

## Effect of Concentration and Time on the Antimicrobial Activity of Human Saliva against *Candida albicans*

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### Abstract

Mechanical cleaning is considered most effective in preventing oral mucosal diseases such as denture stomatitis and aspiration pneumonia; however, host saliva also have effective antimicrobial substances. Beta-defensin and histatin reportedly exert antimicrobial effects against *Candida albicans*; however, their optimal concentration in human saliva and optimum duration of activity are unclear. Therefore, this study aimed to investigate the effects of concentration and time on the antimicrobial activity of human saliva against *C. albicans*. Saliva was collected from 20 volunteers (10 men, 10 women;  $25.7 \pm 1.95$  years) twice daily for 1 week, and each antimicrobial substances was quantified. The viable count, ATP activity, and changes in the drug susceptibility test for *C. albicans* were assessed with respect to different concentrations and durations to analyse antimicrobial activity. Viable count tended to increase with an increase in beta-defensin 3 levels, but tended to decrease with an increase in histatin 5 levels. Yeast density and ATP activity level were significantly positively correlated with each antimicrobial substance (beta-defensin 3; 0.991,  $p < .000$ , histatin 5; 0.975,  $p < .000$ ). Beta-defensin 3 and histatin 5 did not yield zones of inhibition with time; however, antifungal drugs reduced the viable count and ATP activity level regardless of time. The present results indicate that histatin 5 reduces the population of *C. albicans* and ATP activity levels and may be a potential marker for risk assessment of oral mucosal diseases and aspiration pneumonia.

### Keywords :

beta-defensin, histatin,  
*Candida albicans*,  
miconazole nitrate

### Introduction

Cleaning of the mouth and dentures is considered most effective in preventing oral mucosal diseases such as denture stomatitis and the development of aspiration pneumonia. Therefore, numerous studies have reported methods of preventing oral contamination and cleaning dentures and denture bases (1-4). However, many patients do not enhance their practice of oral and denture cleaning; hence, they should be motivated to practice oral and denture cleaning. Furthermore, despite antimicrobial substances of host saliva, oral bacterial count and plaques increase owing to poor cleaning. Among 9 antimicrobial substances in saliva,

3 of them, viz., beta-defensin 3, histatin 5, and IgA, reportedly exert antimicrobial effects on oral bacteria and *Candida albicans* (5, 6).

Although antimicrobial substances of saliva are reportedly effective against *C. albicans in vitro* (5, 6), their effective concentrations in human saliva and reaction durations against *C. albicans* are unclear. Elucidation of the effective concentration and reaction duration of antimicrobial substances of saliva against *C. albicans* would render pretreatment saliva testing a marker for assessing the risk of oral mucosal diseases such as denture stomatitis and infection of aspiration pneumonia. The time for saliva collection should be noted during basic saliva examination. Recently for biochemical evaluation, methods to measure ATP activity to detect individual components of organisms

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have gained increasing attention. ATP is reportedly present in all organisms and has been applied to detect trace microorganisms in body fluids and food residues. Furthermore, it is possible to detect ATP activity with high sensitivity using luciferin, a fluorescent enzyme derived from fireflies (7). In addition, the drug susceptibility is confirmed using disc diffusion method (8). It is therefore necessary to compare the antimicrobial activity of naturally occurring proteins on human origin with those of commercial pharmaceutical antimicrobial substances.

This study aimed to clarify temporal changes in the antimicrobial activity of human saliva against *C. albicans*, via determination of their effective concentration and their potential for use as markers for assessing the risk of infection such as oral mucosal diseases and aspiration pneumonia relative to existing commercial antifungal agents.

## Materials and Methods

### *Yeast, antimicrobial substances, and antifungal agents*

*C. albicans* was obtained from the American Type Culture Collection 90028 (Manassas, VA, USA; ATCC 90028) and widely used for antimicrobial assessment. *C. albicans* were cultured at 37°C in Brain Heart Infusion (BHI) liquid medium (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) or BHI agar medium (FUJIFILM Wako Pure Chemical Co., Osaka, Japan).

