DENTAL MATERIALS 24 (2008) 1036–1042

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Plasma-arc generated light inhibits proliferation and induces apoptosis of human gingival fibroblasts in a dose-dependent manner

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ARTICLE INFO

Article history:
Received 22 November 2006
Received in revised form
27 August 2007
Accepted 21 November 2007

Keywords:
Blue light
Human gingival fibroblasts
Growth inhibition
Apoptosis

ABSTRACT

Objective. This study examined the effects of blue light exposure on the proliferation and cytotoxicity of human gingival fibroblasts (HGF). Cellular mechanism by which blue light causes cytotoxic effects was also investigated.

Methods. HGF were exposed to the plasma-arc generated blue light with various energy densities ranging from 2 to 48 J/cm^2. After light exposure of the cells, they were processed for analyzing tritium incorporation, succinate dehydrogenase (SDH) activity, trypan blue exclusion, and DNA fragmentation. In addition, possible mechanism of the light-mediated cytotoxicity was investigated through flow cytometric and Western blot analyses.

Results. Blue light exposure significantly inhibited proliferation and SDH activity of HGF in a dose-dependent manner; exposure more than 12 J/cm^2 had a toxic effect on the cells. The blue light-induced cytotoxicity of the cells resulted from apoptosis, as proven by the migration of many cells to the sub-G_1 phase of cell cycle and the appearance of DNA ladders. Additional experiments revealed that blue light induces apoptosis of HGF through mitochondrial stress and poly (ADP ribose) polymerase cleavage.

Significance. This study suggests that plasma-arc generated blue light exerts some harm to cells, particularly damaging effect to DNA, and thus a long curing time more than recommended can cause biological damage on the oral tissue.

1. Introduction

Various types of photocuring light sources have been developed to polymerize dental composites, which has led to a rapid increase in the number of esthetic restorative techniques. Of the light sources used, a quartz-tungsten-halogen (QTH) source has long been used for dental photocuring. The recommended standard intensity of QTH sources was approximately 600 mW/cm^2, and has been shown to be capable of curing most dental composites to a depth of 2 mm in approximately 40 s [1,2]. However, for large restorations and/or those of greater, the clinical chair time needed for photocuring is being increased. In order to minimize the curing time, light sources with a powerful intensity higher than QTH sources have been

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doi:10.1016/j.dental.2007.11.018

introduced [3]. Plasma-arc lights are the light sources with high intensity (approximately 2000 mW/cm^2), and are routinely used for dental photocuring, even though the sources are considered to cause shrinkage stress to composites during photocuring [4].

The wavelength produced by a photocuring unit is very important because the ultraviolet radiation can have harmful effects on the eyes and skin [5,6]. Therefore, most photocuring sources are filtered to provide blue light of wavelengths between 400 and 500 nm, which are assumed to be innocuous to cells and/or tissues. However, it has been suggested that blue light induces biological hazards to living cells. This has been demonstrated by some considerable evidences showing that blue light has negative effects such as oxidative DNA damage [7], mitosis inhibition [8], mitochondrial dysfunction [9], and antioxidant system disruption [10]. These results strongly suggest that biological damages on oral tissues can be occurred during dental photocuring. However, in these reports, the experimental conditions such as the doses of, and the times for light exposure, and the types of cells examined have not been the same as those used in clinical photocuring. There are also reports demonstrating that blue light affects cellular responses differentially in tumor versus normal cells [11,12]. In addition, the cellular mechanisms by which blue light causes cytotoxic effects are not completely understood.

Therefore, we have investigated the influence of blue light on the cellular responses using a clinically relevant light source and cells. To this end, we used the primary cultures of human gingival fibroblasts (HGF) and plasma-arc unit as the model cell and light source. In this study, we examined the levels of proliferation and cytotoxicity of the cells exposed to blue light with various energy densities. In addition, we determined whether or not blue light-induced toxicity occurs through apoptosis. Our data shown in this study demonstrate for the first time that blue light inhibits proliferation and induces apoptosis of HGF through mitochondrial stress and poly (ADP ribose) polymerase (PARP) cleavage in an energy density-dependent manner.

