# *In vitro* and *in vivo* phototoxicity on gastric mucosa induced by methylene blue

Hui Yeong Oh*, Hyun Ho Choi*, Eui Jin Kim, Ji Hye Choi, Sung Sook Choi[1], Hae Kyung Lee[2],
Hyung-Keun Kim, Sang Woo Kim, Won Sang H. Park[3], Hiun Suk Chae

Departments of Internal Medicine and [2]Laboratory Medicine, Uijongbu St. Mary's Hospital, College of Medicine,
The Catholic University of Korea, [1]College of Pharmacy, Sahmyook University, [3]Department of Pathology, College of Medicine,
The Catholic University of Korea, Seoul, Republic of Korea
*Contributed equally as first authors

**Abstract**

**Background:** Methylene blue (MB) is used endoscopically to demarcate tumors and as a photosensitizer in photodynamic therapy (PDT). However, there are few *in vivo* studies about its toxicity in healthy stomach tissue. We performed sequential *in vitro* and *in vivo* analyses of MB-induced phototoxicity.

**Methods:** We performed *in vitro* experiments using the AGS human gastric cancer cell line treated with light-emitting diode (LED) irradiation (3.6 J/cm$^2$) and MB. Cytotoxicity was evaluated using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. *In vivo* toxicity was evaluated in the stomach of beagles using the same dose of fiber-optic LED via gastroscopy, after spraying 0.1% and 0.5% MB solutions. Stomach tissue was also evaluated using the TUNEL assay.

**Results:** *In vitro,* increased concentrations of MB led to higher TUNEL scores. However, cell viability was significantly lower after MB plus LED irradiation than after treatment with MB alone ($P < 0.001$). *In vivo*, the TUNEL score was highest immediately after treatment with 0.1% or 0.5% MB plus light irradiation, and the score was significantly higher in the LED illumination plus MB group than in the control group ($P < 0.05$). The elevated TUNEL score was maintained for 3 days in the MB plus light irradiation group but returned to normal levels on day 10.

**Conclusions**: Endoscopic light application with MB 0.5% concentration to the stomach may be regarded as a safe procedure despite some DNA injuries in the early period.

**Keywords:** Chromoendoscopy, cytotoxicity, gastric mucosa, methylene blue, photodynamic therapy

## INTRODUCTION

Methylene blue (MB), a phenothiazine dye that dissolves well in water, has been positively used in many medical fields to promote human health, such as the treatment of diseases like methemoglobinemia and morphologic imaging of cells.[1] Endoscopically, MB has been frequently utilized as a dye of chromoendoscopy to discriminate between gastrointestinal tumor and non-tumor tissue, by using rapidly absorbable characteristics into intestinal

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| | **DOI:** 10.4103/sjg.sjg_315_22 |

tissue.[1-6] For chromoendoscopy with MB, it is sprayed evenly on the mucosal surface as a solution of below 0.5% concentration under the direct endoscopic view.[1-6] Additionally, photodynamic therapy (PDT) to kill either pathogens or tumor cells, is another available area of MB in medicine, as a kind of photosensitizer by producing reactive oxygen species (ROS), as intrinsically it has light absorption peak to be activated around 670 nm.[1,7-9]

Even if MB is useful, the application of MB in the endoscopic field has been sometimes criticized. MB is regarded as a kind of carcinogen because MB can integrate into intracellular DNA leading to human DNA damage in spite of clinically non-toxic or safe usage.[10-15]

Moreover, most clinical cases applying MB were premalignant lesions such as Barrett's esophagus and intestinal metaplasia, which have already acquired genetic instability of DNA.[16-18] It is presumed that these lesions are more susceptible and fragile to ROS produced by MB-induced photodamage than normal tissue.[18]

Some researchers have reported the development of MB-induced phototoxicity immediately after an endoscopic procedure.[10] To date, there have been few *in vivo* studies on the phototoxicity of MB to normal gastrointestinal mucosa during endoscopy. There has been only one study in the colon mucosa of normal persons,[15] but not in gastric mucosa. The safety of MB during endoscopy remains unadressed, especially along with time sequence. When performing PDT, relatively higher-energy light may be required to produce a more profuse amount of ROS for killing pathogens and cancer cells than that needed in chromoendoscopy.[7-9] Therefore, we carried out this experiment about MB-related phototoxicity to normal gastric mucosa of dogs, with the passage of time, by emanating even higher energy from light-emitting diode (LED) light than from endoscopic light. Phototoxicity was evaluated with *in vivo* toxic effects in the dog gastric mucosa in parallel with cellular toxicity *in vitro,* using the AGS human gastric cancer cell line.