The antimicrobial substances of human saliva were assessed by using recombinant mouse beta-defensin 3 protein (R&D systems, MN, USA) and histatin 5 (PEPTIDE INSTITUTE, Osaka, Japan) at concentrations equivalent to those in human saliva for effective antimicrobial assessment.

Antifungal drugs also used as oral fungicides in dentistry and miconazole nitrate, an imidazole antifungal drug with comparatively less side effect than other antifungals, were also used. Miconazole nitrate is hydrophobicity; therefore, miconazole nitrate was dissolved in methanol (FUJIFILM Wako Pure Chemical Co., Japan) and used for experiments.

### *Measurement items and methods*

#### *Antimicrobial and antifungal concentrations*

Since the indicated concentrations of antimicrobial substances had not been clarified, the maximum, median, and minimum concentration of antimicrobial substances in saliva were determined by ELISA from the results (n=280) obtained upon collecting saliva from 20 volunteers (10 men,

10 women; mean age, 25.7 ± 1.95 years) twice daily for one week. This study was approved by the Human Ethics Committee of Nihon University School of Dentistry at Matsudo (#EC16-019).

The control (hereinafter referred to as CONT) comprised only the *C. albicans* culture suspension. Beta-defensin 3 concentrations were 233.8, 24.3, and 2.4 ng/ml, and histatin 5 concentrations were 114.1, 12.3, and 1.1 µg/ml. Myconazole nitrate was used as an antifungal agent and at the concentration (10 mg/ml) indicated on the package insert.

### *Determination of the yeast viable count*

The viable count of yeast was determined using a yeast culture suspension adjusted to  $1.0 \times 10^8$  CFU/ml with each antimicrobial substance and CONT. Yeast cultures were prepared (n=24) for each antimicrobial concentration and CONT, and the mean was obtained from the three samples (n=72). The antibacterial effect against *C. albicans* over time was investigated using a 10-fold dilution method based on the viable count (7). The viable count was determined 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after mixing the antimicrobial solution in the culture medium.

### *Determination of ATP activity*

Bacterial culture suspensions were prepared at  $1.0 \times 10^5$  CFU/ml to determine ATP activity and the mean of three samples was obtained (n=24). The Lucifer HS kit (Kikkoman BioChemifa Co., Ltd., Japan) was used to determine ATP activity. Antimicrobial substances at different concentrations and CONT antifungal agents were added to the yeast culture and dispensed into 1000-µl mill tubes. From the mixing solution, 100 µl ATP-scavenging reagent (ATP-degrading enzyme) was added and stirred and incubated at 27°C for 30 min. Thereafter, 100 µl of ATP extraction reagent was added to the luminescent tube and after 20 s, 100 µl of luminescent reagent was added and stirred. Luminescence (RLU: Relative Light Unit) within 10 s after stirring was measured with a luminescence tester (Kikkoman BioChemifa Co., Ltd.). The lapsed time was determined immediately after mixing 0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after mixing the antimicrobial substances, and the ATP activity level was measured.

### *Disc diffusion method*

The drug susceptibility test was determined via the disc

Table 1. Change in the viable count of yeast over time in different antimicrobial substances and miconazole nitrate concentrations.

		BL	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h	6 h
A	Maximum conc.	1.0	2.5	4.0	4.3	13.5	27.3	124.5	283.4	306.2
	Median conc.	1.0	1.4	3.2	4.2	8.4	26.8	88.4	259.2	277.4
	minimum conc.	1.0	1.2	1.4	4.0	8.2	25.9	97.2	264.0	275.2
B	Maximum conc.	1.0	0.6	0.9	3.2	4.0	6.7	9.9	28.8	66.0
	Median conc.	1.0	0.7	1.0	4.0	4.2	6.8	12.5	26.7	72.0
	minimum conc.	1.0	0.7	0.9	4.0	4.5	6.9	15.8	43.2	79.2
C	Specified conc.	1.0	0.5	0.5	0.4	0.6	0.7	1.1	1.2	1.6
	CONT	1.0	1.0	3.6	4.7	9.2	27.0	63.7	245.2	268.0

Note. BL: Base line. Units are  $\times 10^8$  CFU/ml. A, B, C shows Changes in the viable count of yeast over time in different beta-defensin, histatin 5, miconazole nitrate concentrations. Compared with CONT, the yeast viable counts in Histatin 5 and Miconazole decreased.

diffusion method. Each antimicrobial substance at different concentrations and the CONT antifungal agent were immersed in 70  $\mu$ l of a filter paper (9 mm) (Grade AA DISCS, Whatman, Japan) on the culture BHI agar medium, and the diameters of the zones of inhibition were measured at 37°C after 24 h, and 48 h.

