International Journal of Oral Biology, Vol. 34, No. 4, December 31 2009, p. 185~190
Copyright ⓒ 2009, *The Korean Academy of Oral Biology*

International Journal of Oral Biology

Inhibition of Cell Growth and Mitochondrial Activity in Human Gingival Fibroblasts by LED-Generated Red Light Exposure

Hee-Jeong Kim, Jung-Min Hwang, So-Yeong Kwak, Jong-Ghee Kim, Young-Mi Jeon, and Jeong-Chae Lee*

Department of Orthodontics, Institute of Oral Biosciences and BK 21 program, Chonbuk National University, Jeonju 561-756, Korea

(received October 30, 2009 ; revised November 09, 2009 ; accepted November 13, 2009)

This study examined the effects of red light generated from a light emitting diode (LED) upon proliferation and mitochondrial stress in human gingival fibroblasts (hGFs). Cells were exposed to LED-generated red light at a clinically relevant intensity and distance with a 610-630 nm wavelength for various times (0-48 min). At different exposure times, cells were processed for the analysis of succinate dehydrogenase (SDH) activity, proliferation, mitochondrial membrane potential (MMP) and cytotoxicity. Cell cycle progression was also investigated by flow cytometry after staining with propidium iodide. Red light exposure was found to inhibit SDH activity and DNA synthesis in hGFs in a time-dependent manner. Light exposure also reduced the MMP levels in these cells and this was closely associated with a G_0/G_1 arrest. In contrast, exposure of hGFs to red light for 48 min led to a dramatic loss of MMP with an attendant increase in cytotoxicity. These findings demonstrate that LED-generated red light may cause mitochondrial stress and growth inhibition in hGFs during tooth whitening therapy, depending on the length of the exposure.

Key words: red light, human gingival fibroblasts, proliferation, mitochondrial stress.

Introduction

A discolored teeth can be successfully whitened by an in-office bleaching therapy using highly-concentrated bleaching regimens (Buchalla *et al*., 2007; Dahl *et al*., 2003). Hydrogen peroxide (H_2O_2) has been widely used as bleaching agent for the therapy. This is due to that H_2O_2 oxidizes a wide variety of organic and inorganic compounds, although the mechanisms involved in these reactions are differed according to the substrate and the reaction environment (Joiner, 2006). In the whitening treatment, H_2O_2 causes a change of organic pigment molecules and eventually leading to whitening of teeth through the production of a highly reactive oxidant, hydroxyl radical ($^{\bullet}OH$) (Kashima-Tanaka *et al*., 2003).

A variety of light sources such as quartz-tungsten-halogen (QTH) lamps, plasma arc lamps, metal halide lamps, and light emitting diode (LED) are used in dental clinics for teeth whitening as well as photolight-curing and root canal treatment (Dostalova *et al*., 2004).

Most photocuring sources are filtered to provide blue light of wavelengths between 400 and 500 nm. This is because that the ultraviolet radiation causes harmful effects on the eyes and skin. Notwithstanding, bluelight exposure is considered to induce negative effects on cells by inducing mitosis delay or blockage (Winkler *et al*., 1999), DNA lesions (Zigman, 2000) and/or oxidative stress (Pflaum *et al*., 1998). In contrast, red light generated from LED is thought to be more innocuous to oral tissues and/or cells than blue light, because the red light provides longer wavelengths and less scattered coefficient with a low temperature than blue light. This facilitated the use of red light-generating LEDin dental clinics for tooth whitening treatment.

Although the precise mechanism by which red light exposure promotes teeth whitening is unclear at present, many studies have documented the involvement of $^{\bullet}OH$ which is produced from H_2O_2 by Fenton and/or Haber-Weiss reactions (Antunes *et al*., 2001). Since $H_2O_2$is used widely in dental clinics and the persistent and prolonged presence

*Corresponding author: Jeong-Chae Lee. Institute of Oral Biosciences and BK 21 program, Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, Korea. Tel.: +82-63-270-4049; Fax.: +82-63-250-2139; E-mail: leejc88@jbnu.ac.kr

