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Stress Response in *Candida albicans* Induced by Boric Acid

Tyler Beach[1], Benjamin Hart[1] and Bryan Larsen[1*]

[1]Marian University College of Osteopathic Medicine, Indianapolis Indiana 46222, USA.

Authors' contributions

This work was carried out in collaboration between all authors. All research was carried out in the laboratory of author BL. Authors BH and TB handled all laboratory technical procedures and processes and contributed to the presentation of the results and statistical analysis of the data. All three authors contributed to the manuscript and agree with its contents.

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ABSTRACT

Background: Topical boric acid preparations have been employed in treatment of *Candida*-associated vaginal infections resistant to typical antifungal treatments; however, a mechanism of boric acid's antifungal properties is not fully understood.
Aims: Investigate the antifungal properties of boric acid associated with *Candida albicans* and cell number and geometry through flow cytometry.
Study Design: Twelve clinical isolates of *Candida albicans* were grown in Sabouraud broth with varying concentrations of boric acid followed by measurements of cell characteristics.
Methodology: Absolute counts, cell size data, and fluorescence of test organisms were determined by flow cytometry. All microbial count and growth experiments were conducted in at least duplicate and averages were reported.
Results: Each of the twelve strains showed susceptibility to boric acid with ED50 values below 2400 mg/L. Boric acid decreased cell survival in growth media and distilled water (P<0.05), but disintegration of cells occurred in boric acid and water but not in water alone. Boric acid reduced cell volume implying apoptosis which was supported by annexin V staining. Cell involution and cell number were used to determine relative biomass which showed that biological effects were

**Corresponding author: E-mail: blarsen@marian.edu;*

apparent even at 0.05% (500 mg/L). Mean autofluorescence of test organisms grown in boric acid increased in a dose-dependent manner. Inhibition of catalase could contribute to proapoptotic activity making *Candida albicans* more susceptible to ROS internally.
Conclusion: Boric acid was shown to effectively decrease cell size, induce cell autofluorescence, decrease catalase activity, and initiate programmed cell death. Further experimentation should investigate specific mechanisms of boric acid-induced apoptosis and its role in altering catalase activity.

Keywords: Vaginal therapy; boric acid; Candida albicans; Gardnerella vaginalis.

1. INTRODUCTION

Boron compounds occur naturally in the form of minerals and also in biological systems relevant to plant physiology [1] but their role in medicine is limited to a few examples. Boromycin is a macrolide compound with activity against Human Immunodeficiency Virus [1], bortezomib is a dipeptide boronic acid analog approved for use in multiple myeloma [2], and a recently-approved drug for onychomycosis, tavaborole, is a polycyclic boron containing compound [3] with antifungal properties. Topical boric acid has been used to treat vaginal symptoms associated with *Candida species*, especially when the organism is refractory to commonly used antifungal drugs such as the azole class of ergosterol synthesis inhibitors [4]. More recently, the Centers for Disease Control has suggested that boric acid may be used in some instances to treat bacterial vaginosis [5] due to its inhibitory effect on bacteria, including *Gardnerella vaginalis*. While some summary of clinical studies of boric acid as treatment of *Candida* vaginitis indicates increased side effects, they were not generally considered troubling. It is not known if these are due to adverse effects on cell physiology of the underlying tissue [4,6]. Despite decades of use of boric acid [7], limited information regarding the mechanism of antifungal or antibacterial action of this inhibitor has been available.

Previous work from our laboratory [8,9] indicated that some of the effects of boric acid include a slow increase in cell permeability as indicated by propidium iodide intrusion and decreases in the amount of cellular ergosterol production and suppression of yeast-hypha transformation. Others have indicated that boric acid may have an influence on cell wall biosynthetic mechanisms [6,10].

In this report we investigated the possibility that stress response may play a role in the inhibitory activity of boric acid. Stress responses are important both in increasing fitness leading to survival, but may also result in apoptosis leading to death of affected cells [11]. Various antimicrobial compounds [12-15] induce apoptosis and environmental conditions [16,17] have been demonstrated to induce a stress response in yeast and we here report that this is also a characteristic of boric acid. This experiment utilized a generalized phenomenon to evaluate any source of stress.