#### Statistical analyses

Pearson's correlation analysis was performed to analyze the correlation between viable count and the ATP activity of the yeast. All statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). A *P*-value less than 0.05 was considered statistically significant.

## Results

#### Evaluation of the yeast viable count

As shown in Table, when beta-defensin 3 was mixed with the yeast culture medium, the viable count of the yeast tended to increase relative to the CONT. Histatin 5 tended to decrease the viable count relative to the CONT, and beta-defensin 3 and histatin 5 increased in the viable count after 3 h. Antifungal drugs did not alter the viable count regardless

of the elapsed time.

#### Analysis of ATP activity

As shown in Figure 1, treatment with beta-defensin 3 for 6 h resulted in a maximum concentration of  $8.1 \times 10^8$  RLU, median concentration of  $7.7 \times 10^8$  RLU, and minimum concentration of  $7.6 \times 10^8$  RLU, showing an increasing trend relative to the CONT. Histatin 5 treatment for 6 h resulted in a maximum concentration of  $1.9 \times 10^8$  RLU, median concentration of  $2.0 \times 10^8$  RLU, and minimum concentration of  $2.3 \times 10^8$  RLU, showing a decreasing trend relative to the CONT. Treatment with the antifungal drug for 6 h at  $0.4 \times 10^7$  RLU yielded minor changes and no increase at 0 h.

#### Disc diffusion method

All of concentrations (beta-defensin 3 and histatin 5) treatment did not result in zones of inhibition regardless of the elapsed time. Antifungal agents yielded zones of inhibition of 30 mm after 24 h and 31.6 mm after 48 h.

#### Correlation analysis

Figure 2 shows the correlation between the yeast viable count and ATP activity. The correlation coefficient with the

