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ORIGINAL ARTICLE

Rheological properties of hyaluronic acid and its effects on salivary enzymes and candida

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OBJECTIVE: To investigate the viscosity and wettability of hyaluronic acid (HA), its effects on lysozyme and peroxidase activities, and its candidacidal activity.

MATERIALS AND METHODS: Human whole saliva, HA, hen egg-white lysozyme (HEWL), and bovine lactoperoxidase (bLPO) were used. Viscosity was measured with a cone-and-plate digital viscometer, while wettability was determined by measuring the contact angle. Lysozyme activity was determined by the turbidimetric method. Peroxidase activity was determined with NbsSCN assay. Candidacidal activity was determined by comparing colony forming units.

RESULTS: The viscosity of HA solutions was proportional to its concentration, with 0.05 mg ml^{-1} of HA in distilled water or 0.5 mg ml^{-1} in simulated salivary buffer displaying similar viscosity values to stimulated whole saliva. The contact angle of HA solutions showed no significant differences according to the tested materials and tested HA concentrations. Contact angles of HA solutions on acrylic resin were higher than those of human saliva. HA did not affect lysozyme or peroxidase activities of whole saliva as well as HEWL or bLPO activities. HA also showed no candidacidal activity.

CONCLUSIONS: The viscoelastic properties of HA compared with human saliva were objectively confirmed, indicating a vital role for HA in the development of effective salivary substitutes.

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Keywords: hyaluronic acid; saliva substitute; viscosity; wettability; lysozyme, peroxidase

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Introduction

Hyaluronic acid (HA), an essential component of the extracellular matrix, is a glycosaminoglycan consisting of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units. The glucuronic acid and *N*-acetyl-D-glucosamine are linked $\beta(1 \rightarrow 3)$, while the *N*-acetyl-D-glucosamine and the glucuronic acid are linked $\beta(1 \rightarrow 4)$. HA is abundant in the eye vitreous humor, in the synovial fluid of articular joints, and in the extracellular matrix. The intrinsic biocompatibility of HA and its unique physical properties make it an important basis for drug delivery, production of biomaterials, and substances applied for the symptomatic relief of osteoarthritis (Almond, 2007; Fam *et al*, 2007). HA has also been used in surgical procedures within the eye and on the surface of the eye to prevent dryness and has been prescribed as artificial tears for patients with dry eyes (O'Brien and Collum, 2004; Balazs, 2008).

The presence of HA in human saliva has been reported and HA in saliva may contribute to the lubricating and healing properties of saliva, and assisting in protecting the oral mucosa (Pogrel *et al*, 1996, 2003). HA has also been reported to display anti-*Candida* activity (Sakai *et al*, 2007). Due to its viscoelastic properties and non-immunogeneity (Almond, 2007), HA can be considered a candidate molecule for saliva substitutes for patients with dry mouth. The wound repair activity and potential anti-*Candida* activity of HA (Chen and Abatangelo, 1999; Sakai *et al*, 2007) can provide additional benefits for patients with dry mouth who are susceptible to developing oral mucosal injuries and candidiasis (Porter *et al*, 2004).

The development of effective saliva substitutes requires an understanding of both the rheological and biological properties of human saliva. Although an ideal saliva substitute should mimic the rheological and biochemical properties of human saliva (Vissink *et al*, 1984; Levine, 1993), few objective data of saliva substitutes exist regarding viscosity and film-forming properties essential to the proper function. Moreover, the oral cavity provides an environment in which substances in saliva substitutes and molecules in saliva exist simultaneously. Therefore, HA molecules in saliva

substitutes may also interact with antimicrobial molecules in human saliva. The formation 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) has already been suggested in the previous reports. However, there is no information as to how HA affects the enzymatic activities of salivary lysozyme and peroxidase.

In the present study, we have investigated physical and biological properties of HA as a candidate molecule for saliva substitutes. For physical properties the viscosity and film-forming property of HA were examined. For biological properties the effects of HA on lysozyme and peroxidase activities and candidacidal activity of HA were examined.