2. METHODS

2.1 Organisms

For this study, 12 strains of *Candida albicans* (CA1 – CA12) which were originally isolated from clinical infections were used. At our request from a community clinical laboratory, subcultures from clinical specimens were sent to us with no patient information. These strains were not tested for resistance to other antifungal drugs. These are maintained in Sabouraud Dextrose Broth (SDB) with 50% glycerol at -80ºC. Prior to use, frozen stocks were thawed and grown overnight in SDB consisting of 1% vegetable peptone and 2% glucose. Cultures were then diluted 1:100 in sterile SDB and 10 uL of the diluted starter culture is added to 1 mL of media with and without boric acid. Hyphae formation was induced by growing organisms in non-shaking 48 well plates of SDB for 48 hours at 37°C. Organisms were visualized by inverted microscopy.

2.2 Growth Studies

After establishing a dose-response curve, some experiments were tailored to lower concentrations of boric acid to evaluate physiological effects on *Candida* at sublethal doses. To assess the inhibitory activity of boric acid, a series of dilutions ranging from 0 to 5% w:v in SDB was prepared in 48 well culture plates inoculated with diluted starter cultures as noted above to produce dose-response data for each *Candida albicans* strain. Inoculated cultures

were placed at 37ºC overnight and contents of the wells were counted by flow cytometry, examined microscopically for morphology or tested for extra-cellular enzymes. Where noted, some experiments involved *Candida* being exposed for very short incubation times in the presence of boric acid or other compounds to assess changes occurring in the first hours of contact with inhibitors.

2.3 Microbial Counts

The BD Acuri C-6 flow cytometer fluidics system permits absolute counts and was used to obtain direct cell counts. Particle (*Candida* cell) counts employ the forward scatter channel (FSC) with a threshold set to exclude counts below the size of yeast cells. In addition, the level of auto fluorescence in fluorescence channel 1 (FL1) was also recorded for organisms from the dose-response studies. Fluorescence for the entire population of *Candida* cells was judged by the mean FL1 fluorescence for the sample.

2.4 Osmotic Challenge Experiments

The following solutions were prepared from analytical grade chemicals in sterile distilled water each at a 5% w:v concentration, boric acid, glucose, sucrose or sodium chloride. Yeast cells grown in SDB were recovered by centrifugation and re-suspended in sterile water. Aliquots of the *Candida* strains were placed into culture wells containing the osmotic challenge agents and viability and cell geometry as judged by flow cytometry were compared to cells incubated in water. Representative pH of media was recorded as follows: water (7.77), water and 5% boric acid (3.85), SDB (5.56), SDB and 5% boric acid (4.70).

2.5 Apoptosis Detection

Annexin V staining was used to identify eversion of membrane phospholipids occurring as part of the process of apoptosis. Reagents were obtained from Takara/Clontech (ApoAlert ®kit) and were used according to manufacturer's directions. Briefly, 200 uL aliquots of *Candida* exposed to 1% boric acid were removed at timed intervals (0, 3 and 6 hours) and cells recovered by centrifugation. *Candida* were resuspended in 200 uL incubation buffer and 5 uL of FITC-annexin conjugate and 10 uL of propidium iodide were added and incubated in the dark for 15 minutes. Flow cytometry was used to monitor the changes in FL-1 fluorescence over time in which FL1 fluorescence above background indicated annexin binding to everted *Candida* membranes.

2.6 Catalase Expression

To determine the amount of catalase expression, cells treated with boric acid or other stress conditions were incubated in 0.1% hydrogen peroxide for 30 minutes at 37ºC and absorbance at 230 nm determined and compared to a standard curve of 0.1 % hydrogen peroxide as in previous studies [13]. No validated method was available to test SOD expression during the experiment.

3. RESULTS

We determined, based on flow cytometry quantitative counts in the FSC channel, that all of the 12 test strains of *Candida albicans* used in this study were susceptible to the growth inhibitory effect of boric acid. The dose response curve, exemplified by data from CA2 (Fig. 1), is an asymptotic curve in which overnight growth plotted against boric acid concentration, demonstrated loss of inhibition began at boric acid concentrations of less than 1% w:v, or 10,000 mg/L. From the 36 dose response studies done (triplicates of 12 strains) we calculated the median effective dose of boric acid and found that susceptibility varied between 1000 and 2400 mg/L as shown in Fig. 1.

