Riboflavin Inhibits Growth of *Helicobacter pylori* by Down-regulation of *polA* and *dnaB* Genes

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Infection of *Helicobacter pylori* on gastric mucosa is associated with various gastric diseases. According to the WHO, *H. pylori* causes gastric cancer and has been classified as a class I carcinogen. Riboflavin is an essential vitamin which presents in a wide variety of foods. Previous studies have shown that riboflavin/UVA was effective against the growth inhibition of *Staphylococcus aureus*, *S. epidermidis* and multidrug-resistant *Pseudomonas aeruginosa* and had the potential for antimicrobial properties. Thus, we hypothesized that riboflavin has a potential role in the growth inhibition of *H. pylori*. To demonstrate inhibitory concentration of riboflavin against *H. pylori*, we performed agar and broth dilution methods. As a result, we found that riboflavin inhibited the growth of *H. pylori*. The MIC was 1 mM in agar and broth dilution test. Furthermore, to explain the inhibitory mechanism, we investigated whether riboflavin has an influence on the replication-associated molecules of the bacteria using RT-PCR to detect mRNA expression level in *H. pylori*. Riboflavin treatment of *H. pylori* led to down-regulation of *polA* and *dnaB* mRNA expression levels in a dose dependent manner. After then, we also confirmed whether riboflavin has cytotoxicity to human cells. We used AGS, a gastric cancer cell line, and treated with riboflavin did not show statistically significant decrease of cell viability. Thus, these results indicate that riboflavin can suppress the replication machinery of *H. pylori*. Taken together, these findings demonstrate that riboflavin inhibits growth of *H. pylori* by inhibiting replication of the bacteria.

Key Words: Riboflavin, *Helicobacter pylori*, *PolA*, *DnaB*, Antibacterial

INTRODUCTION

Helicobacter pylori is a gram-negative spiral-shaped bacterium which possess its characteristic helical appearance.

H. pylori has been known to primarily colonize on human gastric epithelium. Approximately half of the world population harbor *H. pylori* and it has been suggested that infection of *H. pylori* generally occurs in childhood and transmitted from family members (Tharmalingam et al., 2016). Numer-

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ous reports suggested that various gastric diseases such as gastric inflammation, chronic gastritis, peptic ulcer and gastric adenocarcinoma are associated with *H. pylori* infection (Cover and Blaser, 2009). Epidemiologically, over 3 billion people are infected by this bacterium and develop persistent stomach inflammation, which lasts for decades unless treated with antibiotics. Moreover, World Health Organization classified *H. pylori* as a class I carcinogen due to the organism causes gastric cancer (Peek and Blaser, 2002).

Riboflavin (vitamin B2) is an essential water-soluble substance present in a wide variety of foods and required for normal cellular functions, including growth and development in all aerobic forms of life. Riboflavin is known as cofactors for enzymes involved in oxidation-reduction reactions, and has a role in the oxidative folding of proteins in the endoplasmic reticulum (Hoppel and Tandler, 1975; Tu et al., 2000).

Its most important biological forms, flavin adenine dinucleotide (FAD) and flavin mononucleotide, participate in a range of redox reactions, some of which are essential to the function of aerobic cells (Powers, 2003). Previous studies have shown that riboflavin can protect microbial keratitis. Riboflavin/UVA was effective against *Staphylococcus aureus*, *Staphylococcus epidermidis*, drug-resistant *Streptococcus pneumoniae*, methicillin-resistant *S. aureus*, multidrug-resistant *Pseudomonas aeruginosa*, and drug-resistant *Streptococcus pneumoniae* and had the potential for antimicrobial properties *in vitro* (Martins et al., 2008). Moreover, riboflavin has been found to increase the survival rate of mice suffering endotoxin-induced sepsis and Gram-negative and Gram-positive bacterial infection (Shih et al., 2010). In addition, riboflavin was also involved in the enhancement of antitumor activity of many anticancer drugs, as well as in the activation of the immune system to kill tumor cells (Zhu et al., 2006; de Souza Queiroz et al., 2007; Santos et al., 2007).

Based on the previous reports suggesting anti-microbial activity of riboflavin, we hypothesized that riboflavin has a potential role in the growth inhibition of *H. pylori*. To prove this hypothesis, we investigated the effect of riboflavin against *H. pylori* growth. We determined minimal inhibitory concentration (MIC) of riboflavin against *H. pylori* using agar dilution method and broth dilution method. Then expres-

sions of DNA replication genes in *H. pylori* were investigated after riboflavin treatment. We also evaluated cytotoxicity of riboflavin on mammalian cells.

