Published in final edited form as:

Shock. 2020 October ; 54(4): 488–497. doi:10.1097/SHK.0000000000001512.

THERAPEUTIC EFFECTS OF HYALURONIC ACID IN PERITONITIS-INDUCED SEPSIS IN MICE

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Abstract

Intra-abdominal infection is the second most common cause of sepsis, and the mortality rate from abdominal sepsis remains high. High molecular weight (HMW) hyaluronic acid (HA) has been studied in sterile injury models as an anti-inflammatory and anti-permeability agent. This study evaluated the therapeutic effects of intraperitoneal HMW HA administration in mice with peritonitis-induced sepsis. Sepsis was induced in C57BL/6 mice by cecal ligation and puncture (CLP), followed 4 h later by an intraperitoneal injection of HMW HA (20 mg/kg) solution or phosphate buffered saline (PBS). Survival, physiological data, organ injury, bacterial burden, and inflammatory cytokine levels were assessed in the CLP mice. To assess the effect of HA on macrophage phagocytosis activity, RAW264.7 cells, primed with lipopolysaccharide, were exposed with either PBS or HMW HA (500 µg/ml) prior to exposure to 10^7 CFU of *E.coli* bacteria. HMW HA instillation significantly improved blood oxygenation, lung histology, and survival in CLP mice. Inflammatory cytokine levels in the plasma and bacterial burdens in the lung and spleen were significantly decreased by HA administration at 24 h after CLP. At 6 h after CLP, HA significantly decreased bacterial burden in the peritoneal lavage fluid. HMW HA administration significantly increased *E.coli* bacterial phagocytosis by RAW264.7 cells in part through increased phosphorylation of ezrin/radixin/moesin, a known downstream target of CD44 (a HA receptor); ezrin inhibition abolished the enhanced phagocytosis by RAW264.7 cells induced by HA. Intraperitoneal administration of HMW HA had therapeutic effects against CLP-induced sepsis in terms of suppressing inflammation and increasing antimicrobial activity.

Keywords

Hyaluronic acid; Inflammation; Sepsis; Peritonitis; Phagocytosis

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Conflicts of interest: The authors declare that they have no conflict of interests.

INTRODUCTION

Sepsis is a clinical syndrome induced by infection accompanied by a dysregulated host response leading to organ dysfunctions and accounts for >200,000 deaths annually in the United States and 18 million deaths worldwide (1). Following pneumonia, intra-abdominal infection is the second most common cause of sepsis. Despite therapeutic modalities such as appropriate antibiotic use and surgical procedures for source control of intra-abdominal infection, the mortality rates from abdominal sepsis have been reported as high as 36% (2–5). Therefore, new adjuvant techniques to control intra-abdominal infection in addition to surgery for source control and antibiotics are needed to reduce the morbidity and mortality associated with sepsis.

Hyaluronic acid (HA) is a non-sulfated glycosaminoglycan which is a major component of the extracellular matrix. Besides its structural role in all organs, it is reported that HA has various immunomodulatory properties in both physiological and pathological states, such as in regulating innate and adaptive immune cells (6). The responses of the immune system to HA are different according to the molecular weight of HA. Low molecular weight HA (<250 kDa) increases production of inflammatory mediators in macrophages (7–10). In contrast, high molecular weight (HMW) HA (>800 kDa), the predominant size in the extracellular matrix, promotes the production of anti-inflammatory mediators (10). More importantly, intermediate molecular weight HA (approximately 500 kDa) has been reported to enhance phagocytosis by binding to the CD44 receptors on neutrophils (11). CD44 is a transmembrane adhesion molecule, and its major function is to bind and internalize HA (6). The engagement of CD44 with HA or anti-CD44 antibodies can cause cytoskeletal reorganization, leading to enhanced phagocytosis (11–13).

Based on these previous studies, we hypothesized that HMW HA (750–1,000 kDa) could suppress excessive inflammation and help in the elimination of pathogens in part by enhancing bacterial phagocytosis by immune cells following abdominal sepsis. Therefore, the current study was undertaken to determine if intraperitoneal administration of HMW HA would have therapeutic effects in peritonitis-induced sepsis in mice in terms of suppressing dysregulated inflammation and overwhelming bacterial infection.

MATERIALS AND METHODS

Peritonitis-Induced Sepsis in Mice With or Without High Molecular Weight Hyaluronic Acid Treatment

C57BL/6J male mice (10–12 weeks old; Jackson Laboratory) were housed in the animal care facility at the University of California, San Francisco (UCSF). All experimental protocols were approved by the Institutional Animal Care and Use Committee at UCSF.

