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ORIGINAL ARTICLE

Influences of hyaluronic acid on the anticandidal activities of lysozyme and the peroxidase system

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OBJECTIVE: To investigate the fungistatic and fungicidal activity of hyaluronic acid (HA) and the influences of HA on the anticandidal activities of lysozyme and the peroxidase system.

MATERIALS AND METHODS: HA, hen egg-white lysozyme, and the bovine lactoperoxidase system were used. *Candida albicans* ATCC 10231, 18804, and 11006 strains were used in the experiments. The fungistatic activity of HA was determined by measuring the optical densities of the cultures. The candidacidal activity of HA and the influences of HA on the candidacidal activities of lysozyme and the peroxidase system were determined by comparing the numbers of colony-forming units.

RESULTS: Hyaluronic acid displayed inhibitory effects on the growth of *C. albicans*, and the inhibitory effects were proportional to HA concentration. HA did not have any measurable candidacidal activity. HA showed inhibitory effects on the candidacidal activities of lysozyme, and the peroxidase system that was proportional to HA concentration. HA at 1.0–2.0 mg ml^{-1} almost completely inhibited the candidacidal activities of lysozyme and the peroxidase system.

CONCLUSIONS: Hyaluronic acid possesses fungistatic activity but no candidacidal activity. HA showed inhibitory effects on the candidacidal activities of lysozyme and the peroxidase system.

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Keywords: hyaluronic acid; lysozyme; peroxidase; anticandidal activity

Introduction

Hyaluronic acid (HA) is a glycosaminoglycan consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units. The glucuronic acid and *N*-acetyl-D-gluco samine are linked $\beta(1 \rightarrow 3)$, while the *N*-acetyl-D-glucosamine and glucuronic acid are linked $\beta(1 \rightarrow 4)$. HA is abundant in the vitreous humor of the eye, in the synovial fluid of articular joints, and in the extracellular matrix. The intrinsic biocompatibility of HA and its unique physical properties make it important for drug delivery, production of biomaterials, artificial tears for patients with dry eyes, and substances for the symptomatic relief of osteoarthritis (O'Brien and Collum, 2004; Almond, 2007; Fam *et al*, 2007).

The presence of HA in human saliva has been reported, and HA in saliva may contribute to the lubricating and healing properties of saliva, thereby assisting in protection of the oral mucosa (Pogrel *et al*, 1996, 2003). HA has also been reported to display anti-*Candida* activity (Sakai *et al*, 2007). Because of its viscoelastic properties and non-immunogenicity (Almond, 2007), HA can be considered a candidate molecule for saliva substitutes for patients with dry mouth, and a certain range of concentration showing similar rheological properties to those of human saliva has been reported (Park *et al*, 2010). A relationship between decreased salivary levels of HA and the occurrence of dry mouth symptoms has also been reported (Higuchi *et al*, 2009), suggesting the importance of HA in protecting and lubricating the oral mucosa. In addition, the wound repair activity and potential anti-*Candida* activity of HA (Chen and Abatangelo, 1999; Sakai *et al*, 2007) can provide additional benefits to patients with dry mouth who are susceptible to developing oral mucosal injuries and candidiasis (Porter *et al*, 2004).

There have been attempts to enhance or restore saliva's own antimicrobial capacity through the use of commercially available oral healthcare products. The antimicrobial host proteins most widely used in these products are lysozyme and lactoperoxidase (Tenovuo,

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2002). These antimicrobials, either alone or in combination with other antimicrobial molecules, have been incorporated into saliva substitutes to restore the antimicrobial capacity of saliva in patients with dry mouth, whose susceptibility for developing candidiasis is increased (Tenovuo, 2002). In fact, the antifungal activities of lysozyme (Tobgi *et al*, 1988; Wu *et al*, 1999; Lee *et al*, 2010) and the peroxidase system (Wright *et al*, 1983; Lenander-Lumikari, 1992; Welk *et al*, 2009; Lee *et al*, 2010) have been reported. The synergic candidacidal activities of lysozyme and the peroxidase system have also been reported (Lee *et al*, 2010). Although it is not certain that the results obtained *in vitro* can be extended to *in vivo* scenarios, these antimicrobial supplements could reduce the occurrence of candidiasis in patients with dry mouth.

