Effects of Storage Temperatures and Type of Oral Moisturizers on their Antifungal Effects

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Abstract

Purpose: This study aimed to examine the effect of storage temperature and oral moisturizer type on antifungal effects. Materials and Methods: Three oral moisturizers (two liquids and one gel), mixtures of the three moisturizers, and amphotericin B were tested. Antifungal effects were evaluated with moisturizer samples stored at 37°C, 25°C, and 4°C. *Candida albicans* (107 cells/ml) was mixed with trypticase soy agar medium and inoculated on 50% trypticase soy agar plates. Oral moisturizer samples were placed in cylindrical holes in the plates, and antifungal effects were evaluated based on growth-inhibitory zones after 24 hours. The effects of storage temperature and type of moisturizer on the growth-inhibitory zones were evaluated with analysis of variance. Growth-inhibitory zone sizes were compared with multiple comparisons. Results: Growth-inhibitory zones were formed with all moisturizer samples and amphotericin B. Significant differences in antifungal effects were found among the different storage temperatures and moisturizer types. The growth-inhibitory zones of the moisturizer samples stored at 4°C were significantly smaller than those of samples stored at other temperatures. Under the same temperature conditions, the growth-inhibitory zones of liquid-gel mixtures were significantly larger than those of other moisturizer types. The zones of the liquid-gel mixtures stored at 37°C were significantly larger than that of high concentrations of amphotericin B (0.63 μg/ml). However, the growth-inhibitory zones of almost all moisturizers were similar in size to that of low concentrations of amphotericin B (concentration, 0.04 μg/ml) at 4°C. Conclusion: From the viewpoint of the antifungal effect, our findings suggest that oral moisturizers should not be stored at low temperature.

Key Words: Oral moisturizers, Dry mouth, Candida albicans, Oral candidiasis

Introduction

Oral moisturizers, including gel and liquid types, are used for symptomatic treatment to alleviate problems caused by dry mouth, such as subjective dry mouth sensation, the progression of dental caries and periodontal disease, and ulceration in denture wearers [1-4]. To investigate attributes such as ease of application, ability to retain water, and retention force, the physical properties of oral moisturizers (the relationship between viscosity and temperature, transpiration rate, and adhesive strength) have been studied [4-6].

Dry mouth increases the presence and number of *Candida albicans* (*C. albicans*) in the oral cavity and on the mucosal surfaces of dentures [1,2,7,8]. Because *C. albicans* co-aggregates with highly pathogenic bacteria, it acts as a reservoir for bacteria leading to denture stomatitis, oral candidiasis, and aspiration pneumonia [7,9,10]. Therefore, the antifungal effects of oral moisturizers are important to maintain systemic health in dry mouth patients [3,11-13].

Other than the manufacturer's instructions that the moisturizers should not be stored at high temperatures or in direct sunlight, there is no clear information about the optimal storage temperature. From the viewpoint of maintaining the quality of the product, it may be better stored at the refrigeratoralthough a relationship between the temperature and viscosity of oral moisturizers has been reported [4], the effects of storage temperature on their antifungal effects are still unknown. Previous studies have shown that the viscosity of moisturizers falls with a rise in temperature [4]; however, the changes in their physical properties vary depending upon their type [5]. Thus, variations in the temperature and type of moisturizer used might have varying effects on the antibacterial ingredients. Hence, the present study aimed to evaluate the effects of different storage temperatures on the antifungal activity of various oral moisturizers to establish the optimal storage method.

Materials and Methods

Commercially available oral moisturizers with proven antifungal effects against *C. albicans* (JCM1537), which included two types of liquid moisturizers (a; DMX Mist, Rohto Pharmaceutical, Osaka, Japan, b; ConCool mouth rinse, Weltec, Osaka, Japan) and one gel moisturizer (A; Refre-care H, EN Otsuka Pharmaceutical, Iwate, Japan), were used in this study [13]. Additionally, equally mixed samples of the three moisturizers (liquid-gel/liquid-liquid) and different concentrations of amphotericin B (AMPH-B; Wako, Tokyo, Japan) in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Tokyo, Japan) and distilled water (W; Otsuka Pharmaceutical, Tokyo, Japan) were used. The final concentrations of AMPH-B diluted in DMSO were 1.25-0.04 μg/ml.