Sepsis was induced by cecal ligation and puncture (CLP). The mice were first anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine by intraperitoneal injection. After the mice was placed in the supine position on a heating pad at 37°C, their bellies were shaved and prepped with 70% ethanol. A ventral midline incision up to 1 cm was made to allow exteriorization of the cecum. To induce high grade sepsis, the cecum was ligated at a

position of 75% of the cecum from the apex with 5–0 silk sutures and penetrated through- and through with a 20-gauge needle. The abdominal incision was then closed in two layers with 6–0 silk sutures. Immediately after surgery, the mice were administered 1 ml of pre-warmed normal saline and 1 mg/kg of sustained release buprenorphine subcutaneously (Buprenorphine SR-LAB 1 mg/ml, Zoopharm Pharmacy, Winsor, USA) for fluid resuscitation and postoperative analgesia respectively. The mice were kept on the heating pad for 1 h following surgery until recovery.

Sodium hyaluronate powder (molecular weight 750–1,000 kDa; Sodium Hyaluronate, HA1M; Lifecore Biomedical, LLC) was dissolved in pyrogen-free phosphate buffered saline (PBS) to a concentration of 2 mg/ml. The mice were injected with the HMW hyaluronic acid (HA) solution (20 mg/kg of HA; CLP+HA group) or PBS (CLP group) intraperitoneally at 4 h after CLP surgery. The mice were euthanized at the designated time point to collect samples for analyses. The total number of mice used in this study was 101.

Assessment of Body Weight, Pulse Rate, and Blood Oxygenation in Septic Mice

Body weight, pulse rate, and pulse oxygen saturation (SpO_2) in the mice were measured immediately before and 24 h after CLP surgery to assess the physiologic status. Pulse rate and SpO_2 were measured non-invasively with a physiologic monitor for mice (PhysioSuite®, Kent Scientific Corp., Torrington, USA).

Evaluation of Bacterial Burden in Organs

Bacterial burden was assessed in the lung and spleen collected from the mice 24 h after CLP surgery. The lungs and the spleens were homogenized in 1 ml sterile saline, and the number of viable bacteria (per colony forming units (CFU)) in the homogenates was determined by plating 10-fold dilution samples on blood agar plates incubated at 37°C overnight (14).

Assessment of Inflammatory Cytokines and Total HA Levels in the Plasma

Blood samples were centrifuged at 8,000 rpm for 10 min, and the supernatants were collected. Tumor necrosis factor (TNF)- α , interleukin (IL)-10, and total HA levels in the supernatants were measured by using ELISA kits (R & D systems, Minneapolis, USA).

Histology

Twenty-four h after CLP surgery, lungs were excised, gently inflated with 0.5 mL of 10% formalin, and the trachea ligated. Then, the lungs were fixed in 10% formalin and dehydrated through a serial diluted ethyl alcohol baths. Following fixation, lungs were embedded in paraffin, cut into 5 μ m sections, and stained with Hematoxylin & Eosin. A researcher who was blinded to mice groups examined the sections and assessed the levels of lung injury based on semi-quantitative scoring. For each mouse, 4 total fields from both lungs (2 fields from each lung) at $\times 20$ magnification were assessed. The scoring was performed by grading as follows: infiltration or aggregation of inflammatory cells in air space or vessel wall: 1=only wall, 2=few cells (1–5 cells) in air space, 3=intermediate, 4=severe (air space congested); interstitial congestion and hyaline membrane formation: 1=normal lung, 2=moderate (<25% of lung section), 3=intermediate (25–50% of lung section), 4=severe (>50% of lung section); hemorrhage: 0=absent, 1=present (15).

Survival Studies

Survival of the mice undergoing CLP surgery was assessed every 6 h for 48 h after the surgery. All the mice were euthanized at the end of the assessments.

Measurement of Fluorescein-Labelled HA Concentration in the Blood

To track HMW HA administered intraperitoneally, fluorescein-labelled HMW HA solution was intraperitoneally injected into mice at 4 h after CLP. Fluorescein hyaluronic acid powder (fluorescein hyaluronic acid >95% powder, molecular weight 640–1,000 kDa, Millipore Sigma, Saint Louis, USA) was dissolved in pyrogen-free PBS to a concentration of 2 mg/ml for the fluorescein-labelled HA solution. At 6 and 12 h after CLP or SHAM surgery, blood was collected from the mice, and fluorescence intensity was measured in the plasma. In order to calculate the plasma concentration of HA based on a standard curve, serial dilutions of fluorescein-labelled HA were prepared to create a series of dilutions from 1.2 to 120,000 pg/ml. The fluorescence intensity of the plasma samples and the standards were detected in volumes of 150 µl in wells of a MicroFluor-1 plate. The plate was read in a fluorescence reader; excitation wavelength was 485 nm, and emission wave length was 528 nm; sensitivity was 80. The fluorescence intensity values were corrected by the subtraction of fluorescence background of the plasma from a mouse that did not receive the fluorescein-labelled HA.