## MATERIALS AND METHODS

### Reagents and equipment
MB solutions of 0.005–0.5% concentrations were prepared by combining MB powder (Sigma-Aldrich, St. Louis, MO, USA) and distilled water. A Fujinon endoscopy system EPX-4400, EG-590WR (Fujinon Co., Saitama, Japan) was used for all the procedures. A spray catheter (Olympus Co., Tokyo, Japan) was used to uniformly stain the antral mucosa of the beagles with MB. The light energy source was a custom-made fiber-coupled light silver-LED (670 nm, 100 mW, Prizmatix Ltd., Holon, Tel Aviv, Israel) emitting 14.5 mW. The power of the LED, measured with a laser power meter LP10 (Sanwa Co., Tokyo, Japan), in a dark room, was 4.0 mW at a target 2.5 cm from the fiber tip. The LED was turned on for 15 min, and its total energy to reach the target was approximately 3.6 J/cm$^2$.

### *In vitro* study
The AGS human gastric cancer cell line was obtained from the Korean Cell Line Bank (Seoul, South Korea). The cells were cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Sigma Aldrich, St. Louis, MO, USA), at 37°C under 5% $CO_2$. AGS cells were seeded at $5 \times 10^4$ cells/cm$^2$ and grown to 80–90% confluence before treatment with MB and LED. In a dark room, wells containing AGS cells were filled with serum-free medium containing MB, at concentrations of 0%, 0.005%, 0.01%, 0.05%, 0.1%, and 0.5% and irradiated with LED light for 15 min. Next, the medium was removed, and the cells were washed with fresh medium without MB. The MB test without environmental illumination was performed in a dark room, using plates covered with aluminum foil to avoid light. This experiment was repeated three times. After the treatment, cellular viability was determined using a Real-Time Glo MT Cell Viability Assay (Promega Corporation, Madison, WI, USA). The ApopTag® peroxidase *in situ* apoptosis detection kit (Millipore, Temecula, CA, USA) was used for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. The cells were fixed in 4% paraformaldehyde and stained with hematoxylin and 3,3'-diaminobenzidine to determine the proportion of damaged or dead cells. After completion of the TUNEL assay, images were captured using a microscope to distinguish between dead and live cells. The TUNEL score represents the percentage of apoptotic cells in the samples.

### *In vivo* study
Eight male beagles (Orientbio Experimental Animal Center, South Korea), 10 months of age and weighing 11–12 kg, were used in this study. Animal selection, management, surgical protocols, and euthanasia procedures were approved by the Ethics Committee on Animal Experimentation of the Institutional Animal Care and Use Committee of CRNEX Ltd., [CRONEX IACUC, (approval number 201803004)] as a non-clinical contract research organization (CRO). The animals were maintained under standard conditions of room temperature and humidity and fed a standard diet. They were kept under fasting with free access to water for 8 h before the experiment. Preliminary anesthesia

