Hyaluronan-Containing Mouthwash as an Adjunctive Plaque-Control Agent

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Purpose: Hyaluronan, commonly known as hyaluronic acid, has been shown to have anti-inflammatory action, bacteriostatic effect and antioxidant properties, thus making its use as a long-term anti-plaque and anti-gingivitis agent an appealing proposition. The aim of the present study was to evaluate the efficacy of 0.025% hyaluronan-containing mouthwash in comparison with 0.2% chlorhexidine and a water-based mouthwash and also to evaluate its antibacterial efficacy on isolated strains of periodontopathogens.

Materials and Methods: Forty-five volunteers in a hospital setting were recruited. A single-blinded, parallel design, randomised controlled trial was carried out and the 4-day plaque re-growth model was used to study the efficacy of the three mouthwashes. Microbiological and clinical evaluation was performed by culturing and using dental indices, respectively. The three mouthwashes used in the present study were commercially available 0.025% sodium hyaluronate, 0.2% chlorhexidine and a water-based rinse (negative control). Effects of the three mouthwashes were tested on the growth of isolated strains of *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa) and *Prevotella intermedia* (Pi).

Results: *In vitro*, hyaluronan had a distinct effect on the growth of Aa and Pi with no effect on the growth of Pg. *In vivo*, the differences between the individual rinse solutions and the water-based solution showed significantly less plaque re-growth with respect to both chlorhexidine ($P = 0.033$) and hyaluronan ($P = 0.045$) when compared to the negative control. The difference between chlorhexidine and hyaluronan was not statistically significant ($P = 0.69$).

Conclusions: Hyaluronan (0.025%)-containing mouthwash was comparable to chlorhexidine (0.2%) in inhibiting plaque growth *in vivo*, and it significantly reduced the growth of Aa and Pi *in vitro*.

Key words: anti-inflammatory, antioxidant, anti-plaque, hyaluronan, mouthwash

Hyaluronan, commonly known as hyaluronic acid, is a simple but unusual polysaccharide. It is a high molecular weight (10,000–10,000,000 Daltons), non-sulphated polysaccharide consisting of repeating disaccharide units of sugars N-acetylglucosamine and D-glucoronic acid, joined alternatively by β-glucosidic (1 to 3) and β-bonds (1 to 4). Its interaction with other proteoglycans and collagen imparts stability and elasticity to the extracellular matrix of connective tissue (Rabasseda, 2000).

The function of hyaluronan, known for several years, is to serve as a binding agent between various connective tissue components. However over the past two decades, several papers have emerged suggesting its other functions, which include an important role in cell adhesion, migration and differentiation. The CD44 receptor it binds to is involved in the interaction between gingival fibroblasts and T- and B-lymphocytes (Murakami et al, 1996). The production of hyaluronan is also increased *in vitro* by the action of bacterial lipopolysaccharides (Bartold, 1991). Hyaluronan enhances tissue regeneration (Nakamura, 1993), stimulates osteoinduction

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(Sakasi and Watanabe, 1995), is involved in the process of osseointegration (Klinger et al, 1998) and plays an important role in the early stages of wound healing (Weigel et al, 1986). It has adhesive properties, and its topical effect remains localised on the area of gums where it is applied (Rabasseda, 2000). Hyaluronan also possesses anti-inflammatory properties (Vangelisti et al, 1993; Pagnacco et al, 1997; Jentsch et al, 2003) and is widely used in the field of research on rheumatoid arthritis and osteoarthritis. It is shown to have both a bacteriostatic effect (Pirnazar et al, 1999) and antioxidant effect (Fukuda et al, 1999).

Among all the properties of hyaluronan described, its anti-inflammatory action, bacteriostatic effect and antioxidant properties make its use as a long-term anti-plaque and anti-gingivitis agent an appealing proposition. Hence, the aim of the present study was to evaluate the efficacy of 0.025% hyaluronan-containing mouthwash in comparison with 0.2% chlorhexidine and a water-based mouthwash and also to evaluate its antibacterial efficacy on isolated strains of periodontopathogens. The effects of commercially available 0.025% sodium hyaluronate, commercially available chlorhexidine (0.2%) mouthwash and a water-based rinse (negative control) were studied on the growth of isolated strains of *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Prevotella intermedia* (*Pi*). Following this, a single-blinded, parallel design, randomised controlled trial was carried out and the 4-day plaque re-growth model was used to study the efficacy of the three mouthwashes.

MATERIALS AND METHODS

The design of the present study conformed to the Declaration of Helsinki and subsequent amendments (World Medical Organization, 1996).

