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Original Article

Candidacidal activities of zinc compounds based on incubation time, pH, and ionic strength — An in vitro study

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Abstract *Background/purpose:* Given the antifungal activity of zinc compounds against *Candida albicans*, this study aimed to investigate the effects of incubation time, pH, and ionic strength on their candidacidal activity against *C. albicans*.
Materials and methods: Two zinc compounds (zinc chloride and sulfate) and three *C. albicans* strains (ATCC 10231, 11006, and 18804) were used. The parameters considered include incubation times of immediate, 10, 60, and 120 min, pH of 5.5, 5.0, 4.5, and 4.0, and ionic strength of mimicking human saliva, 100, 150, and 200 mM. The candidacidal activity was determined by comparing colony numbers on the experimental and control plates.
Results: The candidacidal activities of the zinc compounds significantly increased with increasing incubation time, decreasing pH, and increasing ionic strength. Significant levels of candidacidal activity were detected even within 10 min of incubation. The zinc compounds provided additional candidacidal activity under each pH and ionic strength condition. However, the effects of adding zinc compounds were less than those of lowering pH or increasing ionic strength. The candidacidal activities at each incubation time, pH, and ionic strength varied depending on the zinc compound and *C. albicans* strain; lower activities in zinc chloride and the 18804 strain.
Conclusion: The candidacidal activity of the zinc compounds occurred immediately. They provided additional candidacidal activities even at low pH or high ionic strength environments. Oral healthcare products containing zinc compounds can provide antifungal activities in a changing oral environment.

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Introduction

With the advent of an aging society, the number of patients visiting dental clinics for oral candidiasis is increasing, and the prescription of antifungal drugs to treat oral candidiasis is increasing.[1–3] In most patients, the prescription of antifungal drugs results in good clinical outcomes; however, oral candidiasis tends to recur frequently due to local or systemic problems, including decreased salivation or reduced immunity.[1,3] Furthermore, repeated administration of antifungal medications increases the physical burden in elderly patients through drug interactions[4,5] and organ toxicity.[6] Repeated topical antifungal treatment may also increase dental caries.[7] Therefore, it is essential to develop topical antifungal agents that can be safely and repeatedly applied to elderly patients without physical burden or oral complications.

Recently, a report on the antifungal activity of zinc compounds against oral *Candida albicans*, the first of its kind, was published.[8] Although it varies depending on the zinc compound and the *C. albicans* strain, the minimum inhibitory concentration (MIC) of zinc compounds was approximately 1.0 mM, yielding fungicidal activities of 17.7%–38.8%. Additionally, zinc compounds reportedly affect the activities of antimicrobial enzymes from various sources, including human saliva.[8,9] Particularly, zinc sulfate has been suggested as a candidate compound for oral healthcare products in terms of lysozyme and antifungal activities.[8]

From an oral health perspective, zinc compounds have several advantages. They are safe[10] and have various useful biological activities.[11,12] For topical administration, zinc compounds reduce inflammation and oxidative reactions and help wound regeneration and healing.[13–15] They also prevent dental caries and periodontal diseases by augmenting tooth remineralization and reducing plaque formation.[16–19] Zinc compounds reduce oral malodor by binding to sulfur compounds.[20,21] These properties and antifungal activity of zinc compounds suggest their suitability as topical agents for elderly patients who have difficulty maintaining oral hygiene.

A previous study on the candidacidal activity of zinc compounds used a simulated salivary buffer (SSB) with similar ionic strength to human saliva at pH 5.5 to ensure the solubility of the zinc compounds, and *Candida* cells were exposed to these compounds for 60 min.[8] However, considering the changing oral environment, information on the antifungal activity of zinc compounds under various conditions is essential. Therefore, this study aimed to investigate changes in candidacidal activity based on incubation time, pH, and ionic strength. The results of this study will help us understand the antifungal activity of zinc compounds in various oral environments.

Materials and methods

Zinc compounds and *C. albicans* strains

The zinc compounds, zinc chloride ($ZnCl_2$, M.W. 136.3), and zinc sulfate ($ZnSO_4 \cdot 7H_2O$, M.W. 287.6), were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Unless other experimental conditions were mentioned, each zinc compound at a final concentration of 1 mM in SSB (1.95 mM potassium phosphate dibasic, 16 mM potassium chloride, 14.5 mM sodium chloride, and 0.96 mM calcium chloride dihydrate) at pH 5.5 was used based on a previous study.[8] pH 5.5 was used to ensure the solubility of the zinc compounds.