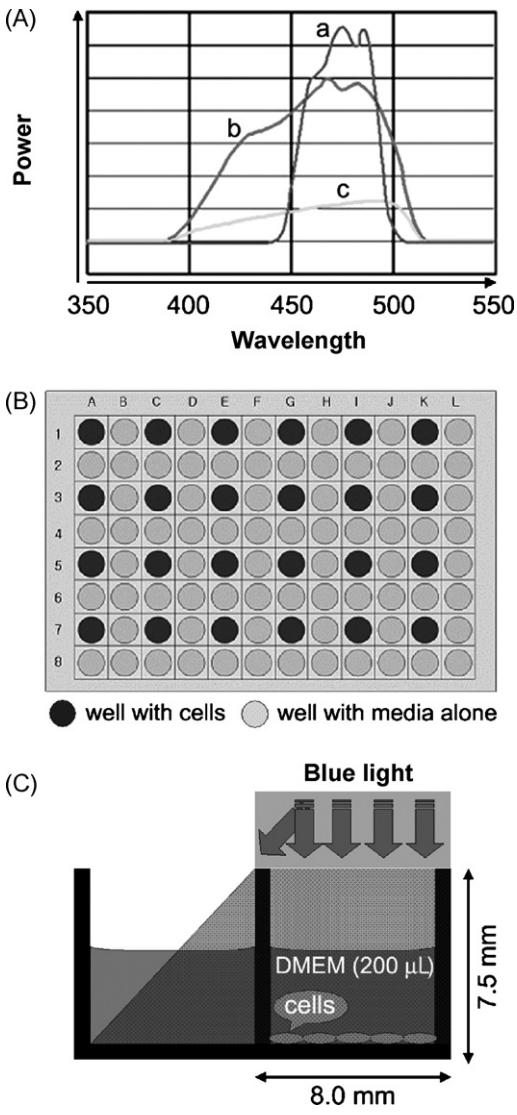

Fig. 1 – Experimental designs for light exposure. (A) Specific characteristics of the spectrum in Flipo. (a), (b), and (c) represent the light sources of the narrow spectrum PAO, Flipo, and halogen ramp, respectively. (B) The design of cell dividing into 96-multiwell culture plates. (C) Diagram showing the condition of light exposure to the human gingival fibroblasts.

2. Materials and methods

2.1. *Chemicals, laboratory wares, and cell culture*

Unless otherwise specified, all the chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. HGF were isolated from the cervical area of a premolar tooth extracted in the course of orthodontic treatment. Informed written consent from donors was obtained for use of the tissues. Patients signed the corresponding informed consent approved by the Review Board of Chonbuk National University Hospital (CNUH) for used of the tissues. Briefly, the tissues were gently dissected and signal cell suspensions were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 IU/mL penicillin G, and 100 μg/mL streptomycin. The cultures were switched to a fresh batch of the same media every 3 days. When the cells had reached confluence, they were resuspended and spread onto each well of 96-well flat-bottomed plates at 20,000 cells/cm^2 in 200 μL medium and were then incubated 24–36 h before blue light exposure. Cells within passages five to seven were used in these experiments.

2.2. *Light source and exposure conditions*

A plasma lamp, Flipo (Lokki, France), was used as the blue light source. The wavelength and energy emitted from the light source were between 400 and 500 nm, and 1900 mW/cm^2, respectively. Fig. 1A gives more detailed information on its spectrum characters.