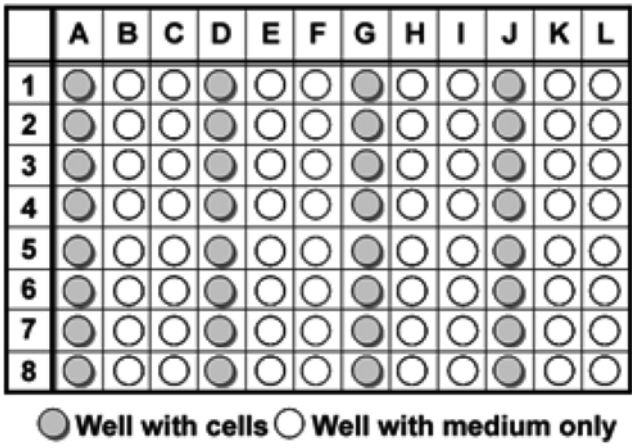

Fig. 1. Design of cell seeding into 96-multiwell culture plates.

of $^{\bullet}OH$ causes oxidative stress, numerous investigators also examined the biological aspects of H_2O_2 using various experimental systems (Buchalla *et al*., 2007 Dahl *et al*., 2003; Kashima-Tanaka *et al*., 2003). In addition, the effect of blue light or laser irradiation to cells have been extremely examined (Buchalla *et al*., 2007 Hwang *et al*., 2008; Kashima-Tanaka *et al*., 2003 Zach *et al*., 1965). Unlike the accumulated evidence on side effects of blue light or laser treatment, however, little information is available on the effect of red light to cells. Here we investigated the effects of LED-generated red light on the proliferation, mitochondria activity, and cell cycle progression using primary cultures of human gingival fibroblasts (hGF).

Materials and Methods

Chemicals and laboratory facilities

Unless otherwise specified, all the chemicals and laboratory facilities were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Cell culture

hGF were cultured according to the method as described elsewhere (Hwang *et al*., 2008). 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 for use of the tissues. When the cells had reached confluence, they were collected and divided into each well of 96-multiwell plates at 20,000 cells in 200 μl medium with a design to avoid an indirect light exposure (Fig. 1) and were further incubated prior to exposure to the red light.

Light source and exposure

When hGF had reached 80% confluence, they were exposed for various times (0-48 min) to the red light generated from

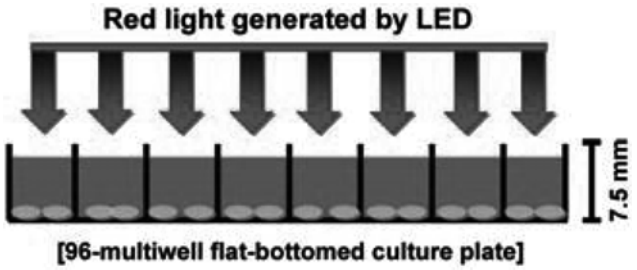

Fig. 2. Diagram showing a condition of light exposure to hGF. When cells had reached 80% confluence, they were exposed to red light for various times (0-48 min) with a clinically relevant energy and distance as described in Materials and Methods.

LED (Quick smile, Lokki, Australia) with the wavelength between 610 and 630 nm and the energy of 200-2,000 mW/cm^2(Fig. 2). These conditions were consistent with those applied clinically in Chonbuk National University Hospital for in-office bleaching treatment. At various times of light exposure, cells were processed for further analyses.

Measurement of succinate dehydrogenase (SDH) activity

3-(4,5-Dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate the SDH activity of hGF and this assay was carried out according to the methods described elsewhere (Lee *et al*., 2008). In this assay, the concentration of the blue product is proportional to the SDH activity to reduce MTT and the result indicates the viability of cells.

DNA synthesis assay

The level of DNA synthesis by hGF after exposure to red light was measured by adding 1 μCi of [*methyl*-^{3}H] Thymidine deoxyribose (TdR; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) 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 TdR content was determined using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL, USA).

Trypan blue exclusion assay

The trypan blue exclusion assay was used to determine the level of cytotoxicity caused by exposing the cells to the red light generated by LED. After various times 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%.

