Escherichia coli Sterilization Using a Light-Emitting Diode and Methylene Blue

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Background and aims: The prevalence of pathogenic E*scherichia coli* as well as *E. coli* O157 and antibiotic-resistant bacteria has increased. This study aimed to examine the effect of methylene blue (MB) with sodium bicarbonate ($NaHCO_3$) against *E. coli* using photodynamic antimicrobial chemotherapy (PACT).
Materials and methods: MB was basified using $NaHCO_3$. *E. coli* and basic MB were smeared on the culture media followed by irradiation using a red light-emitting diode (LED) at 660 nm. Energy densities of 5, 10, 15, and 20 J/cm^2 were applied to the culture medium.
After 24 h, the bactericidal effect of basic MB with LED irradiation was determined based on the bacterial growth.
Results: The basic effect was observed with 1%–6% of $NaHCO_3$ at 5 J/cm^2.
This effect increased between 1% and 2% of $NaHCO_3$ at 10 J/cm^2 and 15 J/cm^2, whereas decreased at the $NaHCO_3$ concentrations of > 2%. Moreover, this effect decreased at an energy density of 20 J/cm^2. The biphasic basic effect on bactericidal activity was observed between pH 8.6 – 9.0.
Conclusions: Thus, PACT using basic MB may be an effective method for pathogenic *E. coli* sterilization.

Key words: *E. coli* O157 • methylene blue • sodium bicarbonate

Introduction

Escherichia coli are susceptible to a variety of antibiotics, which have been used worldwide to sterilize it. However, due to an increase in the antimicrobial resistance in *E. coli*, complete sterilization of this bacterium is no longer possible.

Hemorrhagic enterocolitis caused by *Escherichia coli* O157: H7 and its complication of hemolytic-uremic syndrome (HUS) are well known from large outbreaks caused by contaminated meats and vegetables [1].

Infection with *Escherichia coli* O157 can lead to the development of hemolytic uremic syndrome (HUS) [2]. The use of bactericidal antibiotics, particularly ß-lactams, to treat O157 infection was associated with the subsequent development of HUS [2].

Accordingly, a new method of sterilization that is independent of antibiotic resistance is required to destroy resistant bacteria. Numerous studies have investigated this using a variety of photosensitizers.

One study showed that *E. coli* can be inactivated using a combination of Rose Bengal as a photosensitizer and a blue light-emitting diode (LED), resulting in a colony reduction of 6.60 log10 in the number of CFU/ml after 120 sec of irradiation [3].

Another study reported the use of a red LED combined with methylene blue (MB) as a photosensitizer, resulting in a colony reduction of 93.7% in *E. coli* [3, 4].

These results demonstrate the efficacy of using a photosensitizer and light of a specific wavelength to sterilize *E. coli* under *in vitro* conditions.

The present study investigated this effective method for developing a potential phototherapy for use in clinical practice.

MB is a widely used bacterial stain that generates active oxygen in the presence of light energy [5]. Adding $NaHCO_3$ to MB results in a basic staining solution, which was prepared for use in a new photochemical method.

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Photodynamic antimicrobial chemotherapy (PACT) was used to verify the bactericidal effect of this technique on *E. coli*.

Materials and methods

Materials and preparation

E. coli strain (Japan Collection of Microorganisms: JCM No.1649) obtained from the National Research and Development Agency, Institute of Physical and Chemical Research (RIKEN, Japan) was used for in experiments. *E. coli* were identified using the BD BBL CRYSTAL E/NF ID kit (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan).

A 500-ml solution containing 25 g of MB diluted in ethanol (FUJIFILM Wako Pure Chemical Corporation, Japan) as the stock mixture and $NaHCO_3$ powder (Nichi-Iko Pharmaceutical Co., Ltd., Japan) were used.

A series of solutions containing MB at the concentrations of 0.01%, 0.05%, 0.1%, 0.2%, 0.5%, and 1% in sterile water for injection were prepared in sterile test tubes. $NaHCO_3$ solutions at the concentrations of 1%, 2%, 3%, 4%, 5%, and 6% were similarly made. *E. coli* count was adjusted to 2.0×10^8 cells/ml using sterile water for injection and stored in a sterile test tube.