The oral cavity provides an environment in which substances in saliva substitutes and molecules in saliva can exist simultaneously. Therefore, HA molecules in saliva substitutes may also interact with antimicrobial molecules in human saliva as well as saliva substitutes. The formations of complex molecules between HA and lysozyme (Van Damme *et al*, 1991, 1994; Moss *et al*, 1997) and between HA and peroxidase (Green *et al*, 1990) have already been suggested, and the influences of HA on the enzymatic activities of lysozyme and peroxidase have been reported (Park *et al*, 2010). However, there is no information as to how HA affects the anticandidal activities of lysozyme and the peroxidase system. In the present study, we have investigated the fungistatic and fungicidal activities of HA and the influences of HA on the anticandidal activities of lysozyme and the peroxidase system.

Materials and methods

Hyaluronic acid solution

Hyaluronic acid (1630 kDa; Sigma-Aldrich, St Louis, MO, USA) was solubilized with simulated salivary buffer (SSB, 0.021 M Na_2HPO_4/NaH_2PO_4, pH 7.0, containing 36 mM NaCl and 0.96 mM $CaCl_2$) (Bennick and Cannon, 1978) or RPMI 1640 medium at three different concentrations (0.5, 1.0, and 2.0 mg ml^{-1}).

Lysozyme and peroxidase

Hen egg-white lysozyme (HEWL) and bovine lactoperoxidase (bLPO) (Sigma-Aldrich) dissolved in SSB served as lysozyme and peroxidase sources, respectively. A concentration of 30.0 μg ml^{-1} HEWL or 25.0 μg ml^{-1} bLPO was used for the assay.

Fungistatic activity of HA

Candida albicans strains ATCC 10231, 18804, and 11006 were used in the experiments. One colony of *C. albicans* grown on Sabouraud dextrose agar (SDA) was inoculated into 10 ml Sabouraud dextrose broth and incubated with shaking for 18 h at 37°C. Cells were then harvested, washed, and resuspended to a concentration of 1×10^5 cells per ml in RPMI 1640 medium. To monitor the fungistatic activity of HA, HA was solubilized in 100 ml RPMI 1640 medium containing *C. albicans* at various concentrations (0.5, 1.0, and 2.0 mg ml^{-1}), and the cultures were incubated with shaking at 37°C. The growth phase was determined by measuring the optical densities of the cultures at 600 nm in 1-h intervals and comparing the results with those of the culture without HA. The experiment was performed four times.

Candidacidal activity of HA

One colony from each of *C. albicans* ATCC 10231, 18804, and 11006 was inoculated into 10 ml RPMI 1640 medium and incubated with shaking for 18 h at 37°C. Cells were then harvested, washed, and resuspended to a concentration of 1×10^5 cells per ml in SSB. For the determination of candidacidal activity, 20 μl cell suspension was added to 40 μl HA at different concentrations (final concentrations of 0.5, 1.0, and 2.0 mg ml^{-1}) in sterile tubes. The samples were incubated at 37°C for 1.5 h and mixed every 15 min. At the end of the incubation period, samples were diluted 10 times, and 50 μl (167 cells) of the diluted cells was plated onto SDA plates in triplicate and grown overnight at 37°C. Candidacidal activity was determined by comparing the number of colonies on experimental plates with that on control plates (no HA). The percent loss of cell viability (one minus the ratio of the number of colonies on the test plate to that on the control plate) was also calculated. The experiment was performed six times.

Influence of HA on the candidacidal activities of lysozyme and the peroxidase system

Hyaluronic acid preincubated with candidal cells and then treated with antimicrobial enzymes. Cell suspensions of *C. albicans* grown in the manner discussed above were adjusted to a concentration of 1×10^5 cells per ml in SSB and then 20 μl cell suspension was added to an equal volume of HA (final concentrations of 0.5, 1.0, and 2.0 mg ml^{-1}). The samples were incubated with shaking at 37°C for 1 h. The 40 μl of cell suspension was mixed with 20 μl of HEWL (a final concentration of 30 μg ml^{-1}) or 20 μl of the peroxidase system (final concentrations of 25 μg ml^{-1} bLPO, 1 mM potassium thiocyanate (KSCN), and 100 μM H_2O_2) and then incubated with shaking at 37°C for 1 h. At the end of the incubation, samples were diluted 10 times, and 50 μl (167 cells) of the diluted cells was plated onto SDA plates in triplicate and grown overnight at 37°C. Candidacidal activity and the percent loss of cell viability were calculated. The experiment was performed eight times.

