Anti-bacterial and therapeutic effects of hydrogen mineral disinfectant treatment on *Helicobacter pylori* infected *in vitro* and *in vivo* model

Md. Habibur Rahman

The Graduate School

Yonsei University

Department of Global Medical Science

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Directed by Prof. Kyu-Jae Lee

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Prof. Kyu-Jae Lee, M.D., Ph.D.: Thesis Supervisor

Prof. Soo-Ki Kim, M.D., Ph.D.: Committee Member

Prof. Seo-Bo Am, Ph.D.: Committee Member

Prof. Dong-Heui Kim, Ph.D.: Committee Member

Prof. Yon-Chul Park, M.D., Ph.D.: Committee Member

The Graduate School

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ABBREVIATIONS

ACC: Available chlorine concentration

ANOVA: Analysis of variance

Ca^{2+}: Intracellular calcium

DCFH-DA: Dichlorodihydrofluorescein diacetate

EDTA: Ethylenediaminetetraacetic acid

GM-CSF: Granulocyte/macrophage colony-stimulating factor

GPx: Glutathione peroxidase

HOCl: Hypochlorous acid

IACUC: Institutional animal care and use committee

IL: Interleukin

IFN-γ: Interferon-gamma

iNOS: Nitric oxide synthase

MMPs: Matrix metalloproteinases

MALT: Mucosa-associated lymphoid tissue

NC: Normal control

NED: N-(1-Naphthyl) ethylenediamine

NO: Nitric oxide

OS: Oxidative stress

ORP: Oxidation-reduction potential

PW: Purified water

RIPA: Radioimmunoprecipitation assay

ROS: Reactive oxygen species

SD: Standard deviation

TNF-α: Tumor necrosis factor-alpha

TIMP-1: Tissue inhibitor of metalloproteinases-1

WBC: White blood cell

ABSTRACT

Antibacterial and therapeutic effects of hydrogen mineral disinfectant treatment on *Helicobacter pylori* infected *in vitro* and *in vivo* model

Md. Habibur Rahman

Department of Convergence Medicine

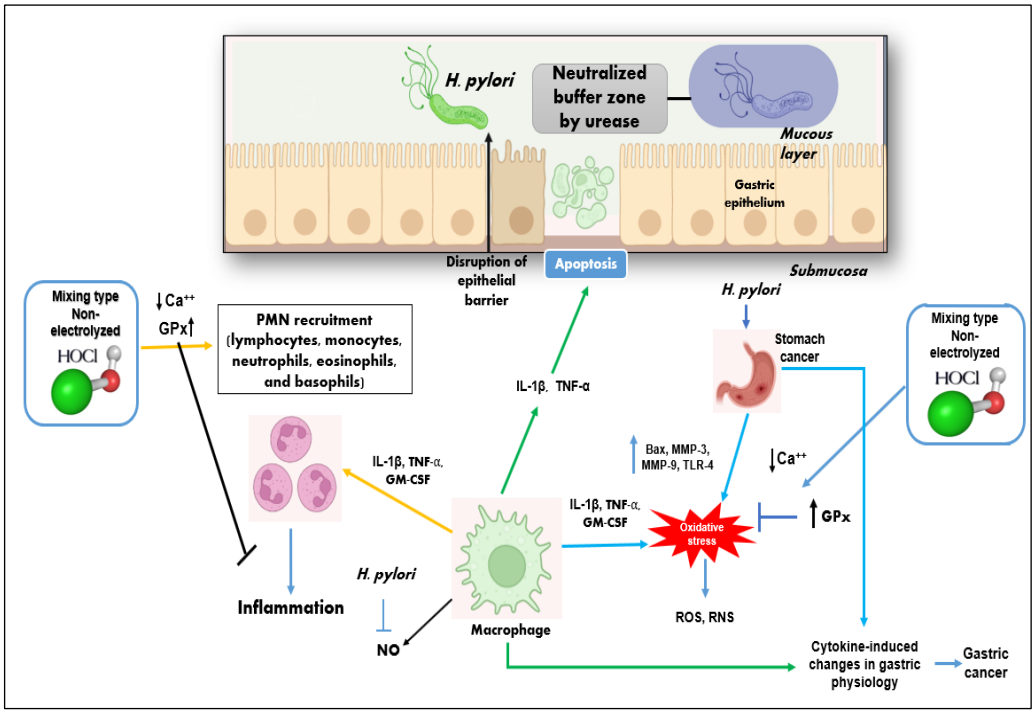
The Graduate School, Yonsei University

Directed by Prof. Kyu-Jae Lee

Background: Hypochlorous acid (HOCl) is a naturally occurring chemical that is a part of the innate immune response in humans. Recently, non-electrolyzed HOCl water has gained the attention of researchers as a new disinfecting agent owing to its high sterilization power, easy accessibility, and safety. Non-electrolyzed HOCl water was developed through mixing at a specific ratio based on hypochlorite and mineral supplements, which revealed a high oxidizing power. In this study, we investigated the effects of non-electrolyzed HOCl water on *Helicobacter pylori* (*H.*

pylori) infection in C57BL/6 mice over 10 weeks. Mice were divided into three groups: normal control (NC) group supplied with purified water (PW) without infection, PW + *H. pylori* group supplied with PW after *H. pylori* infection, and HOCl + *H. pylori* group supplied with HOCl after *H. pylori* infection. Water was supplied to the mice using a mouse water bottle and was exchanged once a day. The antibacterial activity was evaluated by using optical density measurement and Z-score. Our study showed that HOCl + *H. pylori* group completely inhibited bacterial growth as compared to PW + *H. pylori*. Our heatmap results also showed HOCl + *H. pylori* group has excellent antibacterial activity as compared to the PW + *H. pylori* group. Body measurement for each group was done weekly for 10 weeks to obtain baseline data. We found that there were no significant changes among all groups. Our results showed that WBC and its differential counts, including total WBC, neutrophils, lymphocytes, monocytes, and eosinophils, were significantly inhibited in the HOCl + *H. pylori* group compared to those in the PW + *H. pylori* group. We evaluated oxidative stress markers such as reactive oxygen species (ROS) and nitric oxide (NO). In line with this, the level of ROS and calcium activity showed a significant reduction in both serum and stomach lysates in the HOCl + *H. pylori* group compared to those in the PW + *H. pylori* group. In contrast, HOCl water treatment enhanced GPx activity compared to PW treatment after *H. pylori* infection in both serum and stomach lysates. In addition, we evaluated anti-

inflammatory stress markers such as interleukin (1β), granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor-alpha (TNF-α,), etc. As a result, we found that the levels of GM-CSF, IL-1β, and TNF-α cytokine levels were significantly decreased in the HOCl + *H. pylori* group compared to those in the PW+ *H. pylori* group in the stomach lysate; however, there was no significant difference in serum. In addition, the expression levels of Bax, MMP-3, MMP-9, and TLR-4 were found to decrease after HOCl water treatment, whereas the expression level of Bcl-2 was found to be enhanced after HOCl water treatment in the stomach lysate. RT-PCR and q-PCR were performed to check the mRNA level among the three groups. We found that our HOCl + *H. pylori* group significantly decreased mRNA level as compared to the PW + *H. pylori* group. Taken together, our results suggest that drinking non-electrolyzed HOCl water has positive anti-oxidative, anti-inflammatory, and anti-apoptotic effects in *H. pylori*-infected mice through redox and immune regulation. Further studies are required to fully clarify the therapeutic applications of non-electrolyzed HOCl water in *H. pylori*-induced stomach problems.



Keywords: *Helicobacter pylori*, anti-oxidative, anti-inflammatory, anti-apoptotic, non-electrolyzed hypochlorous acid, immune redox modulation

I. INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral-shaped, gram-negative, microaerophilic pathogenic bacterium that is a persistent colonizer of the human stomach and can cause peptic ulcer disease, gastritis, dyspepsia, and stomach [1] [2]. Despite its medicinal importance and extensive distribution, little is known regarding the natural history of *H. pylori* [3]. One of the main reasons for cancer-related deaths worldwide is gastric cancer. The complex etiology of this condition includes Helicobacter pylori as well as environmental and genetic risk factors [4]. A group of patients had gastric ulcer disease and mucosa-associated lymphoid tissue (MALT) lymphoma as a result of infection with virulent *H. pylori* strains. In addition to the bacteria, it is thought that smoking, eating a salty diet, and the genetic susceptibility of the host all contribute to an increased risk of carcinogenic transformation in the human stomach [5]. According to studies, the presence of neutrophils, lymphocytes, macrophages, and dendritic cells in the stomach mucosa during *H. pylori* colonization results in an inflammatory response [6]. In addition, increasing evidence has shown that microbial pathogens, such as *H. pylori*, induce oxidative stress (OS) in the host cell environment, which further plays an important role in epithelial injury [7]. It is a well-known fact that *H. pylori*-induced OS contributes to altered epithelial proliferation in the gastric mucosal layer, increased apoptosis, and increased oxidative damage to DNA [8] [9]. One study reported increased levels of reactive oxygen species (ROS) in the mucosal layer of *H. pylori*-infected patients [10]. Studies reported that *H. pylori* bacteria itself have a tendency to generate ROS and accumulate in gastric epithelial cells [11] [12]. Additionally, proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and interferon-gamma (IFN-γ), were found to induce ROS in the oxidative environment and lead to an inflammatory response in gastric epithelial cells [13] [14]. Infection with *H. pylori* can cause the release of proinflammatory mediators and abnormally high levels of ROS and

NO in stomach mucosal epithelial cells [15]. Additionally, research has demonstrated that the development of cellular injury is significantly influenced by the elevation of intracellular calcium (Ca^{2+}) [16]. Because intracellular Ca^{2+} has numerous important roles in intracellular signaling, the Ca^{2+} overload can affect cellular functions such as cellular proliferation, cytokine secretion, and phagocytosis [17]. However, little is known about the regulation of intracellular Ca^{2+} by *H. pylori* in gastric cells. According to previous studies, *H. pylori* causes apoptosis in the gastric epithelium by upregulating the proapoptotic protein marker Bcl-2-associated X (Bax) and downregulating the anti-apoptotic protein Bcl-2 [18]. Numerous studies have discovered that *H. pylori* infection is connected to changes in Bax and/or Bcl-2 expression patterns [19] [20]. Additionally, matrix metalloproteinases (MMPs) have an impact on both the inflammatory environment and structure of infected tissues [21]. Proton pump inhibitors and antibiotics reduced total and active MMP-9 levels in the gastric mucosa of the antrum and corpus in individuals with *H. pylori*-associated gastritis. Additionally, *H. pylori* infection results in stomach inflammation, which is marked by a significant influx of immune cells, particularly polymorphonuclear leucocytes and macrophages, to the gastric mucosa [22]. MMPs have an impact on the tissue's architectural modifications and inflammatory environment [23]. In the context of *H. pylori*-associated gastric inflammation, MMP-2, -7, and -9 are the most often investigated MMPs employing a range of techniques, including IHC (immunohistochemical analysis), RT-PCR (real-time polymerase chain reaction), zymography, and ELISA (enzyme-linked immunosorbent assay). In gastric mucosa biopsies from persons with H. pylori-associated gastritis, it was discovered that MMP-2, -7, and -9, as well as MT1-MMP and TIMP-2 and -4, were elevated. According to the severity of the disease, flow cytometry showed that these MMPs and TIMPs were elevated on the surface of infiltrative mucosal lymphocytes rather than epithelial cells [24]. There is a wealth of data, sometimes contradictory, on the upregulation of individual MMP members in *H. pylori*-associated gastritis. A therapeutic target,