Flow cytometry events do not indicate whether the counts obtained were viable cells. Thus, counts may have been the result of inability of a viable inoculum to grow in the presence of boric acid (microbistatic action)creating differences between control cultures and those containing boric acid, or alternatively due to death of the yeast (microbicidal action). A follow up experiment was performed in which the 12 strains of *Candida albicans* were inoculated into water, water with 5% boric acid or Sabouraud Dextrose Broth (SDB) with 5% boric acid to employ direct plating to distinguish growth arrest from cell death. After overnight incubation, drop plates were performed with 10 uL aliquots of each of these cultures or with 1:10 dilutions of these cultures. In addition, flow cytometer counts were also performed to compare the number of particle counts (flow cytometry events) with drop plate data. This establishes the proportion of the flow cytometer counts that were due to viable cells.

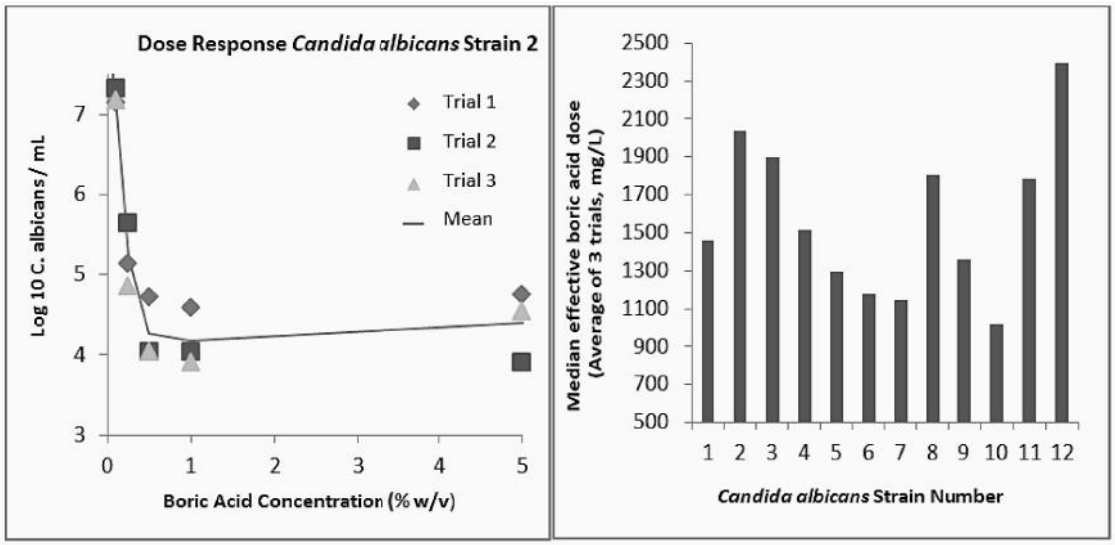

Fig. 1. Left panel shows the result of three dose response curves for *Candida albicans* strain 2 over a concentration range of 0 to 5% boric acid in Sabouraud dextrose broth. Geometric symbols indicate data from each of the 3 iterations, and "—" is the geometric mean for these three trials. The right panel indicates the median effective dose of boric acid based on triplicated dose response curves for the 12 test strains showing variation in susceptibility of strains but all having endpoints below 2500 mg/L or 0.25% w:v

Fig. 2 presents the mean FSC counts (expressed as events / uL) for the 12 strains of *Candida albicans* incubated in 5% boric acid in water or SDB after overnight incubation at 37ºC. These conditions were compared to yeast incubated overnight in distilled water, a condition known to allow persistent survival of yeast [17]. In this experiment, flow cytometry revealed the average number of yeast in the water control was 5.8 x 10^4 / mL, slightly higher that the number in SDB with 5% boric acid (4.2 x 10^4 / mL mean for 12 yeast strains). In strong contrast, the number of yeast from aqueous boric acid was nearly at the lower level of flow cytometry detection with 8.1 x 10^3 / mL. The drop plate data was revealing, as all of the cultures in water show survivors ranging from 23% - 100% of the number detected by flow cytometry, confirming the expected survival in water alone. The cultures incubated with boric acid had no survivors with a detection limit of 1 x 10^2 counts / mL. Together, the viable counts and flow cytometer data indicated that boric acid in SDB resulted in loss of *Candida* viability with modest loss in flow cytometry counts. This indicates that cell bodies remained intact despite decreasing viability in boric acid. Perhaps the effect of boric acid in water was exacerbated by low osmolarity. Boric acid in water not only rendered yeast non-viable, but also caused their disintegration which suggested morphologic evaluation may provide additional insights into the process of yeast cell damage.

