Bacteriostatic Effects of Hyaluronan-Based Bioresorbable Membrane

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Abstract

Purpose: The purpose of this study is to determine the presence of bacteriostatic effects of hyaluronan-based bioresorbable membrane (HA/CMC) on selected major bacterial strains in digestive organs. Methods: We first evaluated the growth inhibition effect of HA/CMC for E. coli and S. aureus by determining the optical density (OD)_{650} in the incubation medium. At second, to determine the viable counts of bacteria, total adenosine triphosphate (ATP) were measured with five groups; several concentrations of HA/CMC and control. Results: OD curve gradually elevated and reached to plateau at 4 hours in E. coli and 6 hours in S. aureus. After reaching plateau, the growth inhibition of both strains was statistically significantly correlated to the concentrations of HA/CMC. The ATP productions had statistically significant differences at 6 hours after incubation and inhibited in dose-dependence of a well-dissolved HA/CMC. Conclusion: HA/CMC may have dose-dependently bacteriostatic effects on S. aureus and E. coli.

Keywords: HA/CMC, Bacteriostatic, Hyaluronan, Adhesion, Surgery

1. Introduction

Clinical effectiveness and safety of hyaluronan-based bioresorbable membrane (HA/CMC) on preventing intraperitoneal adhesions has been widely accepted in abdominal and pelvic surgical use [1-4]. Recently, a few cases with postoperative intraperitoneal infection and inflammation after HA/CMC placement have been reported [5,6]. Though the relationship between HA/CMC and such morbidities was not clear, several clinical and animal studies demonstrated that HA/CMC neither resulted in the propagation of infection, nor affected abscess formation, intraperitoneal septic condition and systemic inflammatory response adversely [7-10].

A previous study at our institute [11] demonstrated that HA/CMC did not aggravate an inflammatory cytokine response or produce extensive intra-abdominal adhesions, even in patients with intraperitoneal septic complications and rat models with bacterial peritonitis induced by cecal ligation and puncture. In experimental basic study, K. Otake et al. [12] revealed HA/CMC itself does not affect the functions of isolated human Polymorphonuclear neutrophils in in vitro experimental models of inflammation and endotoxin-stimulation. Although our results showed that HA/CMC can be safely used even in contaminated conditions, further basal experiments about HA/CMC against infectious pathogens are needed.

HA/CMC is a bioresorbable membrane made of sodium hyaluronate (Hyaluronic acid, HA) and another polyanionic poly saccharide, carboxymethylcellulose (CMC). These components are chemically modified to reduce water solubility and provide longer in vivo residence time than the native polymers. Preclinical evaluation demonstrated that HA/CMC is nontoxic, nonmutagenic, nonimmunogenic, nonirritating and nonpyrogenic [13]. HA is a connective tissue glycosaminoglycan that has a number of embryonic and wound healing properties, including the facilitation of cell migration and differentiation during tissue formation and repair [14,15]. Previous studies suggested that HA has great bacteriostatic effects on the common orthopedic and periodontal bacterial pathogens [16,17]. The purpose of this study is to determine the presence of any bacteriostatic or bactericidal effects of HA/CMC on selected microorganisms such as Staphylococcus aureus (S. aureus) and Escherichia coli.
coli (E. coli).

2. Materials and Methods

2.1. Bacterial Preparations

The two bacterial strains, S. aureus (ATCC27217) and E. coli (ATCC25922) were purchased from the ATCC (American Type Culture Collection). S. aureus and E. coli were cultured in Dulbecco’s modified Eagle’s media (D-MEM) (Invitrogen Co., Tokyo, Japan) for 10 h at 37°C. The culture medium was centrifuged at 1700 × g for 10 min at room temperature to pellet the S. aureus or E. coli, and resuspended in sterile normal saline. A 100 μL quantity of the suspension was serially diluted with sterile saline, applied on 1.5% Agar-B (Qbiogene, Inc.) and 0.8% Difco Nutrient Broth (Difco Laboratories, USA.) plate, and incubated for 18 h to determine the bacterial concentration. The remainder was stored at –80°C until use. Just before use, the bacterial suspension was diluted in medium to achieve a final concentration of 5.0 × 10⁶ colony-forming units (cfu)/mL. The study was approved by the local ethics committee.