Materials and methods

Participants and collection of saliva

Saliva was collected from three healthy non-smokers, one male (25-year-old) and two females (26-year-old) between 8:00 a.m. and 11:00 a.m. to minimize variability in salivary composition. The participants were postgraduate students in the School of Dentistry, Seoul National University. All participants had no history of serious illness and had not taken any medications known to affect the salivary flow rate for several months. Oral hygiene status and periodontal conditions of all participants were good. Their salivary flow rates were within normal ranges. The participants refrained from eating, drinking, and tooth brushing for at least 2 h before saliva collection. Unstimulated whole saliva (UWS) was collected for 10 min by spitting. Saliva was placed in a chilled centrifuge tube in which phenylmethylsulfonylfluoride (PMSF) was added immediately to a final concentration of 1.0 mM. The saliva sample was centrifuged at 3500 *g* for 10 min at 4°C and the resulting clarified supernatant fluid was used immediately for assays. The research protocol was approved by the Institutional Review Board of the University Hospital (#CRI09002).

Hyaluronic acid solution

Hyaluronic acid (1,630 kDa, Sigma-Aldrich, St Louis, MO, USA) was solubilized with distilled deionized water (DDW) or 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) at various concentrations (0.05, 0.1, 0.2, 0.5 and 1.0 mg ml^{-1}).

Lysozyme and peroxidase

Hen egg-white lysozyme (HEWL) and bovine lactoperoxidase (bLPO) (Sigma-Aldrich, St Louis, MO, USA) dissolved in SSB with PMSF (final concentrations of 1.0 mM) served as lysozyme or peroxidase sources, respectively. Clarified whole saliva samples were also used as experimental samples. A preliminary experiment showed that PMSF did not affect the enzymatic activities of lysozyme or peroxidase. A concentration of 10 μg ml^{-1} HEWL or 12.5 μg ml^{-1} bLPO was used for the assay.

Measurement of viscosity

Viscosity measurements were performed with a model LVT Wells-Brookfield cone-and-plate digital viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA). Shear rates were varied incrementally from 11.3 to 450.0 s^{-1} at six different speeds. All measurements were carried out at 37°C, and 0.5 ml volume of fluid was used in each test. The viscosity of each sample was measured six times.

Preparation of test specimens for contact angle measurement

Heat-cured acrylic resin, Paladent® 20 (Herareus Kulzer, Wehrheim, Germany), and cobalt-chromium alloy, Biosil® f (DeguDent, Hanau, Germany) were used as surface phases. Ten specimens of each material ($30 \times 30 \times 1.5$ mm) were prepared to have highly flat surfaces. For acrylic resin specimens, a sheet of wax 1.5 mm thick was pattern-adapted between two plates of glass. The sandwiches of glass and wax were inserted into dental flasks, boiled for 5 min to soften and eliminate the wax, and heat cured. The samples were ground with 600 and 800 grit silicon carbide sandpapers, followed by a felt disc with pumice. Cobalt-chromium alloy specimens (composition in mass %; Co 64.8, Cr 28.5, Mo 5.3, Si 0.5, Mn 0.5, and C 0.4) were cast and finished in the same manner as would be the tissue surface of a removable partial denture framework, according to the manufacturer's instructions.

Measurement of contact angle

Measurement of contact angle and surface tension was performed with a Phoenix 300 (Surface Electro Optics Co., Ansan, Korea). Contact angles were measured on the photographs as follows: 10 μl droplets of each liquid were positioned on the test specimens by means of a 1 ml syringe with a blunt point. After 30 s, a tangent to the droplet was drawn from the point of air–fluid–solid phase intersection. Contact angles between this tangent line and the dental material surface were calculated from enlarged photonegatives of the droplets. Measurements of contact angles were performed ten times for each test solution.

Measurement of lysozyme and peroxidase activity

Lysozyme activity was determined by the turbidimetric method (Grossowicz and Ariel, 1983). Samples were placed in a lyophilized cell suspension of *Micrococcus lysodeikticus* ATCC 4698, starting OD_{450} = 0.65–0.70, so that HEWL and salivary lysozyme could degrade the bacterial substrate. Peroxidase activity was determined by measuring the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5′-dithiobis(2-nitrobenzoic acid) $(Nbs)_2$ by $OSCN^-$ ions generated during the oxidation of SCN^- by bLPO and peroxidase in saliva (POS) (Månsson-Rahemtulla *et al*, 1986). Lysozyme and peroxidase activities were expressed as Units ml^{-1} and mUnits ml^{-1}, respectively.

Influence of HA on lysozyme or peroxidase activity

The effects of HA on lysozyme activity were examined by incubating 250 μl of HA in SSB (0.05, 0.1, 0.2 and

0.5 mg ml^{-1}) with 250 μl of HEWL (10 μg ml^{-1}) or clarified whole salivary samples for 10 min at room temperature (RT). The incubated mixture was placed in a suspension of *M. lysodeikticus* and an incubated mixture of buffer with HEWL, or clarified salivary sample, was used as a control. Either an incubated mixture of HA with buffer, or an incubated buffer alone was used as a blank.

