L-histidine augments the oxidative damage against Gram-negative bacteria by hydrogen peroxide

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Abstract. Excessive damage to DNA and lipid membranes by reactive oxygen species reduces the viability of bacteria. In the present study, the proliferation of *recA*-deficient *Escherichia coli* strains was revealed to be inhibited by 1% L-histidine under aerobic conditions. This inhibition of proliferation was not observed under anaerobic conditions, indicating that L-histidine enhances oxidative DNA damage to *E. coli* cells. Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the expression of *recA* in *E. coli* MG1655 increased ~7-fold following treatment with 10 mM hydrogen peroxide (H_2O_2) plus 1% L-histidine, compared with that following exposure to H_2O_2 alone. L-histidine increased the genomic fragmentation of *E. coli* MG1655 following exposure to H_2O_2. In addition, L-histidine increased the generation of intracellular hydroxyl radicals in the presence of H_2O_2 in *E. coli* cells. Next, our group investigated the disinfection properties of the H_2O_2 and L-histidine combination. The combination of 100 mM H_2O_2 and 1.0% L-histidine significantly reduced the number of viable cells of extended-spectrum-β-lactamase-producing *E. coli* and multi-drug-resistant *Pseudomonas aeruginosa*, and this treatment was more effective than 100 mM H_2O_2 alone, but this effect was not observed in methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant *Enterococcus faecium*. The combination of L-histidine and H_2O_2 may be a useful strategy to selectively increase the microbicidal activity of oxidative agents against Gram-negative bacteria.

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Introduction

Aerobically growing bacteria utilize oxygen species for energy metabolism. Reactive oxygen species (ROS), including superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), are generated during this process (1,2). These ROS impair cellular functions by damaging DNA, proteins, and lipid membranes. Aerobic bacteria have evolved oxidative stress responses, including enzymes to scavenge ROS (catalases, superoxide dismutases and alkyl hydroperoxide reductases) (3,4) and the *rec* system for DNA repair (5). However, oxidative stress that overwhelms the capacity of these stress response systems kills the bacteria. Therefore, oxidative antimicrobials are widely used for sanitation purposes in healthcare facilities.

A compromised DNA repair system has been reported to render microbes vulnerable to oxidative stress (3,5). In the present study, L-histidine suppressed the proliferation of *recA*-deficient *Escherichia coli* laboratory strains under aerobic conditions, but not anaerobic conditions. This indicated that L-histidine enhances the oxidative DNA damage of *E. coli* cells. L-histidine has been reported to enhance the genotoxicity of hydrogen peroxide (H_2O_2) in mammalian cells by increasing ROS production (6-9).

H_2O_2 is produced in hostile eukaryotic hosts (10) or from resident microbes (11), and eradicates pathogenic bacteria or competitors in the same habitat, respectively. H_2O_2 is a non-polar molecule that penetrates lipid membranes and oxidizes intracellular molecules (12,13). H_2O_2 generates hydroxyl radicals (OH·) through reacting with iron in a process known as the Fenton reaction (14). OH· is a powerful oxidant that reacts with a wide range of organic substances (1).

In previous decades, the microbicidal activity of H_2O_2 has been utilized for wastewater disinfection and the sanitation of hospital environments. For example, H_2O_2 vapor is used

for bedroom sanitation in hospitals, and treatment of rooms occupied by patients colonized with multidrug-resistant microorganisms (MDROs) reduces the risk of carriage of these MDROs by 64% (15). In addition, a combination of H_2O_2 and low concentrations of anionic and/or nonionic surfactants has been commercialized as a disinfectant for healthcare settings (16) H_2O_2 is recognized as an environment-friendly reagent that does not leave unfavorable environmental effects (17-19).

The main disinfection mechanism of H_2O_2 involves hydroxyl or hydroperoxyl radical production (20). The combination of H_2O_2 and metallic ions, including ferrous iron (Fe^{2+}) or copper (Cu^{2+}) enhances the disinfection potential via the Fenton reaction, or Fenton-like reactions (21). H_2O_2 combined with copper or silver synergistically enhances the disinfection efficiency of wastewater (22,23). In addition, modifications of the disinfection efficacy of H_2O_2 through combination with other components have been reported, including surfactant (16), chlorhexidine (24), iodine (25), sodium bicarbonate (26), hypothiocyanate (27), rifampicin (28), organic acids (29), neucopropine (30), and UV-irradiation (31). In the present study, L-histidine was also demonstrated to augment the bactericidal activity of H_2O_2 against Gram-negative bacteria.

Materials and methods

Reagents. H_2O_2 (50% w/v in H_2O) and L-histidine were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and Wako Chemicals GmbH (Neuss, Germany), respectively.

