In vitro and *in vivo* antimicrobial activity of water-soluble chitosan oligosaccharides against *Vibrio vulnificus*

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Abstract. *Vibrio vulnificus* is a Gram-negative bacterium that induces severely rapid pathological progress. In this study, we evaluated the antibacterial activity of two water-soluble chitosan oligosaccharides, COS A (MW, 10,000 Da) and COS B (MW, 1,000 Da), from 90-95% deacetylated chitosan, against *V. vulnificus in vitro* and *in vivo*. Treatment with COS A resulted in significantly higher suppressive effects on the growth of *V. vulnificus* than treatment with COS B. The growth of *V. vulnificus* was inhibited within 1 h of treatment with water-soluble COS A in concentrations ranging from 0.5 to 10 mg/ml. Additionally, treatment with COS A completely inhibited *V. vulnificus*-induced cytotoxicity in human intestinal epithelial INT-407 cells, while COS B did not. Furthermore, the administration of COS A (0.1-0.5 mg per mouse) significantly increased the survival period of *V. vulnificus*-infected mice. The number of viable *V. vulnificus* in the spleen, liver, small intestine, and blood was significantly lower in COS A-treated mice than in untreated mice. Here, we clearly demonstrate that COS A is a potential agent for the prevention and treatment of infection with *V. vulnificus*.

Introduction

Vibrio vulnificus is a Gram-negative estuarine bacterium that causes primary sepsis and gastrointestinal illness in humans. In addition, wound infection or oral infection via contaminated shellfish induces severe fulminant septicemia in susceptible individuals such as those with chronic liver disease (1). *V. vulnificus* induces a rapid disease process that results in extensive cellular damage. As a result, mortality exceeds 50% in septic patients, and increases to >90% in patients who go into shock shortly after admission to the hospital. The majority of fatal cases are caused by septic shock, which results from a variety of virulence factors of *V. vulnificus*. Putative virulence factors of *V. vulnifcus*, including capsular polysaccharide (2,3), siderophores (4), hemolysin (5), matrix metalloproteinase, flagella (6), and RtxA toxin (7-9), have been reported *in vivo* and *in vitro*. These virulence factors may persistently activate the production of proinflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1ß, IL-6, IL-8 and nitric oxide in the infected host (10,11). Therefore, successful treatment of fulminant *V. vulnificus* infections requires highly active antimicrobial agents. In this study, we examined the anti-*V. vulnificus* activity of water-soluble chitosan oligosaccharides (COS) *in vitro* and *in vivo*.

Chitin and chitosan are abundant natural polysaccharides. Chitosan has attracted a great deal of interest due to its wide range of functions. Chitosan is a linear polysaccharide comprised of N-acetyl-D-glucosamine and deacetylated glucosamine that shares characteristics with various glucoaminoglycan and hyaluronic acids; therefore, it is likely that chitosan also shares bioactivities associated with these compounds (12). Chitin and chitosan were shown to exert a stimulatory effect on macrophages, as well as chemoattractive effects on neutrophils *in vitro* and *in vivo* (13-16). Furthermore, recent advances provide insight into the health benefits of COS, which are partially hydrolyzed products of chitosan. COS are water-soluble due to their shorter chain lengths and free amino groups in D-glucosamine units. Specifically, many of these compounds were found to have beneficial biological effects including anti-tumor (17-19), antimicrobial (20-22), cholesterol-reducing (23-25), and free radical scavenging (26-28) activity. In addition to its lack of toxicity and allergenicity, the biodegradability and biocompatibility of chitosan and COS make it potentially useful for biomaterial, medical, and pharmaceutical applications (29). Several studies have demonstrated that the degree of deacetylation and molecular weight (MW) strongly influence the action of COS in biological systems (22,29,30). In addition, the anti-microbial activity of COS was shown to be greatly dependent on polymerization or MW. Furthermore, the anti-microbial action of water-soluble chitosan and COS was shown to be dependent on the degree of deacetylation (29,31).

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Previously, we found that two types of water-soluble COS prepared from 90-95% deacetylated chitosan, COS A (MW, 10,000) and COS B (MW, 1,000), exhibited potential anti-inflammatory effects (32). In this study, we investigated the antibacterial activity of COS A and B against *V. vulnificus* as well as their inhibitory effects against *V. vulnificus*-induced cell cytotoxicity *in vitro* and *in vivo*.