Before exposing the HGF to blue light, the cells were resuspended and divided onto 96-multiwell plates with a design to avoid indirect light exposure (Fig. 1B). HGF were exposed to the Flipo-generated blue light with various energy densities ranging from 2 to 48 J/cm^2 in a clinically relevant manner, as described previously [9]. Briefly, the Flipo tip was placed over the opening of the 96-multiwell culture plates and was 7.5 mm from the bottom where cells were attached (Fig. 1C). As shown in the figure, the distance between the tip and cells is quite similar to that in a clinical photocuring. After exposure of the cells to blue light, the cells were analyzed for any blue light-mediated cell responses by determining the level of DNA synthesis, succinate dehydrogenase (SDH) activity, trypan blue exclusion, and DNA fragmentation. In addition, possible mechanism of the light-mediated cytotoxicity was investigated.

2.3. *TdR uptake assay*

The level of DNA synthesis by HGF after exposure to blue light was measured by adding 1 μCi of [methyl-^{3}H] Thymidine deoxyribose (TdR; Amersham Pharmacia Biotech Inc., Piscataway, NJ) to each well for the last 16 h of the culture periods. The cells were then collected using a cell harvester (Inotech Inc., Switzerland), and the TdR content was measured using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

2.4. *Measurement of SDH activity*

3-(4,5-Dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate the SDH activity of HGF. In this assay system, only living cells take up the yellow MTT, which is then converted to a dark blue product by an enzyme, SDH, present in mitochondria. Therefore, the concentration of the blue product is proportional to the SDH activity to reduce MTT, which is also closely related to the viability of cells. After 48 h exposure to blue light with various energy densities, 10 μL of a MTT solution (5 mg/mL in PBS as stock solution) were added into each well, and the cells were incubated for a further 4 h at 37 °C. After removing the culture media, acidic isopropanol (70 μL) was added to each well, and absorbance of the plates was read at 560 nm using a SpectraCount™ ELISA reader (Packard Instrument Co.).

2.5. *Trypan blue exclusion assay*

The trypan blue exclusion assay was used to determine the level of cytotoxicity caused by exposing the cells to the blue light generated by Flipo. After 48 h of exposure, the cells were stained with 0.4% trypan blue and approximately 100 cells were counted for each treatment. The level of cytotoxicity was calculated as follows: %cytotoxicity = [(total cells − viable cells)/total cells] × 100%.

2.6. *PI staining*

The level of DNA fragmentation and cell cycle progression was determined by flow cytometric analysis after propidium iodide (PI) staining. At 48 h of incubation, the cells were collected and fixed with 80% ethanol overnight at 4 °C. After washing with PBS, the cells were incubated in 1 mL of a PI staining solution (250 μL of PBS, 250 μL of 1 mg/mL RNase in 1.12% sodium citrate, and 500 μL of 50 μg/mL PI in 1.12% sodium citrate). Finally, 10,000 cells were analyzed using the FACS Calibur® system (Becton Dickinson, San Jose, CA).

2.7. *Agarose gel electrophoresis*

After blue light exposure, the cells were collected and incubated with a lysis buffer [1% nonidet P (NP)-40 and 1% sodium dodecyl sulfate (SDS) in 50-mM tris, pH 8.0] at 65 °C for 1 h. DNA was extracted with phenol/chloroform/isoamyl alcohol and aqueous phase was precipitated with 2 volumes of ethanol overnight at −20 °C. The pellet was air-dried and resuspended in a TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). The degree of fragmentation was analyzed using 2% agarose gel electrophoresis followed by ethidium bromide staining.

2.8. *Cytofluorimetric analysis of mitochondrial membrane potential ($\Delta\Psi_m$)*

After exposure to light, the cells were collected and resuspended in PBS, and then stained with 50 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) for 20 min at 37 °C. The fluorescence related to $\Delta\Psi_m$ was measured using a FACS Calibur® system (Becton Dickinson). The uptake of $DiOC_6$ was confirmed to be specific to $\Delta\Psi_m$ by treating the cells with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), as described previously [13].

