

# JOE

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# Endodontic Antimicrobial Photodynamic Therapy: Safety Assessment in Mammalian Cell Cultures

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## Abstract

**Objectives:** The purpose of this study was to assess the *in vitro* synergistic effect of methylene blue (MB) and red light on human gingival fibroblasts and osteoblasts with parameters similar to those that may be applied in a clinical setting for endodontic disinfection. **Materials and Methods:** Both cell types were sensitized with 50  $\mu\text{g/mL}$  MB followed by exposure to red light at 665 nm for 5 minutes with an irradiance of 10, 20, and 40  $\text{mW/cm}^2$ . After photodynamic therapy (PDT), cell viability and mitochondrial activity were evaluated by the neutral red and MTT assay, respectively. The assessment of PDT-induced apoptosis was investigated by western blot analysis using cleaved poly(ADP-ribose) polymerase-specific antibodies. **Results:** Light at 20 and 40  $\text{mW/cm}^2$  with MB had modest effects at 24 hours on osteoblasts in both assays, whereas sodium hypochlorite completely eliminated cells. Western blot analysis revealed no signs of apoptosis in either cell type. **Conclusion:** The data suggest that there is a safe therapeutic window whereby PDT can inactivate endodontic pathogens without affecting host cell viability. (*J Endod* 2009;35:1567–1572)

## Key Words

Endodontic disinfection, mammalian cells, methylene blue, photodynamic therapy, safety

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Endodontic therapy attempts to eliminate bacteria within the root canal system by using protocols that combine mechanical instrumentation and chemical irrigation. Although current endodontic techniques are highly predictable, residual bacteria are readily detectable in approximately one half of teeth just before obturation even when chemomechanical debridement is performed at the highest technical standard (1). In addition, the anatomic complexities of the root canal system with its apical ramifications, lateral canals, and isthmuses make complete eradication of bacteria almost impossible (2). It would seem that the goal of complete disinfection of the root canal system will not be achieved even with today's most advanced methods (3). Because of our inability to eradicate bacteria from the canals of infected teeth, a large number of teeth will require retreatment and/or periradicular surgery in order to successfully treat persistent infections. Therefore, new methodologies must be developed as adjuncts to standard endodontic antimicrobial procedures in order to increase the success rate of endodontic therapy.

Photodynamic therapy (PDT), originally developed as a therapy for cancer, is based on the concept that a nontoxic photosensitizing agent known as a photosensitizer (PS) can be preferentially localized in certain tissues and subsequently activated by light of the appropriate wavelength to generate singlet oxygen and free radicals that are cytotoxic to cells of the target tissue (4). Several studies have shown the effectiveness of PDT against oral bacteria (5–7). Recently, PDT has been used to eradicate microorganisms within root canal systems (8–15), suggesting its usefulness as an adjunct to current endodontic disinfection techniques. In several studies, methylene blue (MB) has been used as the PS (8, 10, 11, 13, 14, 15). MB is a hydrophilic PS with low molecular weight and positive charge (16) that has been used in PDT for targeting various gram-positive and gram-negative oral bacteria (17). The dye passes across the porin channels in the outer membrane of gram-negative bacteria and predominantly interacts with the anionic macromolecule lipopolysaccharide resulting in the generation of MB dimers (18). These dimers participate in the photosensitization process (18).