MATERIALS AND METHODS

Bacterial and mammalian cell culture

The *H. pylori* reference strain ATCC 49503 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). *H. pylori* were grown on brucella agar plates (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 10% bovine serum (BRL Life Technologies, Grand Island, NY, USA) under microaerophilic and 100 percent humidity conditions at 37°C and inspected after three days.

AGS gastric adenocarcinoma cells (ATCC CRL-1739) were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM, BRL Life Technologies) supplemented with 10% fetal bovine serum (FBS, BRL Life Technologies) and streptomycin-penicillin (100 µg/mL and 100 IU/mL) (BRL Life Technologies). Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Agar dilution method to determine MIC

H. pylori colonies grown on brucella agar plates were collected and suspended in saline. The number of bacterial particles in the *H. pylori* suspension was set to McFarland 2.0 (6×10^8 /mL), 10 µL of the bacterial suspension was placed on the Mueller-Hinton agar (BD Biosciences) supplemented with 10% bovine serum including indicated concentrations of riboflavin. All solutions were prepared in such a manner that the final dimethylsulfoxide (DMSO) concentration was the same in all treatments. The bacteria were incubated for 72 hours and the MIC was determined based on the lowest concentration at which inhibition of growth was observed in a previous report (Yang et al., 2019).

Broth dilution method to determine MIC

H. pylori suspension set to McFarland 0.33 was prepared in Mueller-Hinton broth (BD Biosciences) supplemented with 10% bovine serum. Various concentrations of riboflavin

Table 1. List of primer sequences and PCR conditions for RT-PCR

Primers	Sequences (5'-3')		Product length (bp)	Annealing temperature (°C)	Cycles	Reference*
	Forward	Reverse				
DnaA	GGGCATGACTTAGCGGTTA	TTAACGAATTGCACGCCAAC	128	55	27	
DnaB	AATGGGCCGTTTATCGTCTC	CAAATCCGCTTGCAACTACG	231	55	27	
DnaE	AATCCACCGGCTCCAAATAC	GCCAAACAAGTGTGGGAGTA	184	55	27	
DnaN	GTTAGCGGTGGTTGAAAACG	CGGTTTCGCTATGCTCAGAA	233	55	27	
DnaQ	CGCATGAAGCTTTGCAAGAA	GCATAGGCTCTATGGCTGAC	244	55	27	
PloA	TCATCATCATTGCCGACTGG	GTCATGCGCAAACACATTCA	134	55	24	*
HolB	GCCCTTGAAATCGTGCTTAC	ATGATGAGAGCTACCCGACA	196	55	25	
RpoB	TTTAGGTAAGCGCGTGGATT	AATCAGCTTTGGATGGAACG	301	59	24	
POLA1	GCCAGTTTGGGCTGGTTG	GTTTCGGTTTGTCACTGCGA	458	57	29	
MCM2	GGCGGAATCATCGGAATCCT	ATCATCCAGAGCCAGTCCCT	295	57	29	
GAPDH	CGGGAAGCTTGTCATCAATGG	GGCAGTGATGGCATGGACTG	349	55	24	(Lee et al., 2015a)

*The primers without reference are designed in this study

were added and the bacteria were grown at 37°C for 72 h in a humidified atmosphere with 10% CO₂. All solutions were prepared in such a manner that the final DMSO concentration was the same in all treatments. The final optical density (at 600 nm wavelength) of the bacterial suspension was measured by spectrophotometry in a previous report (Woo et al., 2020).

RT-PCR (reverse transcription-polymerase chain reaction)

Cultured *H. pylori* were collected and washed twice with PBS. After washing, total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer's instructions. cDNA was synthesized by reverse transcription with 2 µg of total RNA, 0.25 µg of random hexamer (Invitrogen) and 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen) for 50 minutes at 37°C and 15 minutes at 70°C. Subsequent PCR amplification using 0.2 U of *Taq* polymerase (SolGent™, Daejeon, Korea) was performed in a thermocycler using specific primers. The PCR primer sequences used in this study and PCR conditions are described in Table 1. Amplified PCR products were analyzed using 2% agarose gel electrophoresis with Tris-acetate-EDTA (TAE) buffer, stained by ethidium bromide (EtBr) for 10 minutes

and destained in distilled water for 20 minutes. Gel images were taken using Molecular Image Gel Doc™ XR+ system (Bio-Rad, Hercules, CA, USA). The band intensity of PCR product was analyzed with the ImageLab software (Bio-Rad).