For *C. albicans*, three strains (ATCC 10231, 11006, and 18804) with different growth patterns[22] were used. Unless other conditions regarding the candidacidal experiment were mentioned, the incubation time of the zinc compounds with the *Candida* cells was 60 min.

Effects of incubation time on the candidacidal activity of the zinc compounds

The incubation time of immediate, 1, 5, 10, 60, and 120 min were used in preliminary experiments. The candidacidal activities at 1 and 5 min showed a gradual pattern between immediate and 10 min; therefore, the experiment was conducted only at immediate, 10, 60, and 120 min. The sample without the zinc compound served as the control in each experiment. Two concentrations of the zinc compounds (1 and 10 mM) were used. The experiments were performed five times.

Effects of pH on the candidacidal activity of the zinc compounds

The pH values of 5.5, 5.0, 4.5, and 4.0 were used in the experiments. For each pH condition, the sample without any zinc compound served as the control. To investigate the effects of pH, the experimental data obtained without zinc compound at pH 5.0, 4.5, or 4.0 were compared with those without zinc compound at pH 5.5. To investigate the candidacidal activity of the zinc compounds at each pH, the experimental data obtained with and without zinc compounds were compared. The experiments were performed five times.

Effects of ionic strength on the candidacidal activity of the zinc compounds

The ionic strengths of SSB, 100, 150, and 200 mM were used in the experiments. The calculated ionic strength of the SSB

used was 39.2 mM, and the concentration of sodium chloride was adjusted to obtain solutions with specific ionic strengths. At each ionic strength, the sample without zinc compound served as the control. To investigate the effects of ionic strength, the experimental data obtained without the zinc compound at ionic strengths of 100, 150, and 200 mM were compared with those obtained without zinc compound at the ionic strength of the SSB. To investigate the candidacidal activity of the zinc compound at each ionic strength, the experimental data obtained with and without the zinc compounds were compared. The experiments were performed five times.

Determination of candidacidal activity

First, 10 mL of yeast malt (YM) broth inoculated with a single *C. albicans* colony was incubated with shaking at 37 °C for 18 h. The *Candida* cells were then resuspended to 1×10^5 cells/mL. To investigate the candidacidal activity, 20 μL of *Candida* cell suspension was added to 40 μL of the solutions containing reagents suitable for the experimental conditions. The *Candida* cell-reagent mixture solutions were incubated with shaking at 37 °C for the designated time. The mixtures were then diluted ten-fold, 50 μL of the diluted samples were plated onto YM agar plates in triplicate, and grown overnight at 37 °C. The candidacidal activity (%killing) was calculated using the colony numbers on the experimental and control plates. The detailed procedures have been described.[23]

Statistical analysis

Friedman and Wilcoxon signed rank tests were used to analyze differences according to incubation time, pH, and ionic strength. Wilcoxon signed rank test was used to analyze differences according to the presence or absence of each zinc compound. Mann–Whitney U test was used to analyze differences based on the zinc compound type and concentration. Kruskal–Wallis test was used to analyze differences based on *C. albicans* strain, and Mann–Whitney U test was used as a post-hoc. A *P*-value of <0.05 was considered statistically significant.

Results

Effects of incubation time on the candidacidal activity of the zinc compounds

Tables 1–3 show the effects of incubation time on the candidacidal activity of the zinc compounds. Regardless of the *C. albicans* strain, candidacidal activity significantly increased with increasing incubation time for the two zinc compounds at both concentrations. Although there were variations depending on the type of zinc compound and *Candida* strain, at a concentration of 1 mM, the candidacidal activities were approximately 15%–30% at immediate, 25%–50% at 10 min, 35%–60% at 60 min, and 40%–65% at 120 min. The candidacidal activities of the two zinc compounds did not differ significantly based on the concentration of the zinc compounds at all examined incubation

Table 1 Candidacidal activities of the zinc compounds based on incubation time (*Candida albicans* strain ATCC 10231).