Candida exposed to boric acid in SDB and viewed as microscopic wet mount preparations (Fig. 3) indicated that hyphal forms do not readily develop in boric acid treated cells as they do in SDB alone. Under direct microscopic observation, the boric acid-treated cells appeared not only as yeast forms lacking hyphal elements, but also seemed smaller in size than corresponding blastocondia in SDB. While this was a subjective observation, made all the more difficult by the paucity of yeast in boric acid cultures, the FSC channel of the flow cytometer was exploited to demonstrate the relative size of cultured yeast cells.

FSC data available from 12 *Candida* strains challenged with boric acid at 5% in SDB was compared to yeast grown without boric acid because the positive relationship between relative cell size and FSC values. Because the flow cytometer is able to evaluate the individual geometry of thousands of cells in a sample, the power of this technique far exceeds the ability of direct microscopic observation. Fig. 4 which is experimental data from one strain, provides clear evidence that detectable cell involution was reflected by the mean FSC signal of boric acid-treated yeast.

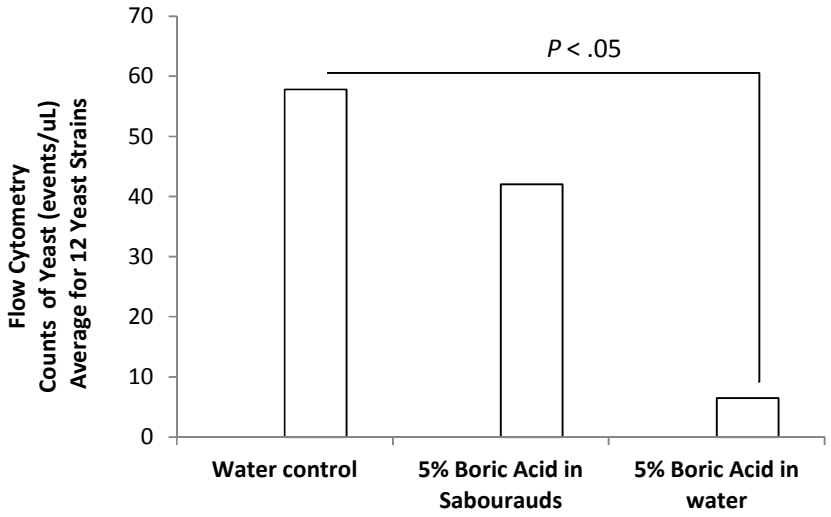

Fig. 2. 12 *Candida albicans* strains were grown overnight in Sabouraud broth as starter cultures and diluted 1:100 in sterile water. For each strain 10 uL of the diluted starter was added to 1 mL of either water, 5% boric acid in water or Sabouraud broth. After overnight incubation at 37ºC counts were made with flow cytometer and by plating on Sabouraud agar. Data is shown for flow cytometer counts and plate counts for both boric acid conditions showed complete loss of viability for all *Candida* strains

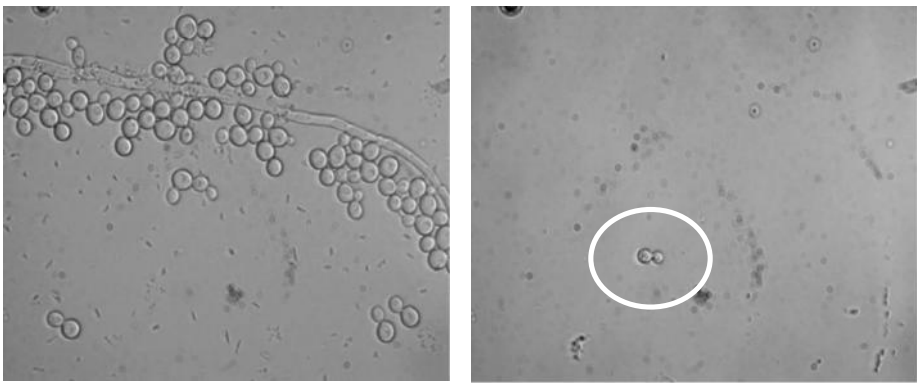

Fig. 3. *Candida albicans*, strain 6 were incubated in Sabouraud broth with and without boric acid. In the absence of boric acid hyphal forms were observed (left). Boric acid reduced the number of cells resulting in few cells per high power field, and cells appeared to be diminished in size. Both images photographed at 400x