toll-like receptors (TLRs) are crucial adaptive immune system activators that control inflammation in response to *H. pylori* infection [25 26 27]. During a 24-hour incubation period in cell culture medium, stomach mucosal biopsies from *H. pylori*-positive patients with chronic superficial gastritis produced more [28]. Antibiotics and proton pump inhibitors reduced total and active MMP-9 levels in the gastric mucosa of the antrum and corpus portions in patients with *H. pylori*-associated gastritis [29]. To overcome this, conventional treatments for *H. pylori*, such as triple therapy (clarithromycin plus amoxicillin/metronidazole with a proton pump inhibitor), are commonly used; however, these treatment modalities are often expensive and are associated with drug resistance or adverse effects [30]. There is an urgent need for new anti-*H. pylori* treatments with high therapeutic effectiveness and few side effects. Researchers have recently become interested in the use of hypochlorous acid water against a variety of bacterial activity, including *H. pylori*. Sodium hypochlorite and HOCl are combined to create the weak acid known as HOCl water, which has a pH adjusted range of 6.0 to 6.4 and a residual chlorine content of about 60 ppm [31]. Evidence suggests that HOCl water is very effective against various microorganisms. Owing to its anti-inflammatory and other biological effects, electrolyzed HOCl water has also been applied in various studies pertaining to the treatment of numerous inflammatory skin diseases, such as seborrheic dermatitis and atopic dermatitis, as well as itching, diabetic ulcers, and wound healing [32 33]. Studies have reported that HOCl is safe for human use [34]. One study reported that intraperitoneal lavage and wound cleaning with HOCl with a residual chlorine concentration of 40–60 ppm had no deleterious effects in a clinical investigation [35]. Additionally, 0.01-0.1% (w/v) HOCl, which is produced by combining hydrochloric acid with sodium hypochlorite, did not cause eye irritation or systemic toxicity, according to a study on animal safety and toxicity tests [36]. In addition to electrolyzed HOCl water, non-electrolyzed HOCl water has been developed as a new disinfection agent owing to its high sterilizing power, ease of accessibility, and safety [37]. By combining hypochlorite and

mineral additions in a precise ratio, non-electrolyzed HOCl water was generated, which exhibited tremendous oxidizing power. Therefore, we evaluated the anti-oxidative, anti-apoptotic, and anti-inflammatory effects of drinking non-electrolyzed HOCl water in *H. pylori* infected C57BL/6 mice. The results of this investigation could serve as a database to create safe and effective treatments for *H. pylori* infection, especially in relation to stomach problems.

II. HYPOTHESIS AND OBJECTIVES

Hypochlorous acid is a weak acid that forms when chlorine dissolves in water, and itself partially dissociates, forming hypochlorite, ClO^-. HClO and ClO^- are oxidizers, and the primary disinfection agents of chlorine solutions. Recently, non-electrolyzed HOCl water has gained the attention of researchers as a new disinfecting agent owing to its high sterilization power, easy accessibility, and safety. Non-electrolyzed HOCl water was developed through mixing at a specific ratio based on hypochlorite and mineral supplements, which revealed a high oxidizing power.

The objectives of the study are the following:

(1) To identify of anti-bacterial effects of non-electrolyzed HOCl on *Helicobacter pylori in vitro* and *in vivo* model

(2) To evaluate the anti-oxidative effects of non-electrolyzed HOCl that suppresses the ROS production on *Helicobacter pylori* Infected C57BL/6 mice

(3) To evaluate the anti-inflammatory and anti-apoptotic effects of non-electrolyzed HOCl by inhibition of *H. pylori* on C57BL/6 mice

III. MATERIALS AND METHODS

3.1 Pour plate method

The pour plate method is typically used to determine how many colony-forming bacteria are present in a liquid specimen. A larger volume was employed than with the spread plate to combine the sample with the molten agar media. Using a sterile pipette, a predetermined volume of inoculum (often 1 ml) from a broth or sample was deposited in the center of a sterile Petri dish. The Petri plate holding the inoculum was filled with molten, cooled agar (about 15mL), which was stirred thoroughly. The plate was inverted and incubated at 37°C for 24-48 hours after the agar has solidified. Both on the surface and within the media, microorganisms proliferate.

The few colonies that develop on the agar surface are of the same size and resemble those on a streak plate; colonies that develop within the medium are typically modest in size and may be confluent. Each colony, big and tiny, was meticulously counted (using magnifying colony counter if needed). A "colony-forming unit" is what each colony stands for (CFU).

The number of microorganisms present in the particular test sample were determined using the formula:

CFU/mL= CFU * dilution factor * 1/aliquot

3.2. Biofilms

The microtiter plate assay, commonly known as the 96-well plate assay, is a technique that enables the observation of bacterial adhesion to an abiotic surface. Bacteria were grown in 96-well microtiter plates for this test. Planktonic bacteria was removed from the mixture after incubation, and the adhering bacteria that are left are stained with crystal violet dye to make the biofilm visible. The dyed biofilms were solubilized and transferred to a 96-well optically transparent flat-bottom plate for quantification.

3.3. Optical Density Measurements

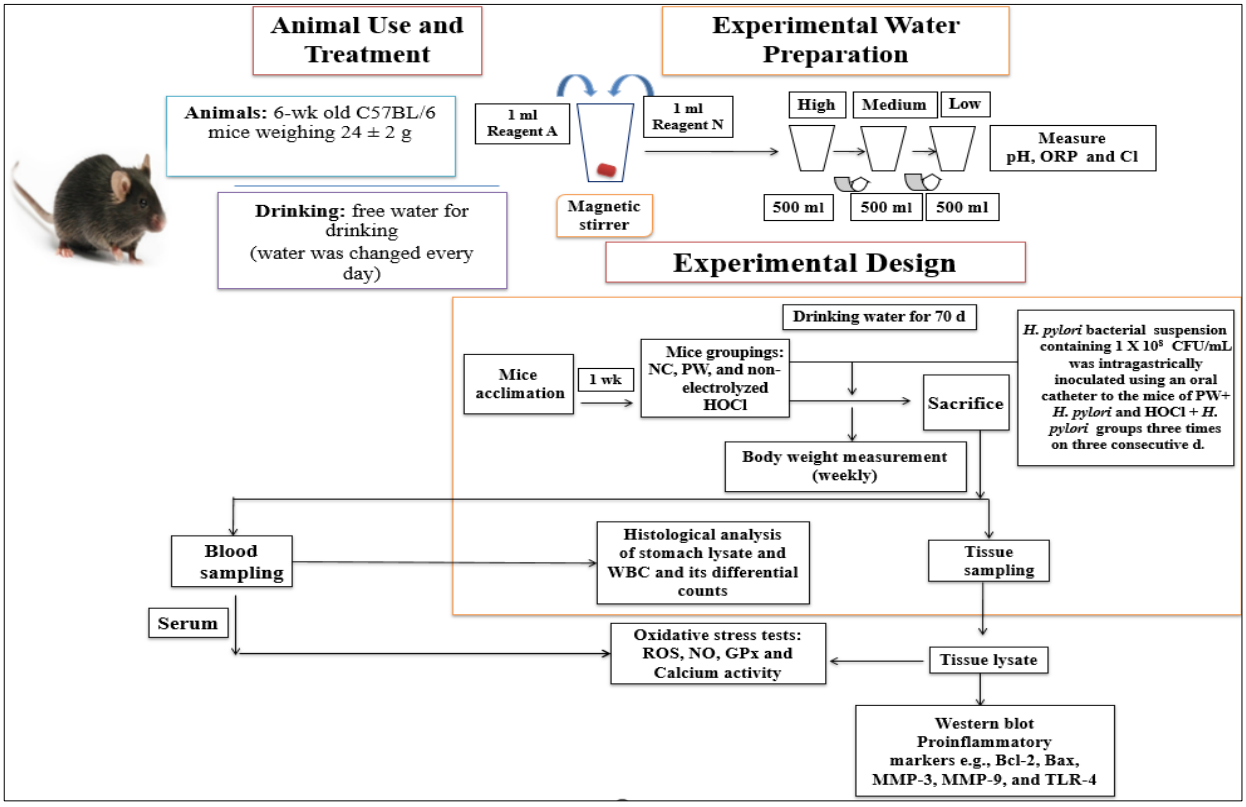
In a SpectraMax® ABS Plus spectrophotometer (Molecular Devices, San Jose, CA, USA) using a Costar Flat Bottom 96-well plate with cover and 200 l per well, OD measurements of bacterial and colloidal bead cultures was taken. At a wavelength of 600 nm and a temperature of 37 °C, absorbance was recorded, with the mean of five values obtained. Bacteria were cultured in 30 wells and cultivated in media until the optical density (OD) is 0.15. To prevent additional cell division or growth, 125 g ml-1 chloramphenicol was added to the combined wells. After then, the cells were gradually diluted to produce a variety of OD measurements. The bright field microscope will then be used to image a single cell dilution for each series, just as was done for the polystyrene beads before. The SpectraMax® ABS Plus spectrophotometer (Molecular Devices, San Jose, CA, USA) was used to report all measurements in the body of the text. The results were given as measured by OD. In RPMI media, cells were cultivated for 16 hours while dilutions for measurement are prepared. At 600 nm (bandwidth 9) with 15 flashes, identical OD measurements will be made at 30 °C. *H. pylori* grown on blood agar plates to midlog as in black and the OD measured in both plate readers was used to calibrate the two spectrophotometers.

3.4. Experimental Design

Prior to treatment, six-week-old C57BL/6 mice with a mean weight of 25 1.2 g were acclimated for 7 days in a controlled environment with a temperature of 22 2°C and 40–60% humidity during a 12:12 h light-dark cycle. These mice were bought from Orient Bio Inc. (Swingman, Republic of Korea).

Fifteen mice were randomly divided into three different groups (n = 5 per group) as follows: normal control (NC) group supplied with purified water (PW) without infection, PW + *H. pylori* group supplied with PW after *H. pylori* infection, and HOCl + *H. pylori* group supplied with HOCl water after *H. pylori* infection. Before each inoculation, all mice were fasted for 6 h with free access to water. Consequently, 100 μL of *H. pylori* bacterial suspension containing 1×10^8 CFU/mL was intragastrically inoculated using an oral catheter into the mice of PW + *H. pylori* and HOCl + *H. pylori* groups three times on three consecutive days. Additionally, experimental water was administered with free access to the mice, and it was exchanged with new water every day during the experimental period. Body weight was measured weekly. After 10 weeks of drinking water, all mice were sacrificed under anesthesia (Isoflurane, Hana Pharm Co., Ltd.. Seoul, Republic of Korea) and blood and tissue samples were collected for hematological and biochemical examinations and western blot analysis. The Institutional Animal Care and Use Committee (IACUC) Wonju College of Medicine, Yonsei University, Gangwon, Wonju, Republic of Korea, authorized the study protocol for this investigation (ethical approval no: YWC-180615-1). Figure 1 shows the experimental design.

Figure 1. Schematic diagram of the experimental process



3.5. Water Preparation and their Properties

Non-electrolyzed HOCl water solution was supplied from Sungjin Farm Co. Ltd. (Gyeongjusi, Gyeongsangbuk-do, Korea), and composed of reagent A and N. In brief, 1000μL of reagent A and N (ratio 1:1) was mixed with 2L of tap water (TW). A pH and ORP meter (DKK-TOA Corporation, Japan) was used to test pH and ORP, while a chlorine meter (Lutron Electronic Enterprise, Co., LTD, Taiwan) was used to measure available chlorine concentration (ACC). The water properties are shown in Table 1.

Table 1. Properties of experimental water.

Parameters	PW	Non-electrolyzed HOCl water
pH	7.5	6.1
ORP (mV)	310	931
ACC (ppm)	0	51

ORP: oxidation-reduction potential, ACC: available chlorine concentration, PW: purified water, HOCl: non-electrolyzed hypochlorous acid water.