Test products

The products used in the present study were as follows: (i) commercially available preparation containing high molecular weight hyaluronan (Gengigel®, Ricerfarma S.R.L, *via* Egadi, Milano, Italy), in the form of 0.025% sodium hyaluronate and xylitol as a preservative; (ii) commercially available chlorhexidine (0.2%) mouthwash; and (iii) a water-based rinse comprising distilled water with dissolved mint tablets. All of the three mouthwashes were dispensed in similar-looking opaque bottles.

Study design

The present study was carried out between February and March 2009, and was divided into two parts. First, the effects of the three mouthwashes were tested on the growth of isolated strains of periodontopathogens *in vitro*. Second, a single-blinded, parallel design, randomised controlled trial was carried out and the 4-day plaque re-growth model was used to study the efficacy of the three mouthwashes.

Microbiological evaluation

The effects of all the three mouthwashes were studied on isolated strains of *Pg, Aa* and *Pi*. As all of the three microorganisms required an anaerobic environment for optimal growth, these organisms were inoculated in thioglycolate broth enriched with haemin and vitamin K. The three mouthwashes were added to the three tubes containing each organism, respectively. After 1-, 2- and 5-minute exposures to the rinse, the organisms were plated out on enriched blood agar plates and cultured in an anaerobic jar for 48 h. Triplicate cultures were spread on three different agar plates for the three microorganisms, at the three time intervals.

Clinical evaluation

Forty-five volunteers (age: 18 to 21 years) were recruited in the present study. These volunteers were the students of SDM College of Dental Sciences and Hospital. Details of the present study were explained, and informed consent was obtained from the students. Exclusion criteria include consumption of antibiotics or other medication in the last 3 months that might interfere with plaque formation, poor oral hygiene (Sulcus Bleeding Index, SBI > 1), < 20 teeth to be included in the evaluation, presence of crowns or restorations, extensive bridges or prosthetic constructions and orthodontic appliances, known intolerance or allergy to mouthwashes, age below 18 years (18 years is the legal age in India to give informed consent without the permission of a parent/guardian) and pregnant women or lactating mothers.

On day 1, the Turesky et al (1970) modification of Quigley and Hein (1962) Plaque Index using a 3% erythrosine dye (Agent P®, ICPA, India) was recorded. Using this index, plaque was assessed on the buccal/labial and lingual/palatal surfaces of all teeth.

The scoring criteria employed were as follows:

0 = No plaque.
1 = Separate flecks of plaque at the cervical margin of the tooth.
2 = A thin continuous band of plaque (up to 1 mm) at the cervical margin of the tooth.
3 = A band of plaque wider than 1 mm at the cervical margin of the tooth.
4 = Plaque covering at least one-third but less than two-thirds of the crown of the tooth.
5 = Plaque covering two-thirds or more of the crown of the tooth.

The SBI by Mühlemann and Son (1971) was also recorded. This index assesses gingival bleeding on the scale of 0 to 5. The scoring criteria employed were as follows:

0 = Healthy appearance of the papillary and marginal gingiva. No bleeding upon sulcus probing.
1 = Apparently healthy papillary and marginal gingiva showing no colour or contour changes, no swelling, but bleeding from sulcus on probing.
2 = Bleeding on probing and colour change caused by inflammation. No swelling or macroscopic oedema.
3 = Bleeding on probing, change in colour and slight oedematous swelling.
4 = Bleeding on probing with obvious swelling.
5 = Spontaneous bleeding on probing, colour change and marked swelling with or without ulceration.

These indices were recorded by two trained and calibrated examiners. Calibration was performed on three subjects, not included in the present study, and the ensuing scores were analysed by a third examiner. Scaling was performed by the same two calibrated examiners on all of the subjects recruited in the present study such that the areas stained with the disclosing agent were completely cleaned, to ensure zero plaque index scores. The baseline plaque index scores were calculated before scaling.

Volunteers were divided into 15 groups of three each (blocking) as suggested by Chilton and Fleiss (1986). Blocking means the formation of matched sets of patients so that each set consists of as many patients as there are treatments (Chilton and Fleiss, 1986). All of the three volunteers in each group had similar plaque and bleeding index scores. The three volunteers in each group were then randomly allocated to one of the three groups. Group A (positive control) was the chlorhexidine group, Group B (test) was the hyaluronan group and Group C (negative control) was the water-based group.

For the following 4 test days, the volunteers had to refrain from carrying out all mechanical oral hygiene measures. Chewing gum was similarly not allowed. Instead, each volunteer rinsed for 1 min, twice daily (in the morning and evening after eating) with the randomly allocated mouthwash solution. The rinsing was monitored on all occasions by the examiners. The dietary regime of the patients was not altered, but it was ensured that the patients had a similar diet.