The consistency of the effect of diminished cell size is illustrated in Fig. 5 which summarizes FSC data for 12 strains of *Candida* grown in SDB at 5%, 0.5% and 0.05% boric acid concentrations. Data were expressed as the percent of relative cell size based on FSC of *Candida* grown in SDB. Cell involution was apparent across all concentrations and all strains of *Candida*. In addition, the relative cell size coupled with the absolute number of *Candida* cells in culture allowed an estimation of relative biomass of cells exposed to boric acid. When FSC values were multiplied by the number of cells, biomass estimates were at least 100 fold lower in the 5% and 0.5% concentrations of boric acid and ten-fold lower than controls in 0.05% boric acid. Although, 0.05% boric acid is below the median effective dose of boric acid for all yeast strains (cf. Fig. 1) the product of cell count and FSC revealed an effect of boric acid even at concentrations that permitted visible growth (turbidity).

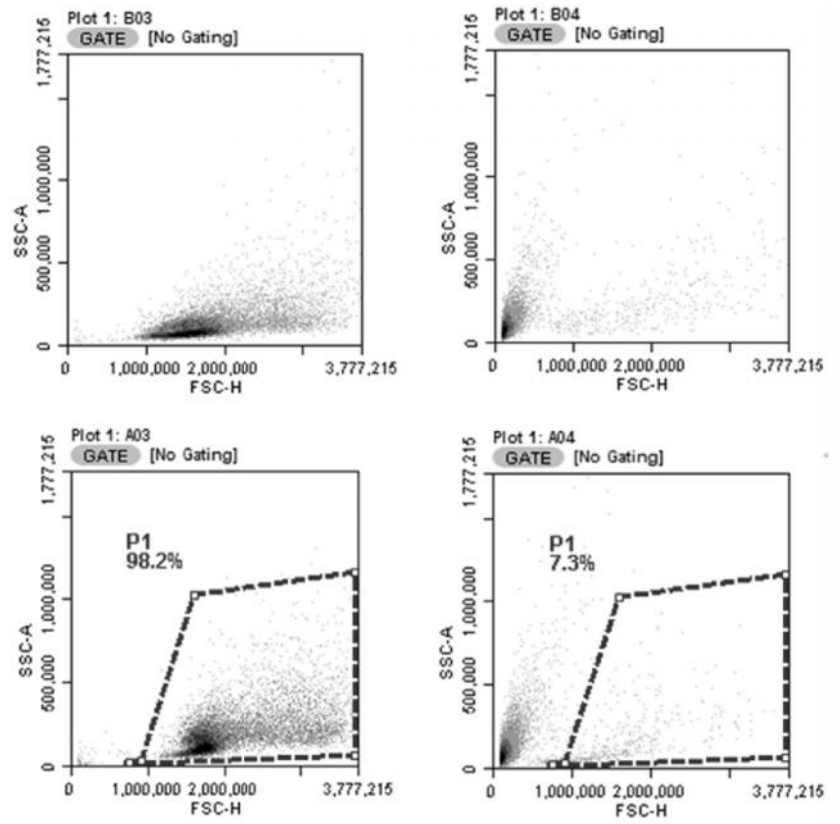

Fig. 4. Flow cytometry data from Sabouraud broth grown *Candida* compared to Sabouraud broth with 5% boric acid. Upper two figures are the raw data from *Candida albicans* 1 with results in boric acid to the right and media without boric acid to the left. The lower figures represent the same experiment with *Candida albicans* 2. A polygon region was drawn around the yeast grown in media (P1) and this region encompassed 98% of events. When the same region was applied to the data for *Candida albicans* 2 grown in 5% boric acid, only 7% of events were in the region indicating the size diminution of boric acid treated cells