Irradiation experiments

MB (100 µL), *E. coli* (20 µL), and $NaHCO_3$ (10 µL) were streaked onto sheep blood agar (Nissui Pharmaceutical Co., Ltd., Japan) using a sterile bacteria spreader. After storing the cultures in the dark for 5 min, the plates were irradiated using a red LED (CCS Inc., Kyoto, Japan) from a distance of 5 cm at 660 nm.

Energy densities of 5 J/cm^2 (1 min 20 sec), 10 J/cm^2 (2 min 40 sec), 15 J/cm^2 (4 min), and 20 J/cm^2 (5 min 20 sec) were applied to the cultures.

The added $NaHCO_3$ was diluted 13-fold before being added to the media, giving final concentrations of 0.07%, 0.15%, 0.23%, 0.30%, 0.38%, and 0.46%, respectively.

Determination of effectiveness

Following irradiation, the cultures were placed in an incubator at 37°C for 24 hours. The bactericidal effects were categorized into the following six grades by observing the areas of *E. coli* growth. There was no effect on bacterial growth in the absence of irradiation and MB: and irradiation in the absence of MB was also ineffective at controlling bacterial growth. (–) indicated no growth (ineffective treatment): (1+), (2+), (3+), and (4+) indicated a decrease in growth area by 20%, 40%, 60%, and 80%, respectively, compared with that in control, comprising an ineffective concentration of MB (0.01%): and (5+) indicated no growth. The control plates each contained a sample of bacteria with each concentration of MB and were not irradiated.

pH measurements

The pH of MB, $NaHCO_3$, and basic MB solutions were measured. The stock mixture of MB at concentrations of 0.01%–1% and $NaHCO_3$ at those of 1%–6% were diluted using sterile water. The pH of 2000 µL of MB solution, 400 µL of sterile water, and 200 µL of $NaHCO_3$ solution were prepared in sterile test tubes. The Seven Compact instrument (METTLER TOLEDO International Inc.) was used to measure pH. The concentration of MB was increased from 0.01% to 1% and that of $NaHCO_3$ was increased from 1% to 6% and pH was measured for each sample in the series.

Results

pH of materials

Table1 shows the pH of MB, $NaHCO_3$, and basic MB solutions. As the concentration of MB increased from 0.01% to 1%, the pH decreased from 7.069 to 5.565.

Moreover, as the concentration of $NaHCO_3$ increased from 1% to 6%, the pH decreased from 8.310 to 8.078. Adding $NaHCO_3$ to MB raised the pH of the solution to 8.583–8.995.

Effects of basic MB

As indicated in **Table 2**, no concentration of MB demonstrated a bactericidal effect of grade 5+ at an energy density of 5 J/cm^2. The MB concentrations of > 0.5% demonstrated a bactericidal activity of grade 5+ after adding 1% of $NaHCO_3$.

Solutions containing 0.2% of MB demonstrated a bactericidal activity of grade 5+ after adding 2%, 3%, and 4% of $NaHCO_3$, respectively.

Solutions containing 0.5% and 1% of MB demonstrated a bactericidal activity of grade 5+ after adding 5% and 6% of $NaHCO_3$.

Table 3 shows that no concentration of MB demonstrated a bactericidal effect of grade 5+ at an energy density of 10 J/cm^2. The bactericidal effect of MB was graded as 5+ after adding 1% $NaHCO_3$; and > 0.05% of MB also demonstrated a bactericidal activity of grade 5+ after adding 2% of $NaHCO_3$.

Solutions containing the MB concentrations of > 0.2%, 0.5%, 0.2%, and 0.5% demonstrated a bactericidal activity of grade 5+ after adding 3%, 4%, 5% and 6% of $NaHCO_3$.