2.2. Bacterial Growth Inhibition

Seprafilm® (HA/CMC, Genzyme Corporation, Japan) is a synthetic product of HA and CMC in the ratio of two to one. A sheet of Seprafilm® (12.7 cm × 14.7 cm, 186.69 cm²) weighs about 1 g including HA 0.7 g and CMC 0.3 g. A quarter sheet of HA/CMC include approximately 175 mg HA [18]. The incubation medium of Group A was 10 ml D-MEM with a well-dissolved quarter sheet of HA/CMC (approximately 175 mg HA). The incubation medium of Group B was 10 ml D-MEM with a well-dissolved one-eighth sheet of HA/CMC (approximately 87.5 mg). The incubation medium of Group C was only D-MEM without HA/CMC. 100 μl bacterial suspension (5.0 × 10⁵ cells) was added to 100 μl medium (total 200 μl) without HA/CMC that is similar to medium of Group C in bacterial growth inhibition assay (Group I). We prepared 100 μl medium with well-dissolved a quarter HA/CMC (Group II, approximately 175 mg HA), a half of HA/CMC (Group III, approximately 350 mg HA), one HA/CMC (Group IV, approximately 750 mg HA), and twice HA/CMC (Group V, approximately 1500 mg HA). So, HA concentrations of medium are approximately 0 mg/ml in Group I, 8.25 mg/ml in Group II, 16.5 mg/ml in Group III, 33 mg/ml in Group IV, and 66 mg/ml in Group V, respectively. Sample sizes (5.0 × 10⁵ cells) of each bacterial suspension were used in determining the initial luminescence. Each sample was incubated using 96-well cell plates (Becton Dickinson Labware, NJ, USA) for 6 hours at 37°C shaking incubator. Trials were conducted for each bacterial strain. At 0, 2, 4, and 6 hours after incubation starts, each sample was mixed well and 100 μl bacterial suspension of each well was transferred to white 96-well cell plates (Thermo Electronic Corporation). An equal volume of the previously prepared BacTiter-Glo reagent was added in each well. The luminescence of the sample was measured with a multi-detection microplate reader (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan) immediately after five minutes incubation. A calibration curve with dilutions of pure rATP (Promega, P1132) was measured before each experiment. ATP value was evaluated in triplicate and the average value was computed.

2.4. Statistics

Statistical analyses were performed using Statview 4.5 (Abacus Concepts, Berkeley, California). Results are presented as the means ± SD. In bacterial growth inhibition assay, two-way repeated measures ANOVA was used to compare the each two groups with respect to bacterial growth over time. In the study of cellular function investigation assay, Spearman’s rank correlation test was used to test the correlation between bacterial growth and the concentration of HA/CMC for 0, 2, 4 and 6 hours. A p value of <0.05 was considered significant.
3. Results

3.1. Bacterial Growth Inhibition

Figure 1 shows the optical density (OD) of broth solutions with *S. aureus* during 12 hours incubation among three groups. OD curve gradually elevated and reached to plateau at 6 hours. There was a significant difference in OD between Group A and B (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Moreover, there was a significant difference in OD between Group A and C (*p* = 0.0020 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). However, there was no difference in OD between Group B and C. (*p* = 0.0550 for HA/CMC concentrations and *p* = 0.5767 for time × HA/CMC concentrations). Figure 2 shows OD of broth solutions with *E. coli* during 12 hours incubation among three groups. OD curve gradually elevated and reached to plateau at 4 hours. There was a significant difference in OD between Group A and B. (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). There was a significant difference in OD between Group A and C (*p* = 0.0020 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Moreover, there was a significant difference in OD between Group B and C (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Statistical analysis was performed by Two-Way Repeated-Measures ANOVA.

3.2. Cellular Function Investigation

Figure 3 shows the serial change of mean ATP value of *S. aureus* in all groups. The mean ATP production was inhibited dose-dependently by HA/CMC and the differences in the value of ATP production are statistically significant among 5 groups at 2, 4, and 6 hours after incubation. Figure 4 shows the serial change of mean ATP value of *E. coli* in all groups. At 6 hours after incubation, the mean ATP production was inhibited dose-dependently by HA/CMC and the differences in the value of ATP production are statistically significant among 5 groups at 6 hours after incubation.

4. Discussion

Recently, as the usage of HA/CMC increases in abdominal surgical scene, the relationship between HA/CMC and postoperative infectious complications has become the center of attention [9,11]. In the presence of infection, fibrinolysis in the abdominal cavity is even more depressed and intraabdominal adhesion becomes more severe. After contaminated or dirty operations, abscesses arising from contaminated fibrin are frequent complications. In the treatment of infection development in the abdominal cavity, elimination of the source of contamination is the cornerstone of abdominal surgery.

Figure 1. The optical density (OD) of broth solutions with *S. aureus* during 12 hours incubation among three groups. HA concentrations of medium are approximately 8.25 mg/ml in Group A, 4.125 mg/ml in Group B, and 0 mg/ml in Group C, respectively. There was a significant difference in OD between Group A and B (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Moreover, there was a significant difference in OD between Group A and C (*p* = 0.0020 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). However, there was no difference in OD between Group B and C (*p* = 0.0550 for HA/CMC concentrations and *p* = 0.5767 for time × HA/CMC concentrations). Statistical analysis was performed by Two-Way Repeated-Measures ANOVA.