Zinc compounds	n = 5	Control	0 min	10 min	60 min	120 min	Significance
Zinc chloride	CFU	101.5 [99.8–104.5]^{abcd}	85.5 [76.0–89.5]^{aef}	68.0 [63.8–71.8]^{bg}	51.0 [42.8–54.5]^{ceg}	60.0 [43.8–64.0]^{df}	0.001**
1 mM	%killing	–	18.2 [11.4–25.4]	32.8 [29.5–37.9]	49.3 [47.9–57.5]	40.9 [37.5–57.2]	
Zinc chloride	CFU	98.5 [91.0–104.8]^{abcd}	63.0 [58.5–84.5]^{aefg}	59.5 [53.0–69.5]^{behi}	51.5 [46.5–64.8]^{cfhj}	39.5 [35.0–59.0]^{dgij}	<0.001**
10 mM	%killing	–	32.6 [19.3–37.4]	41.1 [29.3–44.9]	48.4 [34.8–51.5]§	55.6 [43.4–63.4]	
Zinc sulfate	CFU	93.0 [82.3–102.5]^{abcd}	71.5 [59.3–76.8]^{aef}	52.5 [47.0–55.8]^{bg}	47.0 [33.0–55.3]^{ce}	41.5 [33.0–49.0]^{dfg}	0.001**
1 mM	%killing	–	23.5 [16.4–35.7]	48.5 [35.9–49.5]	53.9 [43.6–59.9]	54.9 [49.4–62.4]	
Zinc sulfate	CFU	86.5 [73.5–95.5]^{abcd}	70.5 [54.5–75.0]^{aefg}	47.5 [37.5–54.3]^{behi}	31.0 [23.5–38.0]^{cfh}	34.0 [24.5–38.8]^{dgi}	0.001**
10 mM	%killing	–	14.0 [10.4–35.8]	45.1 [41.1–50.3]	61.5 [55.0–72.3]§	58.5 [57.0–70.0]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on incubation time. **$P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference at the same concentration of each zinc compound. $P < 0.05$.
The Mann–Whitney U test was used to analyze differences in %killing based on the type of zinc compound at the same concentration at each incubation time. $^{\S}P < 0.05$.

Table 2 Candidacidal activities of the zinc compounds based on incubation time (*Candida albicans* strain ATCC 11006).

Zinc compounds	n = 5	Control	0 min	10 min	60 min	120 min	Significance
Zinc chloride	CFU	100.5 [95.0—104.3]^{abcd}	70.5 [56.0—81.8]^{aefg}	53.0 [46.3—72.3]^{beh}	50.0 [39.8—64.0]^{cf}	40.0 [37.3—60.0]^{dgh}	0.001**
1 mM	%killing	—	32.1 [17.7—43.3]	45.5 [29.5—53.2]	46.2 [38.6—59.8]	59.8 [41.3—62.1]	
Zinc chloride	CFU	98.5 [95.5—104.0]^{abcd}	58.0 [54.8—69.3]^{ae}	53.5 [41.8—68.5]^{bfg}	42.0 [36.0—58.3]^{cef}	40.0 [34.0—61.0]^{dg}	0.002**
10 mM	%killing	—	40.9 [33.0—43.4]§	43.0 [34.2—57.5]	57.4 [42.5—63.7]	58.1 [41.6—65.2]	
Zinc sulfate	CFU	93.5 [79.0—103.8]^{abcd}	62.5 [60.5—71.3]^{aefg}	57.5 [48.8—61.5]^{behi}	52.0 [41.5—52.8]^{cfhj}	37.5 [33.3—44.0]^{dgij}	<0.001**
1 mM	%killing	—	28.0 [18.2—38.0]	38.5 [34.7—44.8]	49.0 [43.7—50.8]	61.3 [51.1—62.9]	
Zinc sulfate	CFU	92.5 [74.0—96.5]^{abcd}	65.0 [50.0—71.5]^{aefg}	51.5 [39.5—56.3]^{beh}	42.0 [33.5—46.5]^{cfh}	40.0 [29.0—42.0]^{dg}	0.001**
10 mM	%killing	—	27.0 [25.5—33.8]§	43.8 [39.6—48.7]	53.5 [47.7—58.8]	56.1 [47.6—67.4]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on incubation time. $^{**}P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference at the same concentration of each zinc compound. $P < 0.05$.
The Mann—Whitney U test was used to analyze differences in %killing based on the type of zinc compound at the same concentration at each incubation time. $^{\S}P < 0.05$.

Table 3 Candidacidal activities of the zinc compounds based on incubation time (*Candida albicans* strain ATCC 18804).