All unopened samples were stored in an incubator (Direct heat CO_2 incubator #310, Thermo Fisher Scientific KK, Tokyo, Japan) at 37°C, in a sealed room maintained at 25°C by an air conditioner (F28STES-W, Daikin Industries Ltd, Osaka, Japan), and in a refrigerator (SJ-PW35Y-W, SHARP, Osaka, Japan) at 4°C, respectively for 72 h.

C. albicans (JCM1537) at a concentration of 107 cells/ml (100 μml) was mixed with 5 ml of Trypticase Soy Agar (TSA) medium (0.8% agar) and inoculated on to 50% TSA plates

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(2% agar) [13]. After hardening of the medium, 20-µl samples were placed into cylindrical holes (diameter, 5 mm; depth, 5 mm) prepared in the TSA plates [13,14]. The diameter of the growth-inhibitory zones was measured after 24 h. Each sample was measured five times (n= 5)

The effects of storage temperature (4℃, 25℃, and 37℃) and oral moisturizer type (gel, liquid, and mixture) on the size of the growth-inhibitory zones were evaluated using the analysis of variance (ANOVA) test. The sizes were analyzed using Tukey's multiple comparisons. SPSS Statistics 19 (Japan IBM, Tokyo, Japan) was used for all statistical analysis, and the significance level was set at $p<0.05$.

Results

Growth-inhibitory zones were formed following the use of the two liquid moisturizers (a, b), gel moisturizer (A), two liquid-gel mixtures (a+A, b+A), one liquid-liquid mixture (a+b), and each concentration of AMPH-B and DMSO.

TOP

Type	Code	Growth-inhibition zone (Mean ± SD : mm)		
		4°C	25°C	37°C
Distilled water	W	0	0	0
Liquid moisturizer	a	9.16 ± 0.08 #	9.78 ± 0.1 $	10.31 ± 0.12 ᵀ
	b	9.29 ± 0.07 #	10.06 ± 0.1 $	10.89 ± 0.08 ᵀ
Gel moisturizer	A	9.10 ± 0.15 #	9.60 ± 0.1 $	10.04 ± 0.18 ᵀ
Liquid+Gel moisturizer	a+A	10.00 ± 0.08 #	10.79 ± 0.08 $	12.31 ± 0.28 ᵀ
	b+A	9.43 ± 0.07 #	10.11 ± 0.09 $	11.98 ± 0.32 ᵀ
Liquid+Liquid moisturizer	a+b	9.24 ± 0.08 #	9.98 ± 0.14 $	10.89 ± 0.08 ᵀ

Horizontally, different capital letters denote significant differences between temperatures (ᵀ:37°C>$: 25°C>#:4°C). (Tukey:$p<0.05$)

BOTTOM

		a	b	A	a+A	b+A	a+b
Liquid moisturizer	a						
	b	ᵀ,$					
Gel moisturizer	A	ᵀ	ᵀ, $				
Liquid+Gel moisturizer	a+A	ᵀ, $, #	ᵀ, $, #	ᵀ, $, #			
	b+A	ᵀ, $, #	ᵀ	ᵀ, $, #	ᵀ, $, #		
Liquid+Liquid moisturizer	a+b	ᵀ		ᵀ, $	ᵀ, $, #	ᵀ	

Capital letters (ᵀ:37°C, $:25°C, #:4°C) denote significant differences between moisturizers (Liquid+Gel>Liquid, Gel, Liquid+Liquid)

Table 1. *Mean and standard deviation of growth-inhibition zones (mm) at 4°C, 25°C, and 37°C for each sample. (Pictorial illustration)*

Table 1 shows the mean values and standard deviations of the growth-inhibitory zones found for each sample

Table 2 shows the results of the ANOVA tests. Two-way ANOVA results indicated that storage temperature, type of moisturizer, and their interactions had significant effects on the size of the growth-inhibitory zones (*Table 2*, top). One-way ANOVA on all conditions combined with the storage temperature and type of moisturizer indicated that significant differences existed (*Table 2*, bottom).

