**Candida albicans Sterilization Using a Light-Emitting Diode and Methylene Blue**

Kouji Ogasawara

*Sarufutsu Village National Health Insurance Hospital, Japan*

**Background and aims:** Conventional fungal treatment using external medication requires considerable time and effort. We herein examined the basic effect of methylene blue (MB) with sodium bicarbonate (NaHCO₃) on *Candida albicans* sterilization using photodynamic antimicrobial chemotherapy (PACT).

**Materials and methods:** NaHCO₃ was added to MB to establish a basic pH. Then, *C. albicans* was smeared on a medium with basic MB followed by irradiation using a red light-emitting diode (LED) with a wavelength of 660 nm. The applied energy fluencies were 5, 10, 15, and 20 J/cm². After 48 h of culture, the effects of the intervention were determined according to the fungal growth area.

**Results:** The basic effect appeared at a pH range of 8.6 to 8.9 and at 10 and 15 J/cm², while the NaHCO₃ concentration was between 1% and 2%.

**Conclusions:** Our results suggested that PACT using basic MB was effective for *C. albicans* sterilization.

**Key words:** *C. albicans* • LED • PACT • methylene blue • sodium bicarbonate • basic effect
A 500-mL stock solution containing 25 g of MB (FUJIFILM Wako Pure Chemical Corporation) and NaHCO₃ (Nichi-Iko Pharmaceutical Co., Ltd.) were utilized. MB (0.01%, 0.05%, 0.1%, 0.2%, 0.5%, and 1%) and NaHCO₃ (1%, 2%, 3%, and 4%) concentrations were regulated in a sterile test tube using sterile water for injection. The *C. albicans* count was adjusted to 2.0 × 10⁸ cells/mL in a sterile test tube using sterile water for injection.

**Irradiation experiments**

Using a sterile pipette tip, 100 µL of MB, 20 µL of *C. albicans*, and 10 µL of NaHCO₃ were drawn from the test tubes and were smeared onto a Sabouraud agar medium (Nissui Pharmaceutical Co., Ltd.) using a sterile bacteria spreader.

After placing the fungal culture in the dark for 5 min, irradiation was performed from a distance of 5 cm using a red LED (CCS Inc., Kyoto, Japan) with a wavelength of 660 nm. The energy fluencies were 5 J/cm² (1 min 20 s), 10 J/cm² (2 min 40 s), 15 J/cm² (4 min), and 20 J/cm² (5 min 20 s). NaHCO₃ concentration was diluted 13-fold before being added to the medium, subsequently obtaining final concentrations of 0.07%, 0.15%, 0.23%, and 0.30%, respectively. After irradiation, the culture was placed in an incubator at 37°C for 48 h.

**Determination of effectiveness**

The fungicidal effect was evaluated according to the fungal growth area of *C.albicans*, which was categorized into six grades.

The criteria for effectiveness were as follows:

The bactericidal effect in the absence of irradiation and MB was ineffective; the bactericidal effect of irradiation in the absence of MB was ineffective.

This blank test included the fungus and each MB concentration without irradiation. Accordingly, (-) indicated ineffectiveness, while (1+), (2+), (3+), and (4+) indicated a decrease in growth areas by 20%, 40%, 60%, and 80%, respectively, compared to blank test using an ineffective concentration of 0.01% MB. Moreover, (5+) indicated no growth area.

**pH measurement of available materials**

Each test tube contained 2000 µL of MB, 400 µL of sterile water for injection, and 200 µL of NaHCO₃. The Seven Compact instrument (METTLER TOLEDO International Inc.) was used to measure pH.

| **Table 1:** pH of methylene blue with NaHCO₃ |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 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 |

| **Table 2:** Basic effect of methylene blue with NaHCO₃ at 5 J/cm² |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| NaHCO₃ concentration (%) | MB concentration% | 0 | 0.01 | 0.05 | 0.1 | 0.2 | 0.5 | 1 |
| 0 | — | (−) | (+) | (−) | (−) | (+) | (+) |
| 1 | — | (−) | (+) | (−) | (−) | (−) | (−) |
| 2 | — | (2+) | (4+) | (4+) | (4+) | (4+) | (4+) |
| 3 | — | (−) | (−) | (−) | (+) | (−) | (−) |
| 4 | — | (−) | (−) | (−) | (−) | (−) | (−) |
| blank test (no irradiation) | — | (−) | (−) | (−) | (−) | (−) | (−) |
Results

pH of available materials

Table 1 shows the pH of MB (C₆H₇ClN₃S)₅, sodium bicarbonate (NaHCO₃)⁶, and MB with NaHCO₃.

As the MB concentration increased from 0.01% to 1%, the pH decreased from 7.069 to 5.565.

Moreover, as the NaHCO₃ concentration increased from 1% to 4%, the pH decreased from 8.310 to 8.093.

Adding NaHCO₃ to MB increased the pH of the solution to 8.583–8.970.

Basic effect of MB with NaHCO₃

Table 2 shows that none of the MB concentrations were graded as 5+ at an energy fluency of 5 J/cm² and with the addition of 1%–4% NaHCO₃.