Evaluation of Bacterial Burden, Differential Cell Count, and Inflammatory Cytokine Levels in the Peritoneal Lavage Fluid.

To evaluate the effects of HMW HA administered intraperitoneally on peritoneal infection and inflammation following CLP, peritoneal lavage fluid (PLF) was collected in the mice at 6 h after surgery. Five ml of sterile PBS was used to lavage the peritoneal cavity. The number of viable bacteria in the PLF was determined by plating 10-fold dilution samples on Luria-Bertani (LB) agar plates which were incubated at 37°C overnight, and then CFU was counted (16). The number of white blood cells with their differentials were measured using a multi-species hematology system (Hemavet® HV950, Drew Scientific, Inc., Dallas, USA), and the level of inflammatory cytokines in the PLF were determined with ELISA.

E.coli Challenge to Lipopolysaccharide-Stimulated RAW264.7 Cells

To determine the effect of HMW HA on macrophages to understand some potential mechanisms underlying the therapeutic effects, we evaluated the response of lipopolysaccharide (LPS)-stimulated RAW264.7 to *E.coli* bacterial inoculation with HMW HA treatment. Mouse macrophage RAW264.7 cells were cultured in high-glucose Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. The RAW264.7 cells were plated at a concentration of 250,000 cells/well and primed with LPS (*E.coli*-0111: B4 endotoxin, Sigma-Aldrich, St. Louis, Missouri; 1 µg/ml). Thirty minutes after LPS priming, the RAW264.7 cells were treated with either negative control (PBS) or HMW HA (500 µg/ml). In experiments to evaluate the role of CD44 or Ezrin proteins, CD44 blocking antibody (anti-mouse CD44, clone KM201, EMD Millipore Corp., Temecula, CA; 5 µg/ml) or ezrin inhibitor (NSC668394, Merck KGaA, Darmstadt, Germany; 10 µM) was added to the HMW HA-treated RAW264.7 cells. At 24 h after LPS priming, cell culture

supernatants were collected to assess inflammatory cytokines levels including TNF α and IL-10 levels with ELISA kits, and 10^7 *E.coli* bacteria (K1 strain) were added to each well of the RAW264.7 cells for 1.5 h. The supernatants were then plated on LB agar plate overnight for *E.coli* CFU count.

To test if the *E.coli* clearance in the supernatants was due to increased bacterial phagocytosis, intracellular *E.coli* bacteria in RAW264.7 cells were measured. The cells were infected as described above. At 1 h after *E.coli* inoculation, the infection was terminated by adding gentamicin at a concentration of 100 μ g/ml into each well for 30 min. Then, the cells were extensively washed and lysed in 1% triton X-100 in PBS. The aliquots of the lysates were plated on LB agar plates for counting *E.coli* bacterial CFU counts.

Assessment of HMW HA Effect on *E.coli* Bacteria Proliferation

Previous studies have reported that HMW HA has bacteriostatic properties (17). To determine if HA can suppress the proliferation of the *E.coli* bacteria strain that we used in the *in vitro* experiment, we performed an assay that assessed *E.coli* bacteria proliferation in two different types of media - one was the medium used for RAW264.7 culture in our experiment and the other was LB broth. The assay was performed in 96-well microplates (Corning Inc., NY, USA); 10 μ l/well of PBS or HA solution, at the final concentration of 500 μ g/ml, was added to 100 μ l/well of the medium – the medium for RAW264.7 or LB broth. Then, 5×10^6 CFU of *E.coli* bacteria were added into each well. The blank wells contained only media with PBS or HA at the above concentration. Every condition was tested in 5 wells. The plates were incubated at 37°C in a moist chamber. *E.coli* bacterial proliferation was assessed by measuring the optical density at 600 nm by means of a microplate reader. The optical density values were read immediately and at several time points for 48 h.

Western Blots

To assess the effect of HMW HA on the activation of ezrin/radixin/moesin (ERM) protein family, phosphorylated ERM expression in the LPS stimulated RAW264.7 cells with or without HA treatment were measured by using Western blots. ERM proteins can be phosphorylated via CD44 activation and is involved in phagocytosis, specifically phagosome formation (18–20). LPS and HMW HA treatment was performed in the RAW264.7 cells as described above. One h after the treatment, the cells were lysed in a cell lysis buffer (Invitrogen™ NP40 Cell Lysis Buffer, Thermo Fisher Scientific, Inc., Waltham, USA) with protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Inc., Denver, USA). The samples were centrifuged at 13,000 rpm for 10 min at 4°C, and supernatants were collected. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, followed by blocking with 5% bovine serum albumin in Tris-buffered saline supplemented with 0.05% Tween 20. Blots were incubated overnight at 4°C with anti-phospho-ERM (1:2000; Cell Signaling Technology) or anti-ERM antibodies as a loading control (1:1000; Cell Signaling Technology). The blots were washed and developed with horseradish peroxidase labeled secondary antibodies (1:5000; Thermo Fisher Scientific) using a chemiluminescence system (ChemiDoc™ imaging system, Bio-Rad Laboratories, Inc., Irvine, USA).