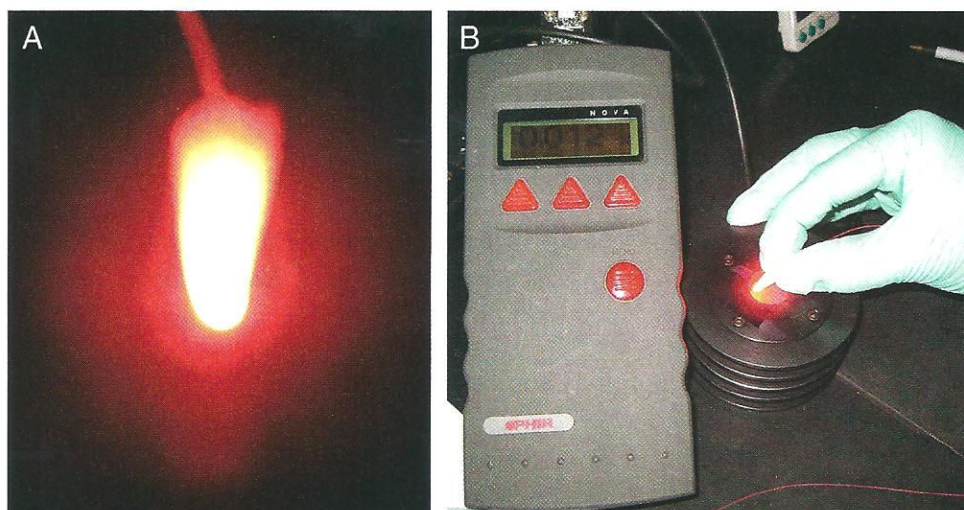
When a photoactive compound is applied in the root canal system, it is taken up by residual bacteria in the main canals, isthmuses, lateral canals, and dentinal tubules. It is also possible that this compound may escape into the periapical tissues. During PDT, light will excite the drug in bacteria within the root canal but could also potentially affect the periapical host cells that have taken up the drug. Therefore, it is important to establish the safety of PDT and to determine the therapeutic window whereby bacteria can be eliminated but host cells are left intact. In this study, we assessed the viability of human gingival fibroblasts and osteoblasts *in vitro* after exposure to MB and red light with parameters similar to those that may be applied in a clinical setting. Sodium hypochlorite was also tested on gingival fibroblasts and osteoblasts in order to have a reference level for the cytotoxicity of current endodontic chemical agents.

## Materials and Methods

### Photosensitizer

MB (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 50  $\mu\text{g/mL}$  (approximately 135  $\mu\text{mol/L}$ ) and was filter sterilized immediately before use. The ultraviolet-visible absorption spectra of MB in PBS were recorded from 200 to 800 nm using quartz cuvettes with 1-cm path length on





**Figure 1.** (A) Exposure of the root canal system of a tooth specimen to red light with a power density of 100 mW/cm<sup>2</sup>. (B) The power of light emitted by the specimen was found to be 0.012 W (12 mW), which corresponded with a power density of 4 mW/cm<sup>2</sup>. The maximum power density obtained was approximately 10 mW/cm<sup>2</sup>.

a diode-array spectrophotometer and were characterized by a long-wavelength maximum at 665 nm as previously shown (8).

## Cells and Culture Conditions

The human gingival fibroblast cell line, HGF-1 (CRL 2014), was obtained from the American Type Culture Collections (ATCC) (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle's Medium with a high glucose content (4.5 g/L) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), L-glutamine (2 mmol/L), sodium bicarbonate (1.5 g/L), penicillin G (100 U/mL), and streptomycin (100 µg/mL) (all reagents from Life Technologies Inc, Gaithersburg, MD). The medium was changed twice a week. Cells were subcultured weekly using 0.25% trypsin and 0.02% EDTA (Gibco, Grand Island, NY).

Normal human osteoblasts were purchased from Lonza (Walkersville, MD). Cells were cultured in osteoblast growth medium, which contains 10% FBS and 100 µg/mL ascorbic acid in osteoblast basal medium (Lonza). The medium was changed every 3 days, and cells were passaged weekly using trypsin-EDTA. All cells were maintained in 10-cm diameter Petri dishes with 12 mL growth medium and kept at 37°C in a humidified 95% air, 5% CO<sub>2</sub> atmosphere.

## Light Source

The irradiation source was a diode laser (BWTEK Inc, Newark, DE) with an output power of 1 W and a central wavelength of 665 nm. The system was coupled to a 1-mm optical fiber that delivered light into a lens, which formed a uniform circular spot on the base of the 96-well plate 2 cm in diameter. Power measurements were quantified with a power meter (Ophir Optonics LTD, Danvers, MA).