Tukey's multiple comparison tests was used to compare the growth-inhibitory zones among different temperatures for the same sample, and revealed that moisturizers stored at 4°C had significantly smaller growth-inhibitory zones than those stored at 25°C and 37°C (37°C>25°C>4°C; *Table 1*, top).

Comparison of the growth-inhibitory zones among different types of moisturizers exposed to the same temperatures revealed significantly larger growth-inhibitory zones in the liquid+gel type moisturizers (except for b+A at 25°C and 4°C) than in the other moisturizers (liquid+gel>liquid, gel, liquid +liquid; *Table 1*, bottom).

Table 2. *Results of analysis of variance test.*

TOP					
Source	**d. f.**	**Sum of squares**	**Mean squares**	**F value**	**P value**
Two-way ANOVA					
Type of moisturizers (A)	3	36.56	12.19	191.45	0.00*
Temperature (B)	2	69.09	34.55	542.64	0.00*
A × B	6	11.24	1.87	29.42	0.00*
Error	168	10.7	0.06		
Total	180	18742.21			
BOTTOM					
Source	**d. f.**	**Sum of squares**	**Mean squares**	**F value**	**P value**
One-way ANOVA					
Samples	11	135.87	12.35	194.02	0.00*
Error	168	10.7	0.06		
Total	180	18742.21			
*p<0.05 denotes significant difference					

Tables 3, 4 and 5 shows the results of Tukey's multiple comparison tests for the size of the growth-inhibitory zones between each concentration of AMPH-B and the moisturizers at 4°C, 25°C, and 37°C. Shaded boxes indicate no significant differences between samples.

In the samples stored at 4°C, the growth-inhibitory zones of the liquid-gel mixture (a+A) were the same size as those of AMPH-B at 0.08 µg/ml. The growth-inhibitory zones of the other moisturizers were similar to those of AMPH-B at 0.04 µg/ml and DMSO (*Table 3*).

Table 3. *Comparison of growth-inhibition zones of each concentration of AMPH-B and moisturizers stored at 4*℃.

4°C		**AMPH-B 1.25** **12.68 (0.29)**	**AMPH-B 0.63** **11.54 (0.15)**	**AMPH-B 0.31** **11.00 (0.09)**	**AMPH-B 0.16** **10.71 (0.32)**	**AMPH-B 0.08** **10.22 (0.15)**	**AMPH-B 0.04** **9.38 (0.16)**	**DMSO** **9.30 (0.17)**
Liquid moisturizer	a 9.16 (0.08)	0.00*	0.00*	0.00*	0.00*	0.00*	0.42	0.98
	b 9.29 (0.07)	0.00*	0.00*	0.00*	0.00*	0.00*	1	1
Gel moisturizer	A 9.10 (0.15)	0.00*	0.00*	0.00*	0.00*	0.00*	0.06	0.69
Liquid+Gel moisturizer	a+A 10.00 (0.08)	0.00*	0.00*	0.00*	0.00*	0.46	0.00#	0.00#
	b+A 9.43 (0.07)	0.00*	0.00*	0.00*	0.00*	0.00*	1	0.99
Liquid+Liquid moisturizer	a+b 9.24 (0.08)	0.00*	0.00*	0.00*	0.00*	0.00*	0.98	1
Horizontally, *AMPHB>oral moisturizer, # AMPHB<oral moisturizer. (p<0.05: Tukey test) Shaded boxes indicate no significant difference between samples								

In the samples stored at 25°C, the growth-inhibitory zones of the liquid-gel mixture (a+A) were the same size as those of AMPH-B at 0.31 and 0.16 µg/ml. The growth-inhibitory zones of the other moisturizers were the same size as those of AMPH-B at 0.08 and 0.04 µg/ml (*Table 4*).