The effects of HA on peroxidase activity were examined by incubating 250 μl of HA (0.05, 0.1, 0.2 and 0.5 mg ml^{-1}) with 250 μL of bLPO (final concentration 12.5 μg ml^{-1}) or clarified whole salivary samples for 10 min at RT. To 300 μl of reaction mixture for the NbsSCN assay, 15 μl of KSCN (final concentration of 4.2 mM SCN^-) and 15 μl of sample solution were added, and the reaction was initiated by the addition of 15 μl of H_2O_2 (final concentrations were 50 μM for bLPO and 100 μM for POS). An incubated mixture of buffer with either bLPO or clarified whole salivary sample was used as a control. For the blank reaction, an incubated mixture of HA with buffer, or an incubated buffer alone was used. The experiments using HEWL or bLPO were performed 10 times. Saliva samples from three subjects were collected on four different days (12 total samples) and used for the experiments. All experiments were performed in duplicate.

Candidacidal assay

One colony of *Candida albicans* ATCC 10231 was inoculated into 10 ml of RPMI 1640 medium and incubated with shaking for 24 h at 37°C. Cells were then harvested, washed, and resuspended to a concentration of 3×10^5 cells ml^{-1} in 0.01 M phosphate buffer (pH 7.4). For the determination of candidacidal activity, 20 μl of cell suspension was added to an equal volume of twofold serial dilutions of HA (final concentrations: 1.0 mg ml^{-1} to 31.3 μg 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, samples were diluted 15 times, and 50 μl of the diluted cells was plated on Sabouraud dextrose agar plates in triplicate and grown overnight at 37°C. Candidacidal activity was determined by comparing the number of colonies on experimental plates with control plates (no HA). The experiment was performed seven times.

Statistics

The Mann–Whitney *U* and Kruskal–Wallis tests were used to compare the mean values of viscosity and contact angle. The Wilcoxon signed rank test was used to analyze the effects of HA compared with their controls. *P*-values less than 0.05 were considered statistically significant.

Results

Viscosity

The viscosity values for HA dissolved in DDW followed a pattern typical of a non-Newtonian fluid (Figure 1), whereas the viscosity values for HA dissolved in SSB at low concentrations (0.1, 0.2 and 0.5 mg ml^{-1}) displayed decreased viscoelastic properties at low shear rates (Figure 2). The viscosity values for HA in SSB were lower than those for HA in DDW. An increase in viscosity was found with increasing HA concentration, as expected.

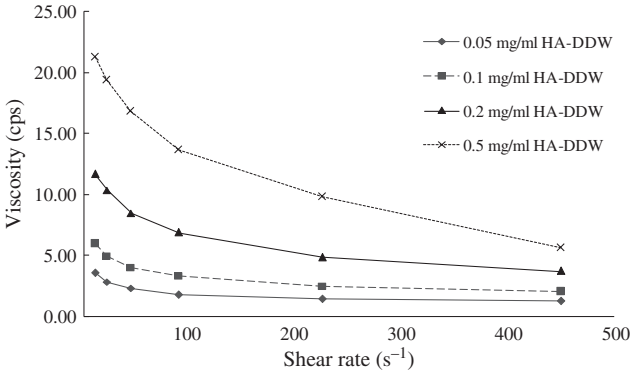

Figure 1 Viscosity values of hyaluronic acid in distilled deionized water (HA-DDW). Viscosity measurements were performed with a cone-and-plate digital viscometer at six different shear rates. The viscosity values for HA-DDW followed a pattern of a non-Newtonian fluid. An increase in viscosity was found with increasing HA concentration

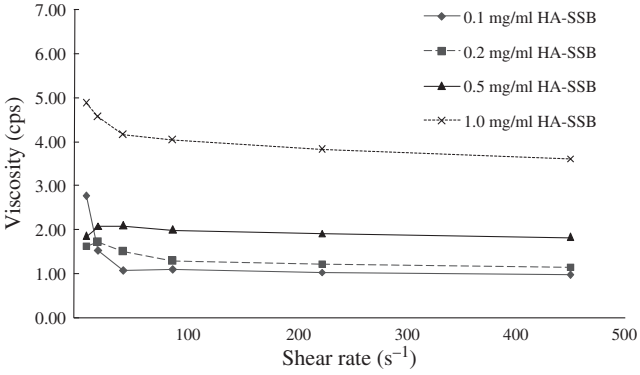

Figure 2 Viscosity values of hyaluronic acid in simulated salivary buffer (HA-SSB). Viscosity measurements were performed with a cone-and-plate digital viscometer at six different shear rates. The viscosity values for HA-SSB at low concentrations displayed decreased viscoelastic properties at low shear rates