## Photodynamic Treatment

Aliquots of  $1.5 \times 10^5$  cells in 0.1 mL growth medium with 10% FBS were seeded in 96-well plates (Corning Incorporated, Corning, NY) and cultured for 24 hours until 70% confluent. Tetraplicate cell cultures were exposed to PBS containing MB (50 µg/mL) for 10 minutes. Cells were washed twice with sterile PBS, and fresh medium was added. All four wells were simultaneously exposed to red light for 5 minutes from above. The power densities were 10, 20, and 40

mW/cm<sup>2</sup>, corresponding with energy fluencies of 3, 6, and 12 J/cm<sup>2</sup>, respectively. In previous studies, we have used light with a power density of 100 mW/cm<sup>2</sup> for killing endodontic pathogens within infected root canal specimens *in vitro* (10, 13). A portion of that light propagates in dentin and escapes from the tooth (Fig. 1A). The power density of light escaping from the root specimen ranges from 5 to 10 mW/cm<sup>2</sup> depending on the morphology of the root canal system (Fig. 1B). In the present study, we used up to fourfold greater power densities. Coomassie blue solution was added in the space between the wells to reduce the scattering effects. All plates were kept covered during the photoirradiation to avoid contamination of the culture. After irradiation, cells were incubated with fresh medium for 24 hours. Controls were (1) cells untreated with MB or light with plates remaining at room temperature covered with aluminum foil during irradiation, (2) cells treated with MB but not exposed to light at room temperature, (3) cells exposed to light (10, 20, and 40 mW/cm<sup>2</sup>) in the absence of MB, and (4) cells exposed to 3% NaOCl for 10 minutes. Three separate experiments were performed.

## Measurement of Cell Viability

Cell viability was evaluated by the neutral red assay, an indirect method for the determination of viability (19). This assay uses neutral red (3-amino-7-dimethylamino-2-methylphenazine-hydrochloride), a water-soluble dye that readily passes an intact plasma membrane and accumulates in lysosomes. A measurement of the uptake of neutral red would be proportional to the number of viable cells. Briefly, immediately and 24 hours after PDT, cells in 96-well microplates were incubated with PBS containing 50 µg/mL neutral red (Sigma) at a final concentration of 0.05% in fresh growth medium for 2 hours in a humidified incubator. Cells were then gently washed twice with sterile PBS. The residual dye was extracted from the cells by the addition of 100 µL extractant solution (51% H<sub>2</sub>O, 48% ethanol, and 1% acetic acid) for 20 minutes at room temperature with constant gentle shaking. The amount of neutral red taken up into the cells was determined spectrophotometrically by measuring the optical density at 550 nm using a VersaMax Tunable Microplate Reader spectrophotometer (Molecular Devices, Sunnyvale, CA).



### Assessment of Mitochondrial Activity

Cell mitochondrial activity was determined immediately and 24 hours after PDT using the 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) microculture tetrazolium assay (Sigma) according to the manufacturer's instructions. An MTT assay is a method of assessing viable cell numbers (20). This assay involves the reduction of a colorless substrate, MTT, to an insoluble dark blue formazan product, which is formed in proportion to the amount of succinate dehydrogenase activity in the mitochondria of living cells. One hundred microliters of growth medium and MTT solution (9:1) was added in each well, and the 96-well plates were incubated at 37 °C in 5% CO<sub>2</sub> for 3 hours. Medium was then removed from all wells and replaced by 100  $\mu$ L dimethyl sulfoxide. After 20 minutes, after thorough formazan solubilization, the absorbance of each well was measured at 570 nm.