Table 4 shows that no concentration of MB demonstrated a bactericidal effect of grade 5+ at an energy density of 15 J/cm^2. The bactericidal effect demonstrated by > 0.1% and > 0.01% of MB was graded as 5+ after adding 1% and 2% of $NaHCO_3$. Moreover, the MB concentrations of > 0.1%, 0.2%, 0.2%, and 0.5% demonstrated a bactericidal activity of grade 5+ after adding 3%, 4%, 5%, and

6% of $NaHCO_3$, respectively.

Table 5 demonstrates that no concentration of MB offered a bactericidal effect of grade 5+, including those basic solutions, at an energy density of 20 J/cm^2.

Figures 1, 2, 3, and 4 demonstrate the effects of adding different concentrations of basic MB to the media containing bacterial cultures.

Appearance of the biphasic effect

Figures 5, 6, and 7 demonstrate the increase in the basic effect followed by a decrease with an increasing pH.

Figure 5 shows that at an energy density of 5 J/cm^2, the basic effect appeared and increased at 1%–4% of $NaHCO_3$. This effect decreased at 4%–6% of $NaHCO_3$ concentrations. The biphasic basic effect was observed between pH 8.6 and 9.0. The strongest basic effect was observed at pH 8.6 and 2%–4% of $NaHCO_3$, which corresponds to an actual concentration of 0.15%–0.30%.

Figures 6 and 7 indicate that at the energy densities of 10 and 15 J/cm^2, the basic effect developed in the range of 1%–2% of $NaHCO_3$. The decreasing phase of the basic effect occurred at 2%–6% of $NaHCO_3$. Biphasic effect was observed at pH 8.6–9.0. The strongest basic effect was observed at pH 8.6 and 2% $NaHCO_3$, which corresponded to an actual concentration of 0.15% $NaHCO_3$.

Boundary of the biphasic effect

Increasing the energy density from 5 to 15 J/cm^2 revealed a gradual enhancement of the basic effect, resulting in a clear biphasic effect.

This indicated that a specific combination of factors induced subsequent observations of the basic effect.

Basic MB exhibited a biphasic effect on *E. coli* and comprised an early phase showing a rise in the effect followed by a later phase showing gradual attenuation. A concentration of 2% $NaHCO_3$ indicated a boundary between these two phases.

Discussion

This study demonstrates the bactericidal effects of MB with $NaHCO_3$. Basic MB was found to exhibit potentially lethal effects on *E. coli* by inducing an acid-base disturbance at a pH of > 8.6. However, this method cannot be applied intravascularly. The mechanism underlying this basic effect was attributed to the features of PACT as well as the dye used.

General features of dyes

MB is widely used to stain microorganisms and is increasingly being utilized as a photosensitizer for use in PACT.

Dyes are generally categorized as acidic, basic and amphoteric [6]. An acidic dye has a negative electric charge in aqueous solution, whereas a basic dye has a positive electric charge [6]. Amphoteric dyes exhibit both charges and maintain a state of equilibrium in solution [6].

MB is a basic, water-soluble dye with an affinity to most bacteria and fungi. [6, 7] MB shows greater staining efficiency under basic conditions [6, 7].

Cell wall and membrane of gram-negative bacteria

In order to elucidate its effect, MB must cross bacterial cell wall and membrane. The cell wall of gram-negative bacteria comprises an outer membrane (envelope), a thin layer of peptidoglycan, and a periplasmic space between the outer membrane and the peptidoglycan/cytoplasmic membrane [8]. Lipopolysaccharides present in the outer membrane of cell wall carry a net negative charge [8].

Because of its cationic charge, MB binds easily to the negatively charged lipopolysaccharides of gram-negative bacteria to cross cell wall and membrane without difficulty [4].

PACT mechanisms

The interaction between a photosensitizer and specific wavelength of light in the presence of oxygen results in a photodynamic reaction [9]. Following the absorption of a photon from light of a specific wavelength, the photosensitizer is promoted to the singlet state [10]. It then converts to a triplet state with a lower energy level and longer lifespan than the singlet state [10]. Cell death is induced by damage to two photo-induced mechanisms [11]. Type I mechanism involves hydrogen abstraction or electron transfer between the excited photosensitizer and nearby biomolecules, yielding oxygenated free radicals, whereas Type II mechanism involves energy transfer between the excited photosensitizer and molecular oxygen, yielding singlet oxygen, $^1O_2$ [12].