The HA solutions at 0.05 mg ml^{-1} in DDW displayed viscosity values similar to those of stimulated whole saliva (SWS) at tested shear rates. HA at 0.5 mg ml^{-1} in SSB displayed viscosity values similar to those of SWS at shear rates of 45 and 90 s^{-1}, and displayed similar viscosity values to UWS at a shear rate of 225 s^{-1}. HA at 1.0 mg ml^{-1} in SSB displayed viscosity values similar to those of UWS at a very low shear rate (Figure 3).

Contact angle

The contact angles of HA solutions showed no significant differences according to either the tested materials or the tested HA concentrations. There were also no significant differences in contact angles between HA-DDW and HA-SSB. Contact angles of HA solutions on acrylic resin were higher than those of human saliva (Figure 4).

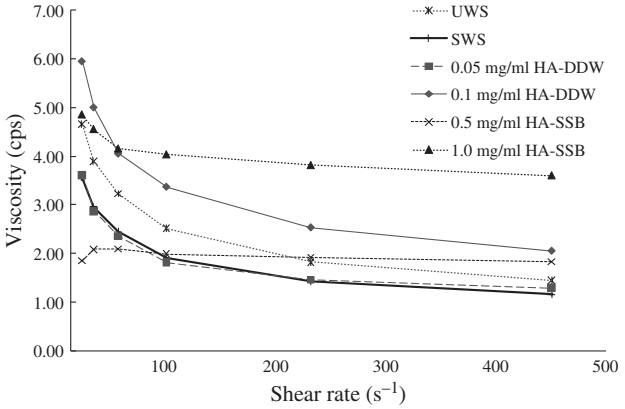

Figure 3 Viscosity values of hyaluronic acid compared with human saliva. The HA solutions at 0.05 mg ml^{-1} in DDW and at 0.5 mg ml^{-1} in SSB displayed viscosity values similar to those of human whole saliva. UWS, unstimulated whole saliva; SWS, stimulated whole saliva; HA-SSB, hyaluronic acid in simulated salivary buffer; HA-DDW, hyaluronic acid in distilled deionized water

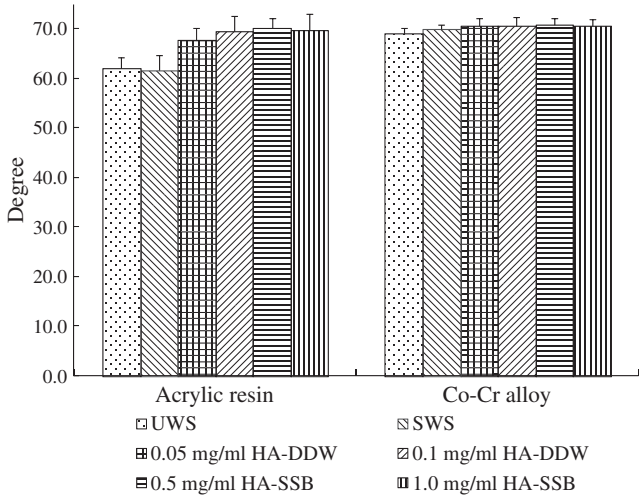

Figure 4 Contact angle of hyaluronic acid compared with human saliva. Contact angles between the tangent to the droplet of each liquid and acrylic resin or cobalt-chromium alloy specimens were calculated from enlarged photonegatives of the droplets. Contact angles of HA solutions on acrylic resin were higher than those of human saliva. UWS, unstimulated whole saliva; SWS, stimulated whole saliva; HA-SSB, hyaluronic acid in simulated salivary buffer; HA-DDW, hyaluronic acid in distilled deionized water

Influence of HA on lysozyme or peroxidase activity

The enzymatic activities of HEWL and bLPO were not affected by HA at four different concentrations tested (0.05, 0.1, 0.2 and 0.5 mg ml^{-1}). Similarly, lysozyme and peroxidase activities in clarified whole saliva were not affected (Tables 1 and 2).