3.6. Infection of *H. pylori* to the Mice

H. pylori strain used in these studies were purchased from the American Type Culture Collection (ATCC® 43504™; PO Box 1549, Manassas, VA 20108 USA) grown, and propagated according to the recommendations for each strain by ATCC. Bacterial cells were harvested at the stationary phase and concentrations were determined by 10-fold dilution as direct colony count. The collection of the microbiological specimens from the surface of the bacterial culture plate was accomplished using an inoculating loop and swab with a transport tube containing 1 mL of Liquid Amies medium. Following overnight shipment of the specimens to the central microbiology lab (Yonsei University), each specimen was processed to culture aerobes and anaerobes with Columbia agar with 5% sheep blood (Becton Dickinson & Company, USA; 18-03 NJ-208, Franklin Lakes, NJ 07417, United States). *H. pylori* were routinely cultured with Columbia agar with 5% sheep blood (Becton Dickinson & Company, USA; 18-03 NJ-208, Franklin Lakes, NJ 07417, United States). at 37℃ for 3-5 days an anaerobic condition. All the mice except for the NC group were fasted for 6 h with free access to water before each inoculation. *H. pylori* were cultured 100 μL *H. pylori* bacterial suspension containing (1×10^8) CFU/mL was intragastrically inoculated using oral catheter to the mice of PW + *H. pylori* and HOCl + *H. pylori* groups three times on three consecutive days.

3.7. Serum and Stomach Tissue Lysates Sample Preparation

Blood was collected in a BD Microtainer tube (Lot9305148, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged at 14,000 rpm for 5 min at 4°C to obtain the serum sample which was stored at -80°C until used. To prepare the stomach tissue sample, stomach tissue (2x2 mm in size) was cut and placed in an ice-cold Radioimmunoprecipitation assay (RIPA) buffer (Pierce Biotechnology Inc., IL, USA) with protease inhibitor compounds (Sigma Chemical Co., St Louis, USA) (Sigma Chemical Co., St Louis, USA). The stomach tissue was homogenized for 10 min at 14,000 rpm, centrifuged for 5 min, and the supernatant of stomach lysate was used as a sample.

3.8. Measurement of Body Weight

Body weight was measured at weekly intervals during the experimental period to obtain baseline data.

3.9. Histological Examination by Giemsa Staining

The body part of stomach (5x5 mm in size) was sectioned for histological investigation. The tissue samples were fixed with 10% formalin solution for 12 h, and embedded in paraffin after the processes of dehydration, clearing and infiltration according to the routine protocol. The sections were cut to a thickness of 5 μm and stained with Giemsa solution (Muto Pure Chemicals Co., Ltd, Tokyo, Japan) after de-paraffin and rehydration processes. The tissues were observed under the light microscope (BX51, Olympus Corp., Tokyo, Japan).

3.10. The Total and Differential Count of White Blood Cells (WBC)

Blood was collected from retro-orbital plexus and placed in tubes coated with ethylenediaminetetraacetic acid. The total WBC and its differential counts including lymphocytes, monocytes, neutrophils, eosinophils, and basophils were measured using an automatic blood analyzer (HEMAVET HV950 FS, Drew Scientific Inc. Texas, USA).

3.11. Measurement of Total ROS

Total ROS formation in serum and stomach tissue lysates was determined using the oxidation of 2-4-dichlorodihydrofluorescein diacetate (DCFH-DA) (Abcam, Cambridge, MA, USA). 50 µL serum and samples of stomach tissue lysates were placed in a 96-well plate. 100 µL of 10 µM DCFH-DA was then put into the well of the plate, and it was then left to react the dark for 30 min. The absorbance was read using a DTX-880 multimode microplate reader (Beckman Counter Inc., Fullerton, CA, USA) at 488nm excitation/525 nm emission.

3.12. Measurement of NO Level

To assess NO production in blood and stomach tissue lysate, Griess reagent (Promega Corp., Madison, WI, USA) was used. The test was carried out in accordance with the manufacturer's instructions. Briefly, standards were constructed, and the stomach tissue lysates samples and 50 µL of serum were added to the wells to assess nitrite. In each well, 50 µL of sulfanilamide solution was added and incubated for 10 min in the dark at room temperature. Then, in each well, 50 µL of N-(1-Naphthyl) ethylenediamine (NED) solution was added and incubated for 15 min in the dark at room temperature. A SpectraMax® ABS Plus (Molecular Devices, San Jose, CA, USA) was used to measure the OD at 520 nm.

3.13. Measurement of Glutathione Peroxidase (GPx) Activity

The Biovision kit (Milpitas, CA, USA) was used to determine the GPx activity in serum and stomach tissue lysates. As indicated by the manufacturer guidelines, the activity of GPx was determined by using normalized protein samples. In brief, the standards of the assay were prepared, the reagents assay were mixed in 96-well microplate. Furthermore, the absorbance was read at 340nm on SpectraMax® ABS Plus (Molecular Devices, San Jose, CA, USA).

3.14. Detection of Intracellular Ca^{2+} Activity

A colorimetric calcium assay kit was used to determine the total intracellular Ca^{2+} activity in accordance with the manufacturer's instructions (Abcam, Cambridge, MA, USA). In brief, a two-fold dilution of the calcium standard was made and placed in the 96-well microplate. After that, the working detection reagent was applied to the wells. The plate was incubated for 5 min before being measured at OD 575 nm (SpectraMax® ABS Plus (Molecular Devices, San Jose, CA, USA).

3.15. Measurement of Proinflammatory Cytokines

Inflammatory cytokines such as GM-CSF, IL-1β, and TNF-α were measured in serum and stomach lysates using a Bead Array Suspension Multiplex Kit (Bio-Rad, San Diego, CA, USA) according to the manufacturer's instructions. Standard curves for each cytokine were generated using the reference concentrations as indicated in the assay kit. Further, standards and samples were measured in a Luminex 200 Bio-plex device (Bio-Rad, Hercules, CA, USA) and 5-parameter logistic technique was used to analyze the raw data.

3.16. Western Blot Analysis

Western blot was performed by the following instruction [38]. The stomach tissue was homogenized for 10 min using the bead milling method (QIAGEN Tissue Lyser II, manufactured by Retsch, Goleta, CA, USA), incubated with 500 mL lysis RIPA buffer at 4°C, and centrifuged at 14,000 rpm for 10 min. The liquid supernatant was collected, and the total protein concentration was determined using a BCA protein assay kit (Takara, Shiga, Japan). On 12% polyacrylamide gels, equal amounts of protein samples (20 μg) were loaded, separated, and transferred to a polyvinylidene difluoride membrane (Pall., Ann Arbor, MI, USA) at 300 mA for 2 h. After transfer, the membranes were blocked for 1 h at room temperature with a blocking buffer (Takara, Shiga, Japan). The membranes were then incubated at a 1:2000 dilution at 4 °C overnight with the primary antibodies β-actin, MMP-3, MMP-9, Bcl-2, Bax and TLR-4 (Cell Signaling Technology, Danvers, MA, USA). The membranes were treated with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (dilution 1:5000; Cell Signaling Technology) in 1X Tris Buffered Saline and Tween (1X TBST) for 2 h at room temperature after being washed three times with 1X TBST. The bound antibodies were detected using the UVP Bio Spectrum 600 Imaging System and an enhanced chemiluminescence kit (ECL Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA) (UVP, LLC, Upland, CA, USA). Band intensity was analyzed using ImageJ software (Version 150-win Java, Bethesda, MD, USA).

3.17. RNA Extraction and cDNA Synthesis

Following the manufacturer's instructions, total RNA was extracted from the co-culture system using TRIzol® Reagent (Invitrogen, Waltham, MA, USA) and the isopropanol–chloroform technique. To make cDNA, 1 μg of total RNA was reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) with gDNA wipeout buffer (7×), RT primer mix, and 1 μL of Quantiscript Reverse Transcriptase (200 units), in a final volume of 20 μL, for 15 min at 42 °C, followed by a 3 min denaturation at 95 °C.

3.18. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) mRNA Assay

Different *H. pylori* genes: GAPDH, MMP-3, MMP-9, TLR-2, TLR-4. All oligonucleotides' primers were purchased from SFC Co., bioproducts. Primers used are seen in Table 2 below:

Table 2. List of Primer

SL No.	Target Gene	Primer Sequence (5'→3')	Amplicon Size (bp)
1	GAPDH	5- AGGTCGGTGTGAACGGATTTG-3 5- TGTAGACCATGTAGTTGAGGTCA-3	123
2	MMP-3	5-ACCGGATTTGCCAAGACAGA-3 5-CAGGCCCATCAAAAGGGACA-3	167
3	MMP-9	5-AAAACCTCCAACCTCACGGA-3 5-CACAGCGTGGTGTTCGAATG-3	196
4	TLR2	5-GCATCCGAATTGCATCACCG-3 5-ACAGCGTTTGCTGAAGAGGA-3	136
5	TLR4	5-TGGTTGCAGAAAATGCCAGG-3 5-TCATCAGGGACTTTGCTGAGTT-3	169

All the primers for 35 cycles of denaturation at 94°C for 30 s, primer annealing at 49°C for 1 min, and extension at 72°C for 1 min to produce a bp of the product. All amplifications began with an initial denaturation of target DNA at 95°C for 10 min, and the final cycles all concluded with extension for 10 min at 72°C to ensure full extension of the product. These four amplifications were performed with the Perkin-Elmer Cetus DNA thermal cycler. The amplified products were characterized by ethidium bromide staining of electrophoresed agarose gels. PCR for the whole gene performed with the Light Cycler as previously described. The all-PCR products were removed from the capillary tubes and analyzed by the same procedure as the other amplified samples.

3.19. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) mRNA Assay

qRT-PCR was carried out in 384-well PCR plates with a final volume of 10 μL, using Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, CA, USA), cDNA template, and RT forward and reverse primers. Triplicate reactions were set up for each primer/cDNA pair. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the internal control. Real-time PCR reactions were initiated in a QuantStudio™ 6 Flex System (Thermo Fisher Scientific, Waltham, MA, USA) by heating to 50 °C for 2 min and then to 95 °C for 10 min, followed by 40 cycles of 95 °C (15 s) and 60 °C (60 s). Relative quantification of gene expression was performed using the threshold cycle (C_t) method.

3.20. Data Management and Statistical Analysis

The statistical analysis was carried out using the Graph Pad Prism version 8.0 software packages (Graph Pad Software, La Jolla, CA, USA), and were compared using one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey). Differences were considered statistically significant at $p < 0.05$.

IV. RESULTS

4.1. Antibacterial Effects of Non-electrolyzed Hyphoclorus Acid Water on *H. pylori in vitro*

First, we determined the antibacterail efficacy of hypochlorous acid (HClO) against *H. pylori* strains growing planktonically and growing in biofilms. As a result we found that *HOCl + H. pylori* group significantly inhibit bacterial growth in (Figure 2A) by measuring OD score) (Figure 2B) observed biofilms methods and (Figure 2C) by observed heatmap method.