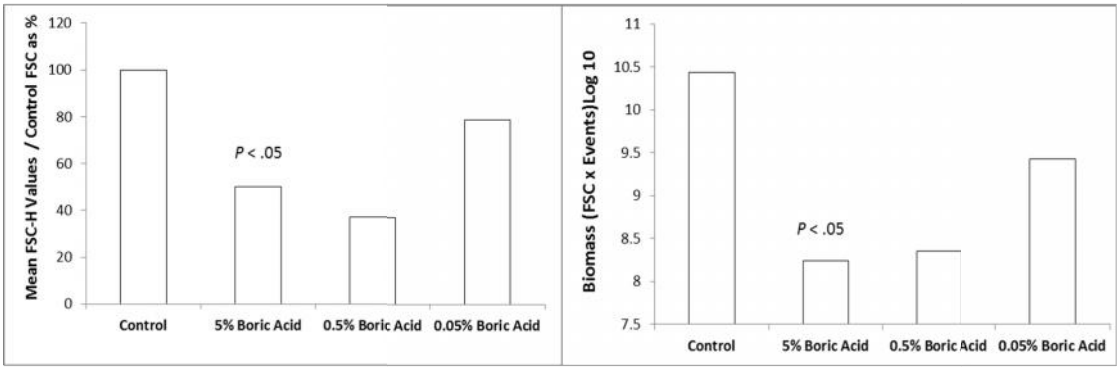

Fig. 5. The consistency of diminished cell size in the presence of boric acid was confirmed by flow cytometry averaged for all 12 *Candida* strains. Upper figure indicates that the mean forward scatter values were less than control values. Cell counts from the same experiment were multiplied by mean FSC values to provide an estimate of the total biomass for each culture (lower figure). For each, paired Student's T test was used to compare controls to 5% boric acid containing cultures

Cell shrinkage occurs in eukaryotic cells undergoing programmed cell death [18] suggesting that changes in cell geometry seen in boric acid exposed yeast may reflect an apoptotic pathway. To investigate whether the cell shrinkage may have been related to apoptosis among cells exposed to boric acid, each of the 12 yeast strains were exposed to 1% boric acid and annexin V staining was monitored over time. Annexin V staining was done at 0, 3, 6 and 24 hours, and increased over the course of the experiment for all strains. As indicated in Table 1 staining intensity nearly doubled over the course of the experiment and the % of yeast strains.

Previous research has shown that osmotic stress through elevated glucose concentrations causes yeast to become apoptotic [19] which prompted an additional experiment in which we exposed 12 *Candida* strains to iso-osmolar (1 M / L) glucose, sucrose, and sodium chloride to determine if these stressors have similar effects as boric acid. Table 2 provides the data obtained which increases in FSC for the sugars and for sodium chloride and boric acid the trend was toward decreasing FSC values, though sodium chloride was not statistically different.

Flow cytometry was also used to evaluate the intrinsic fluorescence signature of boric acid-treated and untreated cells. We incidentally noted that boric acid treated cells had, in addition to showing diminished forward scatter, elevated green channel fluorescence. In Fig. 6, increased fluorescence is evident and is clearly dose-dependent. All three concentrations increased intrinsic fluorescence of the yeast above control levels, including the lowest boric acid concentration, though not as profound as for boric acid concentrations of 5% and 0.5%. This suggested that in addition to boric acid stress initiating apoptotic pathways, it also induced changes resulting in increased cellular auto-fluorescence.

Stress adaptation may also include altered expression of protective enzymes such as antioxidants. A facile method of catalase estimation was used to determine if this stress indicator was induced in the presence of boric acid compared to growth medium. For this experiment, 12 strains of *Candida albicans* were exposed for 2 or 5 hours to SDB or 5% boric acid in SDB alone at 37^{o}C. At the end of the challenge time yeast cells were centrifuged, and medium was removed without disturbing the pellet. The pellet was re-suspended in 0.1% hydrogen peroxide and incubated for 30 minutes at 37^{o}C. Cells were again pelleted by centrifugation and the absorbance of the supernatant fluid at 230 nm was measured and compared to the absorbance of the substrate alone. The results shown in Fig. 7 indicate that in as little as 2 hours exposure, catalase content of the boric acid treated cells was decreased to about half the amount in cells harvested from growth medium without boric acid.