Subsequently, intracellular components are destroyed, resulting in cell death.

Extent of the biphasic basic effect

At an energy density of 5 J/cm^2, the basic effect was extensively distributed between $NaHCO_3$ concentrations of 1% and 6%. Moreover, this effect demonstrated a biphasic pattern with a declining phase following an increasing phase according to the rise in energy density from 10 to 15 J/cm^2.

A concentration of 2% $NaHCO_3$ represented the boundary between the two phases. The actual $NaHCO_3$ concentration was 0.15%.

Furthermore, in this study, relationship between the minimal MB concentration required to achieve a bactericidal effect of grade 5+, $NaHCO_3$ concentration, energy density, and pH were established.

The bactericidal effect of basic MB on *E. coli* growth was associated with the following factors: 1) $NaHCO_3$ concentration, 2) basic pH, and 3) the adjustment and balance of both. It was determined that this effect appeared and increased when the $NaHCO_3$ concentration and basic pH were balanced.

Increasing the energy density to 5–15 J/cm² also enhanced the basic effect and resulted in a decrease in the minimal effective concentration of MB.

At an energy density of 20 J/cm², an expected increase in the basic effect did not occur. Moreover, the basic effect at 20 J/cm² was lower than that at 5, 10, and 15 J/cm², suggesting that increased energy density did not result in an increased basic effect.

Accordingly, the optimal energy density was found to be between 5 and 15 J/cm².

Photochemical pathway underlying the basic effect

It is particularly important that this new photochemical method is performed under basic pH conditions.

This study determined that synergistic interactions between MB, $NaHCO_3$, energy density, and pH were involved in the observed biphasic basic effect.

There appeared to be a specific photochemical pathway that resulted in the generation and enhancement of the basic effect, thereby altering the functionality of basic MB from staining to antimicrobial treatment.

Clinical application

Children with gastrointestinal infections by E*scherichia coli* O157: H7 are at risk for the hemolytic-uremic syndrome [13].

Acute gastrointestinal, and the systemic complica-

Table 1: pH of methylene blue with $NaHCO_3$ solutions

NaHCO3 concentration (%)	MB concentration%						
	0	0.01	0.05	0.1	0.2	0.5	1
0		7.069	6.718	6.868	6.5	6.082	5.565
1	8.31	8.583	8.562	8.591	8.586	8.652	8.716
2	8.187	8.627	8.649	8.623	8.683	8.73	8.884
3	8.16	8.584	8.598	8.627	8.695	8.779	8.914
4	8.093	8.632	8.633	8.66	8.718	8.791	8.97
5	8.093	8.596	8.63	8.628	8.669	8.762	8.931
6	8.078	8.61	8.668	8.671	8.689	8.774	8.995

Table 2: Bactericidal effect of methylene blue with $NaHCO_3$ at an energy density of 5 J/cm²

NaHCO3 concentration (%)	MB concentration%						
	0	0.01	0.05	0.1	0.2	0.5	1
0	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
1	—	(+)	(3+)	(4+)	(4+)	(5+)	(5+)
2	—	(2+)	(3+)	(4+)	(5+)	(4+)	(5+)
3	—	(+)	(3+)	(4+)	(5+)	(5+)	(5+)
4	—	(−)	(+)	(4+)	(5+)	(5+)	(5+)
5	—	(−)	(2+)	(4+)	(4+)	(5+)	(5+)
6	—	(−)	(3+)	(3+)	(4+)	(4+)	(5+)
blank test (no irradiation)	—	(−)	(−)	(−)	(−)	(−)	(−)

Table 3: Bactericidal effect of methylene blue with $NaHCO_3$ at an energy density of 10 J/cm²