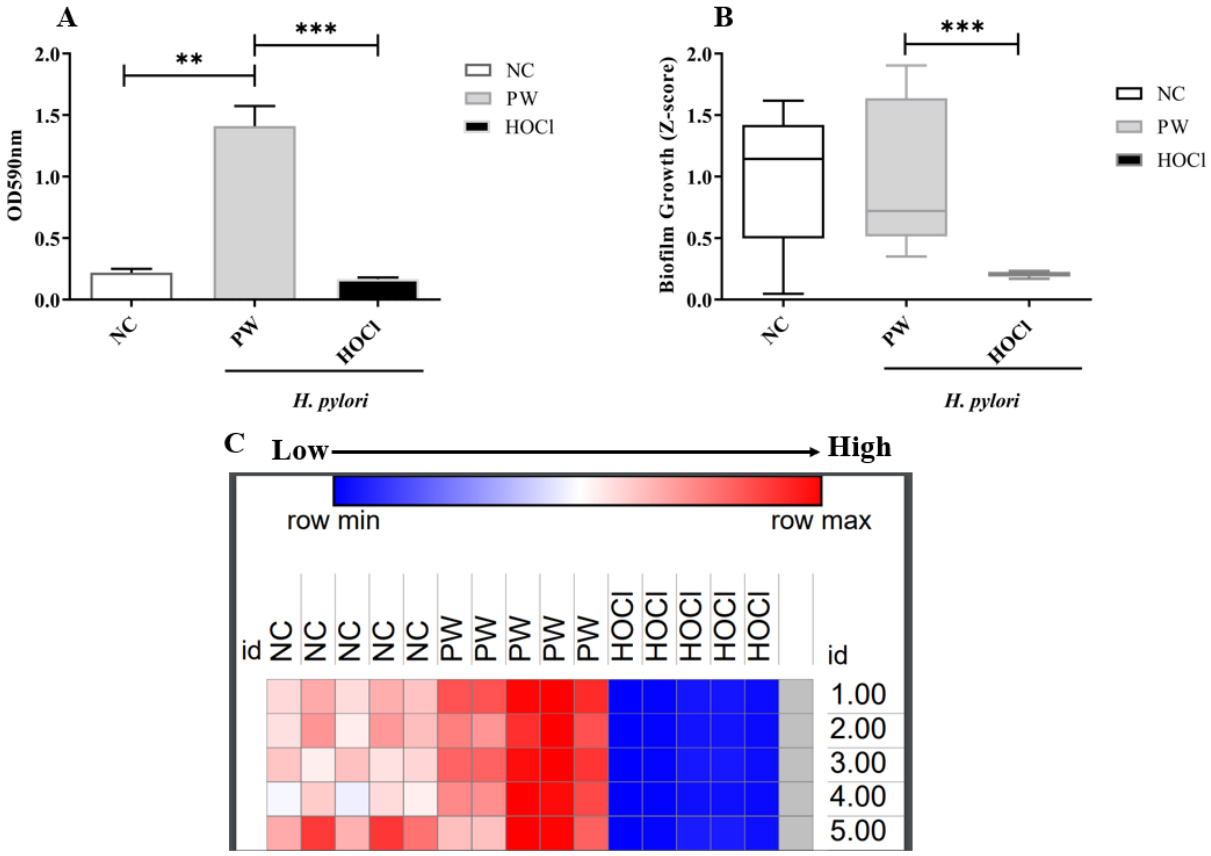


Figure 2. Inhibition of *H. pylori* bacterial growth by flowing biofilm methods *in vitro*. Data are shown as mean ± standard deviation (S.D.). n=5 ** $p < 0.01$, *** $p < 0.001$(one-way ANOVA, Tukey's post-hoc test). **NC:** normal mouse supplied with purified water (PW) without infection, **PW:** PW + *H. pylori*: mousse supplied with PW after *H. pylori* infection, **HOCl:** HOCl + *H. pylori*: mouse supplied with HOCl water after *H. pylori* infection. Data are shown as mean ± standard deviation (S.D.).

4.2. Effects of Non-electrolyzed HOCl Water on the Body Weight of *H. pylori* infected C57BL/6 Mice

To study the effects of consuming HOCl water, the mice's body weights were recorded on a weekly basis. In comparison to the NC group, our findings indicated that *H. pylori* infection caused a minor loss in body weight in both PW- and HOCl-treated groups, particularly from the 3rd to the 10th week; however, the difference was not statistically significant, as shown in supplemental (Figure 3).

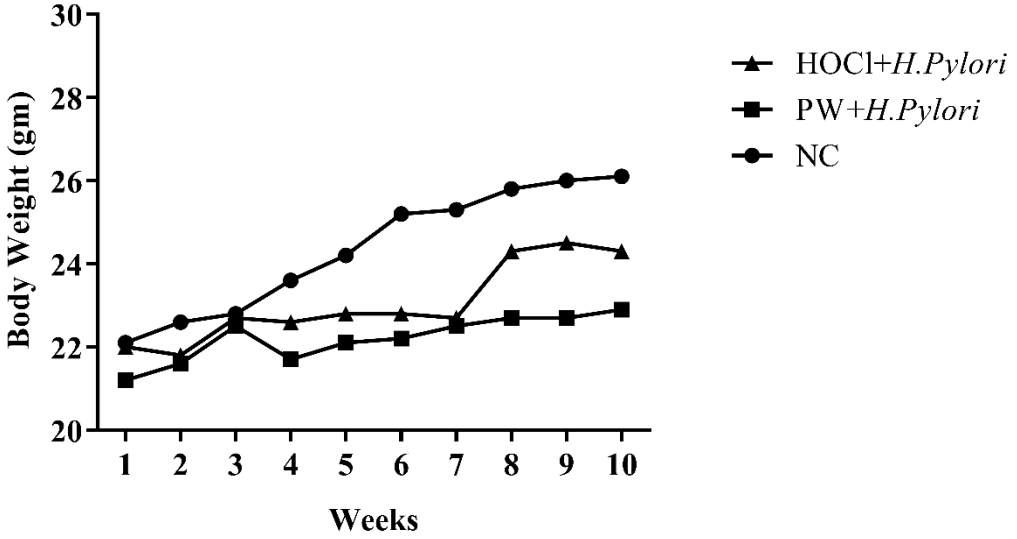


Figure 3. After 10 weeks, the effects of HOCl on body weight measurement on C57BL/6 mice. **NC:** normal mouse supplied with purified water (PW) without infection, **PW:** PW + *H. pylori*: mousse supplied with PW after *H. pylori* infection, **HOCl:** HOCl + *H. pylori*: mouse supplied with HOCl water after *H. pylori* infection. Data are shown as mean ± standard deviation (S.D.).

4.3. Histological Observation of the Stomach of *H. pylori*-infected C57BL/6 Mice

The stomach was cut 10 weeks post-infection, and the sections were observed under a light microscope. All mice were confirmed to be infected with *H. pylori* by observation of *H. pylori* bacteria in the superficial mucous layer and gastric pits of the stomach in both the PW + *H. pylori* and HOCl + *H. pylori* groups (Figure 4). NC mice partially showed inflammatory cells in the stomach tissue, but the level was negligible. Mice in the PW + *H. pylori* and HOCl + *H. pylori* groups showed mild inflammatory cell infiltration in the mucosal layer; however, there was no specific gastric mucosal injury, such as erosion or ulcer.

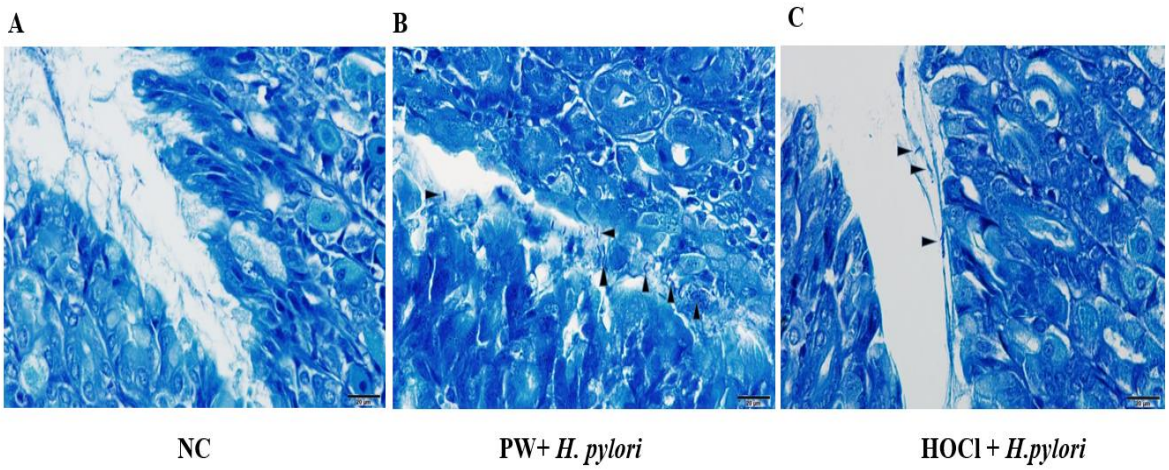


Figure 4. Representative histological images of the stomach after 10 wks. (A) NC: normal mouse supplied with purified water (PW) without infection, (B) PW + *H. pylori*: mousse supplied with PW after *H. pylori* infection, (C) HOCl + *H. pylori*: mouse supplied with HOCl water after *H. pylori* infection. Histological sections were stained with Giemsa solution. Scale bar = 20 μm.

4.4. Effects of Non-electrolyzed HOCl Water in *H. pylori* infected C57BL/6 Mice on Total WBC and its Differential Counts

WBCs are important for both the body's innate and adaptive immune responses. Any foreign invasion into the body causes the number of WBC to increase and the recruited WBCs to perform certain functions. In the total and differential WBC counts, the HOCl + *H. pylori* group showed a significant decrease in the total WBC count ($p < 0.001$), neutrophils ($p < 0.001$), lymphocytes ($p < 0.001$), and monocytes ($p < 0.01$) compared to the PW + *H. pylori* group (Figure 5).

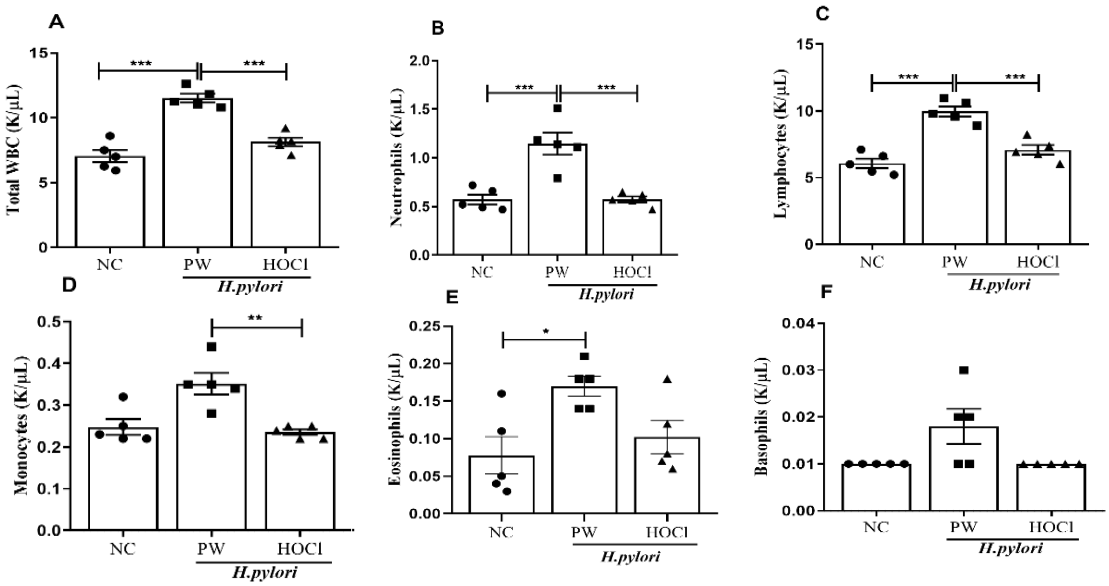


Figure 5. Effects of non-electrolyzed HOCl water on the number of total WBCs and differential WBC counts in *H. pylori*-infected mice. (A) Total WBCs, (B) Neutrophils, (C) Lymphocytes, (D) Monocytes, (E) Eosinophils, (F) Basophils. NC: normal control, PW: purified water, HOCl: hypochlorous acid water. Data are shown as mean ± standard deviation (S.D.), n = 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA, Tukey's post-hoc test).

4.5. Effects of Non-electrolyzed HOCl Water on OS Markers in *H. pylori* infected C57BL/6 Mice

We evaluated ROS and NO levels as well as GPx activity as OS indicators in the serum and stomach lysates to examine the effects of HOCl in C57BL/6 mice with *H. pylori* infection. When compared to the PW + *H. pylori* group, ROS levels in the HOCl + *H. pylori* group significantly decreased in both the serum and stomach lysates ($p < 0.05$) (Figure 6A-B).