### Western Blotting

To determine whether PDT induced apoptosis, we sensitized cells with MB (50  $\mu$ g/mL) for 10 minutes followed by exposure to red light (40 mW/cm<sup>2</sup>) for 5 minutes (energy fluence: 12 J/cm<sup>2</sup>). Cleavage of poly(ADP-ribose) polymerase (PARP) 24 hours after PDT treatment was determined by western blot analysis using the full-length PARP antibody (Cell Signaling Technology, Danvers, MA) that detects full-length PARP-1 (116 kd), the large fragment (89 kd), and the small fragment (24 kd). B-actin (Cell Signaling Technology) was used as a loading control. Protein extracts were generated by lysing cells with 1.5  $\times$  sodium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA). Lysates were then resolved by electrophoresis on 12% tris-glycine gels and transferred to 0.2- $\mu$ m nitrocellulose membranes (Invitrogen). Prestained BenchMark™ protein standards (Invitrogen, product #10748-010) were used to indicate molecular weight. After transfer, the nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (Sigma Aldrich, product #P7949) (TBST) for 60 minutes at room temperature. The membranes were then incubated with gentle agitation overnight at 4°C in the presence of primary antibodies against PARP (dilution 1:500) or  $\beta$ -actin (dilution 1:1000). After incubation, the membranes were washed three times for 5 minutes each in 10 mL TBST, incubated in the presence of an antirabbit horse radish peroxidase-conjugated secondary antibody at dilution of 1:1,000 (Cell Signaling Technology), and washed three times for 5 minutes each in 10 mL TBST. Then, they were incubated in Lumi-GLO solution (Cell Signaling Technology) for 1 minute with gentle agitation at room temperature, exposed to radiographic film (BioMax XAR; Kodak, New Haven, CT), and developed.

### Statistical Analysis

For each pairing of cell type and assay, three separate experiments were conducted. Each experiment included nine independent groups (no light/no MB, only MB, light only at 3 different intensities, MB and light at 3 different intensities, and NaOCl only) with  $n = 4$  independent trials per group. A total of 108 optical density (OD) values were obtained for each pairing at 0 hours and the same number of values at 24 hours.

For each combination of cell type, assay, and time, mean OD values for treatments were evaluated by two-way analysis of variance with eight treatments stratified by three experiments and  $n = 4$  values per treatment group in each experiment. NaOCl was excluded because of its obviously overwhelming effects and minimal variation. Pair-wise comparisons were performed by a Tukey test with overall  $\alpha = 0.05$ .

Change over time for each treatment was evaluated by first obtaining a mean OD value from each experiment and then comparing the overall mean for each treatment from the three experiments at 0 hours with

those at 24 hours by  $t$  tests. Similarly, the mean percent OD level relative to control ( $[(100 \times \text{treatment mean}) / \text{control mean}]$ ) was also calculated for each treatment in each experiment. The overall mean percents and standard errors were then obtained from results of the three experiments. This descriptive summary includes results for NaOCl.

## Results

### PDT Effects on Viability and Mitochondrial Activity

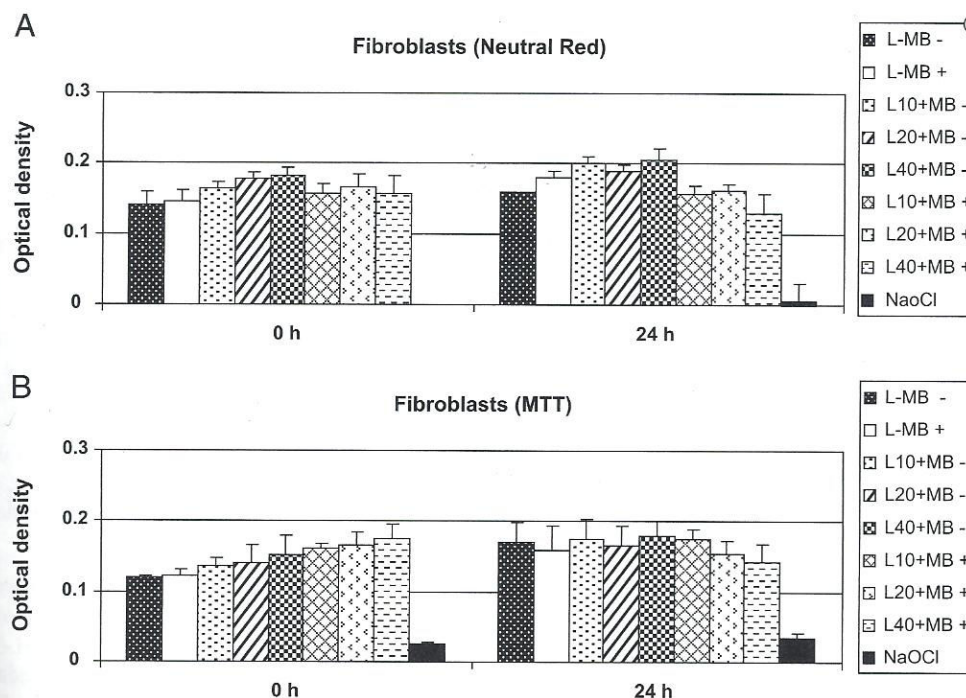
Figures 2 and 3 show the growth of fibroblasts and osteoblasts as determined by the neutral red assay (Figs. 2A and 3A) and MTT assay (Figs. 2B and 3B) after their sensitization with MB and their subsequent exposure to red light. Light alone had no effect on viability and mitochondrial activity of either cell type at 0 and 24 hours. MB alone had no effect on fibroblasts at 0 and 24 hours, whereas it slightly reduced the viability and mitochondrial activity of osteoblasts ranging from 9.4% (0 hours) to 14.6% (24 hours) and from 3.1% (0 hours) to 13.5% (24 hours), respectively. The results for the L + MB+ groups were as follows:

1. Neutral red assay (Figs. 2A and 3A): for fibroblasts, no significant decreases in mean OD levels were observed at 0 hours relative to control (L-MB-) or MB alone (L-MB+). At 24 hours, viability for the L40+MB+ group was reduced by 19.2% and was significantly lower than L40+MB- and L-MB+. For osteoblasts, viability of the L20 + MB+ group was reduced at 0 hours by 12.5% and for the L40 + MB+ group by 33%. Osteoblast viability was significantly lower in these two groups than the control and all other treatment groups. At 24 hours, the mean OD levels for L10+MB+, L20+MB+, and L40+MB+ were reduced by 37.3%, 53%, and 59.2%, respectively. The latter two groups were significantly lower than all other treatment groups, whereas the mean for L10+MB+ was significantly lower than means for the control, L-MB+, and light alone groups.
2. MTT assay (Figs. 2B and 3B): for fibroblasts, all three groups that received light and MB (L10+MB+, L20+MB+, and L40+MB+) were significantly higher than both L-MB+ and L-MB- at 0 hours. At 24 hours, mitochondrial activity for the L40 + MB+ group was reduced by 18.5% and was significantly lower than L40+MB- and control. For osteoblasts, the mean OD levels showed no significant differences among treatment groups at 0 hours. At 24 hours, the mitochondrial activity for the L20+MB+ and L40+MB+ groups was reduced by approximately 34% and was significantly lower than all other treatments.
3. Sodium hypochlorite: treatment with NaOCl resulted in severe reductions to levels ranging from 0% to 20% of control for mean OD levels of fibroblasts and osteoblasts in both assays at 0 and 24 hours.

### Assessment of PDT-induced Apoptosis

Cells were sensitized with MB (50  $\mu$ g/mL) for 10 minutes and then exposed to red light (power density: 40 mW/cm<sup>2</sup>, energy fluence: 12 J/cm<sup>2</sup>). The assessment of PDT-induced apoptosis was performed by western blot analysis using cleaved PARP-specific antibodies. Experimental induction of apoptosis was optimized for each cell type by treating with staurosporine, a known inducer of cellular apoptosis, and assessed by the determination of PARP cleavage. There was no PARP cleavage detectable in either fibroblasts or osteoblasts 24 hours after PDT (Fig. 4). In addition, MB alone and light alone did not result in PARP cleavage (Fig. 4).



