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Comparison of high and low molecular weight chitosan as in-vitro boosting agent for photodynamic therapy against *Helicobacter pylori* using methylene blue and endoscopic light

Eui Jin Kim[a], Ji Hye Choi[a], Hyun Jung Yang[a], Sung Sook Choi[b], Hae Kyung Lee[a], Young-Chang Cho[c], Hyung Keun Kim[a], Sang Woo Kim[a], Hiun Suk Chae[a,*]

[a] Internal Medicine, Uijeongbu St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, South Korea
[b] College of Pharmacy, Sahmyook University, Seoul, South Korea
[c] College of Pharmacy, Chonnam National University, Gwangju, South Korea

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ABSTRACT

Background: We reported in a previous study that photodynamic therapy (PDT) of *Helicobacter pylori*(*H. pylori*) could potentiate bactericidal effect by adding chitosan. As a next step, we compared the bactericidal effects of low molecular weight (LMW) combined with Photodynamic Therapy to high molecular weight (HMW) chitosan.
Method: To perform PDT to kill *H. pylori,* we used endoscopic light as light source, methylene blue (MB) as a photosensitizer and chitosan (310–375, 50–190 kDa). We evaluated bacterial removal rate and its membrane damage by ethidium bromide monoazide PCR method (EMA q-PCR). 8-oxo-2′-dexoyguanosine by ELISA was measured for oxidative stress.
Results: At a chitosan concentration of ≤0.05%, the killing effect did not differ between the two molecular weights, and 100% bacterial removal rate was observed at a light energy ≥ 6.23 mJ/cm^2 powers under 0.02% MB. After 15 min irradiation, LMW chitosan with high concentration of MB (0.004%) showed highest killing effects, which were consistent with the results of EMA q-PCR but not with the level of 8-OHdG. Bactericidal effects of LMW chitosan plus PDT using 0.002 and 0.004% MB for 15 min irradiation were significantly higher than those using HMW chitosan plus PDT.
Conclusion: We found that PDT using methylene blue with LMW chitosan to kill *H. pylori* exerted greater bactericidal effects through bacterial membrane damage than PDT with HMW chitosan. These results suggest that it would be better to choose LMW chitosan to enhance the effect of PDT for clinical application, even at a very low concentration of PS.

1. Introduction

Helicobacter pylori(*H. pylori*) in the stomach causes stomach cancer, chronic gastritis, and gastric ulcers. At present, the treatments for *H. pylori* infection are based on antibiotics and proton pump inhibitors, but the failure rate gradually increases because of the appearance of a resistant strain, intolerance to medication, or recurrent infection [1–5]. Therefore, another elimination method is needed to overcome these problems.

Recently, many treatments for human health using photo dynamic therapy (PDT) and photo thermal therapy (PTT) were reported [6–10]. PDT is a treatment that apply a systemically or locally administered photosensitizer to kill bacteria and cancer cells by irradiation of light for suitable activation wavelength and power [11–19]. The main molecular mechanism is the destruction of targets such as DNA and membrane of bacteria or cancer cells by producing free radicals or singlet oxygen. We reported recently in vitro PDT using endoscopic light as an alternative tool for treating *H. pylori* infection [20]. Generally, an increase in the photosensitizer (PS) concentration is needed to augment the ability to eliminate tumors or bacteria, but this increase is also accompanied by toxicity. Therefore, the use of low concentration PS is required for ensuring safety, despite the intrinsically low toxicity of PSs [21–24]. We adopted methylene blue (MB) as a photosensitizer because it have been frequently used during endoscopic procedure for chromoscopy under 0.5% concentration in spite of DNA damage [25]. Another reason was that time interval of MB penetration into the cells is different between human and bacteria.

Chitosan has been used frequently in food preservation due to

* Corresponding author.
E-mail address: chs@catholic.ac.kr (H.S. Chae).