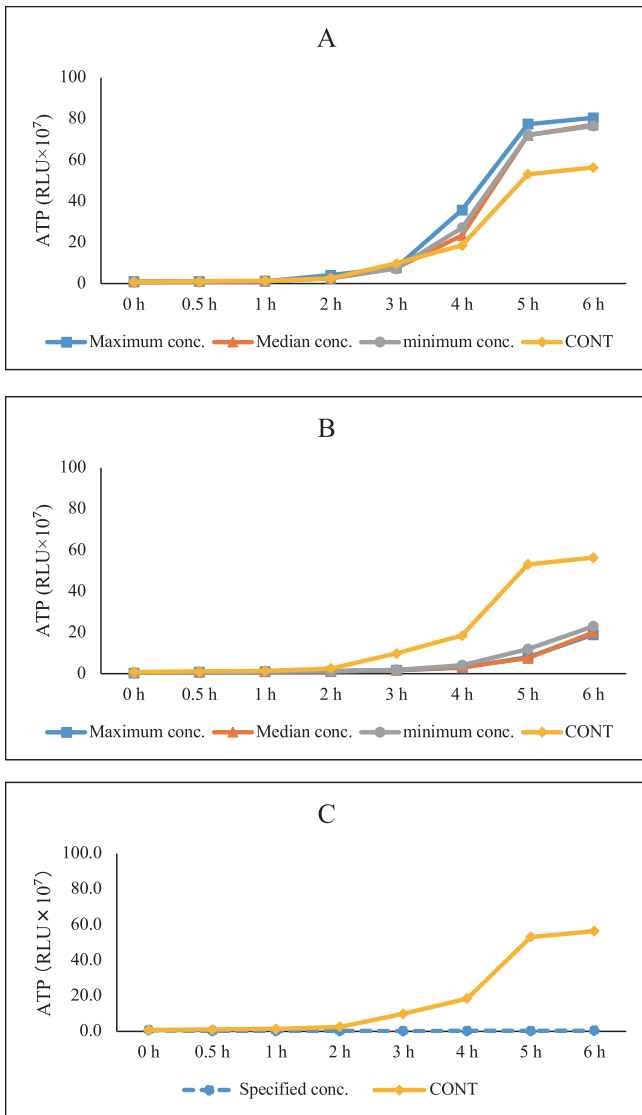


Fig. 1. Changes in Antimicrobial Concentrations and ATP Activity levels Over Time upon Mixing with Antifungal Agents

A shows changes in the ATP activity levels of beta-defensin 3, B shows changes in the ATP activity levels of histatin 5, and C shows changes in the ATP activity levels of Miconazole nitrate. ■:Maximum concentration, ▲:Median concentration, ●:minimum concentration, -●:miconazole nitrate, ◆:CONT shows the change of ATP activity level by 6 h according to each antimicrobial concentration. Histatin 5 and miconazole nitrate showed a decreasing tendency in ATP activity level compared with CONT. Beta-defensin 3 showed an increasing tendency compared with CONT.

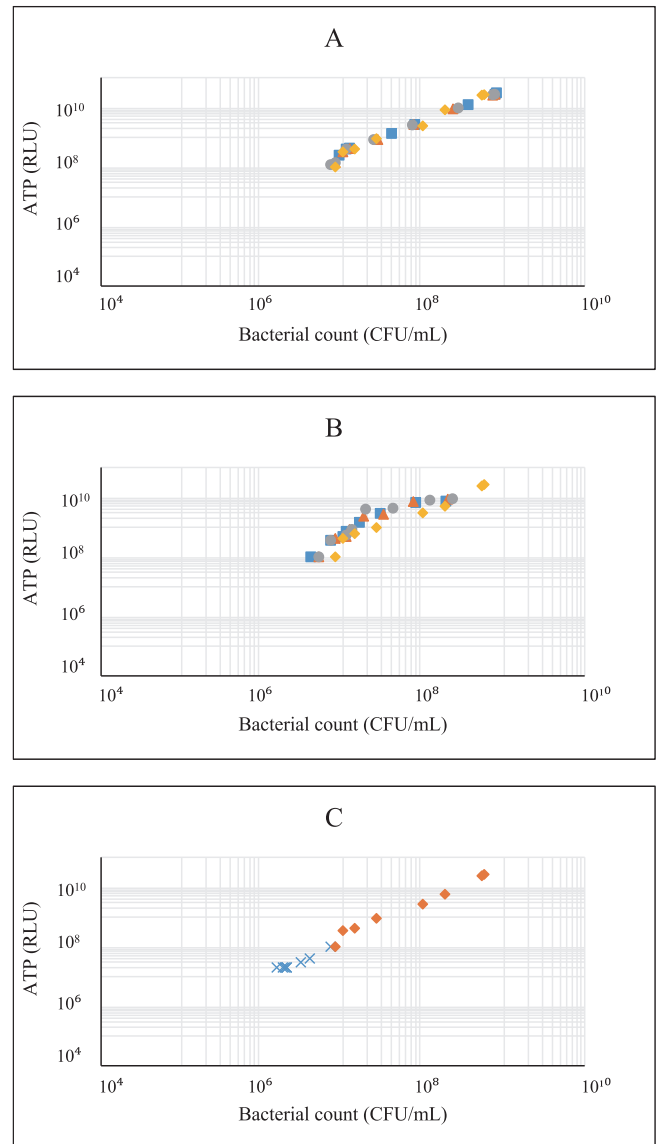


Fig. 2. Relationships between bacterial counts and ATP Activity levels during mixing with antimicrobial and antifungal agents

A shows beta-defensin 3, B shows histatin 5 and C shows miconazole nitrate, and the correlation between the number of bacteria and ATP activity levels is shown. ■:Maximum concentration, ▲:Median concentration, ●: minimum concentration, ×: Miconazole nitrate, ◆: A, B, C Individual antimicrobial concentrations and positive correlations in antifungal agents are shown showing CONT.