On day 5, the indices were re-recorded, and the volunteers were allowed to reinstate their routine oral hygiene procedures. These scores were the plaque re-growth scores and the SBI scores, which were subjected to statistical analysis.
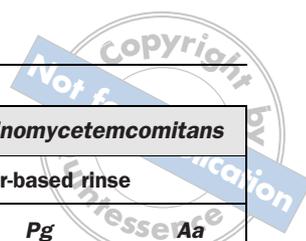
Statistical evaluation

After the completion of re-recording of indices and decoding the mouthwash order, further evaluation was performed with the computer program Statistical Package for the Social Sciences (SPSS) version 11.0. The mean plaque re-growth was calculated for each rinse solution. A Kruskal–Wallis non-parametric test was performed. Differences between the individual rinse solutions and the water-based solution were determined via a Mann–Whitney test.

RESULTS

When tested *in vitro* against periodontopathogens, chlorhexidine continued to remain the gold standard. Hyaluronan had a distinct effect on the growth of colonies *Aa* and *Pi* for 1-, 2- and 5-minute exposures. However, it had no effect on the growth of colonies *Pg* (Table 1).

The *in vivo* results showed that the mean Plaque Index scores were the highest for the water-based rinse (0.797) and the lowest for chlorhexidine (0.354). The mean Plaque Index score for hyaluronan (0.405) was slightly greater than that for chlorhexidine. A Kruskal–Wallis non-parametric test showed a statistically significant difference between the Plaque Index scores ($P = 0.0287$) (Table 2).

The mean SBI scores for chlorhexidine (0.1823), hyaluronan (0.2131) and water-based rinse (0.3678) did not show any statistically significant difference ($P = 0.372$) (Table 2).

Table 1 Effect of the three mouthwashes on the colonies *P. intermedia*, *P. gingivalis* and *A. actinomycetemcomitans*

Minutes	Chlorhexidine			Hyaluronan			Water-based rinse		
	Pi	*Pg*	*Aa*	*Pi*	*Pg*	*Aa*	*Pi*	*Pg*	*Aa*
1	30 c	40 c	50 c	200 c	> 500 c	50 c	> 500 c	> 500 c	> 500 c
	22 c	28 c	32 c	250 c	> 500 c	46 c	> 500 c	> 500 c	> 500 c
	10 c	n/g	21 c	228 c	> 500 c	40 c	> 500 c	> 500 c	> 500 c
2	10 c	2 c	26 c	76 c	> 500 c	38 c	> 500 c	> 500 c	> 500 c
	8 c	n/g	7 c	79 c	> 500 c	30 c	> 500 c	> 500 c	> 500 c
	8 c	n/g	4 c	92 c	> 500 c	28 c	> 500 c	> 500 c	> 500 c
5	n/g	n/g	3 c	40 c	> 500 c	7 c	> 500 c	> 500 c	> 500 c
	n/g	n/g	n/g	27 c	> 500 c	4 c	> 500 c	> 500 c	> 500 c
	n/g	n/g	n/g	38 c	> 500 c	n/g	> 500 c	> 500 c	> 500 c

c, colonies; n/g, no growth.

Table 2 *In vivo* mean Plaque Index and SBI scores; comparison between groups using Kruskal–Wallis non-parametric test

	Sample	M	SD	Sig. ($P < 0.05$)
Plaque Index scores	Chlorhexidine	0.3537	0.3157	**0.0287**
	Hyaluronan	0.4050	0.2038	
	Water-based rinse	0.7968	0.4918	
SBI scores	Chlorhexidine	0.1823	0.1967	0.3721
	Hyaluronan	0.2131	0.1204	
	Water-based rinse	0.3678	0.3555	

Differences between the individual rinse solutions and the water-based solution, determined *via* Mann–Whitney test (Table 3), showed significantly less plaque re-growth with respect to both chlorhexidine ($P = 0.033$) and hyaluronan ($P = 0.045$) as compared to the negative control. The difference between chlorhexidine and hyaluronan was not statistically significant ($P = 0.69$).

Table 3 Comparison between groups for the mean Plaque Index scores using Mann–Whitney test

Sample	Sample	Sig. ($P < 0.05$)
Chlorhexidine		
	Hyaluronan	0.69
	Water-based rinse	0.033
Hyaluronan		
	Chlorhexidine	**0.69**
	Water-based rinse	0.045
Water		
	Chlorhexidine	**0.033**
	Hyaluronan	**0.045**

DISCUSSION

Plaque control is the cornerstone of preventive and maintenance therapy. Mechanical plaque control, though the mainstay in achieving gingival health, is at times not completely efficient. de La Rosa et al (1979) showed that only half of the plaque was removed with brushing for 2 min. Rugg-Gunn and MacGregor (1978) and MacGregor and Rugg-Gurm (1979) suggested that certain tooth surfaces receive little or no attention during the brushing cycle. In combination with toothbrushing, daily use of the tested mouthwashes may result in a higher interproximal plaque reduction than daily flossing (Zimmer et al, 2006). Adjunctive use of chemicals would therefore appear to be a way of overcoming deficiencies in mechanical tooth cleaning habits.