Bacterial strains and culture conditions. Laboratory strains of *E. coli* MG1655 (American Type Culture Collection, Manassas, VA, USA), JM109 (New England BioLabs, Inc., Ipswich, MA, USA), and DH5α (Thermo Fisher Scientific, Inc., Waltham, MA, USA), *Pseudomonas aeruginosa* PAO1 (Japan Collection of Microorganisms, Tsukuba, Ibaraki, Japan) and clinical isolates of multidrug resistant *P. aeruginosa* (MDRP) TUP1 from human blood (Tokushima University Hospital, Tokushima, Japan), methicillin-resistant *Staphylococcus aureus* (MRSA) TUM1 from human sputum (Tokushima University Hospital), extended-spectrum β-lactamase (ESBL)-producing *E. coli* KUM1 (corresponding to the isolate E6 reported by Uemura *et al* (Kagawa University Hospital, Miki, Japan) (32), and vancomycin-resistant *Enterococcus faecium* (VRE) FN1 (Dr Koichi Tanimoto, Gunma University, Maebashi, Japan) (33) were used in the present study. Glycerol stocks (-70˚C) of these strains were streaked onto brain heart infusion (BHI; Eiken Chemical Co., Ltd., Tokyo, Japan) agar plates, which were incubated at 37˚C for 24 h in the dark. A single colony from the BHI agar plate was inoculated into 3 ml BHI broth and incubated aerobically at 37˚C for 16 h. Then, 1 ml of the culture was centrifuged (10,000 x g, 5 min, 4˚C), and the collected cells were washed once with 2.5 ml phosphate-buffered saline (PBS; pH 7.4). The bacterial cells were then suspended in 4 ml PBS and used as the inoculum in the bactericidal test. Ten-fold serial dilutions of bacterial suspension were prepared and 0.1 ml of this suspension was spread on BHI agar plates to enumerate the viable cell counts in the suspension.

Monitoring of E. coli cell proliferation in the presence of L-histidine. The *E. coli* K12 strain MG1655 and the *recA*-deficient strains JM109 and DH5α were cultured in 3 ml BHI with shaking at 37˚C overnight in the dark. Each overnight culture (30 µl) was used to inoculate two sets of 3 ml BHI supplemented with or without 1% L-histidine. One of the tubes was grown aerobically with shaking at 37˚C in the dark, and the other was grown anaerobically at 37˚C in the dark, using an anaerobic chamber conditioned with mixed gas (N_2, 80%; H_2, 10%; CO_2, 10%). The proliferation of *E. coli* strains was monitored every 2 h by checking the optical density at 600 nm (OD_{600}) until 10 h following inoculation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of recA genes. Total RNA was extracted using the hot-phenol method (34) from the mid-logarithmic-phase cultures (OD_{660}, 0.4-0.6) of *E. coli* MG1655 strains with or without exposure to 10 mM H_2O_2 (30 min at 37˚C) and/or 1% L-histidine in the dark. The RNA was further purified using the RNeasy CleanUp kit (Qiagen GmbH, Hilden, Germany) and was treated with TURBO DNA-*free* (Ambion; Thermo Fisher Scientific, Inc.) to remove contaminating DNA. Total RNA (400 ng) was reverse transcribed using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) with random hexamers at 37˚C for 15 min. Reverse transcription was terminated by heating the mixture at 85˚C for 5 sec. The cDNA products were subsequently amplified using SYBR Premix Ex Taq II (Takara Bio, Inc.) under the following conditions: Preheating at 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec in a StepOne Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The oligonucleotide primers used for monitoring *recA* expression were as follows: Forward, 5'-GTGAAGAACAAAATCGCT GC-3' and reverse, 5'-TCTGCTACGCCTTCGCTAT-3' (35). All samples were run in triplicate. Threshold cycle values were normalized to the levels of *rrnH* transcripts, and changes were calculated using the $2^{-\Delta\Delta Cq}$ method (36). The PCR primers used for *rrnH* were as follows: Forward, 5'-AGTCGAACGGTA ACAGGAAGA-3' and reverse, 5'-GCAATATTCCCCACT GCTG-3'.