Hyaluronic acid preincubated with antimicrobial enzymes and then treated with candidal cells. Twenty microliters of HA solution (final concentrations of 0.5, 1.0, and 2.0 mg ml^{-1}) was added to 20 μl of HEWL (a final concentration of 30 μg ml^{-1}) or 20 μl of the peroxidase system (final concentrations of 25 μg ml^{-1} bLPO, 1 mM KSCN, and 100 μM H_2O_2) and then incubated with shaking at 37°C for 1 h. The mixtures were added to the 20 μl cell suspension and incubated with shaking at 37°C for 1 h. At the end of the incubation, samples were diluted 10 times, and 50 μl (167 cells) of the diluted cells

was plated onto SDA plates in triplicate and grown overnight at 37°C. Candidacidal activity and the percent loss of cell viability were calculated. The experiment was performed eight times.

Statistics
The Wilcoxon signed rank test was used to analyze significances between variables. *P*-values <0.05 were considered statistically significant.

Results

Fungistatic activities of HA
Hyaluronic acid displayed inhibitory effects on the growth of *C. albicans* that was proportional to the HA concentration used. The inhibitory effects of HA were the greatest in the *C. albicans* ATCC 11006 strain and were the weakest in the ATCC 18804 strain (Fig. 1a–c).

Candidacidal activities of HA
Hyaluronic acid (final concentrations of 0.5, 1.0, and 2.0 mg ml^{-1}) did not have any measurable candidacidal activity, as there were no significant differences in the numbers of colonies on the experimental plates compared with those of control plates (no HA) in all three strains (data not shown).

Influence of HA on the candidacidal activities of lysozyme and the peroxidase system
Hyaluronic acid preincubated with candidal cells and then treated with antimicrobial enzymes. Hyaluronic acid showed inhibitory effects on the candidacidal activities of lysozyme and the peroxidase system, and the inhibitory activities were proportional to the concentration of HA. Although the levels of inhibition were different according to the *C. albicans* strain, HA at 1.0–2.0 mg ml^{-1} almost completely inhibited the candidacidal activities of lysozyme and the peroxidase system used in the experiments (Tables 1 and 2).

Hyaluronic acid preincubated with antimicrobial enzymes and then treated with candidal cells. In the case of HA preincubated with antimicrobials, HA showed similar inhibitory effects on the candidacidal activities of lysozyme and the peroxidase system. HA at 1.0–2.0 mg ml^{-1} also incapacitated the candidacidal activities of lysozyme and the peroxidase system used in the experiments (Tables 3 and 4).

Discussion

The development of effective saliva substitutes requires an understanding and mimicry of both the rheological and biological properties of human saliva (Vissink *et al*, 1984; Levine, 1993). Therefore, a practical way of developing effective salivary substitutes for xerostomic patients is to identify or develop substances with a viscoelastic pattern similar to that of human whole saliva and to supplement important antimicrobials that restore the decreased biochemical properties. According to our previous report (Park *et al*, 2010), HA displays

