

Bacteriostatic Effects of Hyaluronic Acid

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Background: This investigation is one of a series of projects seeking to ascertain whether hyaluronic acid (HA) is therapeutically effective in tissue regeneration procedures. The rationale for these investigations is to test the hypothesis that HA can serve as a bioabsorbable carrier for other substrates as well as itself actively promote the regeneration of tissue.

Methods: In this paper, we report on the bacteriostatic and bactericidal properties of 3 molecular weight formulations of recombinant HA (low, 141 kD; medium, 757 kD; and high, 1,300 kD) on selected oral and non-oral microorganisms in the planktonic phase. Three concentrations of each HA formulation were screened, 0.5, 1.0, and 2.0 mg/ml, using a standard broth culture assay.

Results: Recombinant HA exerted varied bacteriostatic effects on all the bacterial strains tested depending on its molecular weight (MW) and concentration. The high concentrations of the medium MW HA had the greatest bacteriostatic effect, particularly on the *Actinobacillus actinomycetem-comitans*, *Prevotella oris*, *Staphylococcus aureus*, and *Propionibacterium acnes* strains. The 1.0 mg/ml concentration of high MW HA had the greatest overall bacteriostatic effect, inhibiting the growth of all 6 bacterial strains tested. Among the bacterial strains studied, HA was found to have no bactericidal effects, regardless of concentration or molecular weight.

Conclusions: The results of this study suggest that HA in the MW range of 1,300 kD may prove beneficial in minimizing bacterial contamination of surgical wounds when used in guided tissue regeneration surgery. *J Periodontol* 1999;70:370-374.

KEY WORDS

Hyaluronic acid/therapeutic use; periodontal regeneration; bacteriostatic agents.

Hyaluronic acid (HA), also known as hyaluronate or hyaluronan, is a connective tissue glycosaminoglycan that has a number of embryologic and wound healing properties, including the facilitation of cell migration and differentiation during tissue formation and repair.^{1,2} HA is composed of repeated nonsulfated disaccharide units consisting of D-glucuronic acid and N-acetyl-D-glucosamine linked by $\beta(1-3)$ and $\beta(1-4)$ glycoside linkages, respectively.³

HA shares bone induction characteristics with osteogenic substrates such as a calcitonin gene-related peptide (CgRPa)⁴ and bone morphogenic protein.⁵ Recent studies demonstrated that HA aids in the repair process of both soft and hard tissue (bone).^{2,6}

Because of its unique molecular structure, HA can be assembled into various molecular weights (MW) and lyophilized or esterified into a variety of different structural configurations such as sponges and membranes. The rate of biodegradation of these materials can be manipulated by altering their degree of lyophilization or esterification. Thus, HA may be of benefit as a resorbable grafting material in regenerative surgical procedures.

Recent studies of regenerative surgical procedures using guided tissue membranes indicate that bacterial contamination of the membrane and the surrounding wound following its exposure into the oral cavity may adversely affect the formation of new connective tissue attachment and bone.^{7,8} Reduction of the bacterial burden at the wound site may improve the clinical outcome of regenerative therapy. As a first step in the evaluation of HA prior to its use in regenerative therapy, we conducted this investigation to determine the effect of HA on the growth of relevant bacteria. The possible existence of antibacterial properties may prove HA membranes superior to conventional membranes, which have been shown to become contaminated during the wound healing period.⁹ Thus, the specific goals of this study were to

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determine the presence of any bacteriostatic or bactericidal effects of various formulations of HA on selected oral and non-oral microorganisms and to evaluate any possible difference in the effect of HA on those strains which have been shown to possess hyaluronidase activity and those that do not. Both the *Prevotella oris* and *Propionibacterium acnes* strains have been reported to possess hyaluronidase activity,¹⁰ while the rest of the bacterial strains do not possess any hyaluronidase activity¹⁰ and are commonly found in oral gingival regions and periodontal wounds.¹¹ *P. acnes*, although not normally found in the oral cavity, was included in this study to allow for comparison of effect of HA on bacterial strains with hyaluronidase activity to those without it. In this paper, we report on the bacteriostatic and bactericidal properties of various bioabsorbable formulations of HA on selected oral and non-oral microorganisms.