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antimicrobial activity and a medical field for wound healing and tissue engineering in spite of allergic reactions in a few patients [26]. It also shows antibacterial effects with little toxicity because its own positive charge can perturb negatively charged microbial membrane integrity to easily penetrate PS for killing bacteria as shown in the previous report. We recently reported that the combination of PDT using methylene blue with chitosan showed synergistically bactericidal effects against *H. pylori* which compared methylene blue with PDT. It is thought that chitosan of attaches to *H. pylori* membranes, which contributes to decrease the toxicity of the PS by reducing its dose needed [23,27,28]. With some controversies, low molecular weight (LMW) chitosan below 5000 kDa has greater bactericidal effects against gram-negative bacteria compared with high molecular weight (HMW) chitosan [29,30]. However, few studies have been investigated about *H. pylori*-killing effects of chitosan according to molecular weights. In this work, we investigated the bactericidal effects of two molecular weights of chitosan combined with PDT against *H. pylori*.

2. Materials and methods

2.1. Bacterial strain and culture condition

A standard strain of *H. pylori* (ATCC 700392) was purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) for use as a test strain. *H. pylori* was cultured at 37 °C in a standard micro-aerobic atmosphere (5% O_2, 10% CO_2, and 85% N_2 gas) on blood agar plates (Asan Pharmaceutical, Seoul, Korea).

2.2. Chemicals and instruments

MB and chitosan were purchased from Sigma-Aldrich (St. Louis, MO, USA). The molecular weight of HMW chitosan is 310–375 kDa and the molecular weight of LMW chitosan is 50–190 kDa. The stock solution of MB was prepared in water at a concentration of 40 mg/ml (4%) and chitosan solution was dissolved using 1% acetic acid. All of chemicals were stored for a maximum of 2 weeks at 4 °C in the dark before use. The endoscopic equipment used for the irradiation light source was an Olympus EVIS (Endoscopic Video Information System) Lucera spectrum imaging system and GIF-H260 gastroscope (Olympus Medical Systems Co., Tokyo, Japan). The irradiation procedure was performed in a dark room, and *H. pylori* culture plates were placed 10 cm from the light source. The amount of endoscopic light energy was measured as 7.5 mJ/cm^2 with an optical power meter system (PM 100 Analog Power Meter and PM 30–120 detector; Thorlabs Inc., Newton, NJ, USA).

2.3. Bacterial removal rate

The starting colony counts of all samples were 10^6 CFU/ml, and the removal rate was calculated as:

$$Re\ moval\ rate = 100 - \left[\frac{c}{c_0} \times 100\right]$$

The samples were treated with HMW or LMW chitosan for 30 min and at each of the MB concentrations. The irradiation procedure was performed in a dark room 0 to 15 min. The *H. pylori* culture plates wereplaced10 cm from the light source. The bacterial count was performed using the colony-counting method after 3 days of incubation at 37 °C in the CO_2 chamber. The response surface method (RSM) was used for analysis using SigmaPlot (version 10.0; Systat Software Inc, San Jose, CA, USA). In the control test for evaluation of the effects of chitosan only, the samples were treated at each chitosan concentration for 30 min, centrifuged at 3000 rpm for 15 min, and incubated in a suspended cell culture plate for 3 days at 37 °C under the CO_2 chamber micro-aerobic condition mentioned above, and the number of colonies was counted. All experiments were repeated three times and their measurements were averaged.

2.4. Ethidium bromide monoazide method with quantitative real-time polymerase chain reaction to evaluate H. Pylori membrane damage

Damage to the membranes of *H. pylori* was estimated using the ethidium bromide monoazide(EMA) method and quantitative real-time polymerase chain reaction (qRT-PCR). After PDT treatment of each sample, the cell suspension was treated with 100 μg/ml of EMA. The samples were incubated for 5 min and then exposed to light using a 650 W halogen lamp 20 cm above the tube for 1 min. The sample was placed on ice before light exposure to minimize elevation of the temperature.