use of beta-defensin 3 in the culture medium was 0.999 at the maximum concentration ( $p < .000$ ), 1.000 ( $p < .000$ ) at the median concentration, and 1.000 ( $p < .000$ ) at the minimum concentration, and 0.998 ( $p < .000$ ) for the CONT. The

correlation between total beta-defensin 3 and the viable count of yeast in the culture medium with ATP activity was 0.991 ( $p < .000$ ), yielding a significant positive correlation. The correlation coefficient with the use of histatin 5 in the culture medium was 0.913 at the maximum concentration ( $p = .002$ ), 0.907 at the median concentration ( $p = .002$ ), and

0.900 at the minimum concentration ( $p=0.002$ ), and 0.993 for the CONT ( $p<.000$ ). The correlation between the yeast viable count and ATP activity when the culture medium with total histatin 5 was 0.975 ( $p<.000$ ), thereby yielding a significant positive correlation. The correlation coefficient with the use of antifungal drugs in the culture medium was 0.982 ( $p<.000$ ) and 0.995 for the CONT ( $p<.000$ ). The correlation between the yeast viable count and ATP activity when the antifungal drug and the bacterial culture solution were mixed was 0.995 ( $p<.000$ ), thereby yielding a significant positive correlation.

### Discussion

The yeast viable count tended to increase with an increase in beta-defensin 3 levels with time, relative to the CONT, and beta-defensin 3 did not show antimicrobial action due to the inhibition of adhesion to the cell wall. Histatin 5 showed a tendency to decrease the yeast viable count with an increase in its concentration, this effect being most pronounced after 3 h of treatment. The antimicrobial effect of histatin is believed to result from its interaction with the fungal plasma membrane, thus disrupting membrane architecture compared with the CONT. As antifungal agents remain in the oral cavity while described in the accompanying text, the present experimental form suggests that antifungal agents were present in the BHI liquid culture medium without diluting the concentration of antifungal agents. Therefore, regardless of the time elapsed, antifungal agents continued to act on *C. albicans* with no change in the viable count. Beta-defensin 3 and histatin 5 did not yield zones of inhibition upon analysis of the drug susceptibility, suggesting that *C. albicans* is not susceptible to salivary antimicrobial substances and that they are naturally occurring proteins of human origin with no pharmacological effects. The antifungal drug recognized the inhibition zone in 24 h. Since it took time for the fungus to colonize, the inhibition zone was not confirmed in the early stage, and the inhibition zone was confirmed after 24 h.

Beta-defensin 3, histatin 5, and the antifungal drugs showed significant positive correlations at all concentrations between the viable count and ATP activity, via the 10-fold dilution method. ATP activity also increased in the yeast with an increase in its viable count.

These findings indicate that histatin 5 decreases *C. albicans*, and ATP activity thus may suggest that these indices will become a potential marker to assess oral

mucosal diseases and contagion aspiration pneumonia. There are several limitations to this study. The studied age limits the application old age individuals. Therefore, it is necessary to investigate the optimal concentration of the antimicrobial substance in saliva according to ageing. Beta-defensin is said to exhibit antimicrobial action mainly by inhibiting adhesion to the cell wall. In the case of higher concentration (skin) it is said to destroy the cell wall. Based on the above, we think that the result of this study is a limitation in the experiment method. The bacterial culture and antimicrobial substances were put in the tube and the bacterial count and ATP levels over time were performed in that state. For that reason, we think that the antimicrobial action of the original beta-defensin is not demonstrated. We are also studying beta-defensin and histatin using test specimens for future study. Although within the limitation of the study, the results derived on the effective time on *C. albicans*, will apply to the future study to clarify the timing to clean oral environment and dentures.

### Conclusion

From the concentration of antimicrobial substances; histatin 5 is effective against *C. albicans* as the salivary examination in a healthy volunteers' saliva. Its potential as a marker for risk assessment and the prevention of oral diseases such as oral mucosal diseases and aspiration pneumonia is indicated. It is necessary to investigate the optimal concentration of the antimicrobial substance in saliva according to aging.

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