Candidacidal assay

Hyaluronic acid (final concentrations from 0.0313 to 1.0 mg ml^{-1}) did not have any measurable candidacidal activity, as there were no significant differences in the number of colonies on experimental plates compared with control plates (no HA) (Figure 5).

Discussion

The lubricative and hydration functions of saliva are dependent on saliva quality such as viscosity and film-forming property as well as quantity (Collins and Dawes, 1987; Waterman *et al*, 1988; Dawes, 2008). Therefore, the practical goal of developing effective salivary substitutes for xerostomic patients is to achieve a viscoelastic pattern similar to that of human whole saliva. According to our results, HA displayed viscoelastic properties, which is characteristic of macromolecular solutions. Solutions containing 0.5 mg ml^{-1} HA dissolved in SSB and 0.05 mg ml^{-1} HA dissolved in DDW had viscosity values similar to those of human SWS at shear rates that would exist during oral functions, such as swallowing or speech (from 60 to 160 s^{-1}) (Balmer and Hirsch, 1978).

The difference in viscosity between HA-DDW and HA-SSB is attributable to the difference in ionic strength of the two solutions. This has been previously established by a study on the relationship between ionic strength and viscosity wherein decreases in intrinsic viscosity of canine tracheal mucin or bovine submaxillary mucin were found upon increasing the ionic strength (Litt *et al*, 1977; Park *et al*, 2007). Further, it was reported elsewhere that increasing the ionic strength from 35 to 235 mM resulted in an approximately 50% decrease in specific viscosity (Veerman *et al*, 1989).

Because the wettability on oral structures and dental materials is indispensable for the maintenance of lubrication and denture retention, the film-forming property

Table 1 Influence of hyaluronic acid on lysozyme activity

n = *10*	*HEWL (Units ml^{-1})*	*HEWL with HA (Units ml^{-1})*	*Significance*
HA (0.05 mg ml^{-1})	259.4 ± 21.0	264.0 ± 22.3	NS
HA (0.1 mg ml^{-1})	244.0 ± 13.6	247.2 ± 14.9	NS
HA (0.2 mg ml^{-1})	270.2 ± 17.1	264.4 ± 18.8	NS
HA (0.5 mg ml^{-1})	253.6 ± 15.0	254.9 ± 25.8	NS
n = *12*	*UWS (Units ml^{-1})*	*UWS with HA (Units ml^{-1})*	*Significance*
HA (0.05 mg ml^{-1})	1167.0 ± 367.0	1146.7 ± 369.3	NS
HA (0.1 mg ml^{-1})	1134.8 ± 328.7	1152.2 ± 348.1	NS
HA (0.2 mg ml^{-1})	1149.8 ± 351.5	1157.3 ± 363.1	NS
HA (0.5 mg ml^{-1})	1130.0 ± 333.9	1099.0 ± 344.1	NS

HEWL, hen egg-white lysozyme; UWS, unstimulated whole saliva; HA, hyaluronic acid.

Table 2 Influence of hyaluronic acid on peroxidase activity

n = *10*	*bLPO* (*mUnits ml*$^{-1}$)	*bLPO with HA* (*mUnits ml*$^{-1}$)	*Significance*
HA (0.05 mg ml^{-1})	1.47 ± 0.23	1.45 ± 0.17	NS
HA (0.1 mg ml^{-1})	1.55 ± 0.16	1.57 ± 0.21	NS
HA (0.2 mg ml^{-1})	1.41 ± 0.33	1.48 ± 0.30	NS
HA (0.5 mg ml^{-1})	1.56 ± 0.15	1.56 ± 0.14	NS
n = *12*	*UWS* (*mUnits ml*$^{-1}$)	*UWS with HA* (*mUnits ml*$^{-1}$)	*Significance*
HA (0.05 mg ml^{-1})	1.60 ± 0.41	1.65 ± 0.47	NS
HA (0.1 mg ml^{-1}	1.65 ± 0.31	1.60 ± 0.27	NS
HA (0.2 mg ml^{-1})	1.74 ± 0.41	1.75 ± 0.46	NS
HA (0.5 mg ml^{-1})	1.69 ± 0.37	1.68 ± 0.35	NS

bLPO, bovine lactoperoxidase; UWS, unstimulated whole saliva; HA, hyaluronic acid.

seems to have a greater impact on the clinical efficacy of saliva substitutes than does viscosity alone (Vissink *et al*, 1986; Christersson *et al*, 2000). The present study demonstrates that the contact angles of HA solutions show no significant differences according to either the tested materials or the tested HA concentrations. Specifically, HA solutions displayed inferior wettability on acrylic resin versus human saliva, as do animal mucins (Park *et al*, 2007). Considering the importance of wettability in the retention of dentures made of acrylic resin (Monsenego *et al*, 1989), modification of HA for enhancing wettability is a potentially important consideration for improving their effectiveness in xerostomic patients.