in all dogs was performed by intramuscular injection of 2 ml/kg tiletamine hydrochloride plus zolazepam hydrochloride (Virbac S.A., France) and xylazine hydrochloride (Bayer) in a 1:1 ratio (Zoletil 50: Rompun). Each animal was then moved to the operating room, and general anesthesia was induced through inhalation of Terrell solution (isoflurane; Piramal Critical Care Inc., Seoul, Korea) and oxygen at a 2:1 ratio, after securing the airway with an intubation tube (8.5 Fr) via a laryngoscope. During the entire experiment and follow-up period, a professional veterinarian treated and handled the animals. Dogs anesthetized with Zoletil 50 and Rompun, as described above, were sacrificed by intramuscular injection of 50 mg suxamethonium chloride hydrate (Komipharm Co., Siheung-Si, Gyeonggi-Do, South Korea). The beagles were randomly divided into three groups: controls ($n = 2$), 0.1% MB ($n = 3$), and 0.5% MB ($n = 3$). The dogs were kept under general anesthesia during the entire procedure, as outlined in Figure 1a. About 15 ml of a 0.1% or 0.5% MB solution was sprayed evenly on the mucosal surface of the antrum using a spraying catheter [Figure 1b], and the stomach was irradiated for 15 min with an LED light source. The dogs underwent repeated endoscopic examinations 3 and 10 days later for the acquisition of gastric mucosal tissue. Gastric tissue biopsies were performed at three sites in the antral mucosa using biopsy forceps (FB 230K; Olympus Co., Tokyo, Japan). To assess the cytotoxicity, the number of dead cells in the biopsy specimen were counted by a pathologist using a slide scanner (3DHISTECH; Budapest, Hungary), which was expressed as the TUNEL score.

### Statistical analysis

Data are presented as the mean ± SD. The statistical significance ($P < 0.05$) of differences between groups was analyzed using Student's *t*-test or one-way analysis of variance when more than two groups were compared. All statistical analyses were performed using SPSS v13.0 (SPSS Inc., Chicago, IL, USA).
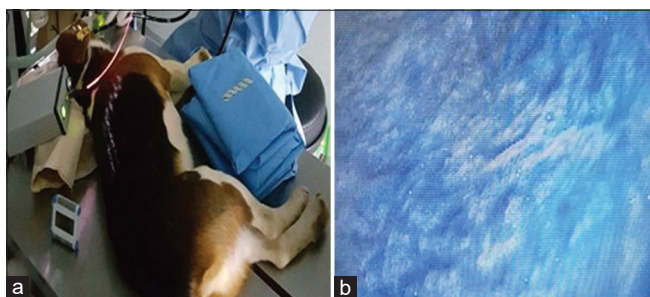


**Figure 1:** (a) Endoscopic procedure under general anesthesia to evaluate the phototoxic effect of methylene blue. LED light was delivered via a pink colored-fiber around the dog's neck. (b) Antral mucosa stained with methylene blue

## RESULTS

### Cell viability assay

To determine the viability of AGS cells after treatment with MB and LED light, we used a luminometer to measure cell viability after treatment with 0%, 0.005%, 0.01%, 0.05%, 0.1%, and 0.5% MB with or without irradiation by LED light. Cell viability decreased as the concentration of MB increased, irrespective of LED irradiation [Figure 2]. Further, the percentage of viable cells was significantly higher in cells treated with MB cells than in cells treated with MB plus LED irradiation cells at all concentrations ($P < 0.001$) (no LED vs. LED: 0.005% MB, 76.4% vs. 28.0%; 0.01% MB, 67.6% vs. 12.4%; 0.05% MB, 59.2% vs. 6.0%; 0.1% MB, 44.6% vs. 4.2%; and 0.5% MB, 3.0% to 0.5%).

### *In vitro* TUNEL assay

The degree of apoptosis caused by treatment with MB and irradiation is shown in Figure 3. The TUNEL score showed an increasing trend with 0%, 0.005%, 0.05%, and 0.5% MB plus LED irradiation. Therefore, LED irradiation with a higher concentration of MB resulted in a higher proportion of apoptotic cells.