Zinc compounds	n = 5	Control	0 min	10 min	60 min	120 min	Significance
Zinc chloride	CFU	122.5 [106.8—134.8]^{abcd}	104.0 [93.5—122.3]^{aefg}	84.5 [80.0—99.5]^{be}	82.0 [69.0—90.3]^{cf}	58.5 [52.0—82.0]^{dg}	0.002**
1 mM	%killing	—	13.7 [3.7—18.2]	24.4 [22.7—31.4]	33.2 [31.5—36.7]	45.3 [37.6—54.8]	
Zinc chloride	CFU	113.0 [98.3—122.5]^{abcd}	96.0 [91.0—101.8]^{aef}	89.5 [77.5—94.0]^{bg}	75.0 [65.5—78.5]^{ceh}	55.5 [50.3—61.3]^{dfgh}	0.001**
10 mM	%killing	—	15.8 [5.7—17.9]	17.9 [13.9—30.8]§	32.0 [24.4—43.2]	50.9 [42.2—55.9]	
Zinc sulfate	CFU	102.0 [93.0—114.8]^{abcd}	82.5 [73.5—85.0]^{ae}	72.5 [47.0—79.3]^{b}	71.0 [51.3—81.0]^{c}	66.5 [50.0—71.5]^{de}	0.007**
1 mM	%killing	—	19.0 [8.6—35.4]	38.2 [17.9—54.1]	35.8 [17.7—49.9]	47.2 [22.6—51.1]	
Zinc sulfate	CFU	103.0 [92.5—107.5]^{abcd}	77.0 [60.0—88.0]^{a}	59.5 [52.0—65.0]^{b}	65.5 [60.0—69.5]^{ce}	52.5 [48.5—57.0]^{de}	0.007**
10 mM	%killing	—	18.1 [8.9—44.0]	39.8 [30.8—51.3]§	35.8 [28.1—41.4]	48.0 [43.8—50.9]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on incubation time. $^{**}P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference at the same concentration of each zinc compound. $P < 0.05$.
The Mann—Whitney U test was used to analyze differences in %killing based on the type of zinc compound at the same concentration at each incubation time. $^{\S}P < 0.05$.

time points in the three *C. albicans* strains. The candidacidal activities of the two zinc compounds at 10 mM differed significantly at 60 min in strain 10231 ($P = 0.008$), immediate in strain 11006 ($P = 0.032$), and 10 min in strain 18804 ($P = 0.032$).

The candidacidal activity based on incubation time differed depending on the *C. albicans* strain, showing relatively lower activity in strain 18804. Zinc chloride at a concentration of 1 mM differed significantly at 60 min (10231 > 18804, $P = 0.008$). Zinc chloride at a concentration of 10 mM differed significantly at immediate (10231 > 18804, $P = 0.016$; 11006 > 18804, $P = 0.008$) and at 10 min (10231 > 18804, $P = 0.032$; 11006 > 18804, $P = 0.032$). Zinc sulfate at 1 mM differed significantly at 120 min (11006 > 18804, $P = 0.032$). Zinc sulfate at 10 mM differed significantly at 60 min (10231 > 18804, $P = 0.008$; 11006 > 18804, $P = 0.008$) and at 120 min (10231 > 18804, $P = 0.008$).

Effects of pH on the candidacidal activity of the zinc compounds

Tables 4–6 show the effects of pH on the candidacidal activity of the zinc compounds. In the absence of the zinc compounds, the candidacidal activity significantly increased with decreasing pH. The candidacidal activities were approximately 15%–50% at pH 5.0, 30%–55% at pH 4.5, and 35%–60% at pH 4.0. The presence of the zinc compounds provided additional candidacidal activity at each pH, especially in strain 18804. However, these effects were less than those of pH itself, and they tended to decrease further as the pH decreased. The presence of zinc chloride significantly increased the candidacidal activity at pH 5.5 ($P = 0.043$) and pH 5.0 ($P = 0.043$) in the three strains and at pH 4.5 ($P = 0.043$) and pH 4.0 ($P = 0.043$) in strain 18804. The presence of zinc sulfate significantly increased the candidacidal activity at pH 5.5 ($P = 0.043$), pH 5.0 ($P = 0.043$),

Table 4 Candidacidal activities of the zinc compounds based on pH (*Candida albicans* strain ATCC 10231).