MATERIALS AND METHODS

Bacterial Cultures and Chemicals

The bacterial strains examined included *Streptococcus mutans* ATCC 10449, *Porphyromonas gingivalis* ATCC 33277, *Prevotella oris* ATCC 33573, *Actinobacillus actinomycetemcomitans* Y4, *Staphylococcus aureus* ATCC 9996, and *Propionibacterium acnes* UD. Bacterial cultures were grown under anaerobic conditions at 37°C on brucella blood agar plates[§] in an anaerobic atmosphere. Sterile brain heart infusion broth containing heme and vitamin K was used for liquid cultures.

Recombinant hyaluronic acid^{||} of low (141 kD), medium (757 kD), and high (1,300 kD) MW was suspended and dissolved in sterile distilled water at concentrations of 0.5, 1.0, and 2.0 mg/ml. Three trials were conducted for each bacterial strain. Each trial consisted of 10 determinations: one for each concentration of each molecular weight HA preparation and one control without any HA.

Bacterial Growth Inhibition

The inocula were prepared by growing the bacterial cultures under anaerobic conditions at 37°C on brucella blood agar plates in an anaerobic atmosphere for 48 hours. Bacterial cells harvested from the solid agar were resuspended to a McFarland standard of 0.5 in the broth solutions containing different concentrations of each molecular weight of HA. Sample sizes of 100 µl were used in determining the initial optical density (OD₆₆₀). Viability of the bacterial cultures was assessed by incubation on agar plates under anaerobic conditions at 37°C for 48 hours. The broth solutions were initially vortexed and incubated undisturbed for 48 hours at 37°C.

To assess the presence or absence of growth, all incubated broth solutions were vortexed and final optical density readings (OD₆₆₀) were taken following the

initial 48-hour incubation period. To determine the viable count, 100 µl samples were plated and incubated on agar plates and total colony counts were measured after 48 hours.

As controls, bacterial growth in the absence of HA was examined. To validate the identification of the bacteria and to rule out the possibility of contamination, 10 colonies for each bacterial strain were randomly chosen and analyzed via Gram staining.

Experimental Definitions

“Bacteriostatic” was defined as inhibition of visible bacterial growth following an experimental condition, whereas “bactericidal” was defined as the absence of bacterial growth during incubation of the strain.

The bacteriostatic properties were determined by comparing the calculated growth index (Grl) value of each sample containing both HA and bacteria to the controls containing only HA without bacteria. The growth index was defined as the natural logarithm of the final absorbance value divided by the initial absorbance value of each tube. Moreover, the “relative growth index” (RGI) of each of the tubes containing HA and bacteria was calculated to allow growth comparison between bacterial strains. The relative growth index was defined as the difference between the Grl for each trial’s control group containing only bacteria and no HA and the Grl for the experimental group containing both HA and bacteria for that trial divided by the Grl for that trial’s control group. Thus, Grl and RGI were defined as:

$$\text{Grl} = \ln(\text{OD}_{\text{final}}/\text{OD}_{\text{initial}}).$$

$$\text{RGI} = (\text{Grl}_{\text{control}} - \text{Grl}_{\text{experimental}}) / (\text{Grl}_{\text{control}})$$

The concentrations and molecular weights of the HA used were abbreviated as follows: L = low MW (141 kD); M = medium MW (757 kD); H = high MW (1,300 kD); 0.5 = low concentration (0.5 mg/ml); 1 = medium concentration (1.0 mg/ml); 2 = high concentration (2.0 mg/ml); ctl = control group. Thus, Grl_{L2} represents the growth index of the high concentration of low MW of HA.

RESULTS

Assessment for the viability of the bacterial strains revealed that all bacterial strains tested, regardless of concentration or molecular weight of HA to which they had been exposed, were viable following the 48-hour incubation period in the absence of HA. No bactericidal effects from HA were detected among the bacterial strains studied.