2.9. *Western blot analysis*

After exposing the cells to blue light for various energy densities, the cells were incubated in a lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, and 1 μg/mL of aprotinin, leupeptin, and pepstatin), and protein content was quantified using the Bradford method [14]. Equal amounts of protein (30 μg/sample) were separated electrophoretically by 12% SDS-PAGE and blotted onto poly vinyl difluoride (PVDF) membranes. Prior to incubation with horseradish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h, the blots were probed with primary antibodies overnight at 4 °C. The blots were then developed with enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to X-ray film (Eastman-Kodak, Rochester, NY). The polyclonal antibody specific to poly (ADP ribose) polymerase (PARP; SC-7250) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.10. *Statistical analyses*

Unless otherwise specified, all the data is expressed as a mean ± standard error (S.E.). One-way ANOVA was used for multiple comparisons using SPSS ver. 10.0 software. A p-value <0.05 was considered significant.

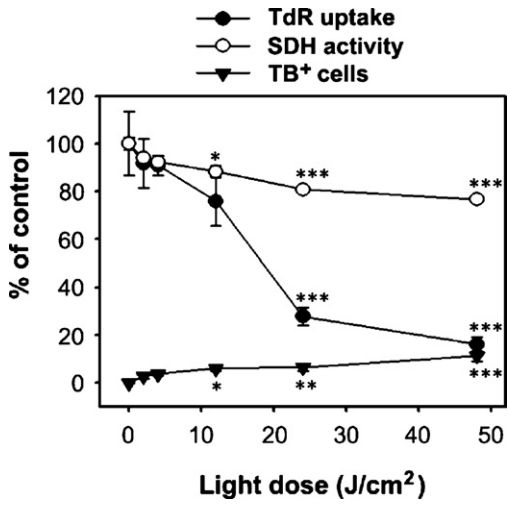

Fig. 2 – Blue light inhibits proliferation of HGF and their viability. The cells were exposed to the Flipo-generated blue light with the indicated doses and processed for analyzing TdR incorporation (closed circle), SDH activity (open circle), and cytotoxicity (closed triangle), as described in Section 2. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ vs. the unexposed control values. TB^{+} cells mean the trypan blue positively stained cells.

3. Results

3.1. *Blue light inhibits proliferation of HGF and reduces their viability*

A compound, phenol red, is known to adsorb blue light. This indicates that caution will be needed when examining the effects of blue light on cells in vitro, because culture media such as DMEM and MEM commonly contain phenol red. However, a recent study showed that phenol red did not affect the blue light-mediated decrease of SDH activity [15]. Thus we used the native DMEM containing phenol red in the present study. We also used the primary cultures of HGF instead of transformed cell lines, because of their homology to the original tissue.

Initially, we determined the effect of Flipo-produced blue light on cell proliferation by examining the level of TdR incorporation by HGF. As shown in Fig. 2, exposure of the cells to blue light reduced the level of TdR incorporated in the cells in a light dose-dependent manner (closed circle). Higher light doses, more than 12 J/cm^2, decreased the level of DNA synthesis in the cells, such that the exposure to blue light with 24 J/cm^2 inhibited the TdR uptake by 27.8%, compared with the untreated control ($p<0.001$). Similar to the TdR uptake assay, exposing the cells to blue light significantly decreased the SDH activity (open circle). Following a 48 h incubation after exposure to blue light (12 J/cm^2), the SDH activity of the cells had decreased to 88.3% ($p<0.05$) of the level in the untreated control cells. However, it decreased to 76.6% ($p<0.001$) after exposing the cells with the energy density of 48 J/cm^2. Subsequently, we examined whether or not the blue light exerted a cytotoxic effect on HGF by monitoring the level of trypan blue exclusion (closed triangle). Blue light had a mild cytotoxic effect on the cells, but there was a difference in the level of significant. This indicates that the Flipo-generated blue light causes a decrease in cell proliferation and mitochondrial function more than direct cell death.