Zinc compounds	n = 5	pH 5.5	pH 5.0	pH 4.5	pH 4.0	Significance
Without Zinc chloride	CFU	93.0 [86.0–96.3]$^{abc\#}$	51.0 [48.5–51.3]$^{a\#}$	50.0 [32.0–53.5]^{b}	33.5 [31.0–51.8]^{c}	0.013*
	%killing	–	45.2 [40.9–49.1]	47.4 [41.5–64.4]	60.5 [44.1–66.9]	
With Zinc chloride	CFU	58.0 [48.8–61.3]$^{\#}$	47.0 [37.5–48.3]$^{\#}$	40.5 [29.0–53.0]	34.0 [27.8–50.5]	0.226
1 mM	%killing	39.3 [35.6–43.3]§	48.9 [44.2–61.1]	58.2 [41.5–67.6]	59.3 [45.4–70.6]	
Without Zinc sulfate	CFU	82.0 [76.0–93.0]$^{abc\#}$	49.5 [43.5–58.3]$^{a\#}$	38.5 [35.5–54.0]$^{b\#}$	40.0 [30.8–53.0]^{c}	0.014*
	%killing	–	38.1 [30.3–49.2]	50.3 [39.8–57.0]	55.1 [41.0–59.8]	
With Zinc sulfate	CFU	47.5 [40.0–50.8]$^{\#}$	40.0 [33.0–48.0]$^{\#}$	36.0 [26.8–51.0]$^{\#}$	34.5 [30.0–45.3]	0.077
1 mM	%killing	45.5 [43.8–47.5]§	55.1 [45.7–56.9]	59.6 [43.4–65.1]	57.1 [51.5–61.0]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on pH. *$P < 0.05$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each pH level. $^{\#}P < 0.05$.
The Mann–Whitney U test was used to analyze differences in %killing based on the type of zinc compound at each pH level. $^{\S}P < 0.05$.

Table 5 Candidacidal activities of the zinc compounds based on pH (*Candida albicans* strain ATCC 11006).

Zinc compounds	n = 5	pH 5.5	pH 5.0	pH 4.5	pH 4.0	Significance
Without Zinc chloride	CFU	71.5 [59.8–86.5]$^{abc\#}$	52.5 [46.8–57.5]$^{ad\#}$	42.5 [41.0–48.8]^{b}	40.0 [36.5–45.8]^{cd}	0.005**
	%killing	–	26.6 [21.8–33.5]	31.6 [28.1–49.7]	41.7 [34.5–51.2]	
With Zinc chloride	CFU	51.5 [42.8–65.0]$^{abc\#}$	42.5 [36.5–51.0]$^{ad\#}$	39.5 [37.8–41.0]^{b}	38.0 [28.8–40.5]^{cd}	0.007**
1 mM	%killing	28.0 [20.4–32.2]	41.3 [34.5–45.1]§	42.7 [36.8–53.1]§	52.5 [41.5–59.0]	
Without Zinc sulfate	CFU	86.5 [75.8–89.5]$^{abc\#}$	44.5 [38.5–52.8]$^{ad\#}$	37.0 [30.3–42.3]$^{b\#}$	36.5 [34.5–40.8]$^{cd\#}$	0.008**
	%killing	–	51.1 [33.8–53.7]	55.1 [49.9–63.0]	57.8 [48.9–59.0]	
With Zinc sulfate	CFU	54.5 [46.5–56.0]$^{abc\#}$	33.0 [28.5–39.3]$^{a\#}$	32.0 [23.3–34.0]$^{b\#}$	34.0 [26.0–34.5]$^{c\#}$	0.012*
1 mM	%killing	37.0 [26.0–48.1]	62.8 [50.3–66.0]§	62.8 [58.0–71.9]§	62.6 [56.6–68.7]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on pH. *$P < 0.05$; **$P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each pH level. $^{\#}P < 0.05$.
The Mann–Whitney U test was used to analyze differences in %killing based on the type of zinc compound at each pH level. $^{\S}P < 0.05$.

Table 6 Candidacidal activities of the zinc compounds based on pH (*Candida albicans* strain ATCC 18804).

Zinc compounds	n = 5	pH 5.5	pH 5.0	pH 4.5	pH 4.0	Significance
Without Zinc chloride	CFU	129.5 [116.8–131.5]$^{abc\#}$	94.5 [78.5–103.5]$^{a\#}$	89.5 [81.0–96.5]$^{b\#}$	75.0 [72.0–87.0]$^{c\#}$	0.014*
	%killing	–	29.2 [12.0–38.5]	30.9 [16.8–38.4]	42.1 [26.4–44.3]	
With Zinc chloride	CFU	73.5 [65.0–77.8]$^{\#}$	67.0 [58.3–81.5]$^{\#}$	71.5 [65.5–73.5]$^{\#}$	61.0 [55.5–66.5]$^{\#}$	0.098
1 mM	%killing	44.9 [33.8–49.1]	46.6 [30.2–55.8]	44.8 [37.6–49.5]	51.3 [46.0–56.2]	
Without Zinc sulfate	CFU	120.5 [107.3–126.5]$^{abc\#}$	91.5 [88.5–110.0]$^{ade\#}$	68.0 [62.0–91.0]$^{bd\#}$	74.0 [63.5–87.5]$^{ce\#}$	0.004**
	%killing	–	13.7 [11.6–23.8]	43.6 [23.5–46.9]	32.0 [28.8–45.6]	
With Zinc sulfate	CFU	64.5 [55.5–69.0]$^{\#}$	54.0 [50.8–59.3]$^{\#}$	57.5 [44.5–65.8]$^{\#}$	51.0 [41.5–57.0]$^{\#}$	0.519
1 mM	%killing	46.6 [38.1–54.3]	51.9 [50.9–56.5]	52.9 [47.9–58.5]	57.7 [51.0–64.1]	