Assessment of DNA damage. *E. coli* MG1655 were statically incubated in 10 ml BHI for 16 h at 37˚C in the dark. The culture was centrifuged (10,000 x g, 5 min, 4˚C) and suspended in 10 ml PBS (pH 7.4) or PBS containing 1% L-histidine. H_2O_2 was added to the tubes (1, 10 or 100 mM) and incubated for 30 min at 37˚C in the dark. Distilled water was used as a control in place of H_2O_2. Following incubation, chromosomal DNA fragmentation of the treated *E. coli* cells was assessed by pulsed-field gel electrophoresis (PFGE) as follows: Treated cells were embedded into 1% agarose to make sample plugs. The *E. coli* cells were then lysed within the agarose plug using the CHEF Bacterial DNA Genome kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. Genomic DNA was prepared *in situ* in agarose blocks. The DNA was resolved by PFGE in 1% agarose (SeaKem GTG agarose, Lonza Japan Ltd., Tokyo, Japan) in 0.5x Tris-borate-EDTA buffer with a CHEF-DR II system (Bio-Rad Laboratories, Inc.) at 14˚C under 6 V/cm of electric field. Pulse time was set to 1-30 sec for the first 17 h,

and changed to 5-9 sec for the last 6 h. Following electrophoresis, the gel was stained with 0.5 μg/ml ethidium bromide for 20 min at room temperature and washed with distilled water. The length of the smear was compared between samples treated with distilled water or H_2O_2 (1, 10 or 100 mM) with or without 1% L-histidine.

Measurement of ROS. Intracellular ROS levels were measured using the OxiSelect Intracellular ROS assay kit (Cell Biolabs, Inc., San Diego, CA, USA). *E. coli* MG1655 was cultured aerobically in 3 ml BHI to the mid-logarithmic phase and collected by centrifugation (10,000 x g, 5 min, 4˚C). The cells were washed once with PBS and resuspended in PBS containing ROS substrate at 37˚C for 60 min. The cells absorbing ROS substrate were collected, washed once with PBS and resuspended in PBS containing 2% L-histidine. An equal volume of 20 mM H_2O_2 was added to the cell suspension and incubated for 10 min at 30˚C. Following incubation, 2',7'-dichlorodihydrofluorescein (DCF) generated by the oxidative degradation of the substrate was monitored at 480 nm excitation/530 nm emission.

Free radical analysis using electron paramagnetic resonance (EPR) spectroscopy. Generation of free radicals in *E. coli* MG1655 following exposure to H_2O_2 and L-histidine was confirmed using an EPR-spin trapping method at room temperature. The spin trapping reagent, 5,5-dimethyl-1-pyrrorine-N-oxide (DMPO), was obtained from Labotec Co., Ltd. (Tokyo, Japan). The *E. coli* MG1655 cell suspension [0.1 ml; $5.6x10^8$ colony forming units (CFU)] containing 2% L-histidine and 500 mM DMPO was incubated for 5 min at room temperature, and 0.1 ml 200 mM H_2O_2 was then added. After 5 min, the mixture was centrifuged at 2,000 x g for 1 min at room temperature and the cell pellets were resuspended in 0.1 ml PBS. EPR measurement was performed at room temperature. The sample was transferred to three sections of glass capillaries (10 μl; Drummond Scientific Co., Inc., Broomall, PA, USA) and set into the EPR cavity for the measurements. A Bruker EMXPlus EPR spectrometer (Bruker Corporation, Billerica, MA, USA) with an X-band cavity (ER 4103TM) was used to collect the EPR signal of the DMPO spin adducts. The typical instrumental conditions were as follows: 10 mW microwave power, 2.0-Gauss modulation amplitude, 0.08-sec time constant, 120-sec scan time, and 100-Gauss scan range. Hyperfine coupling constants and radical concentrations were obtained using the computer program Winsim (37).

H_2O_2 bactericidal test. The final concentrations of H_2O_2 used for the bactericidal test against Gram-negative (MDRP TUP1 and ESBL-producing *E. coli* KUM1) and Gram-positive (MRSA TUM1 and VRE FN1) bacteria were 100 and 200 mM, respectively. Each bacterial suspension (0.25 ml) was added to 0.5 ml of 2% histidine or sterilized distilled water. The suspension was mixed with 0.25 ml H_2O_2 (400 or 800 mM) and incubated at 25˚C for 15 min. Following incubation, the mixture (15 μl) was transferred to 1.5 ml catalase solution (10 mg/ml; Sigma-Aldrich; Merck KGaA) to inactivate H_2O_2. Serial 10-fold dilutions of the mixture were prepared and 0.1 ml of each dilution was spread onto BHI plates in triplicate. Following incubation of the plates at 37˚C for 24 h, the number of surviving cells was calculated from the colony counts grown on the BHI plates.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis of the data was performed with StatFlex 6.0 (Artech Co., Ltd., Osaka, Japan) using one-way analysis of variance to compare the means of all groups, followed by Tukey's test to compare the means of two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of L-histidine on the aerobic proliferation of recA-deficient E. coli strains. The MG1655 *E. coli* strain and the *recA*-deficient strains JM109 and DH5α were cultured in BHI broth supplemented with 1% L-histidine. As presented in Fig. 1, *E. coli* MG1655 grew well in the presence of 1% L-histidine under aerobic and anaerobic conditions. In contrast, JM109 and DH5α did not grow under aerobic conditions in the presence of 1% L-histidine, while they were able to grow under anaerobic conditions in the presence of this amino acid. As RecA plays a crucial role in DNA repair, these results indicated that L-histidine may induce oxidative DNA damage in the presence of oxygen, resulting in a lethal effect on the bacterial strains with impairments in the DNA repair system.