DNA was extracted using a QIAamp Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The target gene was a housekeeping gene (cysS), and the primers to amplify cysS were as follows: (cysS F) 5′GTGCATAATAGCAGCATTGAA3′ and (cysS R) 5′ CTGCATGGATATCAATTTGAT3′. The RT-PCR procedure for cysS was as follows. The reaction mixture contained 2 μl of DNA template mixed with 10 μl of Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY, USA) and 10 pmole of each primer in a final volume of 20 μl A Step-One Plus Real-Time PCR System (Life Technologies) was used with the following reaction conditions: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 60 s as the thermal cycling stage. The program for analytical melting was 15 s at 95 °C and 60 s at 60 °C, and an increase to 95 °C at a ramp rate of 0.3 °C/s. All experiments were repeated three times and their measurements were averaged.

2.5. Measurement of oxidative stress

The genomic DNA of *H. pylori* was isolated using a QIAamp Mini Kit (Qiagen) and stored at −20 °C. The DNA solution was diluted to 200 ng/μl with TE buffer. 8-oxo-2′-deoxyguanosine (8-OHdG) levels were measured with an 8-OHdG ELISA kit (JaICA, Shizuoka, Japan) [31,32] according to the manufacturer's instructions. The standard measurement range was 0.125–10 ng/ml.

Concentrations were calculated by comparing the absorbance intensity with a standard curve. All experiments were repeated three times and their measurements were averaged.

2.6. Statistical analysis

The significance was assessed using analysis of (ANOVA) and significant variation were compared with Dunnett's test

3. Results

3.1. Effects of chitosan on H. Pylori removal rate

The starting colony count of all samples was10^6 CFU/ml, and the colonies were treated with HMW and LMW chitosan for 30 min. At chitosan concentrations of 0.01%, 0.02%, 0.05%, 0.08%, and 0.1%, the *H. pylori* removal rate was4.8%, 4.8%, 11.0%, 41.5%, and 71.4% for HMW chitosan and 6.9%, 8.5%, 11.0%, 76.6%, and 91.7% for LMW chitosan, respectively (Fig. 1). At a chitosan concentration of ≤0.05%, the bactericidal effect did not differ between LMW and HMW chitosan. However, the *H. pylori* removal rate was higher for LMW chitosan at a concentration of 0.08–0.1% than that of HMW chitosan. These findings show that LMW chitosan at higher concentrations was more effective in removing *H. pylori* than was HMW chitosan. Therefore, to identify synergistic effects of the combination of chitosan and PDT, we used chitosan at a concentration of 0.05%.

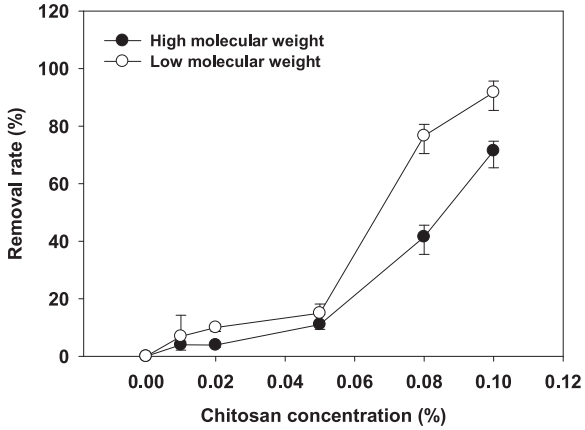

Fig. 1. Comparison of *H. pylori* removal rate between various concentrations of high and low molecular weight chitosan.