When compared to the PW + *H. pylori* group, NO levels also exhibited a decreasing trend, although the difference was not statistically significant (Figure 6C-D). Meanwhile, GPx activity was significantly higher in the HOCl + *H. pylori* group than in the PW + *H. pylori* group in the serum ($p < 0.001$) and stomach lysate ($p < 0.05$) (Figure 6E-F).

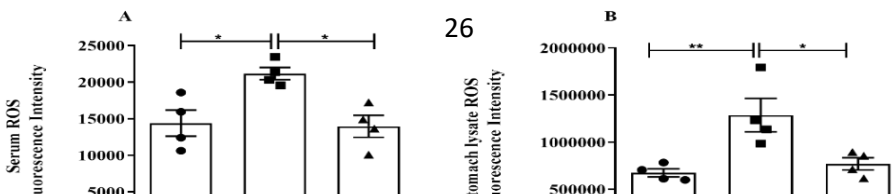


Figure 6. Effects of non-electrolyzed HOCl on the oxidative stress markers in *H. pylori* infected C57BL/6 mice after 10 wk. (A) ROS level in serum, (B) ROS level in stomach lysates, (C) NO level in serum, (D) NO level in stomach lysates, (E) GPx activity in serum, and (F) GPx activity in stomach lysates. NC: normal control, PW: purified water, HOCl: hypochlorous acid water. Data are shown as mean ± standard deviation (S.D.), n = 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$(one-way ANOVA, Tukey's post-hoc test).

4.6. Effects of Non-electrolyzed HOCl Water on the Intracellular Ca^{2+} Activity in *H. pylori* infected C57BL/6 Mice

Intracellular Ca^{2+} activity was assessed in both the serum and stomach lysates to investigate the possible mechanisms of *H. pylori* infection in the stomach. In the serum, the PW + *H. pylori* group showed a significant increase in Ca^{2+} activity compared with the NC group ($p < 0.01$); however, the HOCl + *H. pylori* group showed a significant decrease in Ca^{2+} activity compared with the PW + *H. pylori* group ($p < 0.05$) (Figure 7A). Similarly, in the stomach lysate, the PW + *H. pylori* group showed significantly higher Ca^{2+} activity than the NC group ($p < 0.05$); however, the HOCl + *H. pylori* group tended to show lower Ca^{2+} activity than the PW + *H. pylori* + *H. pylori* group ($p < 0.05$) (Figure 7B).

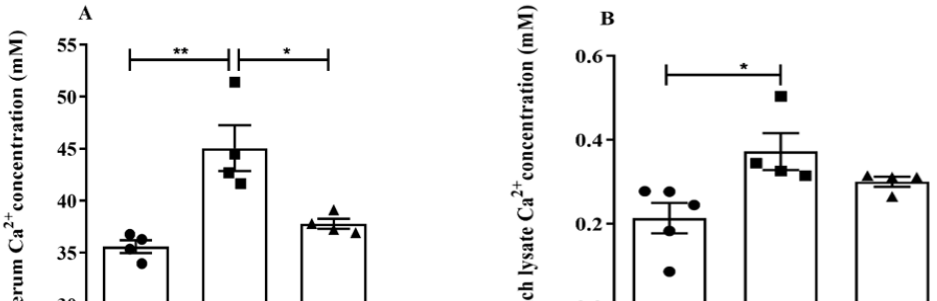


Figure 7. Effects of non-electrolyzed HOCl water on intracellular Ca^{2+} activity in *H. pylori* infected C57BL/6 mice. (A) Ca^{2+} activity in serum, (B) Ca^{2+} activity in stomach lysates. NC: normal control, PW: purified water, HOCl: hypochlorous acid water. Data are shown as mean ± standard deviation (S.D.), n = 5. * $p < 0.05$, ** $p < 0.01$(one-way ANOVA, Tukey's post-hoc test).

4.7. Effects of Non-electrolyzed HOCl on the Level of Inflammatory Cytokines in *H. pylori* infected C57BL/6 Mice

To investigate the impact of HOCl on the inflammatory response, we examined inflammatory cytokines such as GM-CSF, IL-1β, and TNF-α in [illegible] igure 8A-F). The HOCl + *H. pylori* infection group show [illegible] CSF ($p < 0.05$), IL-1β ($p < 0.01$), and TNF-α ($p < 0.001$) lev[illegible] o those in the PW + *H. pylori* group; however, there was no [illegible]

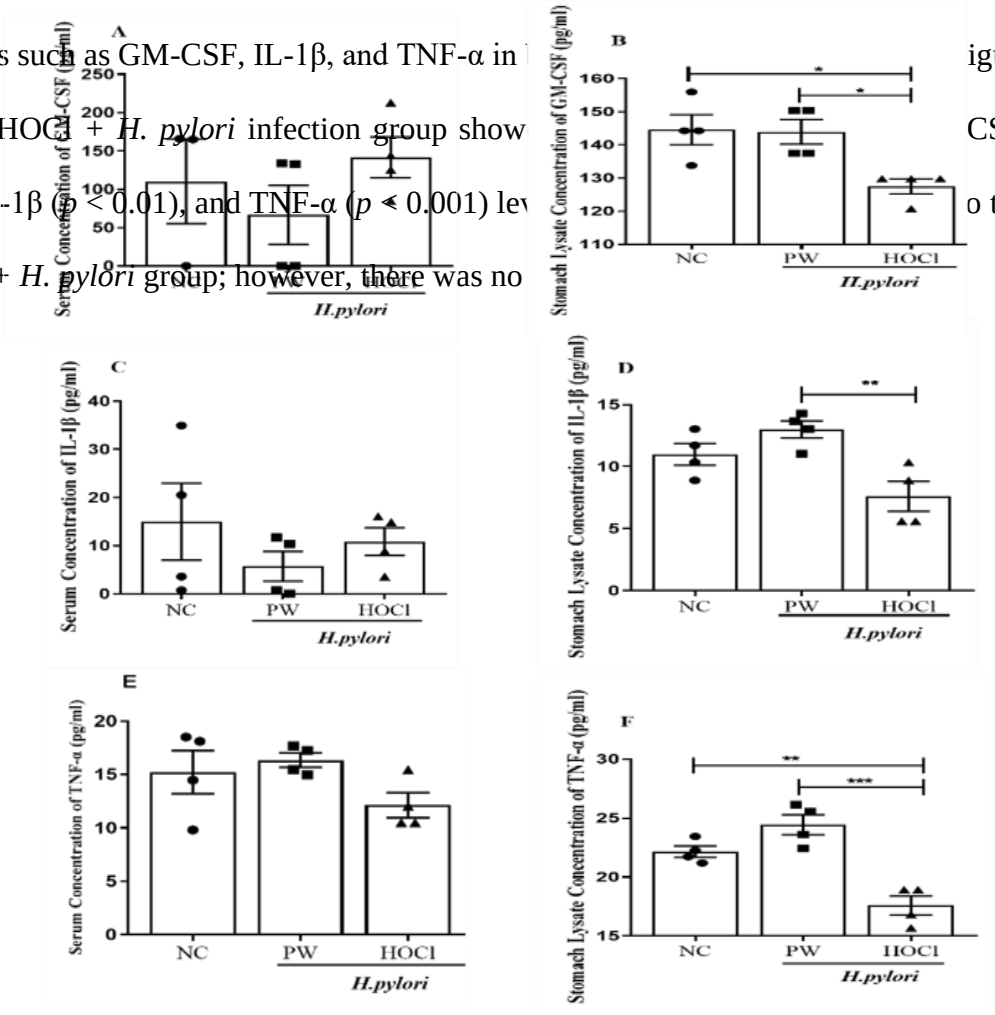
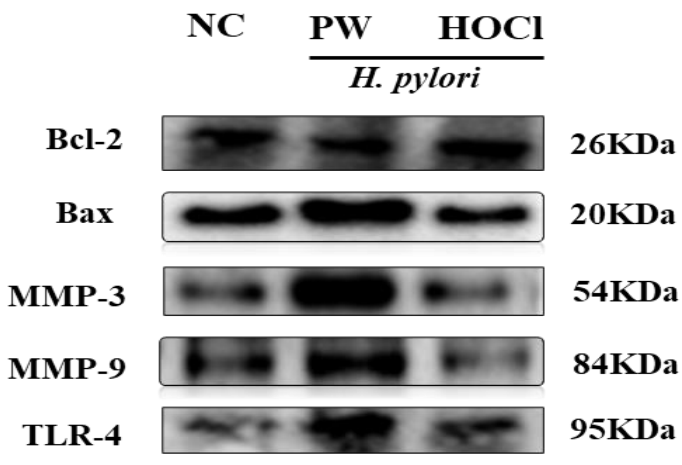


Figure 8. Effects of non-electrolyzed HOCl on the inflammatory response in serum and stomach lysates of C57BL/6 mice. Granulocyte/macrophage colony-stimulating factor (GM-CSF) in serum (A) and stomach lysate (B), IL-1β in serum (C) and stomach lysate (D), tumor necrosis factor alpha (TNF-α) in serum (E) and stomach lysate (F). NC: normal control, PW: purified water, HOCl: hypochlorous acid water. Data are shown as mean ± standard deviation (S.D.). n=5* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$(one-way ANOVA, Tukey's post-hoc test).

4.8. Effects of Non-electrolyzed HOCl on Bcl-2, Bax, MMP-3, MMP-9, and TLR-4 in Stomach Lysates

Through the use of western blot analysis, we investigated the underlying molecular mechanisms of HOCl water therapy against *H. pylori* infection and evaluated the protein

expression levels of Bcl-2, Bax, MMP-3, MMP-9, and TLR-4. We discovered that the PW + *H. pylori* infection group's expression level of Bcl-2 was substantially lower than that of the HOCl + *H. pylori* infection group's ($p < 0.01$) (Figure 9A). However, with HOCl therapy, the expression of Bax was reduced in comparison to that in the PW + *H. pylori* infection group. In contrast, we found that the level of Bax expression was significantly raised in the PW + *H. pylori* infection group ($p < 0.001$) compared to that in the NC group (Figure 9B). Additionally, we discovered that the expression levels of MMP-3 were considerably greater in the PW + *H. pylori* infection group ($p < 0.001$) than in the HOCl + *H. pylori* infection group (Figure 9C). The expression level of MMP-9 was also considerably higher in the PW + *H. pylori* infection group than in the HOCl + *H. pylor*i infection group ($p < 0.01$) (Figure 9D). Similarly, we found that TLR-4 expression levels in the PW group were significantly higher ($p < 0.05$) than in the HOCl + *H. pylori* infection group (Figure 9E). These findings suggest that oral gavage of HOCl in the stomach reduces *H. pylori*-related infection in C57BL/6 mice.