Table 4. *Comparison of growth-inhibition zones of each concentration of AMPH-B and moisturizers stored at 25°C.*

25°C		**AMPH-B 1.25** **12.68 (0.29)**	**AMPH-B 0.63** **11.54 (0.15)**	**AMPH-B 0.31** **11.00 (0.09)**	**AMPH-B 0.16** **10.71 (0.32)**	**AMPH-B 0.08** **10.22 (0.15)**	**AMPH-B 0.04** **9.38 (0.16)**	**DMSO** **9.30 (0.17)**
Liquid moisturizer	a 9.78 (0.1)	0.00*	0.00*	0.00*	0.00*	0.00*	0.00#	0.00#
	b 10.06 (0.1)	0.00*	0.00*	0.00*	0.00*	0.92	0.00#	0.00#
Gel moisturizer	A 9.60 (0.1)	0.00*	0.00*	0.00*	0.00*	0.00*	0.42	0.03#
Liquid+Gel moisturizer	a+A 10.79 (0.08)	0.00*	0.00*	0.49	1	0.00#	0.00#	0.00#
	b+A 10.11 (0.09)	0.00*	0.00*	0.00*	0.00*	1	0.00#	0.00#
Liquid+Liquid moisturizer	a+b 9.98 (0.14)	0.00*	0.00*	0.00*	0.00*	2.75	0.00#	0.00#
Horizontally, *AMPHB>oral moisturizer, # AMPHB<oral moisturizer. (p<0.05: Tukey test) Shaded boxes indicate no significant difference between samples								

In the samples stored at 37°C, the growth-inhibitory zones of the liquid–gel mixture (a+A, b+A) were larger than those of AMPH-B at 0.63 µg/ml, whereas the zones of other moisturizers were similar in size to those of AMPH-B at 0.31, 0.16 and 0.08 µg/ml (*Table 5*).

Table 5. *Comparison of growth-inhibition zones of each concentration of AMPH-B and moisturizers stored at 37°C.*

37°C		**AMPH-B 1.25** **12.68 (0.29)**	**AMPH-B 0.63** **11.54 (0.15)**	**AMPH-B 0.31** **11.00 (0.09)**	**AMPH-B 0.16** **10.71 (0.32)**	**AMPH-B 0.08** **10.22 (0.15)**	**AMPH-B 0.04** **9.38 (0.16)**	**DMSO** **9.30 (0.17)**
Liquid moisturizer	a 10.31 (0.12)	0.00*	0.00*	0.00*	0.00*	1	0.00#	0.00#
	b 10.89 (0.08)	0.00*	0.00*	0.99	0.86	0.00#	0.00#	0.00#
Gel moisturizer	A 10.04 (0.18)	0.00*	0.00*	0.00*	0.00*	0.85	0.00#	0.03#
Liquid+Gel moisturizer	a+A 12.31 (0.28)	0.00*	0.00#	0.00#	0.00#	0.00#	0.00#	0.00#
	b+A 11.98 (0.32)	0.00*	0.00#	0.00#	0.00#	0.00#	0.00#	0.00#
Liquid+Liquid moisturizer	a+b 10.89 (0.08)	0.00*	0.00*	0.99	0.84	0.00#	0.00#	0.00#
Horizontally, *AMPHB>oral moisturizer, # AMPHB<oral moisturizer. (p<0.05: Tukey test). Shaded boxes indicate no significant difference between samples								

Discussion

Pilocarpine and cevimeline are prescribed to induce saliva secretion in patients with dry mouth resulting from radiotherapy or Sjogren's syndrome; nevertheless, these drugs cause side effects such as hyperhidrosis and dyspepsia [2,15,16]. Although salivary gland massage and facial muscle and tongue exercises are thought to improve saliva secretion in patients with dry mouth [13,17], oral moisturizers are commonly used as symptomatic therapy in these patients [1-3,5,13]. Previous studies have described the physical properties of oral moisturizers [4-6]; however, information about their management, including storage temperatures, has not yet been established. Differences in storage temperature may affect the antifungal properties of oral moisturizers.

To our knowledge, this is the first study to examine the effects of various storage temperatures on the antifungal properties of different types of oral moisturizers from the viewpoint of maintaining systemic health in dry mouth patients. In a previous report, the viscosity of moisturizers was found to decrease with rising temperatures [4], indicating the importance of storage temperature. Therefore, we have previously examined the physical [5] and antifungal properties [13] of commercially available oral moisturizers stored in an incubator at 37°C (assumed body temperature).