3.2. *Blue light-induced cytotoxicity in HGF results from apoptosis*

In order to understand the nature of blue light-induced growth inhibition and cytotoxicity in the HGF, the cells were subjected to apoptosis assays. As shown in Fig. 3A, blue light-induced apoptosis was observed by examining the cell cycle progression after PI staining. The results showed that after exposing

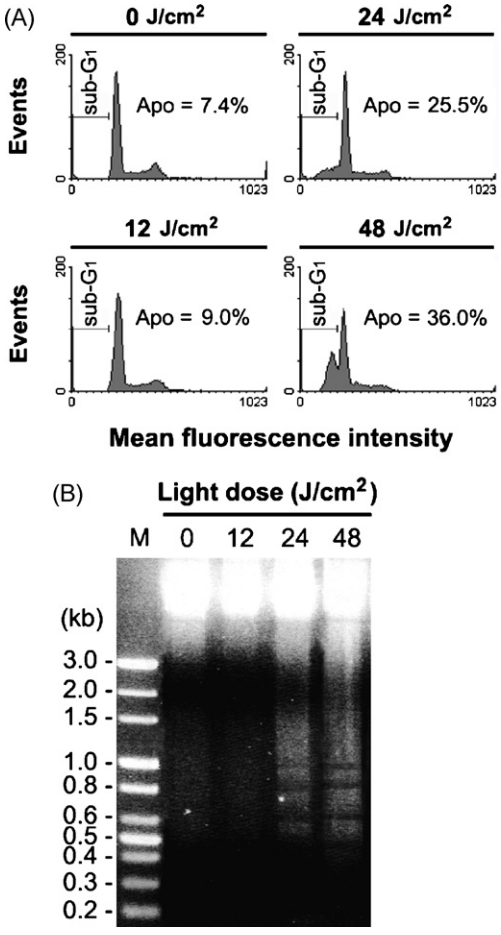

Fig. 3 – Blue light induces apoptosis of HGF in a dose-dependent manner. HGF were exposed to blue light generated from Flipo with the indicated doses and then further incubated for 48 h. (A) The cells were then stained with PI and analyzed by flow cytometry. The figure shows a representative staining profile for 10,000 cells per experiment. Sub-G_1 is the cell population defined as apoptotic. Apo, apoptosis. (B) Genomic DNA was prepared from the cells and analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. A representative result from three independent experiments is shown. M, molecular size marker.

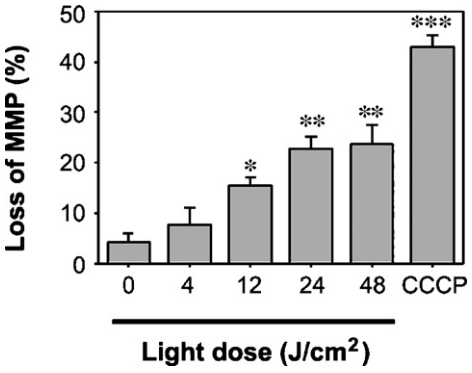

Fig. 4 – Blue light decreases the mitochondrial membrane potential ($\Delta\Psi_m$) in blue light-exposed HGF. HGF were exposed to blue light generated from Flipo with the indicated doses and then further incubated for 48 h. $\Delta\Psi_m$ was examined by $DiOC_6$ staining followed by flow cytometric analysis. The results are reported as a mean ± S.E. of triplicate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the unexposed control value. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 50 μM) was used as a positive control.

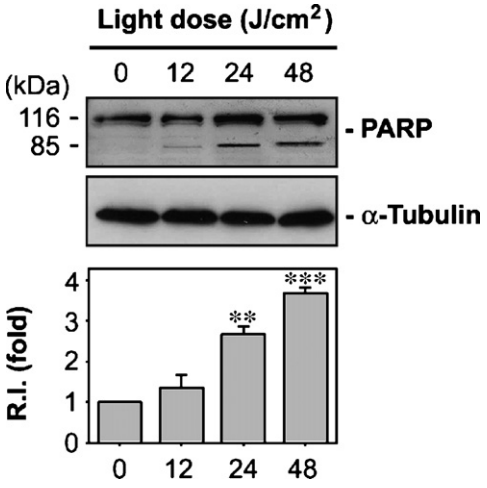

Fig. 5 – Blue light produces the cleaved PARP proteins in a dose-dependent manner. The cells were exposed to blue light with the indicated energy doses. After 24 h exposure, protein extracts were prepared and analyzed by 12% SDS-PAGE followed by immunoblot analysis. Cleaved PARP from three independent experiments was also quantified by densitometry after normalization of the bands based on α-tubulin (relative intensity = 1.0). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the unexposed control value.