Table 1. Annexin V staining of 12 strains of *Candida albicans* exposed to 1% boric acid in SDB

Parameter	0 hours	3 hours	6 hours	24 hours	P= (24 hours vs 0 hours)
Mean FL-1h*	2002±740	2590±937	3015±1170	3933±1075	1.2×10^{-5}
Mean % > $1x10^{3}$**	31.5±15.2	41.5±13.9	43.7±16.0	76.6±740	3.9×10^{-8}

**FL-1h is the FITC signal reflecting Annexin V staining yeast cells averaged for 12 yeast strains.*
***Mean % is derived from flow cytometry histogram in which a marker is set at an FL1-h value excluding the majority of cells unstained by Annexin V and so is an estimate of the percent of cells stained with annexin V. The percent stained cells (FL1-h > $1x10^{3}$) was averaged for the 12 strains.*
P values are based on paired t test comparing mean values for 12 strains at 24 hours vs 0 hours

Table 2. Relative cell size of 12 strains of *Candida* exposed to osmotic stress

Condition	Mean FSC at 4 hours, 25ºC ± SD	P= (compared to water control)
Water	1.896 + 0.163 (x 10^{6})	-
1M glucose	2.178 + 0.436 (x 10^{6})	0.021
1M sucrose	2.691 + 0.384 (x 10^{6})	1.2×10^{-5}
1M NaCl	1.779 + 0.401 (x 10^{6})	0.259
1M boric acid	1.522 + 0.344 (x 10^{6})	0.0023

P values are for two-tailed paired t-test comparing each of 12 strains to the water control

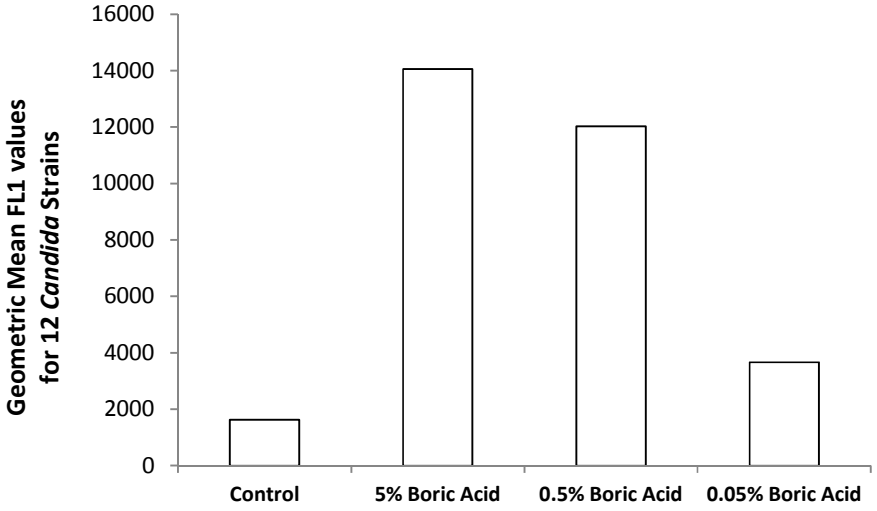

Fig. 6. Fluorescence values recorded for the three concentrations of boric acid in SDB were compared to control cultures for SDB alone. The geometric mean fluorescence for the population for all 12 strains was graphed and all levels of boric acid showed at least double the fluorescence of the controls and for 0.5 and 5% boric acid more than ten-fold increase

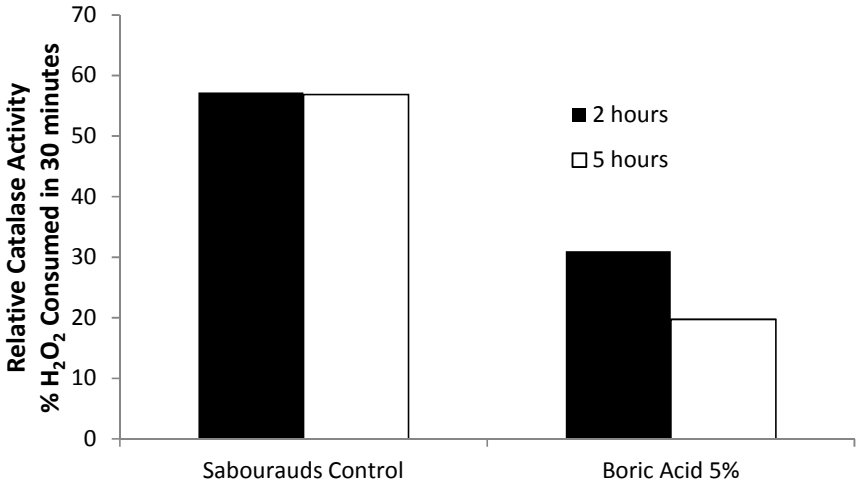

Fig. 7. Relative catalase activity of *Candida albicans* (average for 12 strains). The difference between control and boric acid treated was significant ($p<0.001$ for 5 hour incubation and $p<0.004$ for 2 hour incubation)