Materials and methods

Animal cell culture and chemicals. The human intestinal epithelial INT-407 cell-line (ATCC CCL-6) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained at 37˚C in 5% CO_2 in Minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and antibiotics (10 unit/ml penicillin G and 10 μg/ml streptomycin) (growth medium). Water soluble COS A and B were provided by ECOBIO Inc. (Gwangju, Korea). COS A and B were 90-95% deacetylated and their molecular weights were 10,000 and 1,000 Da, respectively. Eight-week-old female ICR mice (Samtaco Inc, Korea) were maintained and treated under standard conditions according to the animal care committee guidelines of Korea University (KUIACUC-1/5/2009-3).

Bacterial strains and growth conditions. The *V. vulnificus* strain MO6-24/O used in this study was isolated from patients (7,8). The *V. vulnificus* bacteria were grown to log phase at 30˚C in Luria-Bertani medium supplemented with 2% NaCl LBS medium, after which they were diluted to $6x10^8$ CFU/ml in LBS medium and then centrifuged and resuspended in antibiotic-free MEM prior to infection into INT-407 cells. The concentration of bacteria was confirmed via viable cell counts conducted on LBS agar.

In vitro broth cultures of V. vulnificus. The *V. vulnificus* inoculum size was $6x10^8$ CFU/ml. Variable amounts of COS (0.5, 1, 2.5, 5, 10 mg/ml) were solubilized in 20 ml of growth medium (2% NaCl LBS) and then tested for their ability to alter bacterial growth by spectrometry (OD_{600}). The *V. vulnificus* cultures were then incubated with aeration (150 rpm, gyratory shaker) for 5 h at 37˚C.

Infection protocol. Human intestinal epithelial INT-407 cells were infected with *V. vulnificus* as previously described (7,8). Briefly, INT-407 cells were grown in the growth medium at 37˚C in a 5% CO_2 incubator. Next, cells were seeded onto 6- ($8x10^5$ cells/well) and 96-well ($2x10^4$ cells/well) culture plates and then cultured for 24 h in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged for 3 min at 5,000 rpm, resuspended, and adjusted to $6x10^8$ CFU/ml in antibiotic-free MEM medium. The bacterial suspensions were then added to COS-treated or untreated-intestinal epithelial cells at various multiplicities of infection (MOI; the ratio of bacteria number to the number of epithelial cells), after which the infected cells were incubated for 1-4 h in antibiotic-free growth medium at 37˚C in 5% CO_2.

Cytotoxicity assay. The bacteria-infected INT-407 cell cultures were aliquoted into a 96-well tissue culture plate (Nunc, Roskilde, Denmark) as previously described (7,8). The cytotoxicity was then determined by measuring the activity of lactate hydrogenase (LDH) in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany). The cytotoxic level was expressed as a percentage relative to the total LDH activity of cells that were completely lysed by 1% Triton X-100 (7,8).

Morphological study. INT-407 ($8x10^5$ cells/well) cells were incubated with bacteria in a 6-well plate for 3 h at MOI 10, after which the cells were washed with PBS. The cells were then fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature, washed and completely dried. Next, the cells were stained with Giemsa solution (Molecular Probe) for 1 h at room temperature. The cells were then washed twice with distilled water and dried, after which images of the specimens were acquired using a microscope (Olympus IX 71, Japan).

Survival of V. vulnificus-infected mice. Eight-week-old female ICR mice (Samtaco Inc, Korea) housed under specific-pathogen free conditions were used for all experiments. Experiments reported in this study were performed using protocols approved by the Korea University Institutional Animal Care and Use Committee. The mice were intraperitoneally infected with 0.1 ml of 250 μg iron dextran (Sigma Aldrich, St. Louis, MO, USA) 30 min prior to injection with *V. vulnificus*. Next, mice were intraperitoneally injected with $1x10^3$ CFU/0.1 ml *V. vulnificus*. The mice were administered 0.2 ml of chitosan oligosaccharide A solution (0.1, 0.2, 0.5 mg per mouse) or a PBS control intraperitoneally, after which their survival status was assessed once an hour for 24 h.