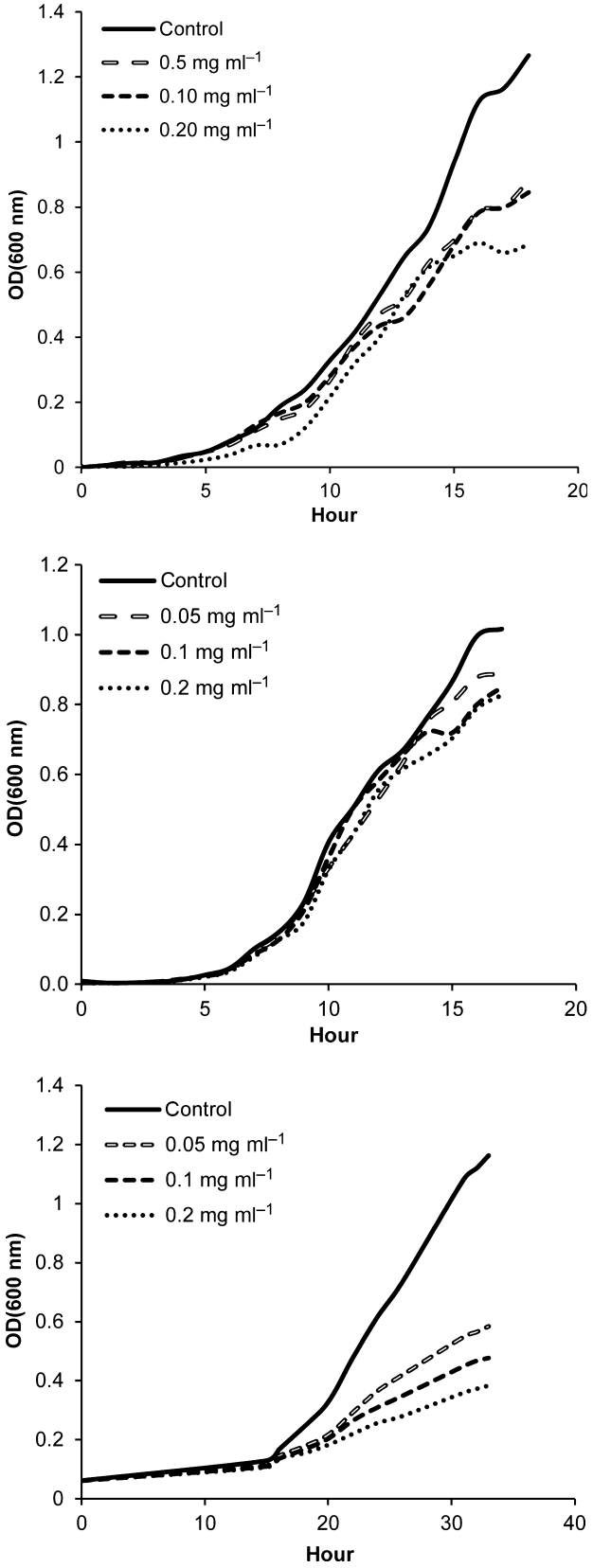

Figure 1 The fungistatic effects of hyaluronic acid on *Candida albicans* at concentrations of 0.5, 1.0, and 2.0 mg ml^{-1}. Fungistatic effects were determined by examining the growth phase of *C. albicans*. The experiment was performed four times. (**a**) ATCC 10231; (**b**) ATCC 18804; (**c**) ATCC 11006

Table 1 Effects of hyaluronic acid (HA) on the candidacidal activity of lysozyme. HA was preincubated with candidal cells and then treated with HEWL

Candida albicans Strain	N = 8	*Control (Group I)*	*HEWL (Group II)*	*HEWL + 0.5 mg ml^{-1} HA (Group III)*	*HEWL + 1.0 mg ml^{-1} HA (Group IV)*	*HEWL + 2.0 mg ml^{-1} HA (Group V)*	*Significance between groups*
ATCC 10231	CFU	154.5 ± 26.5	106.8 ± 15.6	126.8 ± 27.5	120.4 ± 39.7	144.3 ± 24.6	*(I,II)(II,V), (III,V)(IV,V)
	% killing	–	28.7 ± 17.3	15.9 ± 22.3	20.5 ± 25.3	5.3 ± 15.8	
ATCC 18804	CFU	143.9 ± 18.3	104.8 ± 12.8	138.4 ± 24.6	136.6 ± 27.6	155.1 ± 18.8	*(I,II)(II,III)(II,IV)(II,V) (III,V)(IV,V)
	% killing	–	26.9 ± 6.7	3.3 ± 16.2	3.8 ± 23.8	−8.9 ± 16.3	
ATCC 11006	CFU	157.9 ± 18.0	105.8 ± 24.9	150.6 ± 21.8	155.1 ± 19.0	161.3 ± 12.7	*(I,II)(II,III) (II,IV)(II,V)
	% killing	–	32.6 ± 16.2	4.6 ± 9.5	1.4 ± 10.7	−2.8 ± 8.8	

HA inhibited the candidacidal activity of 30.0 μg ml^{-1} HEWL. The assay was performed eight times. Statistical significance was evaluated using the Wilcoxon signed rank test. *$P < 0.05$.
HEWL, hen egg-white lysozyme; CFU, colony-forming unit.