SOS response in E. coli induced by H_2O_2. DNA damage induces the expression of SOS stress response genes in order to repair the injury. The *recA* gene is a representative SOS stress response gene. To assess the level of DNA damage induced by H_2O_2 in the presence of L-histidine, *recA* gene expression in aerobically grown, mid-logarithmic phase *E. coli* MG1655 cells was measured by RT-qPCR (Fig. 2). The relative *recA* expression levels following treatment with 10 mM H_2O_2 increased ~5-fold compared with the distilled water control. The combination treatment with 10 mM H_2O_2 and 1% L-histidine induced a ~7-fold increase in *recA* gene expression compared with 10 mM H_2O_2 alone. Treatment with 1% L-histidine alone did not increase *recA* expression.

Assessment of DNA damage following exposure to H_2O_2. DNA damage induced by H_2O_2 and by the H_2O_2/L-histidine combination in statically grown *E. coli* MG1655 cells was assessed by PFGE. As presented in Fig. 3, treatment of *E. coli* MG1655 cells with 1% L-histidine alone did not induce DNA damage. DNA degradation was not observed in the *E. coli* cells treated with 1 mM H_2O_2 regardless of the presence of L-histidine. In contrast, a clear difference in DNA degradation appeared when >10 mM H_2O_2 was used, as *E. coli* DNA was degraded more extensively when 1% L-histidine was combined with 10 or 100 mM H_2O_2 compared with H_2O_2 treatment alone.

ROS generation in E. coli cells following exposure to H_2O_2. Intracellular ROS levels in *E. coli* MG1655 cells grown to mid-logarithmic phase under aerobic conditions and exposed to H_2O_2 were compared in conditions with or without

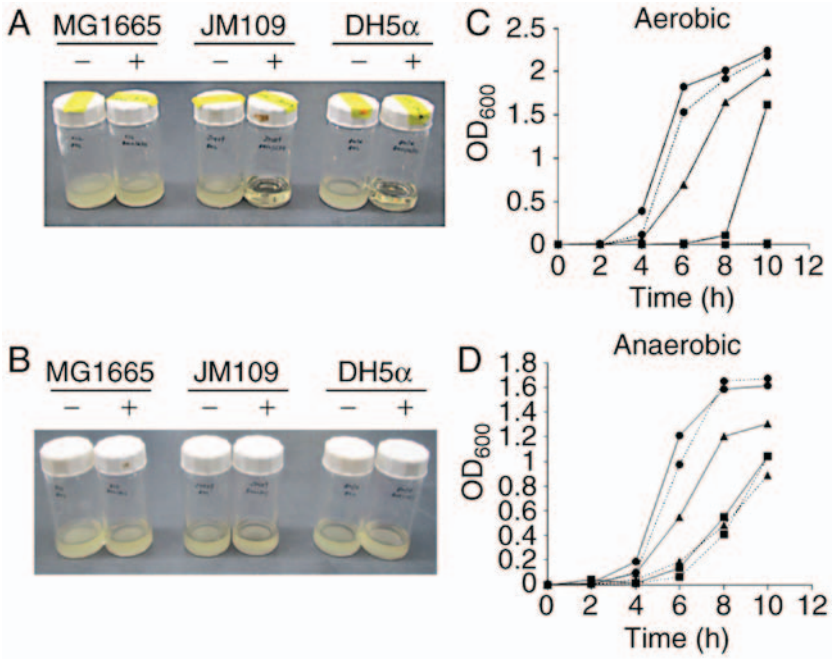

Figure 1. Effect of L-histidine on the growth of *E. coli* laboratory strains. Photographs of the *E. coli* culture tubes after 10-h incubation under (A) aerobic and (B) anaerobic conditions. The presence and absence of L-histidine in the media is indicated by + and -, respectively. Periodic OD_{600} changes of *E. coli* culture under (C) aerobic and (D) anaerobic conditions. Circles, triangles, and squares indicate the strains MG1655, JM109 and DH5α, respectively. Solid and dotted lines indicate the absence and presence of 1% L-histidine in the media, respectively.