Quantitative analysis of bacteria in tissues. The *V. vulnificus*-inoculated mice were sacrificed 7 h after infection, and the spleen, liver, blood, and small intestine lesions were then aseptically removed. The removed specimens were then homogenized in 2 ml PBS using glass tissue homogenizers, after which the homogenates were diluted and plated on 2% NaCl HI agar. The samples were then incubated at 37˚C for 12 h. In addition, blood (50 μl) was collected from the tail vein of the mice and plated on 2% NaCl HI agar plates to determine the number of bacterial colonies.

Statistical analysis. A Student's t-test and one-way analysis of variance (ANOVA) followed by the Bonferroni method were employed to identify statistical differences between the values of the various experimental and control groups. P-values <0.05 were considered of statistical significance.

Results

COS A strongly inhibits V. vulnificus growth in vitro. Two types of water-soluble COS, COS A (MW, 10,000 Da) and COS B (MW, 1,000 Da), were prepared from 90-95% deacetylated chitosan, after which their antibacterial activities against *V. vulnificus* were evaluated *in vitro*. As shown in Fig. 1A, the bacterial numbers of *V. vulnificus* MO6-24/O increased rapidly as incubation time increased. However, treatment with COS A at concentrations of 1, 2.5, 5, and 10 mg/ml completely inhibited the growth of *V. vulnificus* throughout the incubation

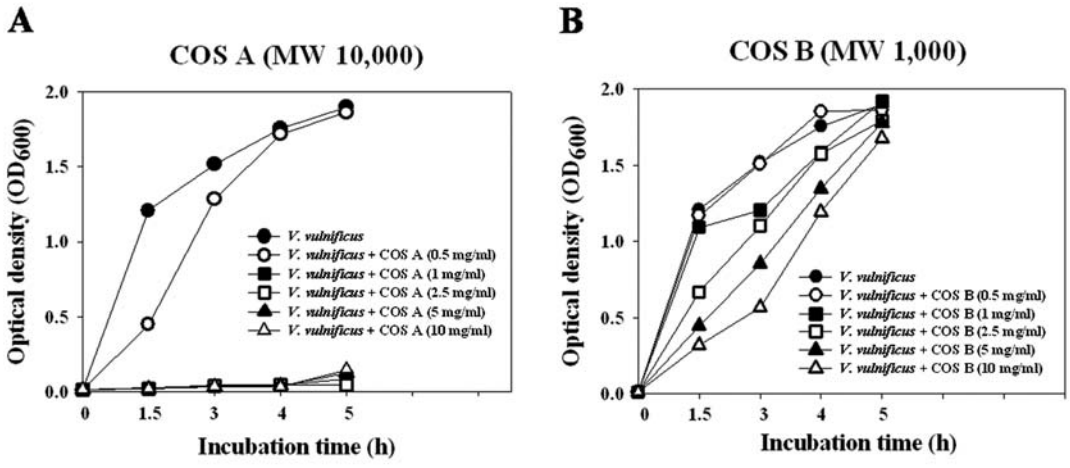

Figure 1. Inhibitory effects of the water-soluble chitosan oligosaccharides, COS A and B, on the growth of *V. vulnificus*. *V. vulnificus* was cultured with various concentrations of COS A (A) and COS B (B) at 37˚C in 2% NaCl LB medium, after which the bacterial growth was evaluated by measuring the OD_{600}.

period (0-5 h). In contrast, treatment with COS B slightly inhibited the growth of *V. vulnificus* for 1-4 h, even when it was administered at a concentration of 10 mg/ml. After 5 h of incubation, the COS A no longer inhibited the growth of bacteria, regardless of the concentration. These findings indicate that COS with an MW of 10,000 Da (COS A) completely inhibits the growth of *V. vulnificus*, while COS with an MW of 1,000 Da (COS B) does not inhibit the growth of *V. vulnificus*.