Moreover, the MB concentration of > 0.5% was graded as 4+ after adding 1% NaHCO₃, while the MB concentration of > 0.05% was graded as 4+ after adding 2% NaHCO₃.

Table 3 shows that none of the MB concentrations were graded as 5+ at an energy fluency of 10 J/cm². Meanwhile, the MB concentrations of > 0.5% and > 0.05% were graded as 5+ after adding 1% and 2% NaHCO₃, respectively.

Moreover, none of the MB concentrations were graded as 5+ after adding 3% and 4% NaHCO₃.

Figures 1, 2, and 3 show the effects on the medium.

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

Table 3: Basic effect of methylene blue with NaHCO₃ at 10 J/cm²

Figure 1: No irradiation

Figure 2: Effect of methylene blue irradiated at a fluence of 10 J/cm²
Table 4 shows that none of the MB concentrations were graded as 5+ at an energy fluency of 15 J/cm². Meanwhile, the MB concentrations of > 0.5% and > 0.1% were graded as 5+ after adding 1% and 2% NaHCO₃, respectively.

Moreover, none of the MB concentrations were graded as 5+ after adding 3% and 4% NaHCO₃.

Figures 4 and 5 show the effects on the medium.

Table 5 shows that none of the MB concentrations, including those with NaHCO₃ were graded as 5+ at an energy fluency of 20 J/cm².

Discussion

The current study was able to clearly present the effects of MB with NaHCO₃. The mechanisms for the basic effect

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

**Figure 3**: Basic effect of methylene blue with 2%NaHCO₃ irradiated at a fluence of 10 J/cm²

**Table 4**: Basic effect of methylene blue with NaHCO₃ at 15 J/cm²

**Figure 4**: Effect of methylene blue irradiated at a fluence of 15 J/cm²

**Figure 5**: Basic effect of methylene blue with 2%NaHCO₃ irradiated at a fluence of 15 J/cm²
were considered to be based on the property of PACT as well as the dye used.

**General features of dyes**

MB \(^5\), the chemical structure of which is shown in Figure 6, has been widely used for the simplest staining of organisms and has been increasingly used as a photosensitizer (PS) for PACT.

Dyes are divided into acidic, basic, and amphoteric types \(^7\). Acidic dyes are negatively charged in an aqueous solution, basic dyes are positively charged, and amphoteric dyes have both properties and maintain an equilibrium state \(^7\).

MB is a basic, water-soluble dye that has a positive electric charge allowing it to bind to a bacterium containing a negative charge \(^7\). MB has an affinity to most bacteria and fungus with its ability to stain increasing in the basic state \(^7,8\).

A PS needs to pass through the cell membrane to penetrate and distribute within \(C.\ albicans\) \(^9\).

Accordingly, MB with light irradiation has been shown to increase membrane permeability \(^9\).

**Mechanism for PACT**

The mechanism for PACT results from the interaction between photons of visible light and a PS \(^10\). Following the absorption of a photon from a specific wavelength of light, the PS is promoted to the singlet state \(^10\). Thereafter, it is converted to the triplet state, which has a lower energy and longer lifetime than the singlet state does \(^10\). Cell death is induced by damages related to two photoinduced mechanisms \(^11\): (1) Type I, triggered by electron transfer between the triplet state PS and biomolecules, generating reactive oxygen species (ROS); and (2) Type II, triggered by energy transfer from the triplet state PS to molecular oxygen, forming singlet oxygen \((^1O_2)\) \(^10,11\).

The Candida species are 25-50 times larger than bacteria and thus contain numerous targets per cell \(^12\).

The mechanism for fungal cell destruction is through perforation of the cell wall and membrane with singlet oxygen and oxygen radicals, allowing the dye to be translocated further into the cell \(^12\). Subsequently, intracellular components are destroyed, resulting in cell death.

Cell membrane damage through light-activated MB and enhanced staining through basic pH have been thought to increase the fungicidal effect.

**Basic phenomenon to basic effect**

At an energy fluency of 5 J/cm\(^2\), indications for a potential basic effect had emerged as a basic phenomenon with the addition of 1% and 2% NaHCO\(_3\). This was considered a preliminary stage for the expression of the basic effect.

**Table 5:** Basic effect of methylene blue with NaHCO\(_3\) at 20 J/cm\(^2\)

| NaHCO\(_3\) concentration (%) | MB concentration% |
|------------------------------|-------------------|
|                              | 0     | 0.01 | 0.05 | 0.1  | 0.2  | 0.5  | 1    |
| 0                            | —     | (+)  | (4+) | (4+) | (4+) | (4+) | (4+) |
| 1                            | —     | (+)  | (3+) | (4+) | (4+) | (4+) | (4+) |
| 2                            | —     | (+)  | (3+) | (4+) | (4+) | (4+) | (4+) |
| 3                            | —     | (+)  | (2+) | (4+) | (4+) | (4+) | (4+) |
| 4                            | —     | (--) | (3+) | (3+) | (4+) | (4+) | (4+) |
| blank test (no irradiation)  | —     | (--) | (--) | (--) | (--) | (--) | (--) |

![Figure 6: The chemical structure of methylene blue](image-url)
The basic phenomenon apparently developed into the basic effect at 10 and 15 J/cm². However, the basic effect observed at 15 J/cm² was slightly lower than that observed at 10 J/cm².