The mean growth index (Grl) and the mean relative growth index (RGI) were calculated using the initial and final optical density as described above. A 2-way analysis of variance (ANOVA) was used in the analysis

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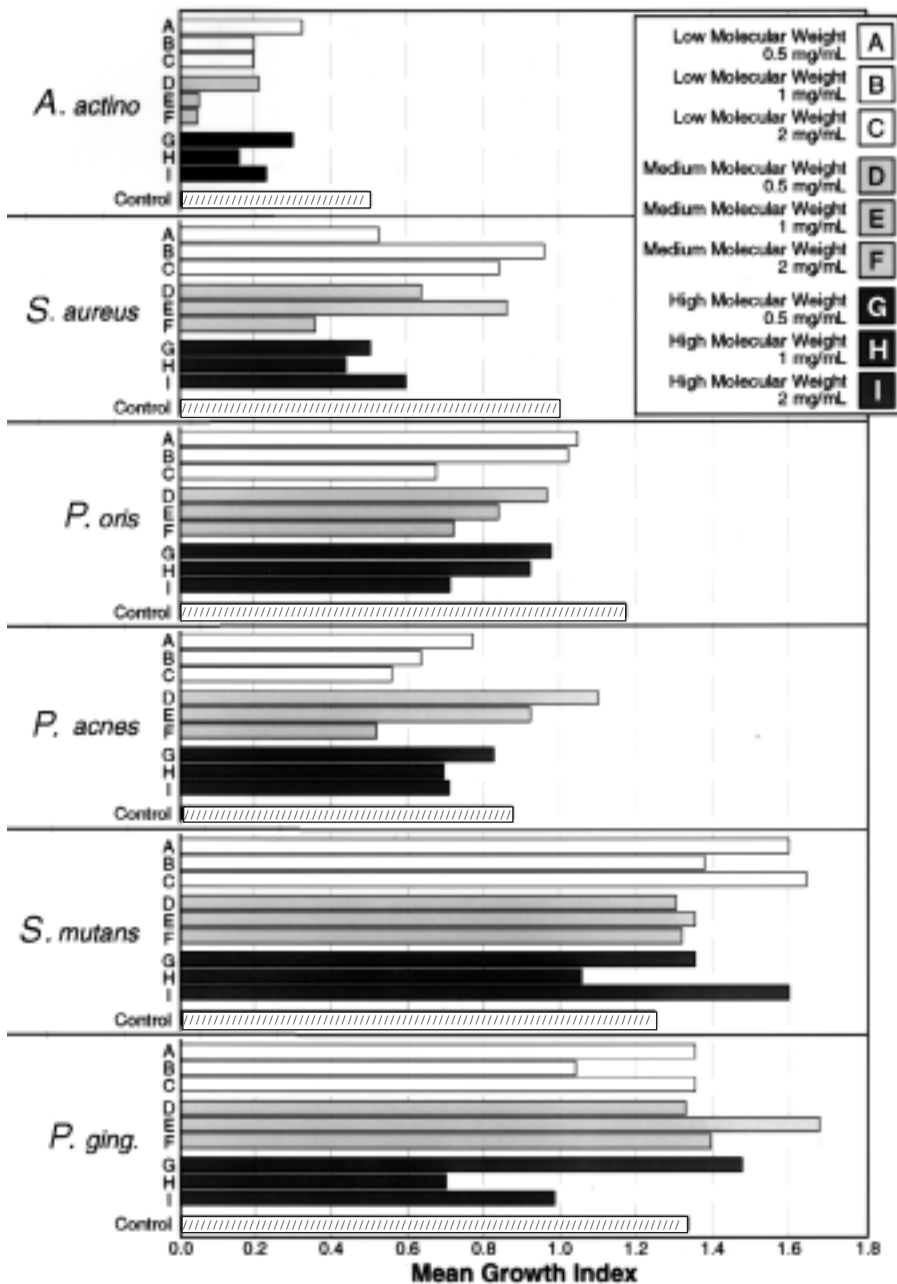


Figure 1.
Mean growth index.

of the bacteriostatic properties of HA by comparing the mean GrI of the experimental group to that of its corresponding control group, containing only broth solution and bacteria and no HA. In this case, HA, depending on its molecular weight and concentration, had bacteriostatic effects on all the bacterial strains tested (Fig. 1).