COS A inhibits the cytotoxicity of V. vulnificus against human intestinal epithelial cells. The cytotoxicity inflicted by a pathogen dictates its virulence. To determine if chitosan oligosaccharides exhibit an inhibitory effect against *V. vulnificus*-induced cytotoxicity, INT-407 cells were infected with *V. vulnificus* at different MOIs and incubation times in the absence or presence of COS A or B, after which the LDH levels released from the damaged cells were determined. As shown in Fig. 2A, the highest level of cytotoxicity was observed in INT-407 cells that were exposed to *V. vulnificus* at MOI 10 or 50. However, treatment with COS A significantly inhibited *V. vulnificus*-induced cytotoxicity of INT-407 cells in a concentration-dependent manner. Indeed, for cells exposed to *V. vulnificus* at MOI 10 or 50 and were treated with 5 mg/ml COS A, the levels of LDH activity from the *V. vulnificus*-infected INT-407 cells were ~3-fold lower than the levels of the *V. vulnificus*-infected cells that were not treated with COS A. Furthermore, the inhibitory activity of COS A against *V. vulnificus*-induced cytotoxicity in INT-407 cells was observed throughout the entire treatment period, although the level of inhibitory activity differed as a function of treatment time (Fig. 2C).

Conversely, COS B exerted significantly lower inhibitory activity against *V. vulnificus*-induced cytotoxicity than COS A (Fig. 2B). Specifically, 22.67±1.51% of the LDH was released from intestinal epithelial cells that were infected with *V. vulnificus* at MOI 10 and then treated with COS A for 3 h, whereas 67.26±1.99% of the LDH was released from *V. vulnificus*-infected cells (MOI 10) that were treated with COS B for 3 h (Fig. 2).

Microscopic analyses were conducted as previously described to confirm the inhibitory effects of COS A on the cytotoxicity of *V. vulnificus* in INT-407 cells (8). Specifically, the size, regularity of the cell margin, and the morphological characteristics of the nuclei in the stained cells were assessed. As shown in Fig. 3A, marked cellular damage was observed in a large number of Giemsa-stained INT-407 cells that were infected with *V. vulnificus*, regardless of treatment with COS B. Furthermore, cytoplasmic loss and cellular damage, which are typical phenotypes of cell death, were observed in intestinal epithelial cells that were infected with *V. vulnificus*. Conversely, many healthy cells with intact cellular shapes were observed in *V. vulnificus*-infected cells treated with COS A. In addition, these cells did not exhibit damaged surfaces or cytoplasmic loss, which suggests that COS A protects the host cell against *V. vulnificus*-induced cytotoxicity (Fig. 3A).

To further examine COS A for inhibitory effects on the growth of *V. vulnificus*, all bacteria were isolated from untreated and treated groups at each incubation time point and plated on agar plates to determine the total numbers of bacterial colonies in each group. As shown in Fig. 3B, the number of bacteria increased sharply as the incubation time increased for epithelial cells infected with *V. vulnificus* in the absence or presence of COS B (5 mg/ml). However, the presence of COS A (5 mg/ml) in the cultures inhibited the growth of *V. vulnificus* throughout the incubation period. Specifically, the number of *V. vulnificus* was (112.5±6.36)x10^5 and (99.6±3.54)x10^5 CFU for the untreated and COS B-treated cell cultures at 4 h post incubation, respectively. Conversely, the number of *V. vulnificus* was (3.95±0.21)x10^5 CFU in COS A-treated cell cultures (Fig. 3B). Taken together, these results indicate that COS A significantly inhibits *V. vulnificus*-induced cell cytotoxicity through inhibition of the growth of *V. vulnificus*.

COS A administration prolongs the survival period of V. vulnificus-infected mice. Mice were infected with *V. vulnificus* and then administered COS A (0.5 mg per mouse) to determine if it increased the survival period. As shown in Fig. 4A, intraperitoneal inoculation with 1x10^3 CFU of *V. vulnificus* resulted in the death of all mice within 10 h. However, treatment with COS A significantly increased the survival of the *V. vulnificus*-