In the present study, the moisturizers were stored at 4℃, 25℃, and 37℃ to simulate the likely temperatures of daily life. However, it is worth noting that the antifungal effects were not evaluated under continuous temperature change; hence, the antifungal effects at temperatures other than those described in this study remain unknown. Although manufacturers' instructions indicate that moisturizers should not be stored at high temperatures, decreased in antifungal effects at 4°C indicate that moisturizers should not be stored at low temperatures. In this study, room temperature was controlled at 25°C using an air conditioner. Changes in antifungal effects caused by temperature variations imply the need to pay attention to storage at room temperature, which may undergo daily and seasonal variations in temperature.

Several in vitro [11] and clinical studies [7,12] have investigated the antifungal effects of a small number of oral moisturizers with inconsistent results. We have previously studied the antifungal effects of commercially available products on *C. albicans* [13]; only 3 out of 17 types of moisturizers demonstrated antifungal activity, which was enhanced in the liquid-gel mixture [13]. Thus, the moisturizers that demonstrated antifungal effects in the previous study were used in the present study. Detailed investigations of changes in antifungal effects at different temperatures were conducted by inoculating *C. albicans* at a concentration of 107 cells/ml. The size of the growth-inhibitory zones in the present study cannot be compared with those in our previous study, where the cells were inoculated with 108 cells/ml of *C. albicans* [13]. However, the moisturizers used in the present study demonstrated antifungal effects under all conditions with the highest effects exerted by the mixtures, which supports the results of our previous study.

It is likely that the larger growth-inhibitory zones observed in the liquid–gel mixture samples when compared with the individual moisturizers were formed as a result of differences in the mechanism of action among the antifungal ingredients in each moisturizer [13]. The antifungal ingredients included in the moisturizers used in this study were hinokitiol [18], protamine [19], whey protein, and lactoferrin [13]. The lowest antifungal effects were obtained at 4°C, suggesting that this is not the optimal temperature for the activation of antifungal ingredients. However, the storage of moisturizers at high temperatures for long periods may deteriorate the quality of the products. In our previous study, we found that the antifungal effects of moisturizers stored in an incubator for 8 hours after opening were significantly reduced relative to those used immediately after opening [13].

Delgado et al. examined the pH of seven commercially available oral moisturizers (different from those used in the present study) and reported values ranging from 3.01 to 9.15 [20]. Additionally, the difference in storage temperature may affect the pH value of the moisturizer. The pH values of CHROM agar and TSA agar mediums are 6.1 and 7.3, respectively. Hence, strongly acidic and alkaline products may have a bacteriostatic effect on *C. albicans*. However, the erosive potential of such products on teeth should be noted.

Oral candidiasis caused by dry mouth is a chronic disease and should be treated with drugs that can be used continuously over a long period of time [13]. However, long-term use of strong and broad-spectrum antimicrobial agents should be avoided because it may cause the emergence of drug-resistant strains [13,21]. The minimum inhibitory concentration of AMPH-B against *C. albicans* has been reported as 0.125-2 μg/ml in healthy individuals, and 0.06-4 μg/ml in HIV-positive patients [22], implying that the minimum inhibitory concentration of AMPH-B against *C. albicans* varies depending upon the systemic condition of the patient. Dry mouth occurs in medically compromised patients who have undergone oral surgical procedures, radiotherapy, and chemotherapy [8,13]. The growth-inhibitory zones of the liquid-gel mixture were greater than those of AMPH-B (0.63 μg/ml) at 37°C, which indicates that choosing the storage temperature and type of moisturizer according to the condition of the patient will encompass a wide range of minimum inhibitory concentrations. Oral moisturizers are considered to be self-care products; patients select and purchase the products by themselves. Therefore, dental practitioners should advise patients about the selection and use of these products based on the characteristics of the moisturizers and the symptoms of the patients.

Conclusion

Within the limitations of this *in vitro* study, we found that the antifungal effects of oral moisturizers vary depending on the storage temperature and the type of moisturizer. From the view point of the antifungal effect, oral moisturizers should not be stored at low temperatures.

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