Values are presented as median [interquartile range].
CFU, colony forming unit.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on pH. *$P < 0.05$; **$P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each pH level. $^{\#}P < 0.05$.

and pH 4.5 ($P = 0.043$) in the three strains and at pH 4.0 ($P = 0.043$) in strains 11006 and 18804. The candidacidal activities of the two zinc compounds differed significantly at pH 5.0 ($P = 0.032$) and pH 4.5 ($P = 0.016$) in strain 11006.

The candidacidal activity based on pH differed significantly depending on the *C. albicans* strain, but only in the absence of the zinc compounds, showing relatively lower activity in strain 18804. The candidacidal activities differed significantly at pH 5.0 (10231 > 18804, $P < 0.001$; 11006 > 18804, $P = 0.019$), pH 4.5 (10231 > 18804, $P = 0.004$; 11006 > 18804, $P = 0.035$), and pH 4.0 (10231 > 18804, $P = 0.004$; 11006 > 18804, $P = 0.035$).

Effects of ionic strength on the candidacidal activity of the zinc compounds

Tables 7–9 show the effects of ionic strength on the candidacidal activity of the zinc compounds. In the absence of the zinc compounds, the candidacidal activity significantly increased with increasing ionic strength. The candidacidal activity was approximately 20%–40% at 100 mM, 30%–50% at 150 mM, and 40%–60% at 200 mM. The presence of the zinc compounds provided additional candidacidal activity at each ionic strength. However, these additional candidacidal activities were less than those resulting from the increase in ionic strength itself and tended to decrease further as the ionic strength increased. The presence of zinc chloride significantly increased candidacidal activity at SSB ($P = 0.043$) in all three strains, at 100 mM ($P = 0.043$) and 150 mM ($P = 0.043$) in strain 10231, and at 100 mM ($P = 0.039$) and 200 mM ($P = 0.042$) in strain 11006. The presence of zinc sulfate significantly increased candidacidal activity at SSB ($P = 0.043$) and 100 mM ($P = 0.043$) in all three strains and at 150 mM ($P = 0.042$) in strains 10231 and 11006. The candidacidal activities of the two zinc compounds differed significantly at SSB ($P = 0.016$) and 200 mM ($P = 0.032$) in strain 10231.

The candidacidal activity based on pH differed significantly depending on the *C. albicans* strain when without zinc compounds and with zinc sulfate, showing relatively lower activity in strain 18804. The candidacidal activities differed significantly at 100 mM (11006 > 18804, $P = 0.002$) in the absence of the zinc compounds and at 200 mM in the presence of zinc sulfate (10231 > 11006, $P = 0.032$; 10231 > 18804, $P = 0.008$).

Discussion

This study aimed to investigate changes in candidacidal activities of zinc compounds based on incubation time, pH, and ionic strength. Although the candidacidal activities of the zinc compounds increased with increasing incubation time, significant levels of candidacidal activity were observed immediately. The candidacidal activity significantly increased with a decrease in pH or an increase in ionic strength. The zinc compounds provided additional candidacidal activity at each pH and ionic strength. However, the effects of the zinc compounds were less than those of the pH or ionic strength itself.

The MIC values and candidacidal activities of zinc compounds against *C. albicans* were extensively reported in our

Table 7 Candidacidal activities of the zinc compounds based on ionic strength (*Candida albicans* strain ATCC 10231).