cells to the light, there was a dose-dependent increase in cell populations in the sub-G_0/G_1 phase of the cell cycle. Approximately 25% and 36% of the cells underwent apoptosis after exposing them to the blue light of 24 J/cm^2 and 48 J/cm^2, respectively. In addition, exposing the cells to blue light produced a clear pattern of ladder formation created by DNA fragmentation (Fig. 3B). As shown in the figure, this formation of genomic DNA ladders was also light dose-dependent.

3.3. *$\Delta\Psi_m$ loss and PARP cleavage are associated with the blue light-induced apoptosis of HGF*

As shown in the present data, blue light significantly reduced SDH activity in HGF. In addition, the loss of $\Delta\Psi_m$ is a classic apoptotic event through mitochondria-mediated pathway. Thus we examined the effects of light exposure on $\Delta\Psi_m$ using a fluorescent dye, $DiOC_6$, which is a voltage-dependent dye. As shown in Fig. 4, blue light decreased the $\Delta\Psi_m$ level in the cells. When the cells were exposed to the energy density of 12 and 24 J/cm^2, the decreased level in $\Delta\Psi_m$ was 16% and 23%, respectively, compared with that of the control cells (4%). In addition, treatment with 50 μM CCCP resulted in a clear decrease in $\Delta\Psi_m$, indicating that $DiOC_6$ had transferred to the mitochondria, and that its uptake really reflected $\Delta\Psi_m$.

The cleavage of PARP is an important marker in the apoptotic process. We next investigated whether or not PARP is associated with the blue light-induced apoptosis using Western blot analysis (Fig. 5). A light dose-dependent increase in the PARP 85 kDa cleavage products was observed in HGF after exposure to blue light. The 85 kDa cleaved proteins were apparently produced after the cells were exposed to blue light with higher energy density than 12 J/cm^2.

4. Discussion

Our data shown in this study suggest that blue light can induce a harmful action in oral tissues. Especially, the results from TdR uptake and SDH activity assays indicate that blue light might induce cytotoxicity of HGF via the induction of mitochondrial dysfunction as well as the damage to DNA, although these effects were only light dose-dependent in that the energy density less than 12 J/cm^2 did not have any toxic effects on the cells (Fig. 2).

We next investigated the nature of cell death induced by exposing the HGF to blue light. Apoptosis is quite distinguishable from necrotic death and a prominent apoptotic feature is the production of characteristic DNA fragments [16]. As evidenced by the migration of many cell populations to the sub-G_0/G_1 phase of the cell cycle progression and the appearance of a clear DNA ladder (Fig. 3), this study strongly suggests that blue light causes cell death by inducing apoptosis. We also examined the cellular mechanism involved in the blue light-mediated apoptosis by determining the level of $\Delta\Psi_m$ (Fig. 4). This is because that a decrease in the mitochondrial transition potential is closely related to apoptosis [17], and that the blue light led to a decrease in the SDH activity (Fig. 2). The data from FACS analysis revealed that blue light reduced the $\Delta\Psi_m$ in a dose-dependent manner, thereby highlighting the involvement of mitochondrial stress in the blue light-mediated apoptosis of HGF.