Statistical Analysis

Data are presented as mean $\pm$ standard error of mean (SEM) if they are normally distributed and median with (interquartile range (IQR)) if not. The normality of the distribution was assessed by using Shapiro-Wilk test. Unpaired t-test was used in comparisons between two groups if the data were normally distributed, and Mann-Whitney U test was used if not. Comparisons between three groups were performed using an analysis of variance with Tukey's post-hoc test if the data were normally distributed. If not, a Kruskal Wallis test with Dunn's post-hoc test was used. The log-rank test was used for comparing survival data for 48 h. A P value less than 0.05 was considered statistically significant. All statistical analysis was performed using GraphPad Prism 7.05 software (La Jolla, USA). N refers to the number of samples or mice and are not the number of replicate experiments of the same sample or mouse.

RESULTS

HMW HA Administration Improved Blood Oxygenation and Survival Following Peritonitis-Induced Sepsis

Body weight, pulse rate, and SpO₂ were assessed before and at 24 h after sham or CLP surgery in mice (Fig. 1). SpO₂ decreased by 11% among mice following CLP, and intraperitoneal administration of HMW HA at 4 h after CLP almost eliminated the SpO₂ decrease (Fig. 1C). The degree of SpO₂ decrease was significantly different between CLP and CLP+HA ($P < 0.05$). Furthermore, HMW HA treatment improved survival by 50% at 48 h after CLP ($P = 0.015$; Fig. 1D). There were no significant differences in body weight or pulse rate changes between groups, although the pulse rate change with HMW HA administration numerically increased, which may reflect the addition of mice who survived compared to the CLP group. The physiologic variables were measured in 25 mice (5 mice for sham surgery, 10 for CLP, and 10 for CLP+HA). A separate group of 20 mice were used for the survival analysis.

HMW HA Administration Decreased Systemic Inflammation, Histologic Damage to the Lung, and Bacterial Burdens Following Sepsis

Among the 25 mice used for physiologic measurements, one mouse with CLP and without HMW treatment died before 24 h after CLP. Therefore, a total of 5 mice for sham surgery, 9 for CLP, and 10 for CLP+HA were evaluated. A separate group of 8 mice were used only for lung histology analysis. The profiles of blood immune cells are shown in Fig. 2A–C. CLP resulted in significant leukopenia and neutropenia when compared to Sham surgery. There were some numerical trends that HMW HA treatment relieved the leukopenia and neutropenia. In addition, HMW HA treatment in CLP mice significantly increased the number of blood monocytes. We measured inflammatory cytokine levels in the plasma to assess systemic inflammation (Fig. 2D–F). The inflammatory cytokines including TNF α , IL-6, and IL-10 were significantly elevated following CLP compared to those following Sham surgery, and HMW HA treatment in CLP mice reduced these elevations. Representative images of the lung in CLP and CLP+HA mice are shown in Fig. 3A and Fig. 3B respectively. As revealed in the representative images, histological severity score was also lower in CLP+HA than in CLP mice (Fig. 3C). CLP resulted in substantial bacterial

burdens to the lung and spleen, which were significantly decreased following HMW HA treatment (Fig. 3D–E).

Intraperitoneal Administration of HMW HA Did Not Increase Total Plasma HA Levels

To test if intraperitoneal administration of HMW HA produced a therapeutic level in the systemic circulation, we first measured the plasma concentration of total HA in the CLP mice with or without HMW HA administration at several time points. To collect plasma samples at 6 h and 12 h after CLP, 20 mice were used for this section (N=5 in each group at each time point). Total plasma HA concentration did not increase in the CLP mice that received intraperitoneal HMW HA compared to the CLP mice (Fig. 4). Rather, HMW HA administration tended to numerically decrease plasma HA levels after CLP, possibly suggesting a reduction in systemic inflammation. To trace the HMW HA administration intraperitoneally, we measured fluorescence intensity in the plasma after injecting fluorescein-labelled HMW HA (F-HA) into the peritoneal space of CLP mice. An additional 8 mice underwent CLP or sham surgery for this measurement. The plasma concentrations of F-HA based on the fluorescence intensities went up from 2 ng/ml at 6 h to 13 ng/ml at 12 h after CLP (Table 1), which implies that intraperitoneal HMW HA was slowly absorbed into the systemic circulation. However, F-HA conc. was less than 3/1000 of the plasma concentration of total HA in the CLP+HA mice at each time point (Fig. 4). Furthermore, only 0.6 – 4.3% of F-HA intraperitoneally administered reached systemic circulation. Therefore, it is unlikely that intraperitoneal instillation of HMW HA produced its therapeutic effect via the systemic circulation.