### TUNEL assay on gastric mucosal tissue of beagles

To evaluate the number of damaged cells after treatment with MB and LED, the TUNEL scores of biopsy samples from the 0.1% and 0.5% groups were compared with that of the control group [Figure 4]. The TUNEL scores of the three groups before light irradiation and MB treatment did not differ significantly (control, 58.35%; 0.1% MB, 60.46%; 0.5% MB, 64.09%; $P > 0.05$). However, the TUNEL scores increased sharply and immediately after the application of 0.1% or 0.5% MB and light (control,
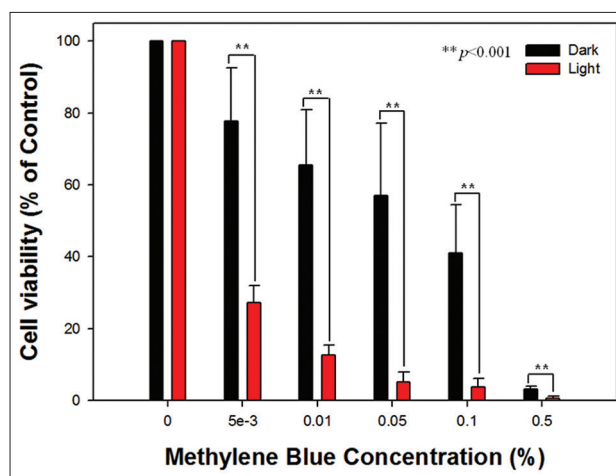


**Figure 2:** Viability of AGS gastric cancer cells treated with various concentrations of methylene blue with or without LED irradiation *in vitro*

54.45%; 0.1% MB, 91.89%; and 0.5% MB, 92.51%; $P < 0.05$). These differences were maintained 3 days after irradiation (control, 58.83%; 0.1% MB, 84.58%; and 0.5% MB, 77.07%; $P < 0.05$). However, 10 days after treatment, the TUNEL scores in the MB-treated samples were lower than those in the control samples (control, 61.08%; 0.1% MB, 42.17%; and 0.5% MB, 43.16%; $P > 0.05$). Figures 5 and 6 show the morphology of stained cells. Significantly, fewer TUNEL-stained cells were observed before treatment [Figure 5a] than on the day of treatment with 0.1% MB [Figure 5b]. However, this effect subsided after 10 days. Similar patterns were observed after treatment with 0.5% MB [Figure 6].

## DISCUSSION

MB has been widely used in medicine for diagnosis via *in vivo* imaging and treatment of septic shock and inflammatory disease.[1] For endoscopy, MB is available as a dye for staining mucosa for chromoendoscopy and a photosensitizer in PDT.[1,2,7-9]

Although MB is considered non-toxic to humans, questions about its potential harmfulness as a cytotoxic agent have been repeatedly raised, especially when MB is used in combination with light irradiation.[10-15] It is thought that current endoscopic procedures for chromoendoscopy using less than 0.5% MB are safe when used for the
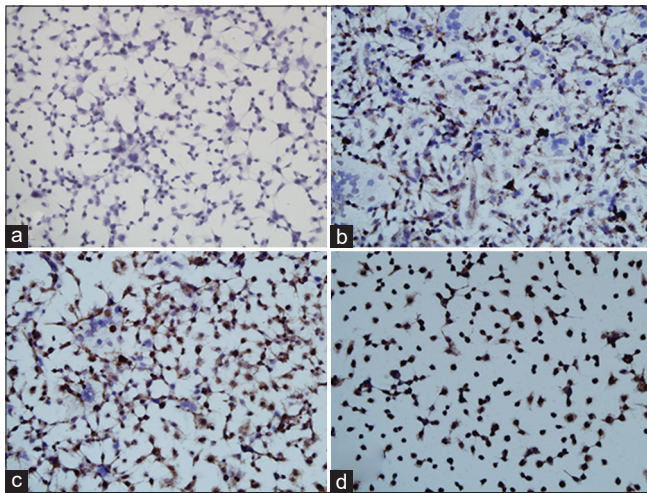