Depending on the concentration and MW of HA, notable effects on the growth of the bacterial strains were observed. When grown in the presence of HA, some strains including *A. actinomycetemcomitans*, *S. aureus*, and *P. oris* showed only inhibition of growth

(negative mean relative growth index; Fig. 2), while other strains, such as *S. mutans* and *P. gingivalis*, showed both growth inhibition and stimulation (Fig. 2), depending on the concentration and MW of HA.

Regardless of concentration or MW of HA, 3 strains (*A. actinomycetemcomitans*, *S. aureus*, and *P. oris*) grown in the presence of HA had a statistically smaller mean GrI than their control groups, grown without HA (Fig. 1). Moreover, the greatest statistical decrease in the mean GrI for *A. actinomycetemcomitans* ($\text{GrI}_{M2} = 0.073$; S.E. ± 0.004 compared to $\text{GrI}_{\text{ctl}} = 0.496$; S.E. ± 0.007 ; $P < 0.05$), *S. aureus* ($\text{GrI}_{M2} = 0.337$; S.E. ± 0.020 compared to $\text{GrI}_{\text{ctl}} = 0.993$; S.E. ± 0.030 ; $P < 0.05$), and *P. oris* ($\text{GrI}_{M2} = 0.521$; S.E. ± 0.014 , compared to $\text{GrI}_{\text{ctl}} = 1.149$; S.E. ± 0.013 ; $P < 0.05$) was observed with the high concentrations of the medium MW HA. The mean RGI, which by definition compares the growth of the experimental group to the control group, had a negative value (representing growth inhibition) for all 3 of these strains regardless of concentration or MW of HA (Fig. 2).

Depending on the concentration and MW of HA, *P. acnes* showed both negative and positive mean RGI values (Fig. 2). The greatest statistical decrease in the mean GrI for this strain was with the high concentration of the medium MW and the high concentration of the low MW HA ($\text{GrI}_{M2} = 0.618$; S.E. ± 0.018 ; $\text{GrI}_{L2} = 0.537$; S.E. ± 0.021 compared to $\text{GrI}_{\text{ctl}} = 0.876$; S.E. ± 0.021 ; $P < 0.05$). Statistically, the largest GrI values were with the low and medium concentrations of the medium MW HA ($\text{GrI}_{M0.5} = 1.101$; S.E. ± 0.043 ; $\text{GrI}_{M1} = 0.941$; S.E. ± 0.031 compared to $\text{GrI}_{\text{ctl}} = 0.876$; S.E. ± 0.021 ; $P < 0.05$).

P. gingivalis showed both negative and positive mean RGI values (Fig. 2), depending on the concentration and MW of HA. The largest statistical decrease in its mean GrI was observed with the high and medium concentrations of the high MW HA ($\text{GrI}_{H1} = 0.709$; S.E. ± 0.021 ; $\text{GrI}_{H2} = 0.988$; S.E. ± 0.042 compared to $\text{GrI}_{\text{ctl}} = 1.336$; S.E. ± 0.033 ; $P < 0.05$). The greatest significant increase in GrI was with the medium concentration of the medium MW HA ($\text{GrI}_{M1} = 1.66$; S.E. ± 0.036 compared to $\text{GrI}_{\text{ctl}} = 1.336$; S.E. ± 0.033 ; $P < 0.05$). There was no significant difference in GrI with most of the other MWs or concentrations.

S. mutans showed the largest mean RGI among the strains tested. Moreover, the mean RGI of *S. mutans* grown in the presence of low and high MW of HA was statistically greater than that of the control group ($GRI_{L0.5}=1.654$; S.E. ± 0.079 and $GRI_{L2}=1.622$; S.E. ± 0.068 compared to $GRI_{ctl}=1.25$; S.E. ± 0.021 ; $P < 0.05$). However, a statistical decrease in RGI was observed with the medium concentration of the high MW HA ($GRI_{H1}=1.066$; S.E. ± 0.048 compared to $GRI_{ctl}=1.25$; S.E. ± 0.021 ; $P < 0.05$).