3.2. Synergic effect of H. Pylori removal rate for methylene blue and chitosan

The amount of radicals generated by PDT is proportional to the PS concentration, irradiation time, and irradiation power. The removal rate for irradiated energy vs methylene blue concentration estimated by the response surface method is shown in Fig. 2 and Table 1.The minimal light energy required to eliminate bacteria with an MB concentration of 0.02% was considered to be 6.23 mJ/cm^2.We tested the synergistic effect of PDT using MB as the PS with HMW or LMW chitosan. To compare the bactericidal effect between the LMW and HMW chitosan, the chitosan concentration was fixed at 0.05%. As shown in Fig. 3, the *H. pylori* removal rates were 67.6%, 81.1%, and 82.8% at 0.002% MB plus HMW chitosan for 5, 10, and 15 min irradiation, respectively. With LMW chitosan under the same conditions, the removal rates were 72.7%, 86.0%, and 91.3% at 5, 10, and 15 min respectively. Statistically, bactericidal effects in 5 and 10 min were not different between 0.002% MB + LMW and 0.002% MB + HMW chitosan but they were higher in 0.002% MB + LMW chitosan for 15 min irradiation than in 0.002% + HMW chitosan. ($P < 0.01$) Bactericidal effects in 5, 10 and 15 min irradiation were 67.1%, 82.9%, and 89.3% in 0.004% MB + HMW chitosan, respectively. And bactericidal effects under same conditions, were 72.8%, 87.7%, and 100% in 0.004% MB + LMW chitosan, respectively. LMW chitosan + 0.004% MB group showed higher killing effects than HMW chitosan + MB group at irradiated 15 min. ($P < 0.001$) (Fig. 3).

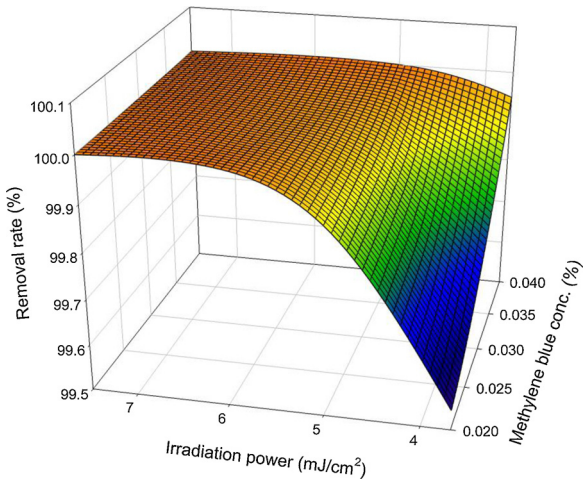

Fig. 2. Removal rate shown for the irradiated energy versus methylene blue concentration estimated by the response surface method.

Table 1
Data obtained using the response surface method to quantify the effects of methylene blue concentration and light energy on bacterial removal rate.

Methylene blue (%)	Light power (mJ/cm^2)	Removal rate (%)
0.02	3.69	99.54
0.02	4.96	99.89
0.02	6.23	100
0.02	7.50	100
0.04	4.96	100
0.04	7.50	100

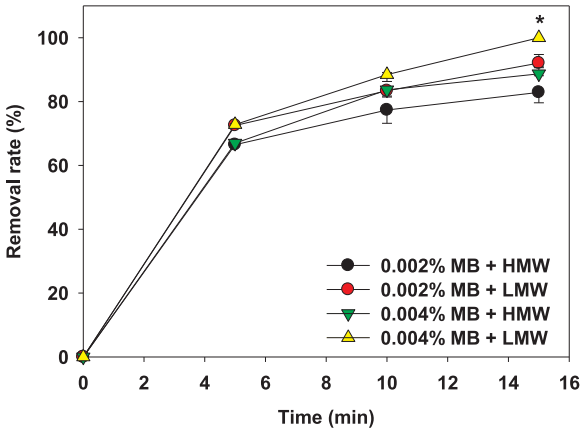

Fig. 3. Bactericidal effect of PDT with methylene blue and chitosan. * $p < 0.001$.