Cell viability assay

AGS cells (1.5×10⁴/well) were plated in 96-well plates. After 24 h, cells were treated with various concentrations of riboflavin dissolved in DMSO. All solutions were prepared in such a manner that the final DMSO concentration was the same in all treatments. The cells were then further incubated for 24 h before cell viability was measured by WST (water soluble tetrazolium salts) assay using EZ-Cytox cell viability assay kit as described in a previous report (Yeon et al., 2019). Briefly, 10 µL of WST solution was added to the cultured media and incubated in the CO₂ incubator for 2 h. Absorbance at 450 nm was measured by spectrophotometer.

Statistical analysis

Data in the graphs have been presented as mean ± standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA, USA). Data were analyzed by

Fig. 1. Determination of the MIC of riboflavin against an *H. pylori* ATCC 49503 strain by broth dilution. (A) *H. pylori* suspended in saline was set to McFarland 2.0 (6×10^8 /mL) and plated on Muller-Hinton agar including indicated concentrations of riboflavin. After 72 h of incubation, MIC was determined. (B) *H. pylori* was set to McFarland 0.33 (1×10^8 /mL) and incubated in Muller-Hinton broth including indicated concentrations of riboflavin for 72 h. And then, absorbance of medium was detected at 600 nm wavelength to determine the MIC. Data were from triplicate experiments and analyzed by Student's *t*-test (*** $P < 0.001$).

unpaired Student's *t*-test and $P < 0.05$ was considered be statistically significant (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

RESULTS

MIC of riboflavin against *H. pylori*

To demonstrate inhibitory effect and MIC of riboflavin against *H. pylori*, we performed agar dilution test. MH agars containing 0.125~2 mM of riboflavin were prepared and 30 μ L of *H. pylori* suspension set to McFarland 2.0 was placed on the MH agar. After then, *H. pylori* was incubated at 37°C and 10% CO₂ condition for 72 h. In the result, we found that *H. pylori* growth was inhibited by 1 mM or higher concentration of riboflavin in the agar dilution test (Fig. 1A). In addition, we further conducted broth dilution test to define the MIC of riboflavin against *H. pylori* in broth condition. *H. pylori* set to McFarland 0.33 was grown in the MH broth containing 0.125~2 mM of riboflavin for 72 h. In the broth dilution test, MIC of riboflavin against *H. pylori* was shown to be 1 mM (Fig. 1B). Based on these results, we demonstrated that riboflavin suppresses the growth of *H. pylori* and MIC was 1 mM in agar dilution test and broth dilution test.

Down-regulation of replication-associated molecules in *H. pylori* by riboflavin

Various factors are involved in the growth of bacteria and, especially the expression of replication-associated molecules is one of the most important factors to the bacterial growth. To explain the mechanism by which riboflavin suppresses growth of *H. pylori*, we investigated whether riboflavin has an influence on the replication-associated molecules of the bacteria. On the basis of the MIC acquired from broth dilution test, *H. pylori* was treated with 0.25~0.5 mM of riboflavin. RNA was isolated from the samples and analyzed by RT-PCR to detect mRNA expression level of replication machineries. In the result, we found that treatment with riboflavin led to down-regulation of mRNA expression levels of *polA*, *dnaB* in a riboflavin dose dependent manner, though expression of the other genes remained constant (Fig. 2A). Reduction of *polA* expression induced by riboflavin was statistically significant in the dose of 0.5 mM ($P = 0.0080$) or higher concentration (Fig. 2B) and *dnaB* expression was statistically significant in the dose of 0.25 mM ($P = 0.0201$) or higher concentration (Fig. 2B). *dnaB* is a bacterial helicase and *polA* is a DNA polymerase I both of which paly the crucial role in a process of bacterial replication.

Therefore, these findings demonstrate that riboflavin may inhibit *H. pylori* growth by down-modulating *polA* and *dnaB* expression.

Evaluation of cytotoxicity of riboflavin on human gastric epithelial cells

As abovementioned, we confirmed that riboflavin suppresses the growth of *H. pylori*. Subsequently, we investigated whether riboflavin has cytotoxicity to human cells. We used AGS, a gastric adenocarcinoma cell line, in this experiment, because *H. pylori* colonizes on the human gastric epithelium. AGS cells treated with 0.25~2 mM of riboflavin were incubated for 24 h. After incubation, we measured cell viability of AGS cells by WST assay. As a result, AGS cells treated with riboflavin did not show statistically significant decrease of cell viability within the range of concentration used in this experiment (Fig. 3A). Additionally, we conducted RT-PCR to elucidate whether riboflavin has an influence on the replication machinery of AGS cells. The target genes were *POLA1* and *MCM2* in eukaryotic cells which are corresponding to the prokaryotic replication machinery, *polA* and *dnaB*. As a result, mRNA expressions of *POLA1* and *MCM2* were not decreased by 0.25~1 mM of riboflavin treatment in AGS cells (Fig. 3B). Thus, this result indicates

that riboflavin can suppress the replication machinery of *H. pylori* but not on the corresponding replication machinery in AGS cells. Taken together, these findings demonstrate that riboflavin does not show cytotoxicity on AGS cells in the range of dose completely inhibited growth of *H. pylori*.