Table 2 Effects of hyaluronic acid (HA) on the candidacidal activity of the peroxidase system. HA was preincubated with candidal cells and then treated with the bLPO system

Candida albicans Strain	N = 8	*Control (Group I)*	*bLPO system (Group II)*	*bLPO system + 0.5 mg ml^{-1} HA (Group III)*	*bLPO system + 1.0 mg ml^{-1} HA (Group IV)*	*bLPO system + 2.0 mg ml^{-1} HA (Group V)*	*Significance between groups*
ATCC 10231	CFU	177.6 ± 54.8	147.3 ± 40.3	162.7 ± 37.7	173.8 ± 64.7	172.9 ± 47.7	*(I,II)(II,V)
	% killing	–	15.9 ± 8.4	6.0 ± 13.9	3.1 ± 15.6	0.9 ± 16.0	
ATCC 18804	CFU	163.4 ± 31.3	120.1 ± 16.6	139.1 ± 27.3	160.8 ± 37.7	162.3 ± 29.8	*(I,II)(I,III)(II,III) (II,IV)(II,V)(III,V)
	% killing	–	25.7 ± 7.3	14.1 ± 12.5	2.0 ± 8.5	0.6 ± 1.1	
ATCC 11006	CFU	156.9 ± 19.2	97.9 ± 15.2	148.1 ± 23.7	154.7 ± 25.4	151.4 ± 22.8	*(I,II)(I,III)(II,III) (II,IV) (II,V)
	% killing	–	37.4 ± 7.7	5.8 ± 6.4	1.4 ± 10.3	2.9 ± 14.7	

HA inhibited the candidacidal activity of the bLPO system (25.0 μg ml^{-1} bLPO, 1.0 mM KSCN, and 100 μM H_2O_2). The assay was performed eight times. Statistical significance was evaluated using the Wilcoxon signed rank test. *$P < 0.05$.
bLPO, bovine lactoperoxidase; CFU, colony-forming unit.

Table 3 Effects of hyaluronic acid (HA) on the candidacidal activity of lysozyme. HA was preincubated with HEWL, then treated with candidal cells

Candida albicans Strain	N = 8	*Control (Group I)*	*HEWL (Group II)*	*HEWL + 0.5 mg ml^{-1} HA (Group III)*	*HEWL + 1.0 mg ml^{-1} HA (Group IV)*	*HEWL + 2.0 mg ml^{-1} HA (Group V)*	*Significance between groups*
ATCC 10231	CFU	160.9 ± 12.1	118.1 ± 16.5	152.4 ± 25.9	161.8 ± 19.4	165.9 ± 24.5	*(I,II)(II,III) (II,IV)(II,V)
	% killing	–	26.6 ± 8.9	5.3 ± 14.2	−0.5 ± 8.9	−3.1 ± 13.1	
ATCC 18804	CFU	143.0 ± 22.7	105.1 ± 26.9	136.2 ± 17.6	143.5 ± 29.4	142.2 ± 22.5	*(I,II)(II,III) (II,IV)(II,V)
	% killing	–	26.9 ± 11.8	3.4 ± 14.6	−0.5 ± 12.4	0.2 ± 9.6	
ATCC 11006	CFU	152.2 ± 20.7	118.3 ± 10.6	148.8 ± 18.9	153.7 ± 19.1	151.9 ± 29.1	*(I,II)(II,III) (II,IV)(II,V)
	% killing	–	21.5 ± 8.9	1.5 ± 11.3	−1.7 ± 11.6	−0.9 ± 19.7	

HA inhibited the candidacidal activity of 30.0 μg ml^{-1} HEWL. The assay was performed eight times. Statistical significance was evaluated using the Wilcoxon signed rank test. *$P < 0.05$.
HEWL, hen egg-white lysozyme; CFU, colony-forming unit.

viscoelastic properties similar to those of human saliva, and HA in SSB at a concentration of 0.5 mg ml^{-1} has viscosity values similar to those of human stimulated whole saliva at shear rates that would exist during oral functions, such as swallowing or speech (from 60 to 160 s^{-1}) (Balmer and Hirsch, 1978). In the present study, we attempted to show the effects of HA on *Candida* with regard to three aspects; fungistatic activities, fungicidal activities, and influences on the fungicidal activities of lysozyme and the peroxidase system. Our results indi-

Table 4 Effects of hyaluronic acid (HA) on the candidacidal activity of the peroxidase system. HA was preincubated with the bLPO system, then treated with candidal cells