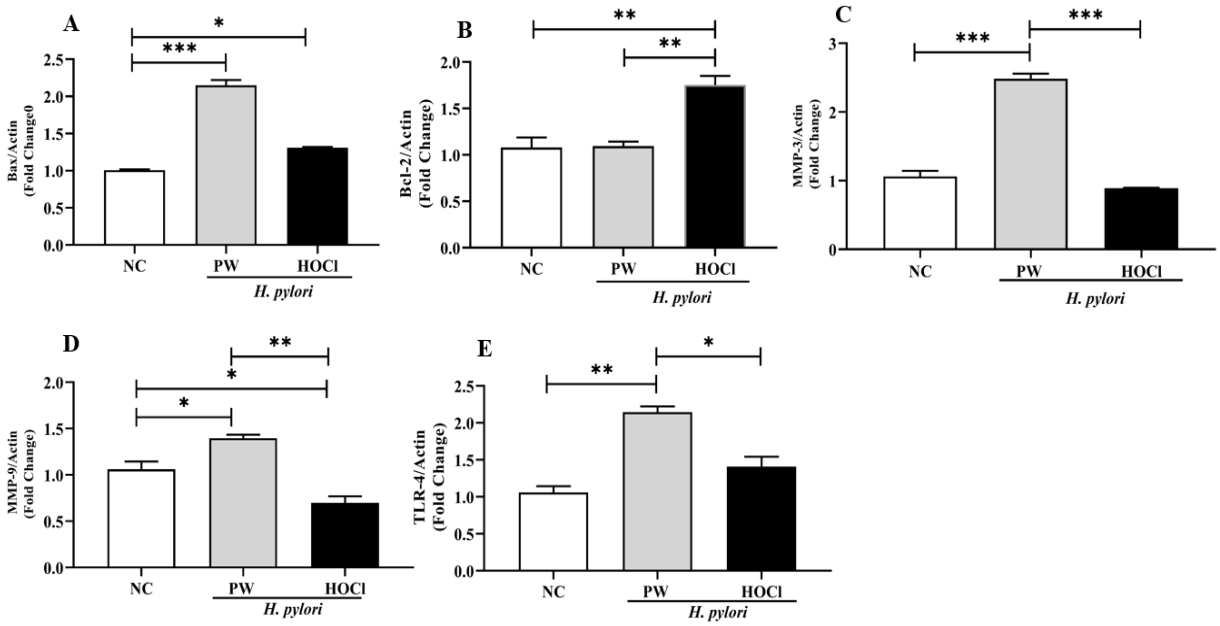


Figure 9. Effects of non-electrolyzed HOCl treatment on Bcl-2, Bax, MMP-3, MMP-9, and TLR-4 protein levels in the stomach lysate of *H. pylori*-infected C57BL/6 mice. (A) Bcl-2 antibody-reactive quantified band intensity in stomach lysates, (B) Bax protein antibody-reactive quantified band intensity. (C) MMP-3 protein antibody-reactive quantified band intensity, (D) MMP-9 protein antibody-reactive quantified band intensity. (E) TLR-4 protein antibody-reactive quantified band intensity. Band intensity of each protein marker is normalized to the total in the bar graphs. NC: normal control, PW: purified water, HOCl: hypochlorous acid water. Data are shown as mean ± standard deviation (S.D.). n=5 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$(one-way ANOVA, Tukey's post-hoc test).

4.9. Anti-inflammatory Effects of Non-electrolyzed HOCl on MMP-3, MMP-9, TLR-2, and TLR-4 in Stomach Lysates *in vivo*

Through the use of western blot analysis, we investigated the underlying molecular mechanisms of HOCl water therapy against *H. pylori* infection and evaluated the protein

expression levels of MMP-2, MMP-9, TLR-2, and TLR-4. We found that the protein expression of MMP-2, MMP-9, TLR-2, and TLR-4 significantly reduced HOCl + *H. pylori* ($p < 0.001$) group in (Figure 10A-D) as compared to PW + *H. pylori* group. We also confirm endpoint RT-PCR of the following gene (GAPDH, TLR-2, TLR-4, MMP-3, and MMP-9) in Figure **11**.

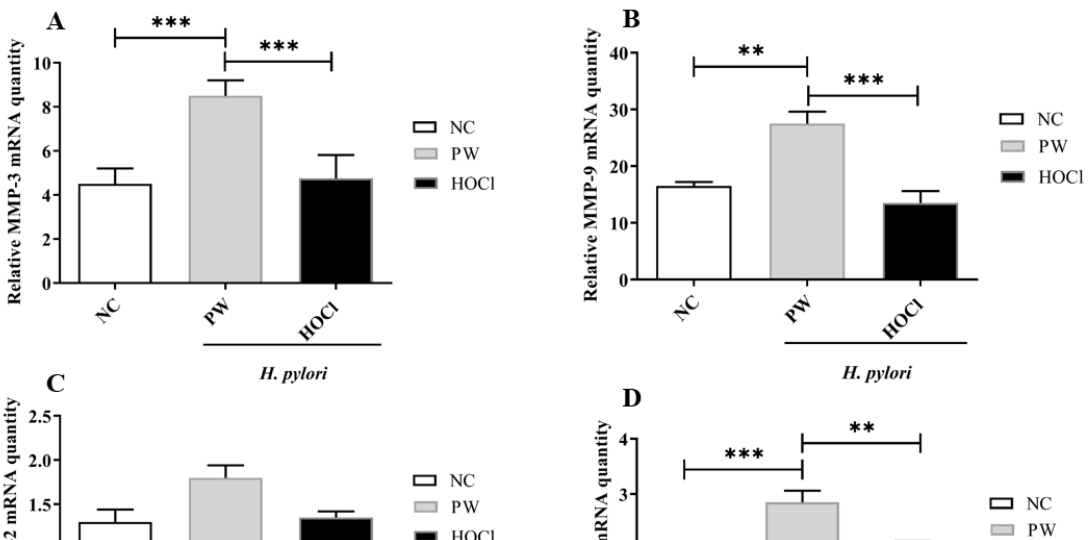


Figure 10. qPCR analysis of the expression of MMP-3 (A), MMP-9 (B), TLR-2 (C), and TLR-4 (D). **NC:** normal mouse supplied with purified water (PW) without infection, **PW:** PW + *H. pylori*: mousse supplied with PW after *H. pylori* infection, **HOCl:** HOCl + *H. pylori*: mouse supplied with HOCl water after *H. pylori* infection. Data are shown as mean ± standard deviation (S.D.). n= 5 ** $p < 0.01$, *** $p < 0.001$(one-way ANOVA, Tukey's post-hoc test).

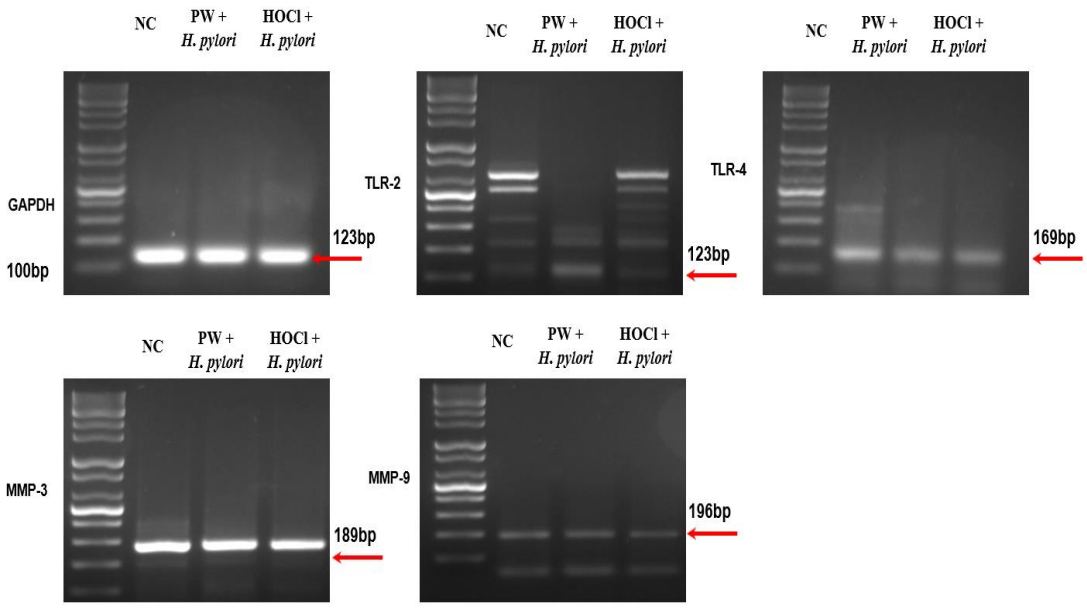


Figure 11. End-point RT-PCR for **GAPDH**, TLR-2, TLR-4, MMP-3 and MMP-9 gene. **NC:** normal mouse supplied with purified water (PW) without infection, **PW:** PW + *H. pylori*: mousse supplied with PW after *H. pylori* infection, **HOCl:** HOCl + *H. pylori*: mouse supplied with HOCl water after *H. pylori* infection.

IV. DISCUSSION

In the present study, we evaluated the anti-oxidative, anti-apoptotic, and anti-inflammatory effects of non-electrolyzed HOCl water treatment in *H. pylori* infected C57BL/6 mice model. In consequence, drinking non-electrolyzed HOCl water significantly suppressed oxidative and inflammatory response in *H. pylori* infected C57BL/6 mice through redox regulation and immune response. *H. pylori* is known to cause a chronic immunological response in the

stomach, including persistent OS. It is known to be the first bacterial pathogen to be identified as a carcinogen [39]. *H. pylori* can cause a long-term inflammatory response that promotes carcinogenesis [40]. However, *H. pylori* can potentially defend itself from the immunological response of the host by triggering macrophage death. OS which is caused by the *H. pylori* infection can cause DNA damage. *H. pylori*'s propensity to cause DNA strand breaks adds to genomic instability and may aid carcinogenesis [41]. In addition, development of antibiotic resistance of *H. pylori* results in failure to eradication of *H. pylori* and associated problems. Towards this, combination of antibiotics including proton pump inhibitors is a popular choice of treatment regarding the eradication of *H. pylori*. However, such combination therapy accompanied with severe side effects such as antibiotic resistance towards *H. pylori* strain [42] [43]. Therefore, non-antibiotics agent such as non-electrolyzed acidic water might be highly effective and safe for the treatment of both antibiotic susceptibility and resistance *H. pylori* bacteria [44]. Recently, studies on HOCl have shown its numerous effects on the eradication of several pathogenic bacteria including *H. pylori* [45] [46].

Towards this, we investigated the bactericidal effect of oral administration of the non-electrolyzed HOCl against *H. pylori*. In our histological observation, the mice both in PW + *H. pylori* and HOCl + *H. pylori* groups showed mild inflammatory cell infiltration in the mucosal layer, however there was no specific gastric mucosal injury such as erosion and ulcer. This suggests that drinking HOCl water is quite safe on the histological damage of mouse stomach compared to drinking PW. Meanwhile, the number of WBCs can be used as an inflammatory indicator to determine severity of various gastric diseases including gastric cancers, and H. pylori infection [47]. In our investigation, WBC count result showed significant decrease in number of total WBC, neutrophils, lymphocytes, and monocytes in HOCl + *H. pylori* group compared to PW + *H. pylori* group. This result implys that drinking HOCl water may affect

systemic immune response in *H. pylori*-infected mice model. The neutrophils in tissue, on the other hand, are unable to eliminate the built colony of *H. pylori* via paracrine secretion, allowing *H. pylori* to stay for a long time. Furthermore, it causes active chronic inflammation of the stomach mucosa. Inducible nitric oxide synthase (iNOS), a crucial enzyme that catalyzes the synthesis of NO and then evolves with O_2 to peroxynitrite has been discovered in the host neutrophils and epithelial cells. This process promotes the creation of more ROS and RNS, aggravating the OS response [48]. When compared to the untreated mice, iNOS gene-deficient mice showed a substantial reduction in the incidence of stomach cancer after *H. pylori* infection. As a result, it's been claimed that iNOS-induced OS is directly associated with the development of stomach cancer [49]. These investigations demonstrated a rise in genomic alterations, indicating that OS develops soon after infection [50]. Our study also revealed that *H. pylori*-infected mice produced more ROS, which could be one of the mechanisms causing infection-related apoptosis. NO is a signaling molecule with strong immunomodulatory properties and is linked to a key host defense component in H. pylori infection. One of the most important findings of our current study is that NO generated by activated macrophages can destroy extracellular *H. pylori*. Although, *H. pylori* have been shown to activate or use several signaling pathways within the host gastric cell, which can result in ulcers or gastric cancer [47] [51]. NO can stop 8-oxoguanine glycosylase from removing DNA mutations [52]. ROS produced by *H. pylori* infection, on the other hand, can damage DNA. These studies also demonstrated that *H. pylori* infection increases intracellular ROS in the gastric epithelial cell [53]. Antioxidants like GPx have been shown to lower ROS production and inhibit *H. pylori*-induced programmed cell death, which has implications for the treatment and prevention of this chronic infectious disease. Therefore, our results indicate that non-electrolyzed HOCl treatment attenuated OS by suppressing ROS, and NO levels, and increasing GPx levels. Additionally, Ca^{2+} level was significantly decreased in HOCl + *H. pylori* group both in serum and stomach lysate as

compared to PW + *H. pylori* group. However, the methods and exact mechanisms of how *H. pylori* infection causes calcium mobilization in gastric cancer cells are still unknown. Most of the studies are concentrated on DNA methylation and tissue damage is caused by OS, however, the specific molecular mechanism is still unknown. The signaling pathways involved in the damage by OS, are highly broad, stimulating not only the generation of inflammatory molecules factors (IL-8, IL-6, and IL-1β) but also the activation of GM-CSF and TNF-α [54]. The exact mechanisms, of how *H. pylori* infection induces inflammation in gastric diseases including cancer cells remain elusive. A variety of gene polymorphisms have been researched, with the IL-1β, GM-CSF, and TNF-α genes being the most commonly studied [55].