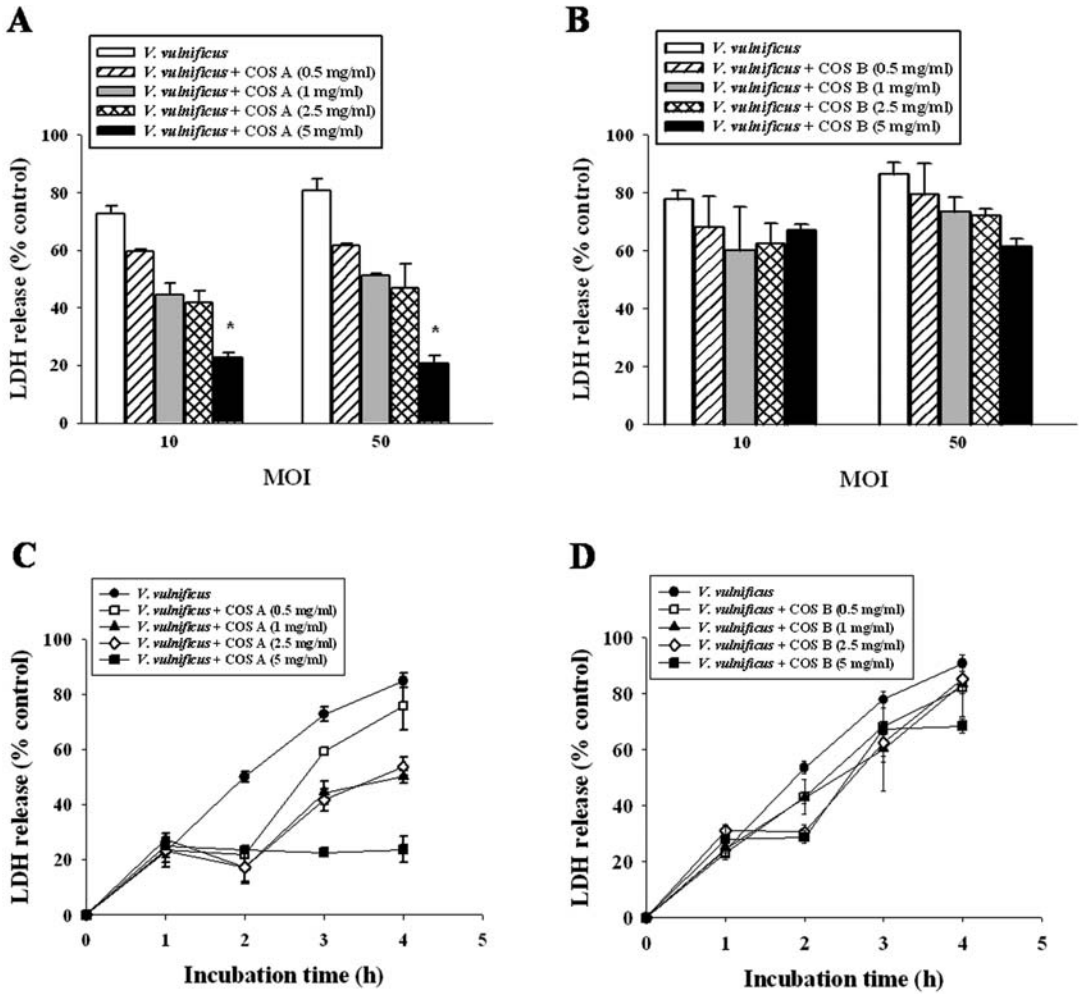

Figure 2. Suppression of the *V. vulnificus*-induced cytotoxicity in human intestinal epithelial cells by COS A and B. (A,B) INT-407 cells ($2x10^4$ cells/well) were infected with *V. vulnificus* for 3 h at MOI 10 and 50 in the absence or presence of COS A or B in a 96-well plate. (C,D) INT-407 cells ($2x10^4$ cells/well) were infected with *V. vulnificus* for 1-4 h at MOI 10 in the absence or presence of COS A or B in a 96-well plate. The cell cytotoxicity was then determined by an LDH release assay, as described in Materials and methods section. The data shown represent the means ±standard errors (n=3). $^*P<0.05$, relative to a group infected with *V. vulnificus* in the absence of COS A.

infected mice. Indeed, 3 of 5 mice were alive for 24 h when the *V. vulnificus*-infected mice were treated with COS A (0.1, 0.2, or 0.5 mg per mouse).

To evaluate the role that COS A plays in the growth of *V. vulnificus in vivo*, we evaluated the post inoculation recovery of *V. vulnificus* isolated from various tissue samples obtained from treated and untreated mice. To accomplish this, mice were injected with $1x10^3$ CFU *V. vulnificus* intraperitoneally, after which they were treated with COS A (0.25 mg per mouse). The spleen, liver, small intestine and blood were then removed from the mice at 7 h post infection, after which the number of *V. vulnificus* in each type of tissue was determined.