Candida albicans Strain	N = 8	*Control (Group I)*	*bLPO system (Group II)*	*bLPO system + 0.5 mg ml^{-1} HA (Group III)*	*bLPO system + 1.0 mg ml^{-1} HA (Group IV)*	*bLPO system + 2.0 mg ml^{-1} HA (Group V)*	*Significance between groups*
ATCC 10231	CFU	155.6 ± 22.9	118.3 ± 19.6	133.0 ± 18.5	144.9 ± 24.7	160.4 ± 16.6	*(I,II) (II,IV)(II,V) (III,V)(IV,V)
	% killing	–	23.2 ± 13.3	13.0 ± 17.6	6.1 ± 16.0	−4.2 ± 12.8	
ATCC 18804	CFU	159.1 ± 5.7	112.6 ± 8.5	139.8 ± 15.2	146.6 ± 13.6	144.8 ± 11.2	*(I,II)(I,III) (I,IV)(I,V) (II,III) (II,IV) (II,V)
	% killing	–	29.2 ± 5.0	12.1 ± 9.8	7.8 ± 8.6	8.9 ± 7.6	
ATCC 11006	CFU	154.0 ± 13.6	101.3 ± 18.6	132.1 ± 20.5	134.5 ± 17.4	143.6 ± 12.9	*(I,II)(I,III)(I,IV) (II,III)(II,IV)(II,V)
	% killing	–	34.0 ± 11.9	14.1 ± 12.1	12.6 ± 8.4	6.4 ± 9.1	

HA inhibited the candidacidal activity of the bLPO system (25.0 μg ml^{-1} bLPO, 1.0 mM KSCN, and 100 μM H_2O_2). The assay was performed eight times. Statistical significance was evaluated using the Wilcoxon signed rank test. *$P < 0.05$.
bLPO, bovine lactoperoxidase; CFU, colony-forming unit.

cated that HA did not have candidacidal activity but had significant fungistatic activity, and these effects were dependent on the concentration of HA. The growths of all three *C. albicans* strains were inhibited, and susceptibility to HA was the most obvious in the ATCC 11006 strain. These results were consistent with those of previous studies (Sakai *et al*, 2007; Park *et al*, 2010). Therefore, it could be concluded that HA retards the growth of but does not kill *Candida* cells. It has been demonstrated that the anti-*Candida* activity of oral epithelial cells was mediated through a carbohydrate moiety (Steele *et al*, 2001). It has also been shown that both oral epithelial-bound and secreted HA had anti-*Candida* activities (Sakai *et al*, 2007). Therefore, the huge carbohydrate structures of HA molecules themselves may be associated with the fungistatic activity.

The antifungal effects of lysozyme and the peroxidase system are well known. Although the precise mechanism by which lysozyme interacts with fungus is not clear, several mechanisms have been suggested: direct binding of cationic lysozyme and yeast cell-wall mannans followed by an influence on yeast viability (Petit and Jolles, 1963; Tobgi *et al*, 1988), the activation or de-regulation of autolytic enzymes (Petit and Jolles, 1963; Tobgi *et al*, 1988), the interaction of lysozyme with other non-substrate fungal components followed by resultant interference with ion transfer and de-regulation of the influx and efflux of cellular constituents (Tobgi *et al*, 1988), the enzymatic hydrolysis of *N*-glycosidic bonds that link the polysaccharides and structural proteins of the yeast cell wall (Marquis *et al*, 1982, 1991), and the modulation of secreted aspartyl proteinase (Sap), a virulence factor of *C. albicans* (Wu *et al*, 1999). Although it has been reported that *C. albicans* is sensitive to $HOSCN/OSCN^-$ (Lenander-Lumikari, 1992), the precise mechanism by which the peroxidase system interacts with the fungus has not been reported. All components of the peroxidase system are needed for its antifungal effects (Lehrer, 1969; Welk *et al*, 2009; Lee *et al*, 2010), and its antifungal activity is affected by the relative proportions of its components and its phosphate concentration (Lenander-Lumikari, 1992). It has also been suggested that the direct binding of peroxidase to cell-wall mannans is important for candidacidal activity (Wright *et al*, 1983). It has been reported that bLPO itself induced an increase in lysozyme activity without causing significant changes in candidacidal activity. The bLPO system with lysozyme exerted synergic candidacidal activity (Lee *et al*, 2010).