NaHCO3 concentration (%)	MB concentration%						
	0	0.01	0.05	0.1	0.2	0.5	1
0	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
1	—	(2+)	(3+)	(4+)	(4+)	(4+)	(5+)
2	—	(4+)	(5+)	(4+)	(5+)	(5+)	(5+)
3	—	(2+)	(3+)	(4+)	(5+)	(5+)	(5+)
4	—	(−)	(2+)	(3+)	(4+)	(5+)	(5+)
5	—	(+)	(3+)	(4+)	(5+)	(5+)	(5+)
6	—	(−)	(2+)	(4+)	(4+)	(5+)	(5+)
blank test (no irradiation)	—	(−)	(−)	(−)	(−)	(−)	(−)

Table 4: Bactericidal effect of methylene blue with $NaHCO_3$ at an energy density of 15 J/cm²

NaHCO3 concentration (%)	MB concentration%						
	0	0.01	0.05	0.1	0.2	0.5	1
0	—	(3+)	(4+)	(4+)	(4+)	(4+)	(4+)
1	—	(3+)	(4+)	(5+)	(5+)	(5+)	(5+)
2	—	(5+)	(5+)	(5+)	(5+)	(4+)	(5+)
3	—	(3+)	(4+)	(5+)	(5+)	(5+)	(5+)
4	—	(+)	(3+)	(4+)	(5+)	(5+)	(5+)
5	—	(4+)	(4+)	(4+)	(5+)	(5+)	(5+)
6	—	(3+)	(4+)	(4+)	(4+)	(5+)	(5+)
blank test (no irradiation)	—	(−)	(−)	(−)	(−)	(−)	(−)

Table 5: Bactericidal effect of methylene blue with $NaHCO_3$ at an energy density of 20 J/cm²

NaHCO3 concentration (%)			MB concentration%				
	0	0.01	0.05	0.1	0.2	0.5	1
0	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
1	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
2	—	(−)	(3+)	(3+)	(4+)	(4+)	(4+)
3	—	(−)	(2+)	(2+)	(4+)	(4+)	(4+)
4	—	(−)	(2+)	(4+)	(4+)	(4+)	(4+)
5	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
6	—	(−)	(2+)	(3+)	(4+)	(4+)	(4+)
blank test (no irradiation)	—	(−)	(−)	(−)	(−)	(−)	(−)

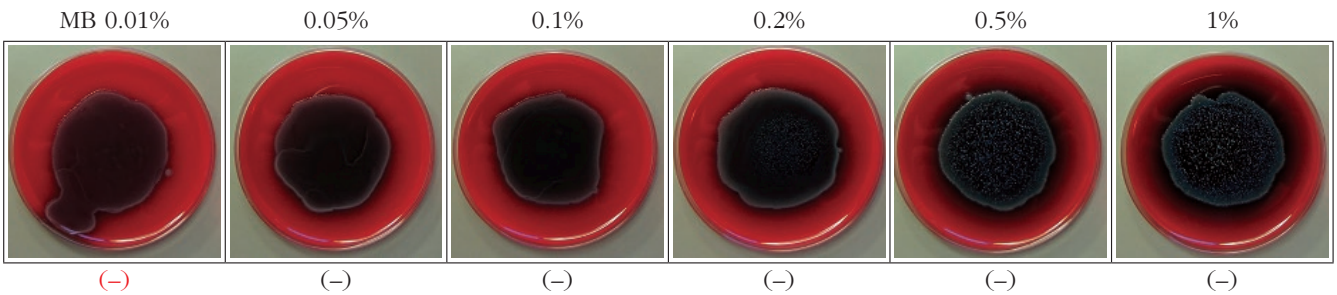

Figure 1: Cultures of *E. coli* under various concentrations of methylene blue without irradiation.

tions hemolytic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), are frequent and severe [14]. Very young or very old patients may be at increased risk [15].

As clinical, pathological and endoscopic findings in *Escherichia coli* O157: H7-assocoated colitis may be similar to the ischemic colitis pattern, differential diagnosis may be difficult [16].