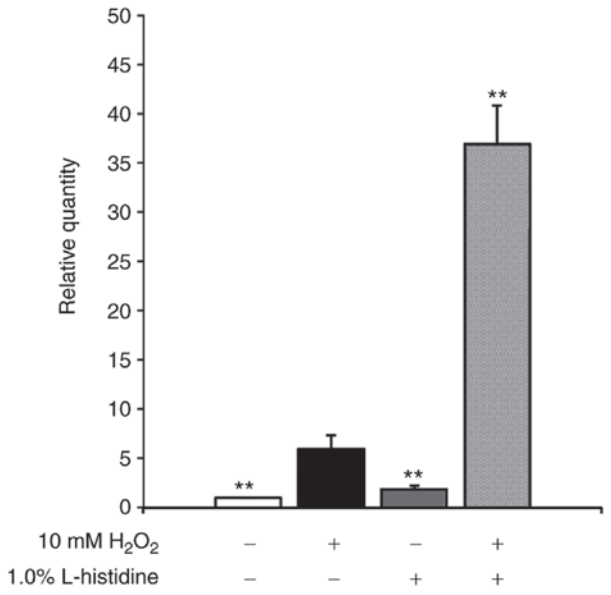

Figure 2. Relative quantification of *recA* gene expression in *E. coli* following exposure to hydrogen peroxide with L-histidine. The *E. coli* strain MG1655, grown to mid-logarithmic phase under aerobic conditions, was incubated in distilled water, 10 mM hydrogen peroxide, 1% L-histidine, or 10 mM hydrogen peroxide plus 1% L-histidine for 30 min at 37˚C. The expression of *recA* relative to the distilled water treatment following exposure to the test reagents are shown. Samples are indicated as follows: H_2O, open column; H_2O_2, closed column; L-histidine, gray column; and H_2O_2/L-histidine combination, hatched column. Data are expressed as the mean ± standard deviation of three independent repeats. $^{**}P<0.01$ vs. H_2O_2 alone.

1% L-histidine. As presented in Fig. 4, intracellular ROS levels were low without H_2O_2. Addition of 1% L-histidine alone did not induce ROS generation. In contrast, exposure to 10 mM H_2O_2 significantly induced ROS generation in *E. coli* MG1655 cells compared with the negative control. In addition, ROS levels in *E. coli* significantly increased when the cells were treated with a combination of 10 mM H_2O_2 and 1% L-histidine compared with the other groups.

EPR analysis. EPR analysis was performed to identify which types of free radical were generated in *E. coli* cells following exposure to 100 mM H_2O_2 and 1% L-histidine. As presented in Fig. 5A, a typical four-line EPR signal attributed to DMPO-OH spin-adduct with an intensity ratio of 1:2:2:1 (hyperfine splitting constant; a^N=15.300 and a^H=14.549 gauss) (38) was observed by Fenton reaction systems containing 20 mM Fe^{2+}, 100 mM H_2O_2 and 500 mM DMPO. The EPR spectrum representing DMPO-OH was observed in *E. coli* cells exposed to 100 mM H_2O_2 and 1% L-histidine (Fig. 5B). On the other hand, this EPR signal was not observed in *E. coli* cells exposed to 100 mM H_2O_2 alone (Fig. 5C). These data indicated that L-histidine promoted intracellular hydroxyl radical formation by H_2O_2 in *E. coli* cells.

Effect of L-histidine on the bactericidal activity of H_2O_2 against Gram-positive MDROs. To examine the sensitivity of MRSA and VRE to H_2O_2, aerobically-grown overnight cultures of these MDROs were exposed to 100, 200 and 300 mM H_2O_2 for 15 min. As presented in Fig. 6, H_2O_2 reduced the number of viable MRSA cells in a dose-dependent manner, and 100, 200 and 300 mM H_2O_2 achieved reductions of 0.29±0.07, 0.58±0.27 and 1.15±0.85 $\log_{10}$ CFU/ml in MRSA. H_2O_2 also reduced the viable numbers of VRE cells dose-dependently, and 100, 200 and 300 mM H_2O_2 achieved reductions of 0.31±0.19, 1.94±1.22 and 2.97±1.40 $\log_{10}$ CFU/ml in VRE.

Next, the effect of the combination of L-histidine and 200 mM H_2O_2 on the viability of these Gram-positive bacteria