Figure 2. The optical density (OD) of broth solutions with *E. coli* during 12 hours incubation among three groups. HA concentrations of medium are approximately 8.25 mg/ml in Group A, 4.125 mg/ml in Group B, and 0 mg/ml in Group C, respectively. OD curve gradually elevated and reached to plateau at 4 hours. There was a significant difference in OD between Group A and B (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). There was a significant difference in OD between Group A and C (*p* = 0.0020 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Moreover, there was a significant difference in OD between Group B and C (*p* < 0.0001 for HA/CMC concentrations and *p* < 0.0001 for time × HA/CMC concentrations). Statistical analysis was performed by Two-Way Repeated-Measures ANOVA.
Figure 3. The serial change of mean ATP value of *S. aureus* in all groups. HA concentrations of medium are approximately 0 mg/ml in Group I, 8.25 mg/ml in Group II, 16.5 mg/ml in Group III, 33 mg/ml in Group IV, and 66 mg/ml in Group V, respectively. The mean ATP production was inhibited dose-dependently by HA/CMC and the differences in the value of ATP production are statistically significant among 5 groups at 2, 4, and 6 hours after incubation. *p = 0.0006, †p = 0.0001, ‡p = 0.0004 (Spearman’s rank correlation test).

Figure 4. The serial change of mean ATP value of *E. coli* in all groups. HA concentrations of medium are approximately 0 mg/ml in Group I, 8.25 mg/ml in Group II, 16.5 mg/ml in Group III, 33 mg/ml in Group IV, and 66 mg/ml in Group V, respectively. At 6 hours after incubation, the mean ATP production was inhibited dose-dependently by HA/CMC and the differences in the value of ATP production are statistically significant among 5 groups at 6 hours after incubation. *p = 0.0002 (Spearman’s rank correlation test).

Fibrin forms deposits that are a matrix for ingrowth of fibrocollagenous tissue. Moreover, fibrin clots protect bacteria against the immunological defences of the abdominal cavity. Following this pathway, intraabdominal fibrin may eventually lead to adhesion and/or abscess formation. Persistence and reinforcement of fibrinolysis is needed after surgery new fibrin deposits will occur.

Animal experiments have demonstrated that enhancement of fibrinolysis reduces intraabdominal abscess formation [19,20]. Several mechanisms may be involved in the reduction of adhesions by HA/CMC, including mechanical separation of wound surfaces, improvement of peritoneal healing and enhanced fibrinolysis. Reijnen et al. [9] reviewed investigations into the pathophysiology of intra-abdominal adhesions and abscess formation, and showed that the potential of hyaluronan-based agents to reduce intra-abdominal adhesions and abscess on abdominal surgery and sepsis is a promising new concept. Previous study at our institute demonstrated that HA/CMC itself does not affect the functions of isolated human Polymorphonuclear neutrophils in *in vitro* experimental models of inflammation and endotoxin-stimulation [12]. In this study, we tried to determine whether hyaluronan-based bioresorbable membrane HA/CMC reduces the bacterial growth. In growth inhibition assay, we investigated the effect of HA/CMC on both the logarithmic growth phase and stationary phase. And we put emphasis on the effect of HA/CMC itself on the total number of bacteria. In this assay, the logarithmic growth phase of *S. aureus* and *E. coli* continued for 6 hours at least. In cellular function investigation, we put emphasis on the association between the viability of bacteria and the concentration of HA/CMC. Therefore, we investigated the effect of HA/CMC to the logarithmic growth phase for 6 hours by measuring total ATP.

HA modulates the inflammatory response; it has free radical scavenging and antioxidant properties [21], and increases interferon γ levels during peritonitis, inhibits inflammatory proteinases [22], and probably induces the production of proinflammatory mediators such as interleukin 1, interleukin 8 and TNF-α [23]. The role of HA in the resistance of bacteria to opsonization and phago-cytic killing is not completely understood [24]. Carlson et al. [5] suggested that the bacteriostatic effect of HA may be due to the saturation of the bacterial hyaluronate lyase by the excess HA, which prevents the bacteria from maintaining elevated levels of tissue permeability and penetrating the physical defenses of the host. This would enhance the ability of the host’s immune system to eradicate pathogens. HA molecules also form a random network of chains that may act as a sieve preventing the spread of the bacteria.

Pirnazar et al. [17] demonstrated the bacteriostatic effects of HA on selected oral and non-oral microorganisms. They suggested that HA has no bactericidal effect, however, the high concentrations (2.0 mg/ml) of the medium (757 kD) molecular weight (MW) HA had the greatest bacteriostatic effect on several bacterial strains compared to the combinations with other concentrations (0.5 or 1.0 mg/ml) and MW (141 kD or 1300 kD). HA in HA/CMC and has been esterified with glucuronic acid residues and is different from native HA in the human body. Based on light-scattering measurements, the modified HA in comprises molecules of approximately 100 kD in MW [18]. HA/CMC is a synthetic product of HA.
and CMC in the ratio of two to one. A sheet of HA/CMC (12.7 cm × 14.7 cm, 186.69 cm²) weighs about 1 g including HA 0.7 g and CMC 0.3 g. A quarter sheet of HA/CMC include approximately 175 mg HA. The concentration of HA formulation used in Group A was 3.5 mg/ml and higher compared to Pirnazar’s study. This study clarified the higher concentrations of HA/CMC introduced, the greater bacteriostatic effects on S. aureus and E. coli using bacterial growth inhibition and cellular function investigation. In conclusion, HA/CMC may have dose-dependently bacteriostatic effects on S. aureus and E. coli. Further investigations are needed about various bacterial strains which could be present in the abdominal cavity.

5. References