Measurement of mitochondrial membrane potential (MMP)

At 12 and 24 h after light exposure, hGF were stained with 50 nM 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$ Molecular Probes, Eugene, OR, USA) for 20 min at 37°C and then resuspended in PBS. The fluorescence related to MMP was

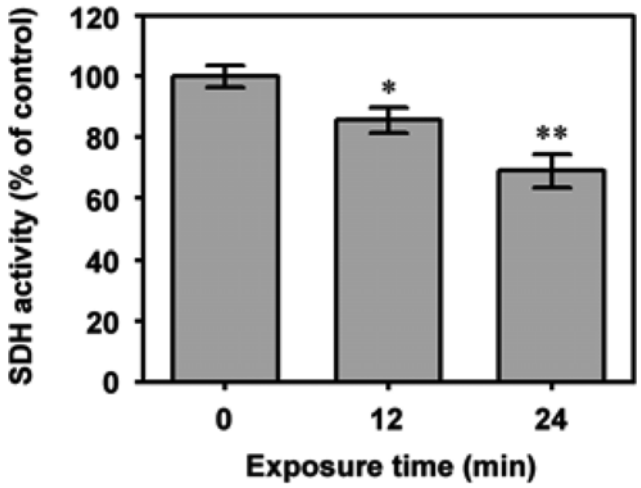

Fig. 3. Inhibitory effect of red light on the SDH activity of hGF. Cells were exposed to red light for the indicated times and processed for the MTT assay. The results represent the mean ± SDof three separate experiments. *$p < 0.05$ and **$p < 0.01$ indicate significant differences between the experiments and the unexposed control value.

measured using a FACS Calibur®system, and the red-light mediated reduction of MMP was surveyed using the WinMDI 2.9 program.

Propidium iodide (PI) staining

Cell cycle progression was determined by flow cytometric analysis after PI staining. In this assay, cells were exposed to red light for the increasing times (0-24 min) and 48 h after exposure, they were stained with 1 ml of PI 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). Ten thousand cells were analyzed by the FACS Calibur system (Becton Dickinson, San Jose, CA, USA).

Statistical analyses

Unless otherwise specified, all data were expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Scheffe's test was used for the multiple comparisons using SPSS version 16.0 software. A p value < 0.05 was considered significant.

Results

Effect of red light on the viability of hGF

Fig. 3 shows that exposure of hGF to red light significantly decreased SDH activity in a time-dependent manner. When the cells were analyzed by MTT assay at 48 h after the light exposure for 12 and 24 min, the SDH activities were decreased to 85.8 ($p < 0.05$) and 69.1% ($p < 0.01$) of the level in the untreated control cells, respectively. This indicates that red light exposure leads to the reduction of mitochondria activity.

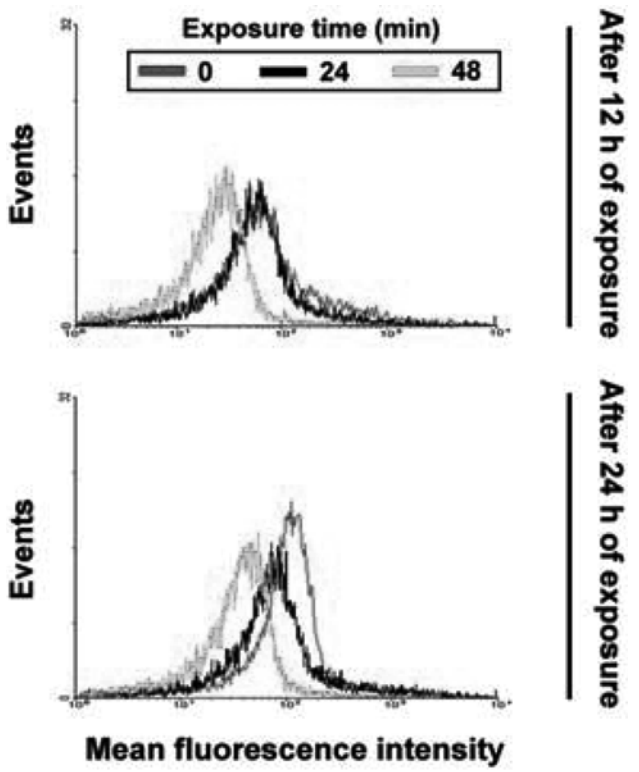

Fig. 4. Effect of red light on the MMP level in hGF. Cells were exposed to red light for various times (0-48 min) and at 12 and 24 h after the exposure, the cells were adjusted to $DiOC_6$ staining followed by flow cytometric analysis.The data show a representative result from triplicate experiments.