As shown in Fig. 4B, the numbers of bacterial colonies were significantly lower in all tissue types collected from mice that had been treated with COS A. Specifically, the number of *V. vulnificus* colonies recovered from the spleen, liver, and small intestine of the untreated mice was $(2.9\pm3.6)x10^9$, $(5.4\pm7.1)x10^9$, and $(2.56\pm12.9)x10^{10}$ CFU, respectively, whereas the number of colonies recovered from COS A treated mice was $(1\pm0.6)x10^8$, $(3\pm1.2)x10^8$, and $(4\pm0.6)x10^8$ CFU, respectively. In addition, the blood collected from the untreated and COS A-treated mice contained (297±12.0), (24±4), and (1±1) CFU *V. vulnificus*, respectively. Furthermore, the necropsy of *V. vulnificus*-infected mice at 7 h post infection revealed that there was edema, hemorrhage, vasodilation, and necrosis in the intestine, liver, and spleen samples collected from the untreated mice, but none of these symptoms were observed in samples collected from the COS A-treated mice. Taken together, these results suggest that COS A significantly inhibits the growth of *V. vulnificus* and *V. vulnificus*-induced illness *in vitro* and *in vivo*.

Discussion

Vibrio vulnificus is a Gram-negative bacterium that causes septicemia in humans who suffer from liver cirrhosis, haemochromatosis, immunocompromised conditions or diabetes (33,34). *V. vulnificus* infection rapidly induces extensive cellular damage, which ultimately results in mortality of >50% of septic patients. Therefore, highly antimicrobial agents are required for successful treatment of *V. vulnificus* infections. Chitosan oligosaccharides (COS) are produced chemically through acid and alkaline hydrolysis, physically through the use of high temperatures or enzymatically with chitinase, lysozyme, cellulase, lipase, or pronase (35,36). The antimicrobial

