (Figure 2D'). Moreover, BA-treated cells showed a typical yeast morphology (Figure 2F'-D'), whereas untreated cells after 24h of infection switched to hyphal morphology (Figure 2D). This observation is consistent with previous analysis showing BA's effect on \textit{C. albicans}' cytoskeletal proteins as a mechanism which inhibits the phenotypic switch [19]. Next, an \textit{in vitro} infection assay was performed to compare \textit{C. albicans} density in HGF-1 in the absence or presence of BA. Lysis of HGF-1 cells with 0.5% Triton X released intracellular \textit{C. albicans}. To assess the number of colonies, extracted \textit{C. albicans} were plated on YPD agar plates and incubated for 12h. Confirming the previous results, the number of colonies isolated from BA-treated HGF-1 cells was visibly lower than that extracted from the untreated control samples (Figure 3A, B).

\textbf{Figure 2:} (A) No signs of cytotoxicity in BA-treated HGF-1 cells. Error bars represent Standard Error (SE). (B-D) BA inhibits Candida infection in HGF-1 oral fibroblasts. Untreated (Unt) (B) and BA-treated (B') HGF-1 cells infected with \textit{C. albicans} for 1.5 h. Inserts show aggregation of \textit{C. albicans} in the supernatant of BA treated (B') but not in Unt HGF-1 cells (B). Untreated (C) and BA-treated (C') HGF-1 cells infected with \textit{C. albicans} for 1.5h fixed and labeled with phalloidin Alexa-568 (Red) and \textit{C. albicans} labeled with calcofluor stain (Blue) show reduced \textit{C. albicans} in BA-treated HGF-1 cells (C') compared to untreated control (C). Unt (D) and BA-treated (D') HGF-1 cells infected with \textit{C. albicans} for 24h show \textit{C. albicans}' switch to invasive pathogenic fungal form in Unt (D) but not in BA-treated (D') HGF-1 cells.
**Figure 3:** BA treatment inhibits *C. albicans* infection of HGF-1 cells. (A) Untreated HGF-1 cells infected with *C. albicans* showing a higher density of fungal colonies on an agar plate. (B) BA treatment reduces *C. albicans* infection and colonization of HGF-1 cells, leading to a decreased density of colonies, compared to the untreated control.

**BA is a potential disinfectant in acrylic-based denture resin:** Consistent with BA’s anti-fungal effect, denture samples pre-soaked in BA solution displayed lower *C. albicans* concentration compared to control samples (soaked in distilled water) (Figure 4A, B). Of note, BA’s effect on *Candida* concentration was directly proportional to the time of incubation: samples incubated for 12h showed a greater decrease in *C. albicans* concentration than those incubated for 6h (Figure 4B). While preliminary, these findings are clinically relevant and highlight BA’s potential to be used as a disinfectant solution to help prevent and to treat OC.

**Figure 4:** BA as a potential disinfectant for acrylic-based denture resin. Identical denture samples (10 x 10 x 5 mm) were sterilized and pre-soaked for 12h in 4 mg/mL BA solution for 6h or 12h. Following treatment, each denture resin was incubated for 12h in 5 mL of fungal solution. Controls consisted of samples pre-soaked in distilled water. On the following day, denture resins with adhered *C. albicans* on the surface were transferred to fresh YPD media and incubated for 12h. Consistent with the anti-fungal effects of BA, *C. albicans* concentration was lower in samples pre-soaked in 4 mg/mL BA compared to the control samples. *C. albicans* growth rate was calculated as described in materials and methods. Error bars represent Standard Error (SE).

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**Citation:** Sonia Maria Rocha-Sanchez, et al. “Protective Effect of Low Concentrated Boric Acid in Oral Candidiasis: An In Vitro Study”. *EC Dental Science* 17.4 (2018): 302-308.
Discussion

BA is widely used in VC in the form of vaginal suppositories [14,20] and is a safe, alternative and economical option for women with recurrent vaginal candida infection [21]. The present study investigates the cytotoxic effects of BA in the oral cavity, which is also susceptible to C. albicans infection.

C. albicans is one of the most common commensals as well as a fungal pathogen in humans, affecting neonates, children and adults alike. In addition to its adverse effects on the individual's quality of life, Candida also poses a grave threat to immunocompromised patients. While it can co-exist as a non-pathogenic commensal in the human body, some factors that alter the normal oral flora (e.g. the use of broad-spectrum antibiotics), can trigger its transformation to a pathogenic form [22]. The fourth most common cause of hospital-acquired bloodstream infections, Candida is recognized as a "serious threat" by the CDC [http://www.cdc.gov/drugresistance/threatreport-2013/pdf/ar-threats-2013-508.pdf#page=63]. A major challenge to successful Candida treatment includes acquired resistance to conventional anti-fungal agents [23,24], emphasizing the need for the investigation and evaluation of new antifungal therapies.

Recent evidence suggests that BA therapeutic properties are related to its effect on Candida's cytoskeleton [19]. The disintegration of hyphal cytoskeleton impairs Candida's ability to switch from harmless yeast morphology to a pathogenic filamentous hyphal form [19]. Our present studies demonstrate that low concentrated (4 mg/mL) BA solution is effective in lowering infection and colonization of oral fibroblasts by C. albicans (Figure 3) without signs of toxicity to the oral fibroblasts (Figure 2). Oral fibroblasts are key components of the oral mucosa and associated with orchestrating immune responses against microbes, including C. albicans [16,17]. Overall, our results point to the antifungal benefits of low concentrated BA solution in acrylic-based denture disinfection and shed light on its potential clinical applications on candidiasis treatment and prevention [9]. Unlike current methods, which focus primarily on treating the established Candida overgrowth in the oral cavity, BA-based denture disinfection is likely to prevent Candida growth in the dental appliances. Whether used alone or in combination with current therapies, BA treatment should be effective in preventing re-infection of the oral cavity by eliminating Candida growth in the dentures. Moreover, the cost of BA is minimal and BA will not alter the color of the acrylic or denture teeth. The present study focused on the effect of BA on C. albicans. However, additional studies investigating the efficacy of BA treatment on other Candida species affecting the oral mucosa, is warranted.

Conclusion

In vitro studies in oral fibroblast and acrylic-based beads provided the results supporting the present study. Likewise, the concentration of BA used (4 mg/mL) was well below the toxic levels and is unlikely to threaten human safety. It is a safe and effective alternative treatment to conventional antifungal denture treatment currently in use. Future studies will explore BA's safety and antifungal properties in human subjects and the possibility of incorporating BA directly in to acrylic during the processing phase of denture fabrication.

Acknowledgements

We would like to thank Mr. Benjamin Pointer for his contribution to the early stages of this study. This work was supported in part by the Creighton University School of Dentistry and the Iowa Dental Association (IDA).

Conflict of Interest

The authors do not have any potential conflict of interest relevant to the present article.

Bibliography


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