Effect of red light on MMP level of hGF

In order to more understand the effect of red light on mitochondria, MMP level of hGF was determined by flow cytometric analysis after staining the cells with a mitochondrion-specific fluorescent dye, $DiOC_6$. When the cells were stained with the dye after 12 h exposure, the fluorescence intensity peak of $DiOC_6$ was changed by red light exposure only in the cells exposed to the light for 48 min (Fig. 4). However, the $DiOC_6$ specific signal was left-shifted in both the cells exposed to the light for 24 and 48 min when they were stained with $DiOC_6$ at 24 h after the exposure. This means that red light exposure can induce the loss of MMP, i.e., mitochondrial stress, and this is to be apparent when red light was subjected to the cells for more than 24 min.

Effect of red light on DNA synthesis of hGF

Figs. 3 and 4 revealed that red light exposure could lead to a damage on mitochondrial functions of hGF. Since mitochondria play a key role in the production of cellular energy required for proliferation, next experiment was focused on the determination of DNA synthesis by the light-exposed hGF. Similar to the SDH activity, red light exposure significantly inhibited DNA synthesis by the cells in a time-dependent manner (Fig. 5). Following a 48 h incubation after exposure to red light for 12 and 24 min, the levels of TdR

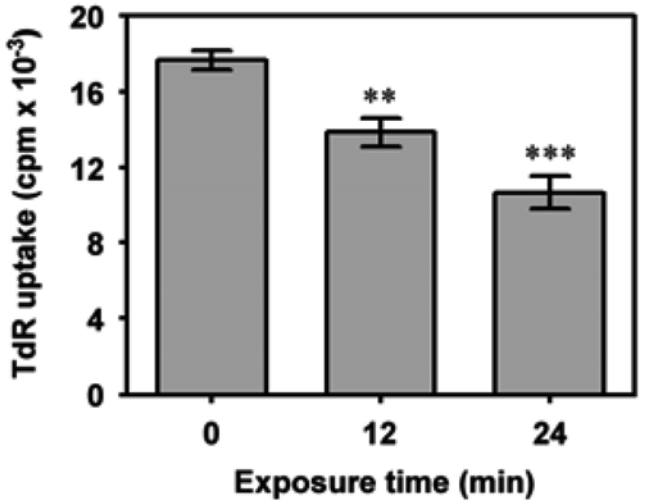

Fig. 5. Time-dependent decrease of hGF proliferation by red light exposure. Cells were exposed to red light for the indicated times and incubated with 1 μCi/ml [*methyl*-^{3}H] TdR for the last 16 h during the 48 h culture period. Each bar represents the mean ± SDfrom three separate experiments. **$p < 0.01$ and ***$p < 0.001$ vs. the unexposed control value. cpm, count per minute.

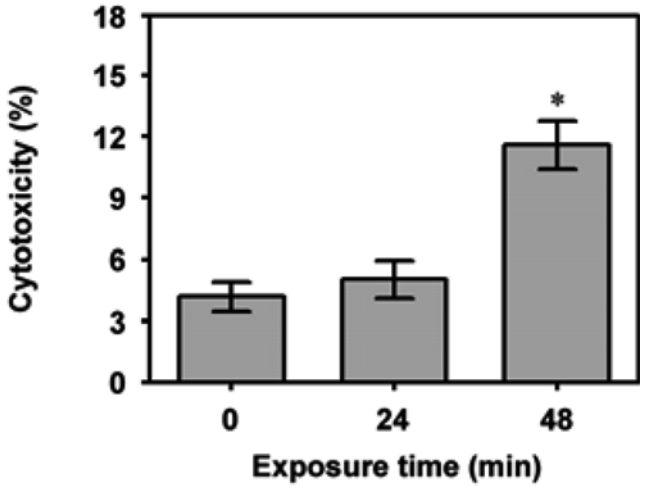

Fig. 6. Effect of red light on cytotoxicity in hGF. Cells exposed to red light for 24 or 48 min were incubated for 48 h and then processed for the trypan blue exclusion assay. The data show the mean ± SD from triplicate experiments. *$p < 0.05$ vs. the unexposed control value.

incorporated by DNA of the cells had decreased to 78.6 ($p < 0.01$) and 60.5% ($p < 0.001$) of the level in the control value (17,624 cpm). This result suggests that the proliferation of hGF is suppressed by red light exposure and this can be associated with the reduction of mitochondrial activity.