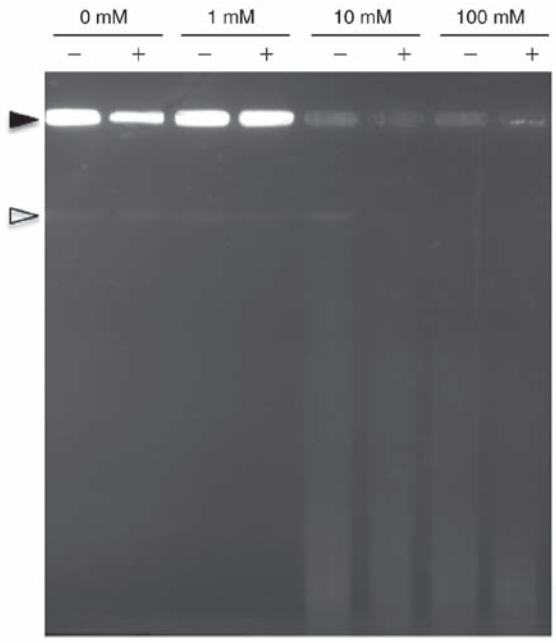

Figure 3. DNA degradation by H_2O_2 with or without 1% L-histidine in *E. coli*. Following exposure to the indicated concentrations of H_2O_2 for 30 min at 37˚C, statically grown *E. coli* MG1655 cells were embedded in an agarose plug and enzymatically lysed. PFGE was performed to assess DNA degradation. The presence or absence of 1% histidine in the H_2O_2 solution is indicated by + or - above the lanes, respectively. Open and closed arrowheads indicate intact and spontaneously fragmented chromosomal DNA, respectively.

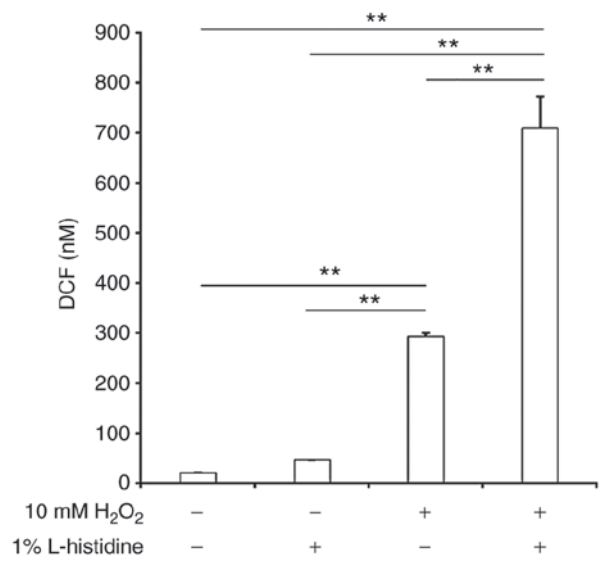

Figure 4. Intracellular ROS generation in *E. coli* induced by H_2O_2 and L-histidine. *E. coli* MG1655 cells were grown to the mid-logarithmic phase under aerobic conditions. After 10 min treatment with the indicated combinations of 10 mM H_2O_2 and L-histidine, intracellular ROS levels were evaluated by measuring the concentration of DCF. Data are expressed as the mean ± standard deviation of three independent repeats. **P<0.01, with comparisons indicated by lines.

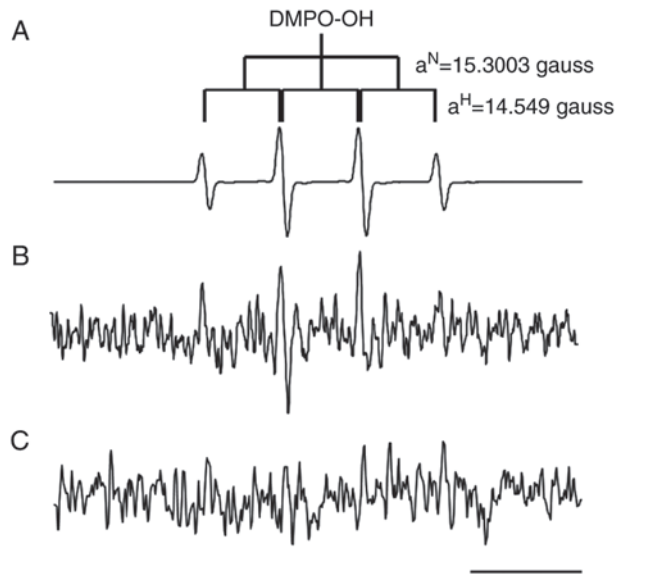

Figure 5. EPR spectrum in *E. coli* exposed to H_2O_2 and L-histidine. (A) Authentic spectrum of standard DMPO-OH. (B) EPR spectrum in *E. coli* exposed to 100 mM H_2O_2 and 100 mM L-histidine. (C) EPR spectrum in *E. coli* following exposure to 100 mM H_2O_2 alone. The bar indicates 20 gauss. EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrrorine-N-oxide.