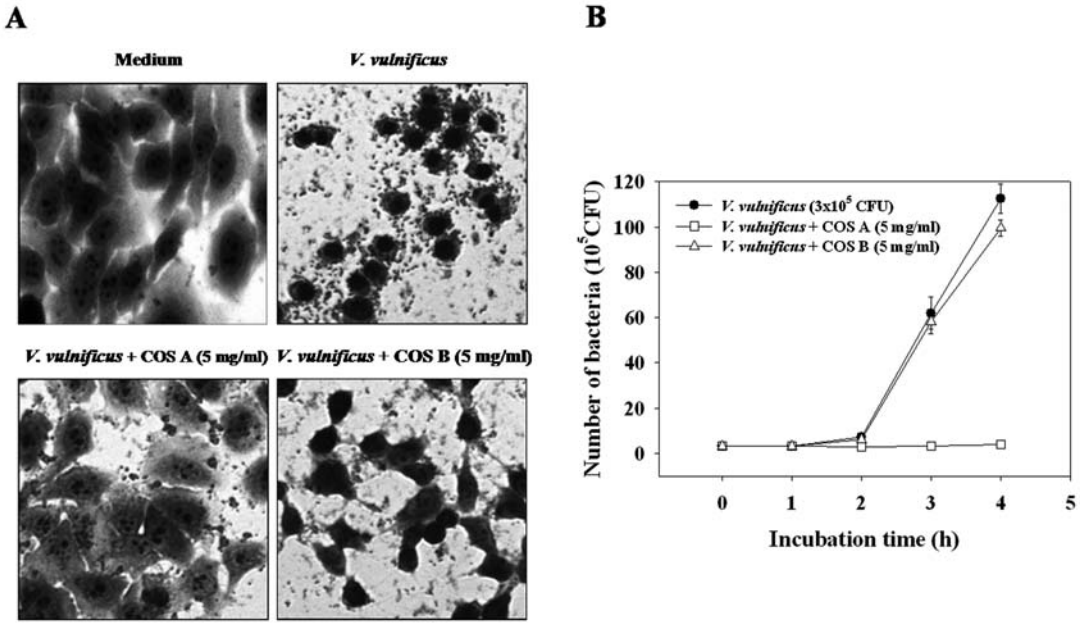

Figure 3. Inhibitory effects of COS A on the growth of *V. vulnificus* in human intestinal epithelial cells. (A) INT-407 ($8x10^5$ cells/well) cells were incubated with *V. vulnificus* for 3 h at MOI 10 in the absence or presence of COS A or B (5 mg/ml) in 6-well plates. The culture plates were then centrifuged and washed twice with pre-warmed PBS (pH 7.4), after which they were fixed with 4% paraformaldehyde. Next, the cells were washed twice with PBS, and then stained with Giemsa solution. The images of the specimens were then acquired using a microscope (Olympus IX 71, Japan). (B) INT-407 ($3x10^4$ cells/well) cells were incubated with *V. vulnificus* for 1-4 h at MOI 10 in the absence or presence of COS A or B (5 mg/ml) in 6-well plates. Both bacteria and INT-407 cells were then harvested and washed twice with pre-warmed PBS (pH 7.4), after which they were lysed with 1% Triton X-100. Aliquots of the cell lysate were then plated on 2% NaCl LB agar after adequate dilution and the numbers of the bacterial colonies were then recorded.