HMW HA Increased Macrophage Counts and Decreased Total Bacterial Burden in the Peritoneal Cavity Following Sepsis

To assess the action of HMW HA in the peritoneal cavity in the early phase of peritonitis-induced sepsis, we lavaged the peritoneal cavity of the CLP mice at 6 h after CLP surgery (2 h after HMW HA administration). Twenty mice were used in this section (N=10 in each group). HMW HA instillation increased the number of macrophages in the peritoneal cavity, which was associated with a decrease in blood monocytes (Fig. 5A–D). However, there were no differences in inflammatory cytokines levels between CLP and CLP+HA mice (Fig. 5E–G). The number of bacterial CFU in the peritoneal lavage fluid was significantly lower in the CLP+HA mice than in the CLP mice (Fig. 5H).

HMW HA Increased Macrophage Phagocytosis of *E.coli* Bacteria In Vitro

To determine a possible mechanism seen with the effect of HMW HA on peritoneal macrophages, we administered HMW HA to LPS-stimulated RAW264.7 cells, a mouse macrophage cell line. HMW HA treatment reduced *E.coli* CFU counts in culture supernatants by approximately 50% compared with PBS treatment 1.5 h after *E.coli* bacterial inoculation (Fig. 6A). However, there were no changes in inflammatory cytokine levels in the supernatants due to HMW HA treatment (Fig. 6B–C). To determine if the decrease in bacterial levels were due to an initial bacteriostatic effect of HMW HA, we measured *E.coli* proliferation in the media of RAW264.7 cells by recording O.D. serially using a spectrophotometer. HMW HA at 500 µg/ml significantly suppressed the O.D. increase in the media containing *E.coli* bacteria for 48 h (Fig. 6D), suggesting a decrease in

bacteria growth, which was similar to bacteria grown in LB media (Fig. 6E). However, there were no significant differences in the O.D. increase during the initial 6 h after *E.coli* inoculation between HMW HA and PBS treatment groups. Therefore, the enhanced *E.coli* elimination in the supernatant of LPS-stimulated RAW 264.7 cells was not due to the bacteriostatic effect of HMW HA on *E.coli* growth because the incubation time of *E.coli* in the experiment was only 1.5 h. Finally, to determine if the decreased level of *E.coli* bacteria in the supernatants was due to macrophage phagocytosis, *E.coli* CFU counts in the RAW264.7 cell lysates were assessed. The number of CFU was significantly greater in the HMW HA-treated RAW264.7 cells than in the PBS-control treated (Fig. 7F), which revealed that HMW HA increased the phagocytosis of bacteria in stimulated mouse macrophages.

Effect of HMW HA on Increased Macrophages Phagocytosis is Through CD44 Activation and Ezrin/Radixin/Moesin Protein Phosphorylation

CD44 is one of major receptors for HA and abundant in macrophages (6, 12). The binding of HMW HA to CD44 receptors promoted phagocytosis in neutrophils (11, 13). Therefore, we tested if the CD44 receptor was involved with the enhanced phagocytosis of bacteria by macrophages following HMW HA administration. We assessed *E.coli* bacterial elimination in the supernatants of LPS-stimulated RAW264.7 cells after adding CD44 blocking antibody. Co-incubation of HMW HA with CD44 blocking antibody largely abrogated the antimicrobial effect of HMW HA (Fig. 7A). Therefore, macrophage phagocytosis was enhanced by HMW HA via CD44 receptor binding. Surprisingly, blocking CD44 receptor did not change the levels of inflammatory cytokines in the supernatants (Fig. 7B–C).

Actin cytoskeletal remodeling is essential for phagocytosis (21). The ERM protein family connects the cytoplasmic domain of CD44 to the submembrane cytoskeleton actin (22), and it is involved in cytoskeleton remodeling (23, 24) and phagocytosis (18, 19). To assess the effect of HMW HA on ERM activation, we measured the expression of phosphorylated ERM (pERM) in LPS-stimulated RAW264.7 cells following HMW HA treatment. When compared to the activated macrophages without HMW HA treatment, HA-treated macrophages showed higher levels of pERM (Fig. 7D–E). To test if the activated ERM is involved in the increased phagocytosis of HMW HA-treated macrophages, the *E.coli* CFU levels in the supernatants were assessed in HMW HA-treated macrophages following Ezrin inhibitor treatment. Ezrin inhibitor suppressed *E.coli* bacterial elimination in the supernatant of HMW HA-treated macrophages (Fig. 7F). Collectively, HMW HA can enhance bacterial phagocytosis in activated macrophages by ERM phosphorylation through CD44 binding.