No specific treatment is currently available for

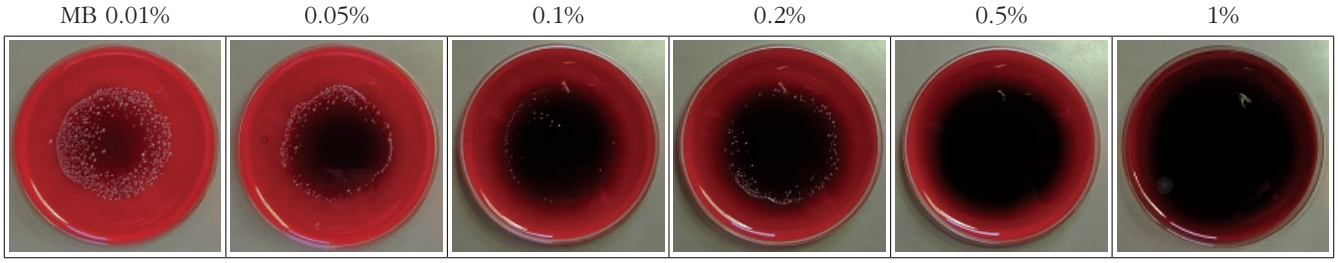

Figure 2: Effect of methylene blue on the growth of *E. coli* cultures irradiated at an energy density of 15 J/cm².

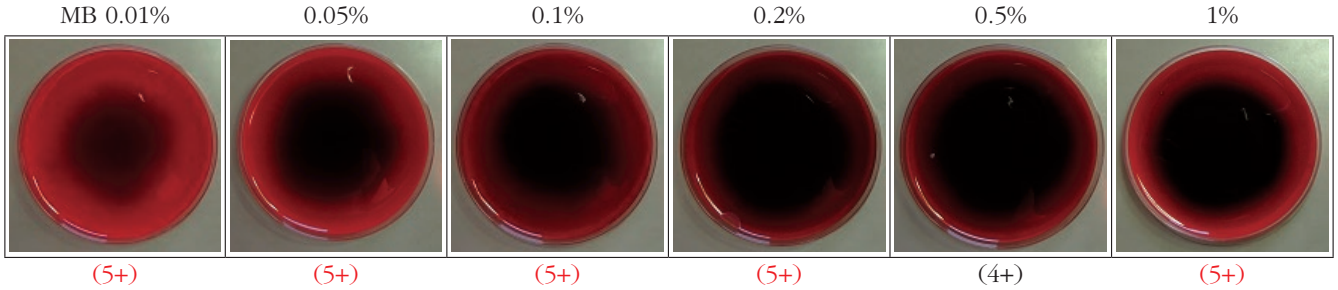

Figure 3: Effect of basic methylene blue (2%$NaHCO_3$) on the growth of *E. coli* cultures irradiated at an energy density of 15 J/cm².

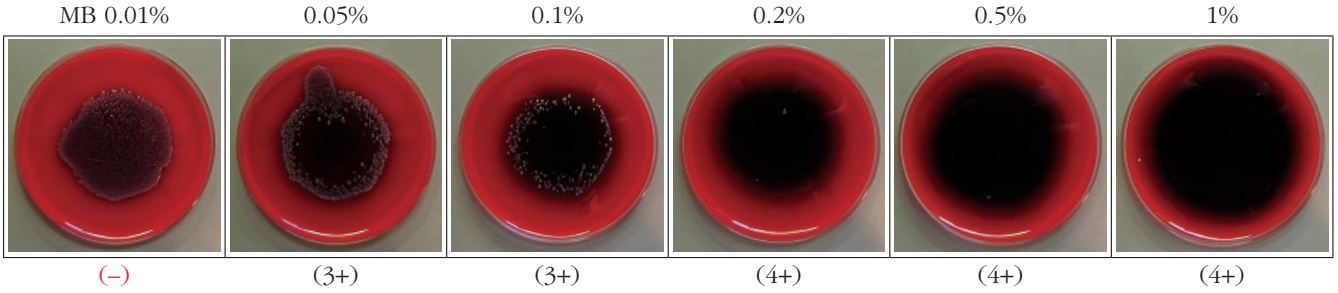

Figure 4: Effect of basic methylene blue (2%$NaHCO_3$) on the growth of *E. coli* cultures irradiated at an energy density of 20 J/cm².