4. DISCUSSION

This study provided several key findings that are of relevance to use of boric acid as an anti-*Candida* topical therapy. First, all of 12 clinical *Candida* isolates tested showed median inhibitory concentration values which, while variable, did not reveal any strains clearly refractory to boric acid inhibition. While this does not preclude intrinsic resistance among *Candida albicans*, a much larger culture collection would need to be tested to establish the absence of resistance for clinical isolates. Manipulation of boric acid sensitivity has been demonstrated through deletion of a putative boron transporter gene [20], but the prevalence of boron resistance in clinical isolates is less well established even though some species such as *Candida glabrata* have higher MICs than do *C. albicans* isolates [21].

We found flow cytometry a useful means of accurately measuring the potency of antimicrobial effect and allowed for evaluation of relatively large experiments. This established the range of concentrations associated with growth inhibition which ranged between 0.1% and 0.25%. According to predictions made previously [8], these concentrations should be readily met with current published treatment regimens [4].

The use of flow cytometry to develop dose-response data led to incidental findings of apparent cell size decrease upon exposure to boric acid and concomitant increases in cellular auto-florescence. Cell involution has been recognized as a hallmark of the apoptotic process in a variety of eukaryotic cell types [18,19] including yeast [22]. Our data use flow cytometry data to confirm the involution of boric acid exposed cells which was suggested by microscopic observation. Because we suspected this change was related to boric acid stress inducing apoptosis, we employed FITC-conjugated annexin V staining to support the possible role of apoptosis. In addition, the inhibition of cell growth coupled (Fig. 1) with cellular involution (Fig. 4) reflected a significant decrease in biomass at all boric acid concentrations tested. The decrease in cell size also revealed that at boric acid concentrations of 0.05%, half that of the lowest median effective doses the biological effect was still observed.

Prior to planned fluorescent staining studies, we measured the auto fluorescence in the FL1 (green) channel and unexpectedly discovered that intrinsic mean cell fluorescence was increased with boric acid exposure. This observation did not reveal the mechanism of increased fluorescence which might be explained as a reflection of cell shrinkage which would be expected to concentrate endogenous fluorescent molecules leading to an increased FL1 signal per cell or may have resulted from up-regulation of fluorescent proteins as part of the stress-response or early stages of apoptosis induction. If additional studies identify the mechanisms involved, the use of cell fluorescence induction could provide a facile measure of stress response.

If programmed cell death occurred in response to boric acid induced stress, it should be possible to demonstrate a loss of microbial viability which we demonstrated *Candida* incubated in 5% boric acid in SDB or 5% boric acid in water. However, in the presence of growth medium there were more surviving cell bodies (flow cytometer events) than in water. Water without boric acid showed higher counts and little loss of viability. Programmed cell death may occur through apoptosis or in some cases autophagy, with the latter dependent on nutrient deprivation [22] which may help explain differences between boric acid effects with and without nutrient.

Among the responses to stress may be the up-regulation of protective substances such as enzymes that inactivate reactive oxygen species. However, when cells enter the apoptotic pathway, protective substances may be decreased and reactive oxygen species accumulate. A facile method to explore this in boric acid-treated *Candida* was measurement of catalase activity which was decreased with boric acid treated cells (Fig. 7). This, along with annexin V data supports the possibility that loss of viability in these experiments involved programmed cell death, though additional methods may be used to further explore and confirm the mechanism.

The mechanism of microbial inhibition by boric acid revealed several interesting biological findings, but our original interest in boric acid originated with its potential use in vaginal infections. This report has provided information about the typical concentrations of boric acid that inhibit a breadth of strains and continues to support the clinical applications and in addition to previous studies that suggested boric acid while limiting *Candida* replication and hyphal transformation, may also have pro-apoptotic activities for which catalase or other protective molecules may have a role.

5. CONCLUSION

Additional work is needed to identify the specific role of boric acid in induction of programmed death of *Candida* and evaluation of the role and kinetics of catalase inhibition in the process. The induction of a programmed death pathway may suggest boric acid treated cultures and possibly infected patients may create a persistent antifungal effect, beneficial to therapy.

CONSENT

No human subjects were involved in this research.

ETHICAL APPROVAL

No animal subjects were employed in the conduct of this research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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