**Figure 3:** *In vitro* TUNEL assay (×100). AGS cells in 0% methylene blue (MB) (a), 0.005% MB (b), 0.05% MB (c), and 0.5% MB (d) with LED irradiation. Live cells are blue; dead cells appear red. TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling
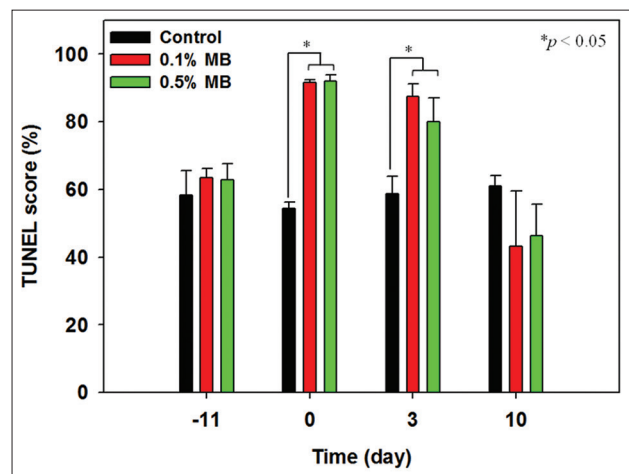


**Figure 4:** TUNEL score for apoptotic cells before, immediately after, 3 d after, and 10 d after LED light irradiation and treatment with 0.1% or 0.5% MB. TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling; MB: methylene blue
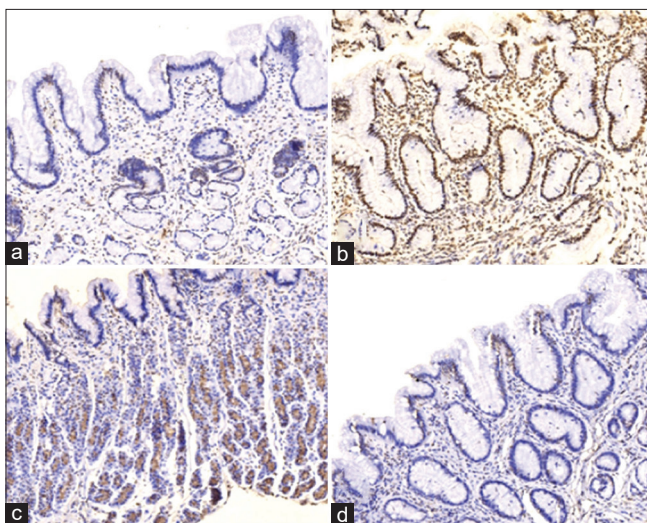


**Figure 5:** Images of TUNEL-stained biopsy samples from gastric mucosa before and after treatment with irradiation and 0.1% methylene blue. Before (a), immediately after (b), 3 days after (c), and 10 days after (d) the treatment. TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling



**Figure 6:** Images of TUNEL-stained biopsy samples from the gastric mucosa before and after LED light irradiation with 0.5% methylene blue. Before (a), immediately after (b), 3 days after (c), and 10 days after (d) the treatment. TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling
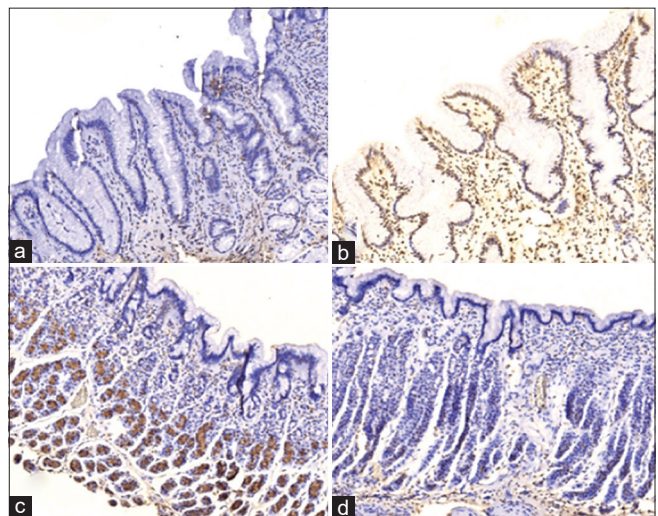
diagnosis of gastrointestinal diseases, because the amount of light energy emitted from the endoscopic light source is low.[7] However, there have been several reports on the *in vitro* or *in vivo* phototoxicity of MB to premalignant lesions in normal intestinal tissue.[10-13] These studies affirmed that chromoendoscopy using MB to delineate Barrett's esophagus led to photodamaging of DNA, the risk of which could be alleviated by lowering and eliminating MB.[11] Because genetic instability is an early step of malignancy in Barrett's esophagus,[16-18] these lesions are more susceptible to genetic insult than other lesions.[10] However, these studies did not examine serial changes in phototoxicity in normal gastric mucosa, or premalignant lesions after initial MB treatment, to determine whether injuries became more severe after the initial injury with time. Therefore, the present study was performed to affirm whether the application of MB in the normal stomach is safe as a staining dye or photosensitizer, and how long initial injuries persist in the stomachs of dogs. We conducted the study using a more powerful LED light to easily produce more phototoxicity than routine endoscopic light. We performed a TUNEL assay to evaluate nuclear DNA injuries as a measure of apoptosis.[19]