Effect of red light on cytotoxicity of hGF

We further determined whether or not the red light-mediated suppression of mitochondrial function and proliferation in hGF led to a direct cytotoxic effect on the cells using a trypan blue staining method.Red light did not increase the number of cells stained positively with trypan blue in a significant level until the cells were exposed to the light for 48 min (Fig. 6). This indicates that the LED-generated red light exposure less than 48 min results in the growth inhibition and mitochondrial dysfunction rather than a direct induction of cell death.

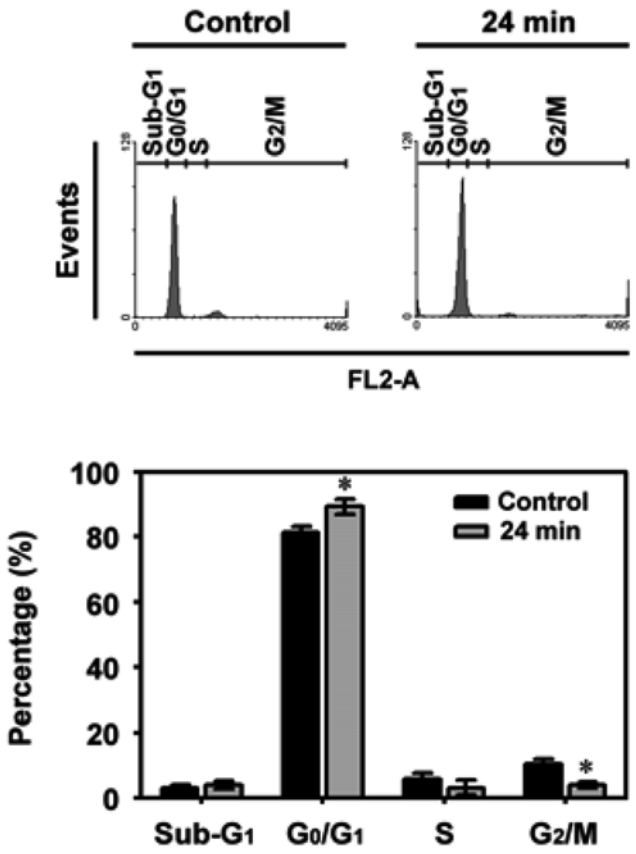

Fig. 7. Cell cycle progression of hGF exposed to the red light. Cells were exposed to red light for 24 min, and after 48 h of exposure, the cells were analyzed by flow cytometer after staining with PI. The figure shows a representative staining profile for 10,000 cells per experiment(upper panel), and cell cycle progression of the cells was calculated from triplicate experiments using WinMDI 2.9 program (below panel).*$p < 0.05$ vs. the unexposed control value.

Effect of red light on cell cycle progression

In order to clarify the nature of red light-induced cytotoxicity in hGF, the cells were subjected to flow cytometric analysis after PI staining. Red light exposure did not increase the migration of cells into the sub-G_1 phase of cell cycle progression under the times of exposure employed (Fig. 7). In contrast, the red light increased slightly the number of cells in the G_0/G_1 phage with the concomitant reduction of G_2/M phase cells. This indicates that red light does not induce a typical apoptotic character such as DNA ladder formation in hGF, whereas it leads to the arrest of cell cycle in the G_0/G_1 phase.