3.3. Damage to the membranes of H. Pylori after irradiation with methylene blue and chitosan

The *cysS* (HP0886) gene is a housekeeping gene of *H. pylori* and was used as the target gene to quantify the number of live cells by assessing bacterial membrane damage. EMA can penetrate only through the damaged membrane of dead cells, where it binds to nuclear DNA and can block DNA polymerization. Table 2 shows the degree of membrane damage by PDT with chitosan. The Ct values by PCR amplification were 18.69, 18.77, 18.87, 20.27, 22.69, and 24.90 in the control, HMW chitosan, LMW chitosan, MB with light, MB and HMW chitosan with light, and MB and LMW chitosan with light samples, respectively. This result suggests that PDT combined with chitosan causes more damage to bacterial membranes than PDT or chitosan alone. LMW chitosan with PDT caused greater bacterial membrane damage than that caused by the combination with HMW chitosan.

Table 2
EMA-qPCR analysis of photodynamically damaged *H. pylori* for the different bactericidal combinations.

Sample	Ct value
Control	18.69 ± 0.035
HMW chitosan	18.77 ± 0.141
LMW chitosan	18.87 ± 0.070
MB + light	20.27 ± 0.035
MB + HMW chitosan + light	22.69 ± 0.141
MB + LMW chitosan + light	24.90 ± 0.820

Control: without light irradiation, MB, and chitosan; LMW chitosan: low molecular weight chitosan (0.05%); HMW chitosan: high molecular weight chitosan (0.05%); MB: methylene blue (0.004%); Light: irradiation for 15 min.

3.4. Oxidative stress induced by methylene blue and chitosan

8-OHdG is a biomarker of DNA oxidative damage. The concentration of 8-OHdGmeasured by ELISA were 0.023, 0.027, 0.024, 0.053, 0.158, and 0.167 ng/ml/μg of DNA in the control, HMW chitosan, LMW chitosan, MB with light, MB and HMW chitosan with light, and MB and LMW chitosan with light samples, respectively. This result suggests also that PDT with chitosan generated more oxygen radicals than PDT or chitosan alone and that LMW chitosan combined with PDT produced slight more oxygen radicals without significance than did HMW chitosan combined with PDT ($P > 0.05$). These results suggest that other factors including oxidative stress may contribute to bactericidal effects.

4. Discussion

Both antibiotics and proton pump inhibiters (PPIs) are the main modality of treatment of *H. pylori* infection; however, strains resistant to antibiotics are constantly emerging [33–35]. PDT may be considered as an alternative to overcome this problem because *H. pylori* contains only one gene to repair injury caused by light and no strain resistant to PDT has appeared [18,36,37]. We recently reported the increased bactericidal effects by the combination of chitosan and PDT [38]. We reasoned that this may help to reduce toxicity by markedly decreasing the amount of PS needed. The mechanism between methylene blue and chitosan is not clear to this day. But, by EMA results of the present study and the previous data showing higher anti-bacterial effect with treatment of chitosan prior to PDT, we confirmed that chitosan would probably display some role in PDT through permitting easy penetration of PS into the bacterial cells by membrane perturbation [38].

The mechanisms responsible for the antibacterial effects of chitosan are different according to bacterial strains and the chemical characteristics of chitosan itself. Therefore, we compared the bactericidal activity against *H. pylori* between LMW (50˜190 kDa) and HMW (310˜375 kDa) chitosan. In this study, the bactericidal effects of the two forms of chitosan against *H. pylori* were similar at a concentration below0.05%. By contrast, at a concentration above 0.05%, bactericidal effect was quite different between HMW and LMW chitosan. Because 0.05% was shown to be a reasonable chitosan concentration for discriminating between LMW and HMW chitosan in our first experiments, our later studies used PDT with0.05% chitosan. Several reports demonstrated that the antimicrobial effect of LMW chitosan was higher than that of HMW chitosan about the gram-negative bacteria like our results but the reason is not clearly until now. We thought probably this mechanism due to the chitosan size and the functional end group of chitosan structure. In Gram negative bacteria, antibacterial activity almost by below 5000 kDa chitosan shows peculiar characteristics and it could occur through changes of bacterial membrane barrier and cytoplasmic membrane breakdown because of electrostatic binding and change of cell wall permeability [39]. In Gram positive bacteria, blockage of nutrient transport almost by over 5000 kDa chitosan is related to bacterial killing [40,41].