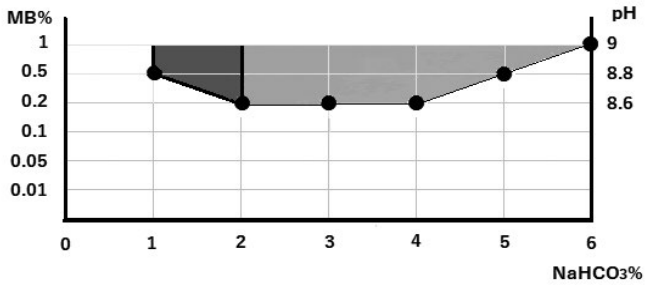

Figure 5: Basic effect on the bactericidal activity of methylene blue (MB) at an energy density of 5 J/cm²

Darker area demonstrates the appearance and increase in the basic effect, and lighter area demonstrates the decrease in the basic effect. ● The minimal MB concentration for achieving a bactericidal effect of grade 5+.

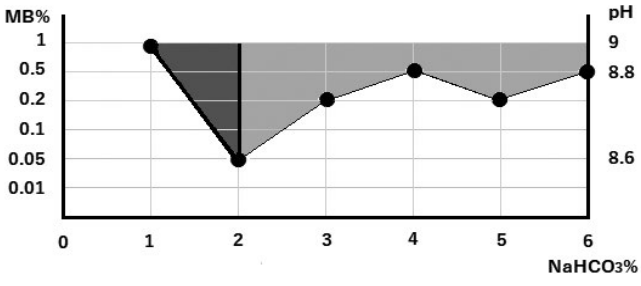

Figure 6: Basic effect on the bactericidal activity of methylene blue (MB) at an energy density of 10 J/cm²

Darker area demonstrates the appearance and increase in the basic effect, and lighter area demonstrates the decrease in the basic effect. ● The minimal MB concentration for achieving a bactericidal effect of grade 5+.

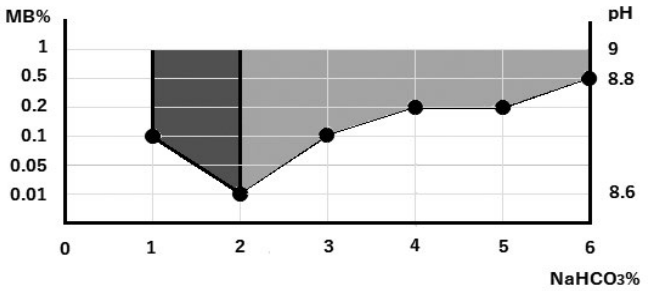

Figure 7: Basic effect on the bactericidal activity of methylene blue (MB) at an energy density of 15 J/cm^2

Darker area demonstrates the appearance and increase in the basic effect, and lighter area demonstrates the decrease of the basic effect. ● The minimal MB concentration for achieving a bactericidal effect of grade 5+.

E. coli O157:H7 infection [15]. Treating O157 infections with antibiotics is a possible factor for HUS development [2].

Antibiotic treatment of children with *E. coli* O157: H7 infection increases the risk of the hemolytic-uremic syndrome [13].

Thus, new treatments to replace antibiotics should be devised. When endoscopic photodynamic antimicrobial chemotherapy (PACT) is performed at an early stage of infection, it can be expected to suppress the progression to HUS/TTP.

Conclusions

This newly developed method of sterilization is effective against *E. coli* and does not result in resistance of bacteria. The bactericidal efficacy of MB can be induced under basic conditions. An acid-base balance at a pH of > 8.6 functioned as an activator of PACT.

In summary, PACT with basic MB may have a potential develop into a viable photochemical sterilization method. Endoscopic treatment of *E. coli* O157 can be expected.

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