Long-term *H. pylori* infection allows these toxins to promote the production of TNF-α by gastric epithelial cells [56]. IL-1β is a powerful inhibitor of stomach acid release [57]. In the presence of *H. pylori*, IL-1β is upregulated, and it is necessary for the initiation and amplification of inflammatory responses to infection [58]. In our study, significant changes were observed in the production of some cytokines in both serum and stomach lysate samples among the three groups. As a result, non-electrolyzed HOCl therapy drastically lowered the levels of inflammatory cytokines such as IL-1β, TNF-α, and GM-CSF, which were previously investigated in C57BL/6 mice in response to *H. pylori* infection. Targeted therapy, on the other hand, might be able to provide a pathological foundation for lowering the risk of early gastric cancer [59].

H. pylori infection contributes to the buildup of molecular genetic defects, and the Bax/Bcl-2 ratio offers potential in screening and early cancer identification among H. pylori-infected patients. In patients with stomach cancer, H. pylori infection has been demonstrated to increase the expression of Bax and Bcl-2 protein markers [60]. As a result, Bcl-2 expression in gastric cancer was higher than Bax expression, and the computed Bax/Bcl-2 ratio in cancer was several

times lower than that in neighboring normal mucosa, as previously reported [61]. According to our findings, Bax protein expression levels were significantly decreased with HOCl treatment in *H. pylori* infection mice whereas Bcl-2 levels were found significantly increased in HOCl treated *H. pylori* infection group, which is consistent with the fact that apoptosis is reduced in stomach tissues with HOCl water treatment.

One of the studies has reported that MMP-9 and tissue inhibitor of metalloproteinases-1 (TIMP-1) production near the ulcer's margin had a strong positive correlation [28]. MMPs' cell surface localization implies that they interact with membrane protein complexes or receptors that sense their surroundings. MMP production is impacted by the lysis of the extracellular matrix (ECM) by MMPs because they alter focal adhesions and activate cellular signaling molecules like ERKs extracellular signal-regulated kinases (ERKs), which encourage cellular migration [62 63]. MMP-9 and MMP-3 expression as well as TIMP-1 expression all increased in individuals with gastric ulcers. Moreover, MMP-9 production has also been linked to a higher risk of recurrence of gastric ulcers [64]. In addition, another study has reported that, MMP-3 and MMP-9 may play a key role in the development of gastric ulcers [65]. Together, MMPs, heterogeneous cell populations, and extracellular matrix reciprocally regulate each other. The proteolysis network in gastric pathology has been thoroughly studied, and some MMPs, like MT1-MMP, have recently attracted attention as potential diagnostic/therapeutic tools [666768]. With this, our results revealed that treatment with non-electrolyzed HOCl significantly decreased the MMP-3 and MMP-9 production in gastric epithelial cells in stomach lysates. Furthermore, evidences have suggested that TLRs are essential as adaptive immunity activators and are involved in the regulation of inflammation during the innate immune response to *H. pylori*. By triggering TLR-4 on the surface of *H. pylori* to create $O_2^{\cdot}$. NOX expression is elevated in gastric epithelial cells by *H. pylori.* According to one of the recent studies

employing mice models of infection, TLR signaling plays a role in modulating *H. pylori*-mediated T cell responses, stomach immunopathology, and colonization *in vivo* [6970]. With this, our results demonstrated that treatment with non-electrolyzed HOCl significantly decreased the TLR-4 production in gastric epithelial cells in stomach lysates. Overall, our results demonstrated that non-electrolyzed HOCl treatment ameliorated *H. pylori* infection from oxidative and inflammatory stress in C57BL/6 mice and might be one of the therapeutic potentials against *H. pylori*-infection related diseases such as gastric cancer, stomach cancer, and peptic ulcer, etc.

V. CONCLUSION

Collectively, based on our results, we demonstrate that non-electrolyzed HOCl water has anti-oxidative, anti-inflammatory, and anti-apoptotic effects through redox and immune regulation mechanism, and might be a good therapeutic potential against *H. pylori*-infection related

diseases. However, the drinking safety of HOCl water has not been sufficiently confirmed, although external use of HOCl is considerably safe and effective for humans. And more studies are needed to fully understand the effects of genetic variants at the immune redox level, as well as their roles and effects on *H. pylori* susceptibility.

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VII. 국문초록

Abstract

차아염소산은 인체 내에서 자연스럽게 생성되는 화학물질로서 선천적 면역반응에 부분적으로 관여한다. 최근, 비전기분해식으로 생성되는 차아염소산수가 소독제로서 연구자들의 주목을 끌고 있는데 이 물질은 강한 살균력과 높은 생체

안전성, 그리고 용이한 접근성을 특징으로 한다. 비전해식 차아염소산수는 차아염소산염과 미네랄을 기본으로 하여 물질들을 특정한 비율로 혼합함으로써 생성되며 높은 산화력을 나타낸다. 본 연구는 *Helicobacter pylori* (*H. pylori*)를 인위적으로 감염시킨 C57BL/6 mice 에게 비전해식 차아염소산수를 10 주 동안 음용하게 한 후 효과를 확인하고자 수행되었다. 실험 동물은 세 군[헬리코박터 감염 없이 정수물(purified water, PW)을 음용한 정상군(normal control group, NC 군), 헬리코박터 감염 후 PW 를 음용한 PW + *H. pylori* 군, 헬리코박터 감염 후 차아염소산수를 음용한 HOCl + *H. pylori* 군]으로 나누어 진행되었다. 음용수는 마우스용 물병을 이용하여 충분히 공급되었고 매일 새 물로 교체하였다.

10 주 음용 후 총 백혈구 수와 각각의 백혈구(호중성구, 림프구, 단핵구, 호산성구) 수는 PW + *H. pylori* 군과 비교하여 HOCl + *H. pylori* 군에서 유의하게 감소된 것으로 나타났다. 마찬가지로, HOCl + *H. pylori* 군의 활성산소와 칼슘 이온 활성은 PW + *H. pylori* 군과 비교하여 혈중과 위장 조직에서 모두에서 유의한 감소를 나타내었다. 이와 달리 차아염소산수를 음용한 동물군의 GPx 활성은 혈액과 위장조직 모두에서 정수물 음용군과 비교하여 유의한 증가를 보여주었다. 또한 granulocyte-macrophage colony-stimulating factor, IL-1β, TNF-α cytokine 수준은 정수물 음용군과

비교하여 HOCl + *H. pylori* 군의 위장 조직에서 유의하게 감소하였다. Bax, MMP-3, MMP-9, TLR-4 수준은 차아염소산수 음용군에서 감소한 반면 Bcl2 은 위장조직에서 증가한 것으로 나타났다. 결론적으로, 본 연구 결과는 *H. pylori* 에 감염된 마우스가 비전해 차아연소산수를 음용하였을 때 항산화 효과, 항염증 효과 및 항세포자살 효과를 나타내는 것으로 확인되었으며 이것은 *H. pylori* 감염 동물 모델에서 비전해 차아염소산수가 산화환원 및 면역 조절 기전에 관여하였기 때문으로 판단된다. 그러나 *H. pylori* 감염에 대한 비전해 차아염소산수의 치료 기전과 생체 적용 방법에 대해서는 더 깊이 있는 연구가 이루어져야 할 것으로 사료된다.

핵심단어: 비전해 차아염소산수, *Helicobacter pylori*, 항산화 효과, 항염증 효과, 항세포자살 효과, 산화환원 및 면역 조절

VIII. PUBLICATION LIST

International Journal

1. **Rahman, M. H**., Bajgai, J., Fadriquela, A., Sharma, S., Trinh Thi, T., Akter, R., ... & Lee, K. J. (2021). Redox effects of molecular hydrogen and its therapeutic efficacy in the treatment of neurodegenerative diseases. Processes, 9(2), 308.

2. **Rahman, M. H.,** Bajgai, J., Fadriquela, A., Sharma, S., Trinh, T. T., Akter, R., ... & Lee, K. J. (2021). Therapeutic potential of natural products in treating neurodegenerative disorders and their future prospects and challenges. *Molecules*, *26*(17), 5327.

3. Jeong, E. S., Bajgai, J., You, I. S., **Rahman, M. H.,** Fadriquela, A., Sharma, S., ... & Lee, K. J. (2021). Therapeutic effects of hydrogen gas inhalation on trimethyltin-induced neurotoxicity and cognitive impairment in the C57BL/6 mice model. *International Journal of Molecular Sciences*, *22*(24), 13313.

4. Bajgai, J., Kim, C. S., **Rahman, M. H.,** Jeong, E. S., Jang, H. Y., Kim, K. E., ... & Lee, M. (2022). Effects of alkaline-reduced water on gastrointestinal diseases. *Processes*, *10*(1), 87.

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6. Sharma, S., Lee, K. J., Bajgai, J., Trinh, T. T., Antonio, J. M., **Rahman, M. H.,** ... & Kim, Y. (2022). Anti-Oxidative and Anti-Diabetic Effects of Electrolyzed Weakly Alkaline Reduced Water on Renal Proximal Tubular Epithelial Cells. *Processes*, *10*(10), 2025.

7. Sharma, S., Bajgai, J., Antonio, J. M., Fadriquela, A., Trinh, T. T., **Rahman, M. H.**, ... & Lee, K. J. (2022). Anti-Hyperglycemic Effect of Magnesium-Enhanced Alkaline-Reduced Water on High Glucose-Induced Oxidative Stress in Renal Tubular Epithelial Cells. *Processes*, *10*(5), 919.

8. Trinh, T. T., Fadriquela, A., Bajgai, J., Sharma, S., **Rahman, M. H.,** Goh, S. H., ... & Lee, K. J. (2021). Anti-oxidative effect of weak alkaline reduced water in raw 264.7 murine macrophage cells. *Processes*, *9*(11), 2062.

9. Trinh, T. T., Fadriquela, A., Lee, K. J., Bajgai, J., Sharma, S., **Rahman, M. H.,** ... & Jeon, H. T. (2021). Development of alkaline reduced water using high-temperature-roasted mineral salt and its antioxidative effect in raw 264.7 murine macrophage cell line. *Processes*, *9*(11), 1928.

Korean Journal

1. **Rahman, M. H.,** Bajgai, J., Fadriquela, A., Kim, C. S., & Lee, K. J. (2019). Characteristics and Anti-bacterial Effects of Mineral Supplement-Hypochlorous Acid Water on Human Pathogenic Bacteria. 한국물학회지 Vol, 7(1).