To calculate the full-powered light energy, we measured the bactericidal rate at an MB concentration of 0.02–0.04%. The slope began to decrease at an MB concentration of 0.02% with6.23 mJ/cm^2 (Fig. 2). In other words, at an MB concentration below0.02%, an irradiation power of 6.23 mJ/cm^{2}was sufficient for killing *H. pylori*.

We next attempted to confirm the booster effect on PDT by addition of LMW or HMW chitosan at a1/10 concentration of MB(0.002 or 0.004%). The bactericidal activity was greater for LMW chitosan than for HMW under the same PDT conditions (Fig. 3). The future *in vivo* studies may help to identify the optimal concentration of LMW chitosan to effectively kill *H. pylori* with phototherapy. And it is also needed in the next study to research exact mechanism of interaction between methylene blue and chitosan. Many studies have used chitosan as a tool of drug (antibiotics) delivery and nanoparticle synthesis, which were not recommended in humans due to biological incompatibility and safety such as allergic reactions [42,43].

Table 3
Quantification of 8-OHdG in DNA of *H. pylori* after PDT with chitosan.

Sample	Concentration of 8-OHdG (ng/ml/μg of DNA)
Control	0.023 ± 0.0047
HMW chitosan	0.027 ± 0.0050
LMW chitosan	0.024 ± 0.0030
MB + light	0.053 ± 0.0173
MB + HMW chitosan + light	0.158 ± 0.0101
MB + LMW chitosan + light	0.167 ± 0.0076

Control: without light irradiation, MB, and chitosan; LMW chitosan: low molecular weight chitosan (0.05%); HMW chitosan: high molecular weight chitosan (0.05%); MB: methylene blue (0.004%); Light: irradiation for 15 min.

We evaluated the killing mechanism against *H. pylori* induced by chitosan plus PDT. We used EMA-qPCR to assess bacterial cell wall damage and oxygen radical generation. EMA can be used to identify dead cells because it enters cells via damaged cell membranes and binds to cell DNA. The Ct value for the *cysS* gene in PCR analysis indicates the degree of damage to cells. Table 2 shows that phototherapy plus chitosan caused greater membrane damage than phototherapy or chitosan therapy alone. However, the EMA-qPCR results did not differ between LMW and HMW chitosan at a concentration of 0.05% (Table 2), which was consistent with the lack of differences in *H. pylori* removal rate between the two chitosan treatments (Fig. 1). However, the Ct value for EMA-qPCR was higher for LMW chitosan plus phototherapy than for HMW plus phototherapy, which was also consistent with the results for *H. pylori* removal rate (Fig. 3).

Oxygen radicals, especially singlet oxygen, are a main cause of oxidative DNA damage to target bacteria or tumors.8-OHdG is a novel biomarker of DNA oxidative damage. Its level was higher in the phototherapy plus chitosan treatment than in treatment with chitosan or phototherapy alone (Table 3). Moreover, at the same concentration of chitosan (0.05%), LMW chitosan produced just a little more 8-OHdGthan did HMW chitosan (Fig. 3). Therefore, besides oxidative damage, another mechanisms including chitosan molecular weights should be found in the future. These findings suggest that LMW chitosan plus phototherapy produced more reactive oxygen radicals and greater bacterial damage than single treatment with chitosan or phototherapy. Furthermore, as a clean therapy, it is necessary to perform PDT using antibody about specific bacterial antigen and DNA aptamers to enhance targeting therapy in the future.

In conclusion, although MB, chitosan, and light irradiation are considered to be safe for humans, it is desirable to use low amounts of PSs to reduce toxicity. PDT with MB and LMW chitosan may provide a new candidate therapy for lowering PS concentration and boosting the bactericidal effect of traditional PDT against *H. pylori*.

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