Zinc compounds	n = 5	SSB	100 mM	150 mM	200 mM	Significance
Without Zinc chloride	CFU	72.0 [66.3–86.3]$^{abc\#}$	43.0 [40.5–58.3]$^{a\#}$	40.5 [32.5–45.0]$^{b\#}$	40.0 [35.5–48.5]^{c}	0.021*
	%killing	–	38.1 [14.8–52.2]	43.8 [39.7–57.6]	42.7 [30.0–57.3]	
With Zinc chloride	CFU	37.5 [34.3–42.8]$^{\#}$	32.0 [28.8–41.3]$^{\#}$	35.5 [27.0–42.3]$^{\#}$	37.0 [33.0–46.8]	0.647
1 mM	%killing	47.8 [43.8–54.7]§	56.0 [40.8–64.0]	50.7 [43.5–64.6]	46.6 [32.6–60.6]§	
Without Zinc sulfate	CFU	76.0 [67.3–85.5]$^{abc\#}$	52.0 [42.5–56.0]$^{ad\#}$	47.0 [35.3–54.5]$^{b\#}$	26.0 [25.0–28.5]^{cd}	0.007**
	%killing	–	31.7 [21.9–46.1]	42.7 [26.0–54.3]	65.8 [58.0–70.1]	
With Zinc sulfate	CFU	49.0 [44.0–56.5]$^{a\#}$	44.5 [34.8–48.5]$^{b\#}$	42.5 [25.5–53.3]$^{\#}$	24.5 [23.0–27.3]^{ab}	0.039*
1 mM	%killing	31.5 [28.0–41.4]§	45.7 [29.5–56.4]	52.2 [27.8–65.3]	67.1 [59.5–73.1]§	

Values are presented as median [interquartile range].
CFU, colony forming unit; SSB, simulated salivary buffer.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on ionic strength. *$P < 0.05$; **$P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each ionic strength level. $^{\#}P < 0.05$.
The Mann–Whitney U test was used to analyze differences in %killing based on the type of zinc compound at each ionic strength level. $^{\S}P < 0.05$.

Table 8 Candidacidal activities of the zinc compounds based on ionic strength (*Candida albicans* strain ATCC 11006).

Zinc compounds	n = 5	SSB	100 mM	150 mM	200 mM	Significance
Without Zinc chloride	CFU	65.0 [57.8–69.0]$^{abc\#}$	40.0 [35.5–40.5]$^{a\#}$	36.0 [29.8–41.5]^{b}	39.0 [30.0–40.5]$^{c\#}$	0.016*
	%killing	–	40.3 [34.7–44.0]	49.3 [32.3–52.1]	41.8 [33.9–53.3]	
With Zinc chloride	CFU	46.0 [36.0–48.0]$^{\#}$	37.0 [28.3–38.0]$^{\#}$	36.0 [27.0–39.3]	37.0 [26.0–39.0]$^{\#}$	0.151
1 mM	%killing	29.2 [16.9–47.9]	43.3 [38.8–55.3]	49.3 [36.0–56.6]	43.3 [37.1–59.3]	
Without Zinc sulfate	CFU	67.0 [60.0–73.5]$^{abc\#}$	37.0 [34.5–50.0]$^{a\#}$	37.0 [35.0–46.8]$^{bd\#}$	31.5 [28.0–37.0]^{cd}	0.005*
	%killing	–	38.2 [24.1–50.8]	45.2 [23.0–51.1]	48.1 [42.3–61.2]	
With Zinc sulfate	CFU	46.5 [38.8–52.8]$^{\#}$	33.0 [29.5–43.0]$^{\#}$	34.0 [32.3–40.3]$^{\#}$	32.0 [27.0–33.5]	0.054
1 mM	%killing	30.6 [18.0–43.2]	47.8 [30.8–59.5]	49.6 [33.7–54.9]	50.7 [45.7–62.7]	

Values are presented as median [interquartile range].
CFU, colony forming unit; SSB, simulated salivary buffer.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on ionic strength. *$P < 0.05$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each ionic strength level. $^{\#}P < 0.05$.

Table 9 Candidacidal activities of the zinc compounds based on ionic strength (*Candida albicans* strain ATCC 18804).

Zinc compounds	n = 5	SSB	100 mM	150 mM	200 mM	Significance
Without Zinc chloride	CFU	119.0 [111.5–127.0]$^{abc\#}$	92.0 [83.5–97.8]^{ad}	73.0 [66.5–81.0]^{b}	67.0 [64.0–73.3]^{cd}	0.005**
	%killing	–	22.7 [19.5–28.2]	40.9 [27.3–46.6]	42.7 [36.9–48.1]	
With Zinc chloride	CFU	75.0 [70.0–91.3]$^{a\#}$	77.0 [74.5–84.8]^{b}	70.0 [67.5–74.3]^{b}	67.0 [63.0–72.5]^{a}	0.041*
1 mM	%killing	35.0 [20.4–44.4]	37.0 [26.0–38.8]	41.2 [33.4–46.8]	44.5 [38.2–47.5]	
Without Zinc sulfate	CFU	109.0 [98.5–127.3]$^{abc\#}$	90.0 [78.0–99.8]$^{a\#}$	96.0 [57.8–108.3]^{b}	66.0 [59.5–78.5]^{c}	0.019*
	%killing	–	20.8 [14.9–25.6]	21.9 [9.9–41.5]	41.7 [25.5–49.3]	
With Zinc sulfate	CFU	85.0 [72.8–88.3]$^{\#}$	69.5 [57.3–75.5]$^{\#}$	75.5 [53.5–96.0]	66.0 [58.5–78.0]	0.241
1 mM	%killing	29.6 [19.4–33.8]	45.1 [28.0–48.8]	34.7 [18.6–48.8]	43.2 [25.9–49.5]	