Knowledge regarding the influences of saliva substitutes on salivary antimicrobials is very important because this information can be a factor in determining the concentrations of antimicrobial supplements in saliva substitutes. This knowledge also provides information on how salivary antimicrobials in the residual saliva of patients with dry mouth are affected by saliva substitutes. Our results showed that HA has inhibitory effects on the candidacidal activities of lysozyme and the peroxidase system, and HA at 1.0–2.0 mg ml^{-1} almost completely incapacitates the candidacidal activities of lysozyme and the peroxidase system.

To find out the mechanisms responsible for HA's inhibition of the candidacidal activities of lysozyme and the bLPO system, two kinds of experiments with different incubation sequences were performed. Irrespective of preincubation sequence, the inhibitory effects of HA were almost the same. Therefore, it can be suggested that the inhibitory mechanism of HA on the candidacidal activities of antimicrobials is not mainly because of direct interactions of HA with *Candida* cells or antimicrobials. It has been shown that the enzymatic activities of human salivary lysozyme and peroxidase as well as HEWL and bLPO were not affected by HA (Park *et al*, 2010). It has also been reported that the formation of HA-myeloperoxidase ionic complex did not affect myeloperoxidase activity (Avila and Convit, 1975; Green *et al*, 1990). Concentrated HA solutions with viscoelastic properties might inhibit the candidacidal activities of lysozyme and the peroxidase system by limiting their diffusion.

The presence of HA in human saliva has been reported, and HA in human whole saliva originates from pure glandular saliva as well as gingival exudate (Last and Embery, 1987; Pogrel *et al*, 1996). Its concentration is approximately 459 ng ml^{-1} in unstimulated whole saliva and 176 ng ml^{-1} in stimulated whole saliva (Pogrel *et al*, 1996, 2003). Based on the results of a previous study (Park *et al*, 2010), the HA concentration in human saliva does not contribute greatly to the viscoelasticity of human saliva. Therefore, the protective property of saliva may be enhanced by HA supplementation, especially in patients with dry mouth. HA also has beneficial effects in the treatment of plaque-induced gingivitis, acts as a carrier for growth factors, including transforming and epidermal growth factors, and is known to protect growth factors from protease digestion (Locci *et al*, 1995; Jentsch *et al*, 2003). Therefore, in the mixture of HA with lysozyme and the peroxidase system, our results showed that HA at a concentration of 0.5 mg ml^{-1}, which simulates the viscoelastic properties of human saliva, inhibits the candidacidal activities of lysozyme and the peroxidase system to a certain degree. Lysozyme might enhance the inhibitory effects of the peroxidase system on *Streptococcus mutans* in these products (Lenander-Lumikari *et al*, 1992). bLPO might increase the enzymatic activity of HEWL, and the bLPO system might increase the candidacidal activity of lysozyme (Lee *et al*, 2010). HA provides viscoelastic and protective properties as well as fungistatic activity, but interferes with the fungicidal activity of antimicrobial supplements and natural antimicrobials in residual saliva. Thus, increases of HEWL and the bLPO system should be considered to counterbalance the effects of HA. In addition, there are several other things to be considered when developing more effective saliva substitutes. In the case of polymers, the molecular size of candidate substances might affect their physical and biological properties. It has also been suggested that the formulation and delivery method of saliva substitutes affect the therapeutic efficacy and composition of oral microflora in patients with dry mouth (Weerkamp *et al*, 1987; Regelink *et al*, 1998).

In conclusion, the present study provides valuable information regarding the properties of HA in terms of anti-*Candida* activity. HA inhibited the growth of *Candida*, but did not have candidacidal activity. HA inhibited the candidacidal activities of lysozyme and the peroxidase system. These interactions among candidate molecules of saliva substitutes and antimicrobials may occur in oral healthcare products and potentially in the oral cavity.

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Author contributions

All authors contributed extensively to the work presented in this paper. Jeong-Hyun Kang played a main role in the study. She designed the study, did experiments, analysed data, and drafted paper. Yoon-Young Kim and Ji-Youn Chang did experiments on the influences of hyaluronic acid on the candidacidal activity of lysozyme and the peroxidase system. Hong-Seop Kho as a corresponding author designed the study, supervised all the experiments, analysed data, and wrote the manuscript.

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