It has been suggested that glycosaminoglycans may modify lysosomal function through the formation of complexes with lysosomal enzymes (Avila and Convit, 1975). An ionic interaction between HA and lysozyme or peroxidase has been suggested (Green *et al*, 1990; Van Damme *et al*, 1991, 1994; Moss *et al*, 1997). It was previously reported that lysozyme activity of leucocytic lysosome was inhibited 30% by HA (Avila and Convit, 1975). However, the enzymatic activity of HEWL and salivary lysozyme was not affected by HA in the present study. The differences in results may be due to the different experimental conditions. It has been suggested that HA-lysozyme interaction was affected by salt concentrations and pH. High binding affinity was previously observed at pH 7.5 and at approximately 10–50 mM salt (Van Damme *et al*, 1991, 1994; Moss *et al*, 1997). 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). HA did not affect the enzymatic activity of bLPO and POS in the present study.

Although the enzymatic activity of lysozyme or peroxidase was not affected by the presence of HA, the rheological properties of HA can be affected by complex formation of HA with antimicrobials in human saliva or saliva substitutes. Depolymerization of HA and decreases in HA viscosity following exposure to myeloperoxidase system has been reported (Baker *et al*, 1989; Green *et al*, 1990; Lindvall and Rydell, 1994). It has also been proposed that disaggregation of proteoglycans occurs due to a specific interaction of lysozyme with HA (Blanco and Pita, 1985). Therefore, the changes of rheological properties of HA-antimicrobial complexes compared with HA or antimicrobials alone need to be explored.

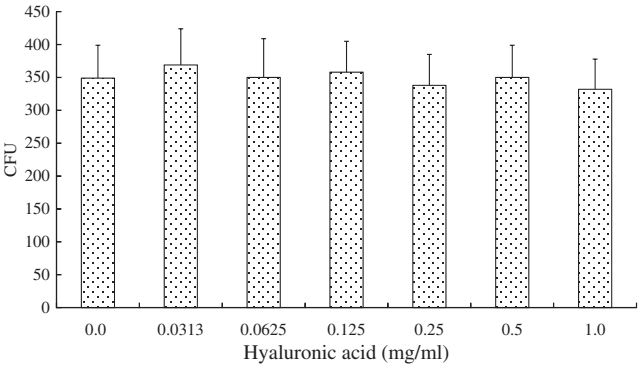

Figure 5 The effects of hyaluronic acid on Candida. Candidacidal activity was determined by comparing the number of colonies on experimental plates with control plates (no HA). HA showed no candidacidal activity. The experiment was performed seven times. CFU, colony forming units

In the present study, HA is shown to possess no candidacidal activity, even at high concentrations. It has been reported that high concentrations of high-molecular-weight HA has fungistatic effects on *C. albicans* (Sakai *et al*, 2007). Therefore, it could be concluded that HA retards growth of rather than kills *Candida*.

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. The maintenance of HA levels in saliva is the result of a steady-state equilibrium between HA and hyaluronidase (Last and Embery, 1987; Pogrel *et al*, 1996). The concentration of HA was higher in whole saliva than parotid saliva and higher in unstimulated than stimulated saliva. 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). Considering the results of the present study, 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 because it also serves as a lubricant. HA also has a beneficial effect in the treatment of plaque-induced gingivitis and acts as a carrier for growth factors, including transforming and epidermal

growth factor, and is known to protect growth factors from protease digestion (Locci *et al*, 1995; Jentsch *et al*, 2003).

In conclusion, the present study provides an objective observation on the properties of HA solutions in terms of their rheological and biological aspects. The HA solution at 0.5 mg ml^{-1} in SSB displays similar viscosity values to stimulated whole saliva. The contact angle of HA solutions showed no significant differences according to the tested materials and HA concentrations. HA did not affect lysozyme or peroxidase activities. HA also showed no candidacidal activity. For the development of effective saliva substitutes, additional studies focusing on the wettability of HA solutions on oral mucosa are needed. The rheological properties of HA-antimicrobial complexes and the effects of HA on the function of other antimicrobials also need to be elucidated.

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