Among all of the anti-plaque and anti-gingivitis agents available, chlorhexidine continues to remain the 'gold standard' (Jones, 1997). However, it comes

at the cost of several side effects. Hence, its long-term use is not advocated. Excluding chlorhexidine containing rinses, only essential oil-containing rinse has been extensively evaluated and subsequently been shown to be of value as an adjunct to mechanical oral procedures (Lamster et al, 1983; Gordon et al, 1985; DePaola et al, 1989). However, the alcohol content of essential oil rinses and its unpleasant taste are unacceptable to a few patients. Hence, the quest for a long-term, ideal and safe, anti-plaque and anti-gingivitis agent still continues.

Hyaluronan has been proven to have a long-term anti-inflammatory action, showing a decrease in the amount of plaque-induced gingivitis (Vangelisti et al, 1993; Pagnacco et al, 1997; Jentsch et al, 2003). de Araújo et al (2007) suggested the use of hyaluronan in peri-implant maintenance. The anti-inflammatory action of hyaluronan is thought to be due to its scavenging action on matrix metalloproteinases and prostaglandins that are the important inflammatory mediators (Laurent et al, 1995).

The bacteriostatic action of hyaluronan is studied in the field of ophthalmology, where its use in artificial tears is shown to have bacteriostatic action (Albert et al, 2008). Also bladder instillations of hyaluronic acid have been shown to decrease the prevalence of urinary tract infections (Lee et al, 2001). With regard to the periodontal literature, only one such study carried out by Pirnazar et al (1999) is available, which has shown the bacteriostatic action of hyaluronic acid on several organisms.

The antioxidant action of hyaluronan has been extensively studied in arthritis research (Foschi et al, 1990). Hyaluronic acid has been used in the treatment of rheumatoid arthritis and osteoarthritis. Campo et al (2003) showed that treatment with hyaluronan and chondroitin-4-sulphate, starting at the onset of arthritis for 10 days, limited the erosive action of the disease in the articular joints of the knee, reduced lipid peroxidation, restored the endogenous antioxidants such as reduced glutathione and superoxide dismutase, decreased plasma TNF-α levels and limited synovial neutrophil infiltration.

The results of the present study showed that hyaluronan-containing mouthwash was comparable to chlorhexidine with respect to its anti-plaque action with no statistically significant difference between the two ($P = 0.69$).

There was no statistically significant difference between the three mouthwashes with respect to the SBI. This could be due to the short time frame of the present study, where any changes in the level of inflammation were not perceptible. It could also be because the hyaluronan-containing mouthwashes

had no action on the growth of *Pg*, one of the red complex species associated with gingival bleeding. The other probable reasons could be that there is really no difference or the SBI is unable to detect a possible difference.

Hyaluronan had no effect on the growth of *Pg*. This was in contrast to the result obtained by Pirnazar et al (1999), where hyaluronan at a higher concentration (0.1%) had a distinct effect on the growth of *Pg*. The concentration of hyaluronan used as a rinse in the present study was 0.025%. Probably, hyaluronan at a higher concentration can limit the growth of *Pg*. However, further research is warranted.

Hyaluronan-containing mouthwash had a perceptible effect on the growth of *Aa* and *Pi*. The colony-forming units of *Aa* were significantly reduced. This could suggest a possible role of hyaluronan in the long-term maintenance of patients with localised aggressive periodontitis.

The limitations of the present study include the short time frame of the study, due to which the effect of the three mouthwashes on gingival inflammation was not perceptible. The rinse has xylitol as a preservative, this in itself may have an anti-plaque action (Lingstrom et al, 1997). Also, the parallel design of the present study could have affected the results, as every individual has a different rate of plaque growth. However, blocking could have eliminated this bias to a certain extent.

The anti-inflammatory and antioxidant action of hyaluronan has been cited in the literature. This was a coherent attempt to study its antibacterial and anti-plaque action. The potential strategies to harness these actions of hyaluronan could be supragingivally in the form of a mouthwash or a gel and subgingivally, especially in patients with localised aggressive periodontitis, in the form of irrigation or local drug delivery system.

CONCLUSIONS

Within the limitations of the present study, it can be concluded that 0.025% hyaluronan-containing mouthwash was comparable to chlorhexidine in inhibiting plaque growth, and it significantly reduced the growth of *Aa* and *Pi in vitro*.

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