DISCUSSION

In this study, we tested the therapeutic effects of intraperitoneal administration of HMW HA for peritonitis-induced sepsis in mice. We demonstrated that intraperitoneal HMW HA administration at 4 h post-CLP surgery suppressed systemic inflammation, partially restored the leukopenia/neutropenia in the systemic circulation, decreased the bacterial burden in the lung and spleen, and improved blood oxygenation and lung tissue damage following CLP-induced sepsis (Fig. 1–3). More importantly, intraperitoneal HMA HA instillation significantly increased the survival rate of the CLP mice. Because the systemic absorption of

the intraperitoneal HMW HA was minimal (Fig. 4), we focused on the therapeutic effects of HMW HA in the peritoneal cavity in the early phase of CLP-induced sepsis. Intraperitoneal HMW HA administration decreased bacterial burden and increased the number of macrophages in the peritoneal cavity of the mice during early CLP-induced sepsis (Fig. 5). To evaluate a possible mechanism of bacterial elimination in the peritoneal cavity, we treated LPS stimulated mouse macrophages with HMW HA *in vitro*. The *in vitro* experiments demonstrated that HMW HA can promote bacterial phagocytosis in activated macrophages by ERM phosphorylation, which is through HA-CD44 receptor binding (Fig. 7).

Due to its immunological properties, HA has been tested in a few studies as a therapeutic agent against sepsis in pre-clinical models. Muto et al. surprisingly found that the intraperitoneal administration of low-intermediate molecular weight HA (wide range of sizes up to 500 kDa) 1 h prior to intraperitoneal administration of LPS reduced inflammatory cytokines levels at 10 h after LPS injection in mice (25). The protective effects of HA were through cell signaling initiated by binding to CD44 and toll like receptor 4. However, HA was administered pre-injury, and the sepsis model was sterile, limiting its clinical impact. Liu et al. demonstrated that LPS injection into the carotid artery combined with mechanical ventilation caused acute lung injury in rats within 4 h, and continuous intravenous infusion of HMW HA (1,600 kDa) starting at 1 h after LPS administration attenuated neutrophil infiltration and histologic injury in the lungs of the rats (26). Despite the protective effects of HA as a therapy, the study was again limited by the use of a sterile injury model. When administered intravenously, HA is known to disappear rapidly from the plasma with a half-life of 2.5–4.5 min due to degradation in the liver and spleen (27). Continuous infusion or repeated injections may be critical for a systemic effect of HMW HA; however, the high viscosity of HMW HA could be a barrier for intravenous HA administration as a therapeutic option in patients with sepsis who often have labile blood pressure and possible lung injury with pulmonary dysfunction. The current study demonstrated that just a single administration of intraperitoneal HMW HA even 4 h after peritonitis induction had a therapeutic effect against polymicrobial sepsis by suppressing inflammation and, surprisingly, by enhancing bacterial clearance; intraperitoneal application of HA solution is already known to be safe in patients undergoing abdominal surgery (28).

Recently, Kuethe, et al (29). evaluated the characteristics of mice that survived CLP compared to mice that died. In their study, mice underwent CLP followed by necrotic cecum debridement, abdominal cavity wash, and intraperitoneal antibiotic administration 24 h after CLP. The mice who survived showed a greater number of inflammatory monocytes in the peritoneal lavage fluid, elevated phagocytosis activity of peritoneal neutrophils, and lower systemic levels of both pro-inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines when compared to mice who died. Their data suggested that the major inflammatory response in the survivors against CLP-induced sepsis was limited to the site of infection, blunting overall the systemic inflammatory response. Furthermore, Osuchowski et al. demonstrated that CLP mice with prolonged survival showed lower plasma levels of both pro- and anti-inflammatory cytokines than those with decreased survival (30). In humans, both pro-inflammatory and anti-inflammatory cytokine levels were also increased in septic patients, suggesting dysregulated inflammation (31–33). Therefore, in order to survive sepsis, blunting the overall systemic inflammatory response may be more critical than either

suppressing pro-inflammatory pathways or enhancing anti-inflammatory pathways as a therapeutic target. Infection control within the site of infection may be an ideal method to prevent the excessive systemic inflammation and the subsequent development of sepsis. Interestingly, in our study, HMW HA-treated mice showed similar characteristics to mice who survived sepsis in the previous studies such as increased number of peritoneal macrophages, decreased bacterial burden in peritoneal lavage fluid, and lower levels of systemic inflammation.