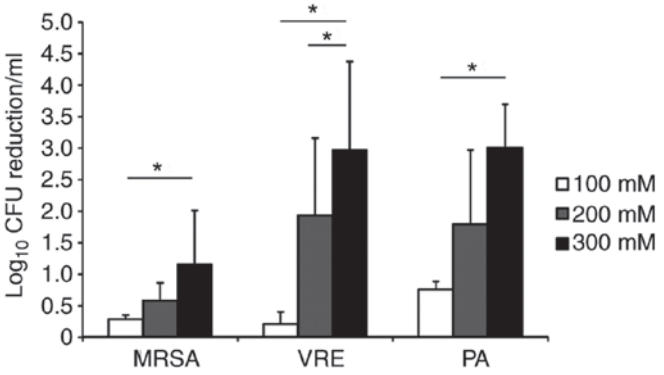

Figure 6. Sensitivities of MRSA, VRE, and PA to H_2O_2. Aerobically grown, overnight cultures of the test strains were exposed to the indicated concentration of H_2O_2 for 15 min at 25˚C, and surviving cells were then enumerated by plating on brain heart infusion agar plates and reported on a logarithmic scale. The reduction of viable cells was calculated by subtracting each value from the value of the distilled water control. Data are expressed as the mean ± standard deviation of three independent repeats. *P<0.05, with comparisons indicated by lines. MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus faecium*; PA, *P. aeruginosa* PAO1; CFU, colony forming units.

was assessed. As presented in Table I, no bactericidal effect was observed in the presence of 1% L-histidine alone. Addition of 200 mM H_2O_2 alone resulted in 0.89±0.35 and 1.22±0.74 $\log_{10}$ CFU/ml reductions in MRSA and VRE, while 200 mM H_2O_2 with 1% L-histidine reduced cell numbers only by 0.40±0.14 and 0.92±0.63 $\log_{10}$ CFU/ml, respectively. Thus, L-histidine did not improve the bactericidal effect of H_2O_2 against MRSA and VRE.

Effect of L-histidine on the bactericidal activity of H_2O_2 against Gram-negative MDROs. The bactericidal activities of 100 mM H_2O_2 against aerobically grown overnight cultures of MDRP or ESBL-producing *E. coli* were assessed in the presence or absence of 1% histidine (Table I). H_2O_2 alone achieved 0.24±0.16 and 0.76±0.08 $\log_{10}$ CFU/ml reductions in MDRP and ESBL-producing *E. coli* cells, respectively.

Table I. Effect of L-histidine on the bactericidal activity of H_2O_2 against multidrug-resistant microorganisms.

Treatment		Organisms (Log_{10} CFU/ml reduction)			
H_2O_2 (mM)	1% L-histidine	MRSA	VRE	MDRP	ESBL *E. coli*
-	+	-0.01±0.13[a,b]	-0.01±0.09[a,b]	0.00±0.12[d,e]	-0.07±0.09[d,e]
100	-	NT	NT	0.24±0.16[e,f]	0.76±0.08[e,f]
	+	NT	NT	1.12±0.36[d,f]	1.14±0.02[d,f]
200	-	0.89±0.35[b,c]	1.21±0.74[c]	NT	NT
	+	0.40±0.14[a,c]	0.91±0.63[c]	NT	NT

Data are presented as the mean ± standard deviation, obtained from three independent experiments. NT, not tested; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus faecium*; MDRP, multidrug resistant *P. aeruginosa*; ESBL, extended-spectrum β-lactamase. [a]P<0.01 vs. 200 mM H_2O_2, [b]P<0.01 vs. 200 mM H_2O_2+1% L-histidine, [c]P<0.01 vs. 1% L-histidine, [d]P<0.01 vs. 100 mM H_2O_2, [e]P<0.01 vs. 100 mM H_2O_2+1% L-histidine, [f]P<0.01 vs. 1% L-histidine.

Addition of 100 mM H_2O_2 along with 1% L-histidine reduced cell counts by 1.12±0.36 and 1.14±0.02 log_{10} CFU/ml in these bacteria, respectively. These results indicated that L-histidine significantly enhanced the bactericidal action of H_2O_2 against MDRP and ESBL-producing *E. coli* (P<0.01).

Discussion

In the present study, L-histidine supplementation in the culture media was revealed to suppress the proliferation of the *recA*-deficient *E. coli* laboratory strains JM109 and DH5α, but this amino acid did not influence the proliferation of strain MG1655, which possesses an intact *rec* system. In addition, this suppression was not apparent when the strains were cultured anaerobically. These results indicate that L-histidine may increase oxidative DNA damage to *E. coli*, because RecA serves a crucial function in DNA repair. This hypothesis was supported by the experimental data, which revealed that *recA* gene expression in *E. coli* MG1655 cells exposed to 10 mM H_2O_2 plus 1% L-histidine increased 7-fold compared with that in cells exposed to 10 mM H_2O_2 alone. In addition, DNA degradation was more evident in the *E. coli* cells exposed to 10-100 mM H_2O_2 plus 1% L-histidine than in cells treated with the same concentration of H_2O_2 alone.