This indicated that certain factors were closely involved in subsequent observations.

Determining factors and extent of the basic effect

The present study demonstrated the relationships between the minimum MB concentration required for achieving a grade 5+ fungicidal effect, NaHCO₃ concentration, energy fluency, and pH (Figures 7 and 8). The pastel portion of the figure is the region where the basic effect of MB had appeared and had increased.

At an energy fluency of 20 J/cm², an apparent basic effect did not appear.

Moreover, the basic effect at 20 J/cm² was even lower compared to that at 10 and 15 J/cm², suggesting that increasing energy fluency did not result in increased basic effect.

Accordingly, the optimal energy range was found to be between 10 and 15 J/cm².

The basic effect had been estimated to have appeared and increased between 1% and 2% NaHCO₃ and a pH of 8.6–8.9 at 10 and 15 J/cm².

The actual NaHCO₃ concentration had ranged between 0.07%–0.15%.

The strongest basic effect after adding 2% NaHCO₃ had appeared at a pH of 8.6 and at a fluence of 10 and 15 J/cm², with the actual NaHCO₃ concentration being 0.15%.

After adding 2% NaHCO₃, the increasing energy elevated MB’s minimum effective concentration from 0.05% to 0.1%.

Accordingly, the basic effect of MB was associated with the following features:

1. NaHCO₃ concentration
2. basic pH
3. adjustment and balance

The basic effect had been estimated to have appeared and increased when the adjusted NaHCO₃ concentration and balance of basic pH were optimized. Moreover, the basic effect also varied with changes in energy fluency.

Photochemical pathway for the basic effect

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

Moreover, a photochemical pathway leading to the generation and enhancement of the basic effect seems to have been present, which could have altered the role of basic MB from staining to therapeutic.

Conclusions

The presented sterilization method had been effective against C. albicans. Accordingly, this new photochemical method using basic MB can be expected to develop into a viable and promising treatment for superficial candidiasis.

In addition to the cost-effectiveness of MB, NaHCO₃, and LED, basic phototherapy has been projected to decrease the time and effort required for treatment.

Therefore, we believe that the inexpensive, safe, and simple phototherapy method presented herein could be a viable alternative to conventional treatment.
References

1: Yoshida S, Yanagi Y, Yoshikai Y.: TODA'S NEW BACTERIOLOGY. 33rd Edition. Nanzando Co., Ltd., Japan, 2010;307.
2: Peloi LS, Soares RR, Biondo CE, Souza VR, Hioka N, Kimura E. Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue. Journal of Biosciences, 2008;33 (2): 231–7.
3: Sousa JN, Queiroga BH, Kocerginsky PD, Marinho PH, and Araki AT: Photoinactivation of Candida albicans using methylene blue as photosensitizer. RGO-Revista Gaúcha de Odontologia, 2015;63 (4):411–417.
4: Sugimori A, Tokita S. FPhotoc hemistry- Reaction and Functionality-. Shokabo Co., Ltd., Japan, 2012,127.
5: CAS Registry Number: 61-73-4
6: CAS Registry Number: 144-55-81
7: Sano Y: Histological Tecnics: -Theoretical and Applied -. Nanzando Co., Ltd., Japan, 1981;223–6.
8: Mizuguchi K, Ito K, Shitara M, Higashi K, Hiroi S, Fujita K: The newest complete guide to staining techniques. Medical Technology Extra Issue. Ishiyaku Publishers Inc., Japan, 2011; 88.
9: Giroldo LM, Felipe MP, de Oliveira MA, Munin E, Alves LP, Costa MS. Photodynamic antimicrobial chemotherapy (PACT) with methylene blue increases membrane permeability in Candida albicans. Lasers in Medical Science, 2009;24:109–112.
10: Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. Microbiological Research, 2008; 163 (1):1–12.
11: Gabrielli D, Belisle E, Severino D, Kowaltowski AJ, Baptista MS. Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. Photochemistry and Photobiology, 2004;79 (5):227–32.
12: Calzabara-Pinton P, Rossi MT, Sala R, Venturini M. Photodynamic Antifungal Chemotherapy. Photochemistry and Photobiology, 2012;88:512–522.

Authors
Kouji Ogasawara: Sarufutsu Village National Health Insurance Hospital, Japan
Asahikawa Medical University, Respiratory Center, Japan
Yoshinobu Oosaki: Asahikawa Medical University, Respiratory Center, Japan
Mizuki Sasaki: Asahikawa Medical University, Parasitology course, Japan
Susumu Nakajima: Moriyama Memorial Hospital, Japan
Hiroshi Sato: Sarufutsu Village National Health Insurance Hospital, Japan
Tadashi Sasaki: Sarufutsu Village National Health Insurance Hospital, Japan

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