Analysis of the RGI, comparing the relative growth inhibition by HA on the various bacterial strains, revealed that *A. actinomycetemcomitans* was overall the most inhibited strain (RGI strain=-0.624, S.E. ± 0.053 ; $P < 0.05$) and that *S. mutans* was overall the least inhibited strain (RGI strain=0.126, S.E. ± 0.049 ; $P < 0.05$) (Fig. 2). The high concentrations of medium MW HA had the overall greatest bacteriostatic effect, particularly with the *A. actinomycetemcomitans*, *P. oris*, *P. acnes*, and the *S. aureus* strains.

DISCUSSION

This study was part of a series of projects to determine the feasibility or applicability of using hyaluronic acid (HA) gels, membranes, and sponges for regenerative surgical therapy. As an initial step, we sought to determine the possible effect of water-soluble HA upon the growth of selected bacteria. The specific goal of this study was to determine whether various HA formulations had bacteriostatic or bactericidal effects on the growth of selected oral and non-oral strains.

The results of this study demonstrated that, regardless of concentration or molecular weight of HA, no bactericidal effects were detected for any of the bacterial strains studied. HA, depending on its MW and concentration, did exhibit bacteriostatic effects on all the bacterial strains tested (Fig. 1). The results demonstrate a varied pattern of bacteriostatic effects on the bacterial strains studied.

HA had the least overall bacteriostatic effect on the *S. mutans* and the *P. gingivalis* strains. A positive mean RGI (growth stimulation) was detected for *S. mutans*, suggesting that the availability of the HA in the environment increased its growth rate. Although the *P. gingivalis* strain also had a low mean RGI, at medium