In peritonitis-induced sepsis model, innate immune cells in the peritoneal cavity such as macrophages have been reported to adjust and orchestrate overall immune response through release of vasoactive mediators, cytokines, and chemokines (34, 35). Furthermore, the severity of peritonitis-induced sepsis can be determined by clearance of peritoneal bacteria by macrophages and neutrophils during the early stages [36–38]. In the current study, intraperitoneal administration of HMW HA into CLP mice increased the number of macrophages and decreased the bacterial burden in the peritoneal cavity without any significant differences in inflammatory cytokine levels. To identify a potential mechanism for the peritoneal immune response, we investigated the effects of HMW HA on activated macrophages *in vitro*. HMW HA did not affect the secretion of inflammatory cytokines in the activated macrophages. However, it promoted increased bacterial phagocytosis, corroborating the *in vivo* findings; HMW HA-enhanced phagocytosis was through CD44 binding and by ERM activation (18, 19, 21–24). Therefore, HMW HA administration could enhance phagocytosis by peritoneal macrophages and preserve its capability to secrete inflammatory cytokines, which might be the underlying mechanisms of the therapeutic effects of HMW HA against peritonitis-induced sepsis.

Contrary to our findings, a few previous *in vitro* studies reported that HMW-HA suppressed phagocytosis in macrophages in a dose-dependent manner (39, 40). However, a recent investigation by Schommer et al. demonstrated that degradation of HA at the infection site enhanced host defense (41). In their study, the phagocytosis of group A streptococcus was enhanced in a hyaluronidase pre-treated murine alveolar macrophage cell line and mouse peritoneal macrophages with hyaluronidase overexpression, which suggested that the breakdown of HMW HA facilitated macrophage phagocytosis. During tissue injury and inflammation, HA is degraded in part due to dysregulated expression of HA synthases and hyaluronidases and/or accumulated reactive oxygen species (6). Peritonitis was also reported to increase HA levels in the peritoneal fluid of peritoneal dialysis patients (42), which implied that inflammation in the peritoneal cavity caused HA degradation. Therefore, it is likely that HMW HA injected into the peritoneal space of CLP mice was degraded into intermediate to low molecular weight HA that had therapeutic properties. Low to intermediate molecular weight HA was reported to increase phagocytosis in neutrophils through CD44 binding (11, 13). Our data showed that HMW-HA degradation can increase phagocytosis in macrophages through CD44. In addition, fragmented HA (100–700 kDa) was reported to increase monocyte chemoattractant protein-1 secretion by peritoneal mesothelial cells (43), which might be a cause of the increased number of peritoneal macrophages in HMW HA treated CLP mice in our study. It is likely that HMW HA added into wells of LPS-stimulated RAW264.7 cells was also degraded because LPS-stimulated

RAW264.7 cells also secreted reactive oxygen species (44). Further studies are needed to identify the role of molecular weight in the antimicrobial properties of HA.

Despite our promising results, our study has some limitations. 1) We did not administer antibiotics in an attempt to isolate the effects of HMW HA. Depending on the severity of the CLP model, such therapeutic modalities as antibiotics may overwhelm the beneficial effects of HMW HA. Consequently, we cannot assert that HMW HA can add some additional beneficial effects to conventional therapeutics for sepsis; 2) HMW HA has known anti-permeability properties (45). Therefore, the beneficial effects of HMW HA on systemic inflammation may be in part attributed to decreased endothelial permeability of the gut or peritoneum, preventing the translocation of bacteria into the systemic circulation.

In conclusion, intraperitoneal administration of HMW HA had therapeutic effects against CLP-induced sepsis. HMW HA increased bacterial phagocytosis in macrophages/monocytes through CD44 receptor binding and by ERM activation. The preclinical results demonstrated that HMW HA may be an adjuvant technique to control intra-abdominal infection in patients at risk of abdominal infection and, subsequently, sepsis.

Funding:

This work was supported by the National Institute of Health National Heart, Lung, and Blood Institute grant number (HL 113022 to Dr. JW Lee) and a faculty research grant of Yonsei University College of Medicine (6-2017-0059 to Dr. JH Lee).

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Figure 1. Physiologic Variables and Overall Survival in Mice Undergoing Sham or CLP Surgery with or without HMW HA Treatment.

Changes in the body weight (A), pulse rate (B), and oxygen saturation (C) at 24 h after CLP surgery. Survival rates in the CLP mice with or without HMW HA treatment for 48 h. CLP, cecal ligation and puncture; HA, hyaluronic acid; SpO₂, pulse oxygen saturation. N=5 in the Sham, N=10 in the CLP and CLP+HA group. *P<0.05 vs. CLP group.

Figure 2. Profiles of Immune Cells and Inflammatory Cytokines in the Systemic Circulation Following CLP with or without HMW HA Treatment.

The number of WBC (A), neutrophils (B), and monocytes (C) in the blood of sham/CLP mice at 24 h after the surgery. The plasma levels of $\text{TNF}\alpha$ (D), IL-6 (E), and IL-10 (F) in the sham/CLP mice at 24 h after the surgery. WBC, white blood cell. N=5 in the Sham, N=9 in the CLP, N=10 in the CLP+HA group. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Figure 3. Lung Injury and Bacterial Burdens in the Lung and Spleen Following CLP with or without HMW HA Treatment.