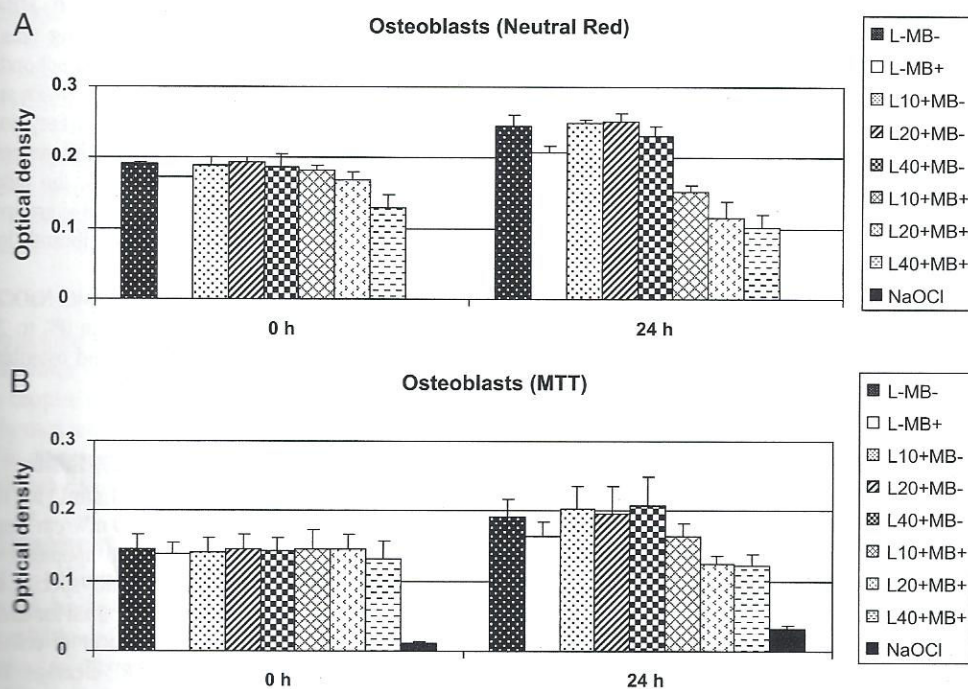


**Figure 2.** Growth of MB-sensitized gingival fibroblasts as determined by the (A) neutral red assay and (B) MTT assay after their exposure to red light. Bars represent the means of optical density ( $\pm$ standard error). L-MB-, no light/no drug; L-MB+, MB-treated cells but not irradiated with light; L10+MB-, L20+MB-, and L40+MB-, MB-untreated cells but irradiated with light of 10, 20, and 40 mW/cm<sup>2</sup>; L10+MB+, L20+MB+, and L40+MB+, MB-treated cells and irradiated with light of 10, 20, and 40 mW/cm<sup>2</sup>; NaOCl, cells treated with sodium hypochlorite.

## Discussion

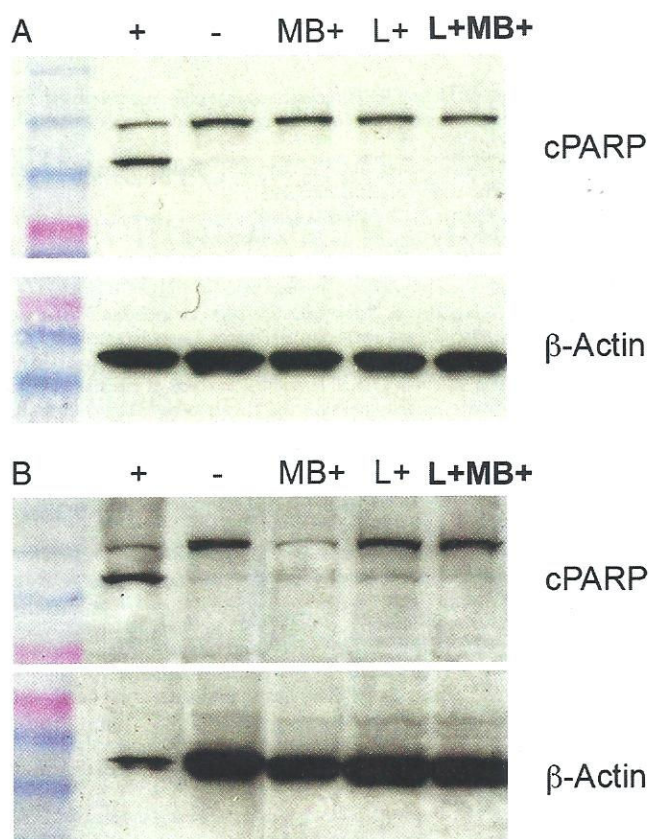
The synergistic effect of light and photoactive drugs to eliminate residual endodontic bacteria in the root canal system has been explored in previous studies (8–15). In a clinical setting, photoactive

compounds will be applied in the root canal system and taken up by residual bacteria in the main canals, isthmuses, lateral canals, and dentinal tubules. Much like current endodontic irrigating solutions and intracanal medicaments, the escape of photoactive compounds