2. **Rahman, M. H.,** Kim, C. S., & Lee, K. J. (2021). Hydrogen Mineral Disinfectant Water and its Application in Agriculture and Livestock Farming. *한국물학회지 Vol*, *9*(1).

3. **Rahman, M. H.,** Bajgai, J., Fadriquela, A., Sharma, S., Thuy, T. T., Hoon, G. S., & Lee, C. S. K. K. J. (2020). 차아염소산수 공중 분무를 통한인체 병원성 황색포도상구균 살균효과 평가. *한국물학회지 Vol*, *8*(1).

4. Bajgai, J., Kim, C. S., **Rahman, M. H.,** Fadriquela, A., Thuy, T. T., Song, S. B., & Lee, K. J. (2020). Application of New Concept Disinfectant, Huureka®, On Livestock Farming. *한국물학회지 Vol*, *8*(1).

5. Sharma, S., Bajgai, J., Fadriquela, A., **Rahman, M. H.,** Thuy, T. T., Goh, S. H., ... & Lee, K. J. (2020). The Effect of a Granule-type Anti-hangover Compound, Quechung, on Acute

Alcohol-induced Hangover in Healthy Subjects: a Randomized Crossover Study. *한국물학회지 Vol*, *8*(1).

National or Other Professional Meetings Attended (Indicate the Meeting Title, Oral or Poster Presentation).

No.	Presenter	Title	Type of Presentation	Name of Conference	Date	Venue
1.	Ailyn Fadriquela, Johny Bajgai**, Md. Habibur Rahman**, Dong-Heui Kim, Cheol-Su Kim, Kyu-Jae Lee	Effects of Drinking Mineral Supplement Hypochlorous Acid Water on Oxidative Stress andLiver Function	Poster	2019, Gangwon Bio International Symposium	Sept, 25th, 2019	Chuncheon Bears Hotel, Chuncheon, Korea
2.	Rahima Begum, Cheol-Su Kim, AilynFadriquela, **Johny Bajgai**, Xingyu Jing, Dong-Heui Kim,	Molecular HydrogenInhibits the MAPK Signaling Pathway via the Nrf2 against Oxidative Stress	Poster (Best Poster Award)	2019, Gangwon Bio International Symposium	Sept, 25th, 2019	Chuncheon Bears Hotel, Chuncheon, Korea

	Soo-Ki Kim and Kyu-Jae Lee	in RAW 264.7 Macrophage Cells				
3.	**Md. Habibur Rahman**, Johny Bajgai, Ailyn Fadriquela, Cheol-SuKim, Kyu-Jae Lee	Anti-Bacterial Effects of Mineral Supplement-Hypochlorous Acid Water on Pathogenic Bacteria for Food Products	Poster	2019, Gangwon Bio International Symposium	Sept, 25th, 2019	Chuncheon Bears Hotel, Chuncheon, Korea
4.	Johny Bajgai, Ailyn Fadriquela, **Md. Habibur Rahman**, Dong HeuiKim, Cheol-Su Kim, Soo-Ki Kim, and Kyu-Jae Lee	Effects of Hovenia dulcis and Stevia rebaudiana Extract on Acute Alcohol-induced Hangover in Sprague-Dawley Rats	Poster	15th International Conference on Toxicogenomics(ICT)	Nov 5-6, 2019	Nest Hotel, Incheon
5.	Ailyn Fadriquela, Johny Bajgai**, Md. Habibur Rahm**an, Dong-Heui Kim, Cheol-Su Kim, Kyu-Jae Lee	Effects of Drinking Mineral Supplement Hypochlorous Acid Water on Oxidative Stress andLiver Function	Poster	15th International Conference on Toxicogenomics(ICT)	Nov 5-6, 2019	Nest Hotel, Incheon
6.	Ailyn Fadriquela, Subham Sharma, Johny Bajgai**, Md. Habibur Rahman**, Goh Seong Hoon, Kyu-Jae Lee, Cheol-Su Kim and Soo-Ki Kim	Redox profiling forprogression of colorectal cancer inTrp53 transgenic mice	Poster (Best Poster Award)	16th International Conference on Toxicogenomics (ICT) (Genome information andNovel biomaterials)	Nov 4, 2020	Online
7.	Johny Bajgai, **Md. Habibur Rahman**, Ailyn Fadriquela, Subham Sharma, Dong Heui Kim, GohSeong Hoon, In Soo You, Yun Su Na, Nam Kyu	Antiamnesic Effectof Molecular Hydrogen in C57BL/6 Mouse Model of TMT-induced Learning and Cognitive Dysfunction	Poster	16th International Conference on Toxicogenomics (ICT) (Genome information and Novel biomaterials)	Nov 4, 2020	Online

	Kong, Soo-Ki Kim, Cheol- Su Kim and Kyu-JaeLee					
8.	**Md. Habibur Rahman,** Johny Bajgai, Ailyn Fadriquela, SubhamSharma, Dong HeuiKim, Goh Seong Hoon, Soo-Ki Kim, Cheol-Su Kim and Kyu-Jae Lee	Safety and Redox Effects of Mineral Supplement Hypochlorous AcidWater on C57BL/6Mouse Model	Poster	16th International Conference on Toxicogenomics (ICT) (Genome information and Novel biomaterials)	Nov 4, 2020	Online
9.	Johny Bajgai, **Md. Habibur Rahman**, Ailyn Fadriquela, Subham Sharma, Dong Heui Kim, GohSeong Hoon, In Soo You, Yun Su Na, Nam Kyu Kong, Soo-Ki Kim, Cheol-Su Kim and Kyu-Jae Lee	Redox Effects of Molecular Hydrogenin C57BL/6 Mouse Model of TMT-Induced Cognitive Dysfunction	Poster	2020 International Conference of Korean Water Society	Nov 6-8, 2020	Tour Complex Andong, South Korea
10.	**Md. Habibur Rahman**, Ailyn Fadriquela, Johny Bajgai, Goh Seong Hoon, Cheol-Su Kim, Kyu-Jae Lee	Long-Term Skin Safety Effect of Chlorine-Rich Sterilized Water Treatment on C57BL/6 mice	Poster	2020 International Conference of Korean Water Society	Nov 6-8, 2020	Tour Complex Andong, South Korea
11.	Trinh Thi Thuy, Johny Bajgai**, Md. Habibur Rahman**, Ailyn Fadriquela, Subham Sharma, GohSeong Hoon, Cheol- Su Kim and Kyu-JaeLee	Application of Hydrogen for the Health Improvementin Vietnam	Poster	2020 International Conference of Korean Water Society	Nov 6-8, 2020	Tour Complex Andong, South Korea

12.	**Md. Habibur Rahman**, Jayson Antonio, Johny Bajgai, Ailyn Fadriquela, SubhamSharma, Trinh Thi Thuy, Yun Ju Jeong, Goh Seong Hoon, Cheol-Su Kim and Kyu-Jae Lee *	Anti-Bacterial Effects of Hypochlorous Acid (HOCl) Produced byNon-Electrolysis MixingMethod Against Salmonella Species	Poster/Oral	45th ISMH CONGRESS, International Society of Medical Hydrology and Climatology 2021	June 10-11, 2021	Dax-France
13.	Thuy Thi Trinh, Subham Sharma, Ailyn Fadriquela, Johny Bajgai, **Md. Habibur Rahman,** YunJu Jeong, Song Sik Khang, Woo Rham Khang, Seong Hoon Goh, Cheol Su	Anti-Inflammatory, Anti-Oxidative andImmune Redox Effects of Alkaline Reduced Water in RAW 264.7 Macrophage Cell Line	Poster/Oral	45th ISMH CONGRESS, International Society of Medical Hydrology and Climatology 2021	June 10-11, 2021	Dax-France
14.	**Md. Habibur Rahman**†, Johny Bajgai†, Cheol-Su Kim and Kyu-Jae Lee *	Therapeutic and Redox Effect of Mixed Hydrogen Mineral Disinfectant Hypochlorous Acid Water on Helicobacter pylori Infected C57BL/6 Mouse Model	Poster	17th International Conference on Toxicogenomics (ICT) (Genome information and Novel biomaterials)	Nov 3, 2021	Online
15.	Vira Kchorng, Subham Sharma, Johny Bajgai, AilynFadriquela, **Md. Habibur Rahman**, Cheol-Su Kim, Kyu-Jae Lee	Post-use Survey Study of Feminine Cleanser	Poster	2021, Northeast Asia Culture and Tourism International Seminar with Korean Water Society	Nov 19-21	Lotus Complex 101,Daegu

16.	Vira Kchorng, **Md. Habibur Rahman**, Johny Bajgai, Ailyn Fadriquela, Subham Sharma, Cheol-Su Kim, Kyu-Jae Lee	Anti-bacterial and Anti-fungal Effects of Feminine Cleanser	Poster	2021, Northeast Asia Culture and Tourism International Seminar with Korean Water Society	Nov 19-21	Lotus Complex 101,Daegu
17.	**Md. Habibur Rahman**†, Johny Bajgai†, Cheol-Su Kim and Kyu-Jae Lee *	Therapeutic and Redox Effect of Mixed Hydrogen Mineral Disinfectant Hypochlorous Acid Water on Helicobacter pylori Infected C57BL/6 Mouse Model	Poster	2021, Northeast Asia Culture and Tourism International Seminar with Korean Water Society	Nov 19-21	Lotus Complex 101,Daegu
18.	Subham Sharma, Jayson M. Antonio, Johny Bajgai**,** ThuyThi Trinh, **Md. Habibur Rahman**, Kchorng Vira, Abdul-Nasir Sofian, Cheol-Su Kim, Kyu-Jae Lee*	Anti-Diabetic andAnti-Oxidative Effect of Alkaline Reduced Water inHK-2 Cells	Poster Presentation	The 29th Federation Meetingof Korean Basic Scientists June (2022)	30 June-1 July, 2022	Gwangju, South Korea
19.	**Md. Habibur Rahman**†, Johny Bajgai†, Yoojin Cho, Ailyn Fadriquela, SubhamSharma, Trinh Thi Thuy, Yun Ju Jeong,Goh Seong Hoon, Cheol-Su Kim, and Kyu-Jae Lee*	Anti-oxidative, Anti-apoptotic, and Anti-inflammatory Effectsof Non-Electrolyzed Hypochlorous Acid Water on *Helicobacter pylori* infected C57BL/6 Mouse Model	Poster Presentation	The 29th Federation Meetingof Korean Basic Scientists June (2022)	30 June-1 July, 2022	Gwangju, South Korea
20.	Subham Sharma, Johny Bajgai, AilynFadriquela, Thuy ThiTrinh, **Md. Habibur Rahman**, Cheol-	Protective Effect of Hydrogen Gas in Airway Epithelial Cells Against Oxidative Stress	Poster Presentation	The 29th Federation Meetingof Korean Basic Scientists June (2022)	30 June-1 July, 2022	Gwangju, South Korea

	Su Kim, Kyu-Jae Lee *					
21.	Vira Kchorng, Jayson M. Antonio, **Md. Habibur Rahman**,Ailyn Fadriquela, Johny Bajgai, Subham Sharma, Trinh Thi Thuy, Abdul Nasir Sofian,Cheol-Su Kim, and Kyu-Jae Lee*	Effects of Cellzymesecret therapy plus against pathogenic fungus (Candida albicans) and somebacteria causing vaginal infection	Poster Presentation	The 29th Federation Meetingof Korean Basic Scientists June (2022)	30 June-1 July, 2022	Gwangju, South Korea
22.	He Wenjing, **Md. Habibur Rahman**, Subham Sharma, Kchorng Vira, Abdul-Nasir Sofian, Mo Chaodeng, Cheol-Su Kim and Kyu-Jae Lee	Hydrogen Medicine Progress Research in China	Abstract	Tourism Institute of Northeast Asia	Nov 21,202	Daegu Hotel