The augmenting effect of L-histidine on H_2O_2-induced DNA damage may be mediated by a Fenton-like reaction initiated by the contact of oxidative agents with metal ions, generating ROS (21). Since the imidazole group in L-histidine is known to bind to metal ions, including Ni^+ or Co^+, this characteristic has been applied to histidine-tagged recombinant protein purification. Prior work has demonstrated that L-histidine increased the genotoxicity of H_2O_2 against Chinese hamster ovary (CHO) cells (8,9) and that this is dependent on the L-histidine transporting activity of the CHO cells (39). Therefore, metal ions may be co-transported inside the *E. coli* cells with L-histidine, and these metal ions may increase the H_2O_2-derived DNA damage by a Fenton-like reaction. In fact, our group detected hydroxyl radicals in *E. coli* cells following treatment with H_2O_2 and L-histidine, but an EPR signal was not observed when the cells were treated with H_2O_2 alone. Schubert *et al* (7) reported that L-hisitidine and H_2O_2 readily form a stable adduct. Their group indicated that L-hisitidine-peroxide adduct may enter bacterial cells more rapidly than H_2O_2 alone, or that the rate of breakdown of the adduct by catalase is slower.

Consistent with the augmenting effect of L-histidine on H_2O_2-derived DNA damage in *E. coli*, L-histidine increased the killing activity against Gram-negative MDROs: ESBL-producing *E. coli* and MDRP. Wide penetration of multidrug resistance (MDR) in Gram-negative bacilli is a serious, global public health concern (40). MDR in highly virulent bacteria including fluoroquinolone-resistant *Salmonella* (41) or ESBL-producing Shiga-toxigenic *E. coli* (42,43) is emerging. In addition, the dissemination of carbapenemase-producing *Enterobacteriaceae* has made it difficult to treat certain infectious diseases with antibiotics (44). Thus, transmission control of Gram-negative bacilli with MDR is an urgent issue. Disinfection techniques using a combination of H_2O_2 and L-histidine may contribute to the efficient eradication of these MDR Gram-negative bacteria from hospital environments. However, H_2O_2 is a highly reactive oxidizing agent and has genotoxic effects. Oosterik *et al* (45) reported that nebulization of 2% H_2O_2 renders chickens more susceptible to avian pathogenic *E. coli*, potentially due to epithelial damage by hydroxyl radicals. The simultaneous use of L-histidine may decrease the H_2O_2 concentration necessary to eradicate Gram-negative pathogens.

In contrast to Gram-negative MDROs, L-histidine did not increase the microbicidal activity of H_2O_2 against Gram-positive bacteria (MRSA and VRE). The reason for this selective augmentation remains unclear but L-histidine transporting activity may be different between Gram-negative and Gram-positive bacteria. This selective effect of L-histidine on the bactericidal activity of H_2O_2 may be advantageous in certain instances, including during the elimination of foodborne pathogens (for example, *Salmonella* and pathogenic *E. coli* from fermented food made with lactic acid bacteria) or for bioremediation of contaminated soil by Gram-positive bacteria. The physiological benefit of resident microflora for human health has also been increasingly recognized. For example, a resident

skin microbe, *Staphylococcus epidermidis*, produces an Esp protease that degrades *S. aureus* biofilms, thereby conferring colonization resistance to MRSA (46). Thus, the use of the H_2O_2/L-histidine combination may be applicable for treatment of skin lesions, including decubitus ulcers infected with *P. aeruginosa*. H_2O_2/L-histidine is expected to exert enhanced toxicity against Gram-negative pathogens, while having a less detrimental effect on colonization resistance by normal resident skin flora.

In the present study, our group demonstrated the selective augmenting effect of L-histidine on the microbicidal activity of H_2O_2 against Gram-negative bacteria. This effect is potentially derived from DNA damage by ROS generated through Fenton-like reactions between H_2O_2 and metal ions bound to the imidazole group of L-histidine. The H_2O_2/L-histidine combination reduces the H_2O_2 concentration necessary to inactivate Gram-negative pathogens. In addition, this selectivity to Gram-negative bacteria may be useful in sanitation processes required to protect Gram-positive bacteria, including lactic acid bacteria in fermented foods or resident skin microbiota. Taken together, the results of the present study may provide valuable information to develop novel H_2O_2-based disinfection techniques.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

TN and HNI analyzed and interpreted the effect of the test reagents on *E. coli* growth. AT and HNI performed *recA* qPCR and ROS measurement. ME and HY performed PFGE analysis. MH and KT performed EPR analysis. TN, ET and KO examined the bactericidal effect of test reagents. TN and TK were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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