Representative images of hematoxylin and eosin-stained lung section of CLP (A) and CLP +HA (B). Scale bars are 50 µm. (C) Lung injury as assessed by semi-quantitative scoring of four fields in each mouse (16 scores from 4 mice in each group). Bacterial CFU counts in tissue homogenates of the lung (D) and spleen (E) of sham, CLP, and CLP+HA mice (N=5 in Sham, N=9 in CLP, and N=10 in CLP+HA group). CFU, colony-forming unit. *P<0.05; ***P<0.001.

Figure 4. Plasma Total Hyaluronic Acid Levels Following CLP with or without HMW HA Treatment.

There were no significant differences in the plasma HA levels between the two groups at any time point. N=5 in each group at 6 h and 12 h; N=9 in CLP, and N=10 in CLP+HA at 24 h after CLP.

Figure 5. Profiles of Immune Cells, Inflammatory Cytokines, and Bacterial Burdens in the Peritoneal Cavity Following CLP with or without HMW HA Treatment.

(A-C) The comparisons of the numbers of WBC, neutrophils, and macrophages in the peritoneal lavage fluid between the CLP and the CLP+HA mice. (D) The number of blood monocytes in the CLP and the CLP+HA mice at the same point. (E-G) Comparisons of inflammatory cytokines levels in peritoneal lavage fluid between the CLP and the CLP+HA mice. (H) Bacterial CFU counts in the peritoneal lavage fluid of the CLP and CLP+HA mice. N=10 in each group; *P<0.05, **P<0.01.

Figure 6. Effect of HMW HA on LPS-Stimulated RAW264.7 Cells.

(A) The effect of HMW HA on *E. coli* bacterial clearance in the supernatant of LPS-stimulated RAW264.7 cells (N=10 in each group). (B, C) The levels of inflammatory cytokines in the supernatant of LPS-stimulated RAW264.7 cells with or without HMW HA administration (N=10 in each group). Optical densities of *E. coli* (a measure of bacterial load) in the media for RAW264.7 cell culture (D) and in the LB broth (E) (N=5 in each group of each time point). (F) The CFU counts of phagocytosed *E. coli* bacteria in LPS-stimulated RAW264.7 cells with or without HMW HA administration (N=12 in each group). LPS, lipopolysaccharide; LB, Luria-Bertani. *P<0.05; **P<0.01; ***P<0.001.

Figure 7. Roles of CD44 and Ezrin/Radixin/Moesin Phosphorylation in the Phagocytosis of Bacteria by LPS-Stimulated RAW 264.7 Cells.

(A) The effect of blocking CD44 on HMW HA-induced enhancement of bacterial elimination in the supernatant of LPS-stimulated RAW264.7 cells (N=14 in each group). (B, C) The effects of HA and CD44 blocking antibodies on the profiles of inflammatory cytokines in LPS-stimulated RAW264.7 cells (N=8 in each group). (D, E) The effect of HA on phosphorylated ERM in LPS-stimulated RAW264.7 cells (N=9 in each group). (F) The effect of ezrin inhibition on HA-induced enhancement of bacterial elimination in the supernatant of LPS-stimulated RAW264.7 cells (N=20 in each group). *P<0.05, **P<0.01.

Table 1.

Absorbance of Fluorescein Labelled HMW HA into the Systemic Circulation

	6 h after CLP (2 h after HA administration)	12h after CLP (8 h after HA administration)
Fluorescence Intensity	601 (400–802)	3680 (3333–4027)
Plasma F-HA concentration (ng/ml)	1.8 (1.1–2.5)	12.7 (11.5–13.9)
*Estimated amount of F-HA in whole plasma (ng)	3.8 (2.3–5.3)	23.8 (22.9–24.7)
Ratio of plasma F-HA over administered F-HA (%)	0.6 (0.4–0.9)	4.3 (3.8–4.7)
	6 h after sham surgery (2 h after HA administration)	12h after sham surgery (8 h after HA administration)
Fluorescence Intensity	556 (490–622)	390 (364–556)
Plasma F-HA concentration (ng/ml)	1.6 (1.4–1.9)	1.1 (1.0–1.2)
*Estimated amount of F-HA in whole plasma (ng)	2.6 (2.1–3.0)	1.7 (1.6–1.9)
Ratio of plasma F-HA over administered F-HA (%)	0.6 (0.5–0.6)	0.4 (0.3–0.6)

Data are median (range); N=2 in each time point of each surgery; F-HA, Fluorescein-labeled HMW HA; The concentration of F-HA was calculated for each plasma sample by applying the equation generated for the standard curve linear regression using the known fluorescence intensity.

* The amount of plasma F-HA was calculated for each mice by multiplying the concentration of plasma F-HA by the plasma volume of each mice (Assumption: mouse plasma volume 67 ml/kg (46)).