On the other hand, the generation of cleaved PARP proteins is an important characteristic in the apoptotic process [18]. Western blot analysis showed that the blue light-mediated apoptosis of HGF was closely related to the cleavage of PARP (Fig. 5). In spite of the reports demonstrating that an arrest

of cell cycle progression is a common event in many cases of apoptotic death [19], there was no sign of the cycle arrest in the apoptosis induced by the blue light generated by FLIPO (see Fig. 3A). Moreover, a temperature increase in the cells or in a media containing cells, which can occur during photocuring, does not appear to be involved in the blue light-mediated toxicity of the HGF [9,20]. Therefore, we suggest that in addition to the mitochondrial stress, DNA damage is responsible event for the blue light-mediated apoptosis. This can be supported by the result showing that blue light sensitively inhibited the DNA synthesis more than the SDH activity (Fig. 2).

Recent accumulating evidences have established that blue light alters cell responses through a photosensitive mediator rather than a direct manner, and that reactive oxygen species (ROS) are one of the major substances responsible for the blue light-induced cytotoxicity. With this regard, the photosensitive molecules such as flavins, porphyrins or other compounds having phenol ring are considered as the important sources of ROS leading to the damaging effect of blue light [21]. In particular, riboflavin (vitamins B_2), which is widely distributed in tissues and fluids in various forms, has been implicated as an important blue light absorber that is capable of transducing blue light into ROS [22,23]. This indicates that a caution will be needed when the role of blue light is evaluated using the cultured cells, because culture medium such as DMEM contains the riboflavin. Consistent with previous reports [24,25], we found that adding riboflavin into the HGF cultures prominently accelerated the blue light-mediated decrease of SDH activity (data not shown). In addition, this acceleration was significantly inhibited by catalase but not by superoxide dismutase (data not shown), thereby supporting that hydroxyl radicals are the key molecules generated by riboflavin in culture medium [21,24]. Notwithstanding, we postulate that the amount of riboflavin contained in the native DMEM do not influence the blue light-mediated cytotoxicity. This is because, under the culture condition containing 10% FBS, riboflavin-mediated formation of ROS and their toxic effect to cells were not apparent [21]. This led us to suggest that at least in this study, intracellular photosensitizing molecules but not extracellular environments are the pivotal mediators of blue light-induced growth inhibition and apoptosis of HGF.

Several studies emphasize that the main endogenous photosensitizers are flavins and porphyrins. The photosensitizing activity of riboflavin is thought to lead to damage of DNA and cytoplasmic membranes, because of its location within cells [23]. This can explain why blue light sensitively inhibits the DNA synthesis and induces the PARP cleavage (Figs. 2 and 5). In addition, it cannot be excluded that mitochondrial stress is related to the blue light-induced apoptosis of HGF, as evidenced by our present study (Figs. 2 and 4). Otherwise, light irradiation produces triplet riboflavin, which is an intermediate in a wide range of photobiological processes and influences intracellular redox state [23]. Intracellular signaling pathways are quite sensitive to the oxido-reductive conditions [26]. In particular, the transcription factors including nuclear factor-κB, activator protein-1, and p53 are regulated by the intracellular redox state and associated with the induction of apoptosis [26,27]. Moreover, the binding sites for these transcription factors are located in the promoter regions of a variety of genes that are directly involved in oxidative stress-mediated diseases [26]. Therefore, we assume that in addition to the direct and/or indirect oxidative damage induced by photosensitizers, changes in the expression of redox sensitive genes are closely involved in the blue light-mediated cell responses.

In summary, our present data show that the blue light exposure inhibits proliferation and induces apoptotic death of HGF in a light dose-dependent manner. These data may provide a convincing argument that blue light causes some harm to the oral tissues according to the exposure conditions. Therefore, a long curing time more than recommended can cause biological hazards to the cells and/or tissues. We are further determining the precise contribution of Flipo-generated blue light to the oral tissues both in vitro and in vivo. We are also investigating the key factors in mediating the cellular responses to blue light.

Acknowledgement

This work was supported by the Korea Research Foundation Grant endowed to the RCBM of Chonbuk National University (KRF-2006-005-J03102)

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