DISCUSSION

In this study, we demonstrated inhibitory function and mechanism of riboflavin against *H. pylori*. MIC of riboflavin against *H. pylori* was 1 mM in the agar dilution test and broth dilution test, respectively. To elucidate the inhibitory mechanism of riboflavin, we investigated expressions of replication-associated molecules of the bacteria and found that *polA* and *dnaB* mRNA levels were down-regulated by riboflavin. Furthermore, riboflavin did not inhibit cell viability and mRNA expressions of *POLAI* and *MCM2* in AGS cells within the range of concentration completely inhibited growth of *H. pylori*.

DNA replication is indispensable for survival and proliferation of living organisms including bacteria. In prokaryotes, DnaA is a highly conserved protein in all bacteria which activates initiation of DNA replication. It binds to OriC site which has AT rich region within the replication origin. DnaA provides docking platform for DnaB, the replicative DNA helicase, in a complex with DnaC (Chodavarapu and Kaguni, 2016). *H. pylori* does not have DnaC but DnaB binds to DNA double strand and function as an helicase without help of DnaC (Soni et al., 2005). DnaB helicases load on to the unwound region and migrates along the single-stranded DNA, unwinding the DNA as it travels in the 5'-3' direction (Nitharwal et al., 2011). The interaction of DnaB with primase (DnaG) leads to the primer formation (Chodavarapu and Kaguni, 2016). The elongation phase begins when DNA polymerase III loads on to the 3' termini of the primer synthesized by DnaG (Nitharwal et al., 2011). DNA polymerase III is composed of core polymerases (DnaE, DnaQ and HolE), sliding clamp (DnaN) and multiprotein clamp-loader (DnaX, HolA, HolB, HolC and HolD) all of which are essential for appropriate function as a single machinery (Nitharwal et al., 2011). DNA polymerase I is encoded by *polA* gene. The major role of DNA polymerase I is to repair

any damage with DNA, and to connect Okazaki fragments by deleting RNA primers and replacing the strand with DNA (Joyce et al., 1982). In our experiment, we found that riboflavin down-regulated mRNA expression of *dnaB* and *polA* genes. Both DNA helicase and DNA polymerase I are indispensable for replication and survival of the bacteria. Thus, the down-regulation of *dnaB* and *polA* explains how riboflavin inhibited growth of *H. pylori*.

Currently, triple therapy using clarithromycin, amoxicillin and proton pump inhibitor are recommended as the first-line regimen for eradication of *H. pylori* (Lee et al., 2015b). However, numerous reports warning the prevalence of clarithromycin resistance and decrease of eradication rate by first-line therapy worldwide (Lee et al., 2013; Ghotaslou et al., 2015; Wu et al., 2015; Zhang et al., 2015). Clarithromycin resistance rate in Asia has been markedly increased from 15.28% in 2009 to 32.46% in 2014 (Ghotaslou et al., 2015). Moreover, resistance rate of other antibiotics such as levofloxacin and metronidazole have also been increased in *H. pylori* (Ghotaslou et al., 2015). Increase of the antibiotic resistance seems unavoidable as long as we use the antibiotics for eradication of *H. pylori*, thus there should be continuous efforts for surveillance on antibiotic resistance rate and appropriate selection of antibiotic regimens. Furthermore, development of new therapeutic agents to help eradication of *H. pylori* is also necessary to overcome the prevalence of antibiotic resistance at this moment and in the future. There have been reports that riboflavin, a natural compound, inhibits the growth of various Gram-positive and negative bacteria, but no studies have been reported on its effect on the growth and inhibitory mechanisms of *H. pylori*. Therefore, this study confirmed the results that riboflavin inhibited growth by downregulation of the replication factors of *H. pylori*. However, as report on the correlation between riboflavin receptors in patients infected with *H. pylori* have been published, various possibilities for inhibitory mechanisms cannot be excluded (Matnuri et al., 2015). Therefore, further studies seem to be necessary to understand the inhibitory function and mechanism of riboflavin in *H. pylori* in detail, and its toxicity and effectiveness need to be confirmed *in vivo*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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