**Figure 2.** Growth of MB-sensitized osteoblasts as determined by the (A) neutral red assay and (B) MTT assay after their exposure to red light. Bars represent the means of optical density ( $\pm$ standard error). The groups are the same as in Figure 2.





**Figure 4.** (A) Osteoblasts and (B) fibroblasts were treated with MB only (lane MB+), light only (lane L+), or light and MB (lane L+MB+). Control cultures were left untreated (lane -) or treated with 200  $\mu\text{mol/L}$  staurosporine (lane +). Twenty-four hours after treatment, cultures were stopped and analyzed by western blot using cleaved PARP- and  $\beta$ -actin-specific antibodies. Neither treatment resulted in PARP cleavage.

beyond the apical foramen into the periapical tissues during clinical treatment is a distinct possibility. During PDT, light will excite the drug in bacteria in the root canal and could also potentially affect host cells in the periapical region. Accordingly, it is important to establish the safety of PDT and to determine the therapeutic window whereby bacteria can be eliminated but host cells are left intact.

The hypothesis of the present study is that PDT doses that can be used for the elimination of root canal microorganisms *in vivo* will not induce significant cytotoxic effects on normal human gingival fibroblasts and osteoblasts in culture. To test this hypothesis, cells were incubated with 50  $\mu\text{g/mL}$  MB for 10 minutes followed by exposure to red light for 5 minutes at power densities of 10, 20, and 40  $\text{mW/cm}^2$  that corresponded with energy fluences of 3, 6, and 12  $\text{J/cm}^2$ , respectively. The rationales for using the previously described drug and light parameters were based on several prior studies. The basis for the MB concentration came from a set of three separate experiments, which showed no cytotoxic effects on both cell types at a concentration of 25  $\mu\text{g/mL}$  of MB in combination with light with the same parameters as described earlier (data not shown). Therefore, we tested the concentration of 50  $\mu\text{g/mL}$  of MB in the present study. In terms of the light parameters, a 250- $\mu\text{m}$ -diameter polymethyl methacrylate optical fiber that uniformly distributed light over  $360^\circ$  was previously used for bacterial killing in experimentally infected teeth (13). The advantage here is that, compared with optical fibers that transmit light over a straight path, less than 20% of light energy delivered by the fiber escapes from the root

apex. The power density of light in the periapical region ranges from 5 to 10  $\text{mW/cm}^2$  depending on the anatomy of the root canal (Fig. 1). In a clinical setting, this may provide the opportunity for enlargement of a safe therapeutic window using greater drug and light parameters. Preliminary studies with 50  $\mu\text{g/mL}$  MB and light with a power density and energy fluency of 100  $\text{mW/cm}^2$  and 30  $\text{J/cm}^2$ , respectively, led to  $>3 \log_{10}$  bacterial killing of infected canals of freshly extracted human teeth with pulpal necrosis and associated periapical radiolucencies.

In this study, we showed that osteoblasts exhibited greater susceptibility to PDT compared with fibroblasts. The synergism of MB and light did not exhibit any significant cytotoxic effects on the viability and mitochondrial activity of human gingival fibroblasts. On the other hand, MB caused a mean reduction in mitochondrial activity of approximately 34% in human osteoblasts 24 hours after treatment with light at 20 and 40  $\text{mW/cm}^2$ . There was a mean reduction in the viability of osteoblasts at 24 hours ranging from approximately 38% (10  $\text{mW/cm}^2$ ) to 59% (40  $\text{mW/cm}^2$ ). There was no reduction of either mitochondrial activity or viability for both cell types after exposure to light alone and MB alone. Sodium hypochlorite was found to inhibit mitochondrial activity and reduce cell viability by 97% to 100%. These results suggest that PDT is much less cytotoxic to mammalian cells than NaOCl, an agent commonly used in clinical practice.