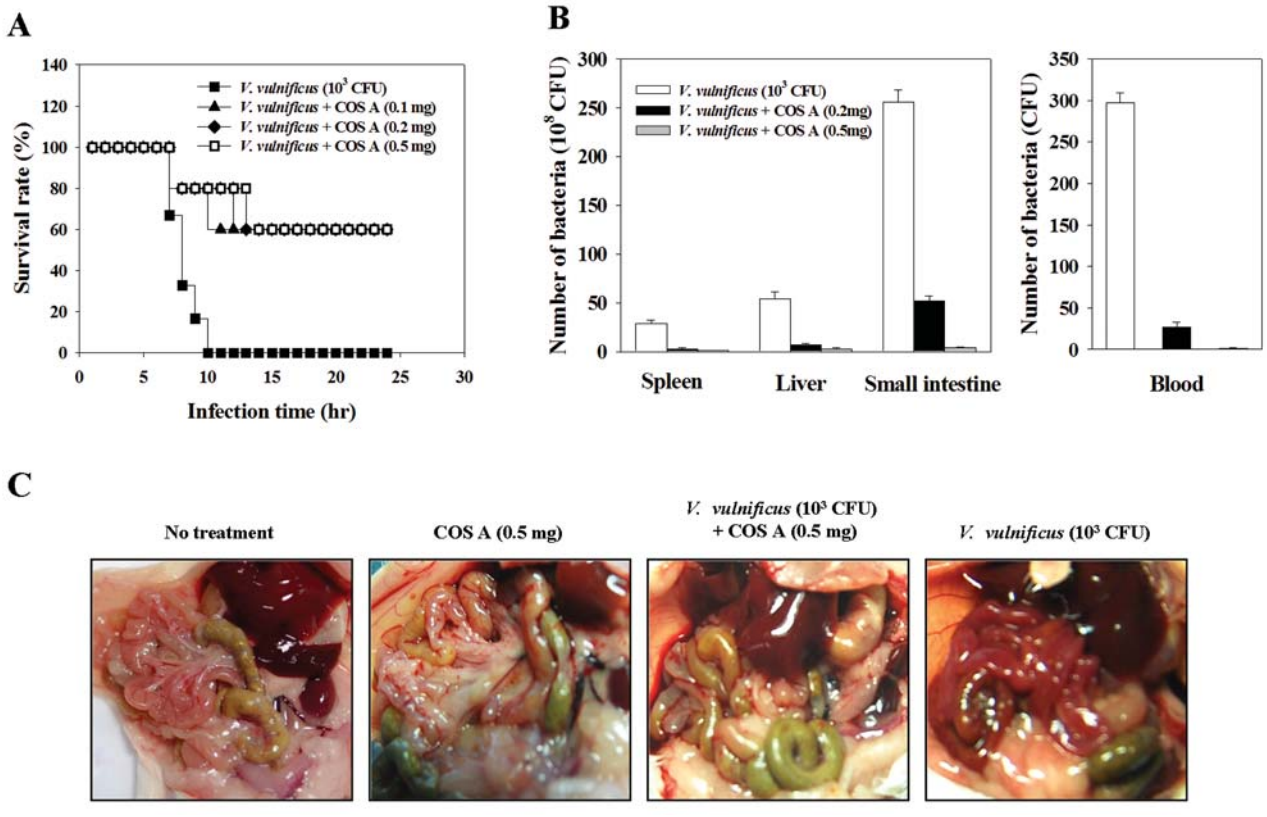

Figure 4. Prolongation of the survival of *V. vulnificus*-inoculated mice in response to COS A treatment. (A) Survival of *V. vulnificus*-inoculated mice. ICR female mice (n=5 per group) were intraperitoneally inoculated with 10^3 CFU *V. vulnificus*, after which they were treated with COS A (0.5 mg per mouse). The survival of the mice was then recorded every hour for 24 h. (B) Determination of the numbers of *V. vulnificus* colonies recovered from various tissues. ICR female mice (n=5 per group) were intraperitoneally inoculated with 10^3 CFU *V. vulnificus*, and then either treated with COS A (0.5 mg per mouse), or left untreated. At 7 h after *V. vulnificus* infection, the spleen, liver, small intestine, and blood of *V. vulnificus*-infected mice were obtained. The specimens were then homogenized, diluted, and plated on 2% NaCl HI agar plates and incubated at 37˚C for 12 h, after which the number of bacterial colonies was counted. (C) The opened abdomen of *V. vulnificus*-infected mice with or without COS A treatment, as observed by a digital camera (Nikon D60).

activity of COS is dependent on the COS and the microorganism (21,24,26). The results of the present study demonstrate that water-soluble COS A has strong anti-*V. vulnificus* activity in intestinal epithelial cells and mice. Several studies have shown the degree that deacetylation and molecular weight influence the antimicrobial activity of COS (29,30). COS A and B in this study were both ~95% deacetylated.

The growth of *V. vulnificus* was more effectively suppressed by treatment with 10,000-Da COS A than with 1,000-Da COS B. Indeed, the growth of *V. vulnificus* was completely inhibited throughout the entire incubation period by COS A at concentrations of 1-10 mg/ml, while COS B had only weak inhibitory activity against *V. vulnificus* growth and did not have any inhibitory effect until after 5 h of treatment (Fig. 1). Furthermore, *V. vulnificus*-infected intestinal epithelial cells treated with COS A exhibited 60% less cytotoxicity than those treated with COS B, which indicates that COS A protected the intestinal epithelial cells from *V. vulnificus*-induced cell death. Moreover, no cytoplasmic loss or cellular damage was observed in intestinal epithelial cells that were infected with *V. vulnificus* in the presence of COS A. Additionally, treatment of *V. vulnificus* infected mice with COS A resulted in an increased survival rate when compared to the untreated and COS B-treated groups (Fig. 4A). Finally, the number of *V. vulnificus* colonies recovered from the spleen, liver, small intestine, and blood of COS A-treated mice was significantly lower than the concentration recovered from COS B-treated mice.

The reason for greater antibacterial activity against *V. vulnificus* observed in response to treatment with 10,000-Da COS A when compared to treatment with 1,000-Da COS B remains unclear. Recent studies show that chitosan malate inhibits exotoxin production in *S. aureus* and *A streptococci*, and that weakens virulence factors such as secreted aspartyl proteinases (SAPs) (20). In addition, *V. vulnificus* has been shown to exert cytotoxicity toward Jurkat cells of T-lymphocytes through production of intracellular reactive oxygen species (ROS) (37). ROS generation is essential for both cell signaling and bacterial defense in innate immunity. However, excessive generation of ROS in host cells that are infected with bacteria is known to induce cell death. Recently, COS was found to have several beneficial biological effects including free radical scavenging activity (26-28), a stimulatory effect on macrophages, and chemoattractive effects on neutrophils *in vitro* and *in vivo* (13-16). Therefore, COS A may have a greater effect than COS B via the inhibition of cytotoxic factors such as cytolysin, metalloproteinase, elastase, and RTX toxin in *V. vulnificus* or through the suppression of excessive ROS by *V. vulnificus*-infected cells. However, further studies are needed to determine the mechanistic reasons for the differential antibacterial efficacy of COS A and B against *V. vulnificus* infection, as well as their therapeutic pathway and optimum treatment conditions.

Taken together, the results of this study clearly demonstrate that water-soluble COS A has strong antibacterial activity against *V. vulnificus in vitro* and *in vivo* and that the efficacy of COS was dependent on their molecular weight. These findings also suggest that the COS A (MW; 10,000) can be applied as a therapeutic agent for the treatment of *V. vulnificus* infectious disease.

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