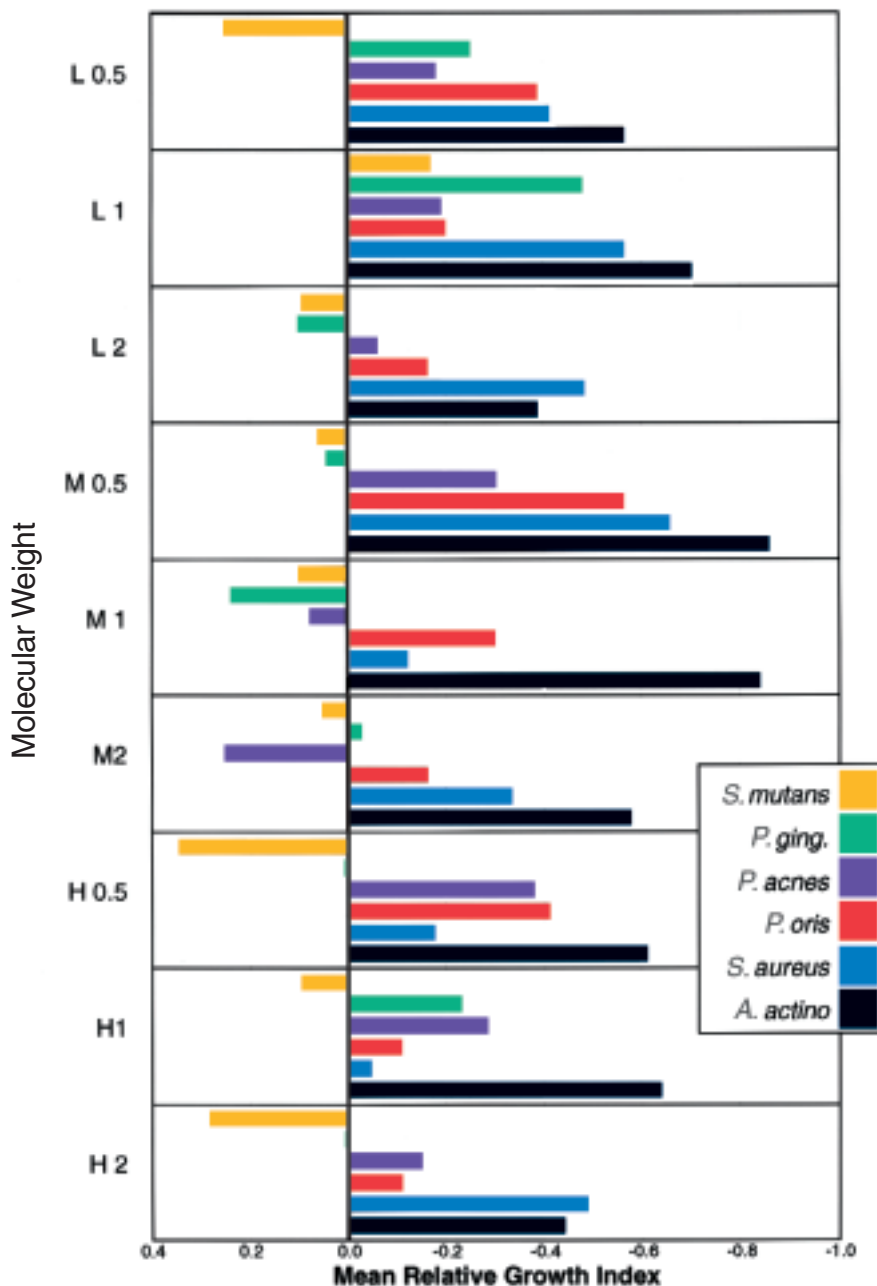


Figure 2. Mean relative growth index.

and high concentrations of high MW HA there was significant reduction in its RGI, indicating that high concentrations of high MW HA showed bacteriostatic effects on this strain.

Depending on its concentration and MW, HA showed both a stimulatory as well as an inhibitory effect on the growth of *P. acnes*. The most significant bacteriostatic effects were observed in the presence of the high concentrations of the medium MW and the high concentrations of the low MW HA. Although this study did not evaluate production of hyaluronidase by either *P. oris* or

P. acnes, production of this enzyme has been reported in previous studies.¹¹ If hyaluronidase was produced by these organisms, it is possible that the enzyme might function in a way that diminishes the bacteriostatic effect of the HA. However, further investigation is needed to examine this issue.

Significant bacteriostatic effects were observed regardless of concentration or MW of HA for *S. aureus* and to a greater extent for *A. actinomycetemcomitans*. Furthermore, the most significant bacteriostatic effects on both these strains were observed with the high concentrations of the medium MW HA. The fact that *A. actinomycetemcomitans* had the least overall RGI may be attributed to reports that growth of this strain in broth media is often barely visible, with little turbidity produced.¹² However, both these strains appear to follow a pattern similar to several other bacterial strains in that the greatest growth inhibition occurs when exposed to high concentrations of HA. Thus, by way of its bacteriostatic activity, the presence of HA in healing tissues may prove to be important in modulating bacterial contamination of the membrane materials.

The high concentrations of the medium MW HA had the greatest bacteriostatic effect, particularly on the *A. actinomycetemcomitans*, *P. oris*, *S. aureus*, and *P. acnes* strains. The middle concentration of the high MW HA, however, had the greatest overall bacteriostatic effect, inhibiting the growth of all 6 bacterial strains tested (Fig. 2). Thus, although several of the bacterial strains were inhibited to a greater extent when exposed to the high concentrations of the medium MW HA, the middle concentration of the high MW HA had a broader inhibitory effect, affecting all of the bacterial strains studied. A capacity for degrading hyaluronate within connective tissues, presumably by fibroblasts, has been inferred, and human cutaneous fibroblasts have been shown to be capable of both binding and internalizing hyaluronate, possibly as a prerequisite for degradation.¹³

The medium concentrations (1 mg/ml) of high MW (1,300 kD) HA may prove desirable when considering the formation of a bioabsorbable membrane for use in regenerative surgery. Such a membrane containing bioactive HA might aid in the reduction of bacterial contamination in surgical wound healing. While the study indicated that the bacteriostatic properties of some forms of HA are reasonable, it is premature to conclude the scope of this effect when considering the variety of bacterial strains which could be present at the healing site. But, it is possible that this observed activity will be found among other bacterial strains as well. Moreover, because of HA's degradation either by fibroblasts¹³ or by other mechanisms within body tissues, an initially high concentration of HA may eventually be degraded into medium and lower molecular weight HA, thereby causing greater growth inhibition on those specific strains which were inhibited to a greater extent by

the medium molecular weight HA. The results of this study suggest that the clinical application of HA membranes, gels, or sponges during surgical therapy may reduce bacterial contamination of the surgical wound site, thereby lessening the risk of postsurgical infection and promoting more predictable regeneration.

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