In the present study, we investigated the *in vitro* phototoxicity of MB using a gastric cancer cell line to assess cell viability and apoptosis. The results showed that cell viability was significantly lower after light irradiation plus any concentration of MB ≥0.005%, than after MB alone, at the same concentration ($P < 0.001$). These data indicate that even low concentrations of MB may cause minimal cell damage, which was markedly potentiated by the application of light, as shown in Figures 2 and 3. We also confirmed that the cellular toxicity of MB increased with increasing MB concentration. This finding is consistent with that of a previous study.[11] Because the concentration of MB may be the principal factor determining cytotoxicity, it would be better to use MB at a low concentration to avoid this side effect, if possible. Therefore, when performing PDT using MB, it is helpful to apply synergistically acting cationic materials, such as chitosan, to decrease the total dose of MB, thereby minimizing toxicity.[20]

We aimed to elucidate the toxicity of 0.1% and 0.5% MB plus LED irradiation during endoscopic procedures *in vivo*. In this animal study, the total dose of light energy was approximately two-fold higher (about 14.5 mW LED) than that of endoscopy light only (below 7 mW).[7] Its energy 2.5 cm apart from the light source was 4.0 mW which was measured with a power meter. In the previous works of PDT against *Helicobacter pylori in vitro* study, illuminating time to eradicate the bacteria took 10 min.[7] We believed that irradiation of LED for 15 min might be sufficient to accomplish the procedure. Therefore, the calculated total energy to the targeting gastric mucosa was as follows: Watt (4 mW) × time (15 min × 60 s) = J/cm² (3.6 J/cm²). The proportions of apoptotic cells in the gastric mucosa of dogs treated with 0.1% and 0.5% MB plus light were immediately and significantly higher than that of the mucosa of control dogs. This finding was in agreement with that of a previous study, in which immediate DNA damage occurred after applying light and MB in Barrett's esophagus.[10] In contrast to the *in vitro* data, there was little difference in the number of apoptotic cells between samples treated with 0.1% and 0.5% MB. This apoptosis was maintained up to 3 days after treatment, but the number of damaged cells was reduced to normal levels by 10 days. These data suggest that endoscopic procedures using MB concentrations of 0.5% or lower may be safe in the normal mucosa, despite some cellular toxicity in the early period. These findings seem to be the first data on serial tests of MB toxicity in healthy gastric mucosa, indicating that the toxic effects of MB and light do not persist longer than 10 days. Despite the absence of a previous report on additional damage by repeated MB-induced phototoxicity, a lag time of >10 days would be needed between two serial endoscopic procedures using MB, to avoid the potential for more serious damage.

In the future, further studies are required to examine several aspects with regard to the cytotoxic effects of MB-induced photodamage: first, phototoxicity by repeated exposure to MB and light; second, serial assessment of phototoxicity, especially in premalignant lesions; third, clinical studies on photodamage to the human stomach; and fourth, development of an endoscopic device reducing the time of the endoscopic procedure to alleviate phototoxicity.

In conclusion, our results indicate that endoscopic examination using 0.5% MB at a concentration of 0.5% or lower is a safe procedure in the normal gastric mucosa, despite photodamage in the early period.

### Conflicts of interest
There are no conflicts of interest.

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