There have been relatively few *in vitro* studies in the literature showing microbial killing under conditions that are safe for mammalian cells. A number of studies that used different photoactive compounds have suggested a possible set of optimal conditions for antimicrobial PDT (21). Short incubation times up to 10 minutes with low concentrations of photosensitizer together with power densities and energy fluences less than 50  $\text{mW/cm}^2$  and 5  $\text{J/cm}^2$  led to bacterial killing while human cells (fibroblasts and keratinocytes) were spared (21). Similar conditions were also applied in the present study. Microorganisms appear to be much more susceptible to PDT induced by phenothiazine dyes, such as toluidine blue O and MB, than mammalian cells (22–25). Toluidine blue O and light at 633 nm fully eliminated *Streptococcus sanguinis* (formerly known as *Streptococcus sanguis*) *in vitro* using parameters that spared human gingival keratinocytes and fibroblasts (22). The killing rates for human keratinocytes after sensitization with MB (100  $\mu\text{g/mL}$ ) and exposure to visible light (400–700 nm) were found to be 18- to 200-fold slower (23) than those of cutaneous microbial species (24). In a recent study, the photodynamic effects of MB dissolved in water were tested on fibroblasts and *Enterococcus faecalis* (25). Concentrations of MB ranging from 10 to 100  $\mu\text{mol/L}$  produced up to 36% and 100% killing for fibroblasts and *E. faecalis*, respectively, after incubation for 20 minutes followed by exposure to red light with a total fluence of 36 J (25). A polycationic conjugate between poly-L-lysine and the photosensitizer chlorin e6 could efficiently target photodestruction of *Porphyromonas gingivalis* and *Actinomyces naeslundii* (formerly known as *Actinomyces viscosus*) while sparing an oral epithelial cell line (6). In another study, selective killing of *P. gingivalis* was achieved in the presence of *Streptococcus sanguinis* or human gingival fibroblasts using an anti-*P. gingivalis* monoclonal antibody conjugated with toluidine blue O (26). The results obtained for NaOCl confirm earlier data obtained from fibroblasts (25, 27) and human periodontal ligament cells (28).

PDT is known to exert oxidative stress and induce apoptosis (4). The involvement of apoptosis has been shown to be an early response to therapy, both *in vitro* and *in vivo* (29, 30). Therefore, the involvement of apoptosis after PDT was explored on mammalian cells in the present study. Western blot analysis of cell protein extracts revealed no apoptosis 24 hours after PDT. In another study, the comet assay was not able to detect any DNA damage in keratinocytes after incubation with MB and exposure to visible light (31). DNA damage was detected



by the comet assay in the human myeloid leukaemic cells immediately after MB-induced PDT (32). However, no residual level of DNA damage was evident 4 hours after treatment (32). Other studies have shown mitochondria-dependent apoptosis induced by MB-mediated PDT (33, 34). Recently, Sturmey et al (35) showed that excitation of MB by white light caused a dose- and time-dependent DNA damage *in vitro*, with approximately 30% of the cell population entering apoptosis in response to treatment. The concentrations of MB in the Sturmey et al study were approximately 10- to 100-fold greater than those used in our study. MB has been used as a photosensitizing agent for almost 9 decades (36) and is used routinely as a marker dye in surgery (37). The clinical use of MB for photodynamic therapy of bladder (38) and esophageal (39) cancer along with its use in photo targeting of *Helicobacter pylori* in the rat gastric mucosa (40) suggest the local use of MB is safe.

In conclusion, our findings suggest that MB-mediated PDT, as an adjunctive technique for endodontic disinfection, may be effectively used in a clinical setting without harming cells in the periapical region. The current study provides evidence that PDT with light parameters used for bacterial targeting in the root canal system (power density: 100 mW/cm<sup>2</sup>; energy fluence: 30 J/cm<sup>2</sup>) displays a safe therapeutic window.

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