Values are presented as median [interquartile range].
CFU, colony forming unit; SSB, simulated salivary buffer.
% killing was calculated as (1 - CFU on the experimental plates/CFU on the control plates) x 100 (%).
The Friedman test was used to analyze differences in CFU based on ionic strength. *$P < 0.05$; **$P < 0.01$.
The Wilcoxon signed rank test was used for post-hoc analysis. Pairs of the same letter denote a significant difference in the same zinc compound. $P < 0.05$.
The Wilcoxon signed rank test was used to analyze differences in CFU based on the presence or absence of each zinc compound at each ionic strength level. $^{\#}P < 0.05$.

previous study,[8] in which the incubation time was 60 min. The results of the present study showed that the candidacidal activities of zinc compounds (zinc chloride and sulfate) were approximately 15%–30% and 25%–50% at immediate and 10 min of incubation, respectively, suggesting that the rapid candidacidal mechanism of zinc compounds is associated with interactions of zinc compounds with the fungal cell wall structure. The rapid mechanism supported the potential use of zinc compounds as topical agents. Although there was a report that zinc compounds affected conidia production, hyphae morphological alterations, and the mortality of food-related fungi,[24] additional reports on the antifungal effects of zinc compounds are very rare. Therefore, further studies on the antifungal mechanisms of zinc compounds targeting the cell wall components of oral *Candida* species are required.

The results of the present study showed that the candidacidal activities of the zinc compounds increased due to low pH, inconsistent with those of previous reports. A low pH environment has been reported to be a favorable condition for oral *Candida* carriage and the occurrence of oral candidiasis.[25] However, the differences in the results were attributed to the differences in the experimental conditions. Previous studies showing that low pH was favorable for the growth of *Candida* were based on the experiments using *Candida* grown in a low pH environment.[26–28] Reportedly, *Candida* grown in a low pH environment exhibited increased adherence to epithelial surfaces[26] and decreased reactivity to salivary IgA.[27] These characteristics have been reported to be due to changes in the expression of the cell wall components of *Candida* grown at low pH, which were believed to affect the innate immune recognition by hosts.[27,28] However, the results of the present study were obtained by suddenly exposing *C. albicans* to a low pH environment, mimicking the rapid environmental changes in the oral cavity. In an environment where the pH changed so rapidly, low pH itself increased the candidacidal activity, and additional candidacidal activity due to the zinc compounds showed a decreasing trend. Although these results could not be fully explained, they were believed to be due to changes in the *Candida* cell wall caused by changes in pH and in the interactions between the zinc compounds and the cell wall components.

The increased candidacidal activity in higher ionic strength environments could be explained by osmotic stress and changes in cell wall mannoproteins. Although cellular mechanisms regulate intracellular water volumes for survival,[29] rapid osmotic stress can cause cell damage, such as cell shrinkage or swelling. Increasing ionic strength could change solubility of proteins and affect their structural stability and functions. Increasing ionic strength reportedly affects the viscoelastic properties of yeast cells and increases yeast cell adhesion.[30] Furthermore, increased ionic strength enhances the inactivation of microorganisms via microwave irradiation[31] and the effectiveness of antimicrobial photodynamic therapy using methylene blue as a photosensitizer.[32] The decreases in the additional candidacidal activities of the zinc compounds in higher ionic strength environments were attributed to the changes in the interactions between the zinc compounds and the cell wall components.

The limitations of the current study include the fact that the results were obtained in vitro and that the concentrations of the zinc compounds did not vary. Although the candidacidal activities of the zinc compounds were reduced at low pH and high ionic strength, the results of the rapid increase in candidacidal activity suggested that zinc compounds could provide beneficial effects when used in oral healthcare products.

In conclusion, the zinc compounds provided additional fungicidal activity against *C. albicans*, even at low pH or high ionic strength environments. The rapid candidacidal activity of the zinc compounds supported the fact that zinc compounds could be useful candidates for effective topical oral healthcare products.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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