Discussion

Light or laser irradiation is thought to be effective in a teeth whitening treatment, but it also can induce pulp irritation as well (Zach *et al*., 1965). Among the various light sources (Rohanizadeh *et al*., 1999 Sun, 2000), red LED light is widely used in a dental bleaching device. This is due to its longer wavelengths and less scattered coefficient. In other word, the most prominent side effects by light exposure are the carbonization and the creation of fissures and cracks in the surrounding tissues and the damage to pulp tissue (Cohen *et al*., 1979; Zach *et al*., 1965). This is believed to be related to the increase of temperature in the adjacent tissue by light exposure. It was reported that light exposure to cell cultures increases the temperature up to approximately 6°C for 1 h and this stimulates the induction of heat shock proteins (HSP) (Noda *et al*., 2002). This is partially in parallel with our finding that red light exposure for 48 min elevated temperature of the culture media to about 2.5°C compared to the control value (data not shown). Additional experiments in order to know whether the expression of HSP is also involved in the responses of hGF to red light exposure will be needed.

In this study, we have used hGF to investigate the effects of red light generated from LED on the mitochondrial activity, proliferation and cell cycle progression. This is because the gingival tissues are mainly consisted of fibroblasts. We showed that red light inhibits SDH activity, MMP level and eventually DNA synthesis of the cells. Red light exposure also causedcell cycle arrest in the G_0/G_1 phase without the accumulation of the sub-G_1 phase cells, although over-exposure (more than 24 min) of red light increased the number of cells stained with trypan blue. This suggests that red light predominantly causes mitochondrial stress in hGF and then reduces proliferation of the cells depending on the times exposed.

It is worthy to consider that the side effect of blue light on cells is more prominent than that induced by red light, although the red light also induced the growth inhibition and mitochondrial stress. We previously showed that the blue light generated from plasma-arc leads toa severe decrease of SDH activity and proliferation and eventually to poly (ADP-ribose) polymerase-mediated apoptosis in hGF in a light dose-dependent manner (Hwang *et al*., 2008). There are also many reports to support that blue light causes damage to cells and alters cellular functions (Gritsch *et al*., 2008 Gorgdize *et al*., 1998Lewis *et al*., 2005; Wataha *et al*., 2004). These indicate that cells and/or tissues can be differently damaged according to both the light source and the wavelength after filtering, and red light is more applicable for teeth whitening purposethan blue light.

It should be considered that in addition to periodontal ligament fibroblasts (Kim *et al*., 2009), gingival fibroblasts play important roles in an orthodontic force-induced tooth movement. hGF also have roles to protect periodontal tissues from extraordinary stresses (Kook *et al*., 2009). As devices for light and laser irradiation are commonly used in dental clinics, the use of LED in teeth whitening treatment is also popular at present. Although blue light seems to induce more mitochondrial dysfunction than redlight, our current data suggest that red light itself could induce a change of cellular function, especially to mitochondria. It has to be also noted that a bleaching treatment involves the use of highly concentrated H_2O_2, where light exposure facilitates the conversion of this agent to a direct toxic mediator, $^{\bullet}OH$. With this regard, we postulate that over-exposure to red light may cause side effects on cells and this is further augmented in the presence of H_2O_2. This could emphasize that a caution should be followed in the process of teeth whitening treatment. Notwithstanding, it is important to consider that the cellular responses to light exposure are differ according to the types of cells and the experimental conditions i.e., *in vivo* and *in vitro*. More detailed experiments will be needed in order to clarify the effects of red light on periodontal cells and tissues. Our current work are focused on determining the effects of red light on cells present in periodontal tissues in the presence of a bleaching agent such as H_2O_2.

Acknowledgement

This study was supported by a grant from the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs,Republic of Korea (A084283).

References

Antunes F, Cadenas E. Cellular titration of apoptosis with steady state concentrations of H_2O_2: Submicromolar levels of H_2O_2 induce apoptosis through Fenton chemistry independent of the cellular thiol state. Free Radic Biol Med. 2001; 30:1008-18.

Buchalla W, Attin T. External bleaching therapy with activation by heat, light or laser-A systematic review. Dent Mater. 2007;23:586-96.

Cohen SC, Chase C. Human pulpal response to bleaching procedures on vital teeth. J Endodont 1979;5:134-8.

Dahl JE, Pallesen U. Tooth bleaching-A critical review of the biological aspects. Crit Rev Oral Biol Med. 2003;14:292-304.

Dostalova T, Jelinkova H, Housova D, Sulc J, Nemec M, Miyagi M. Diode laser-activated bleaching. Braz Dent J. 2004;15:SI3-8.

Gorgdize LA, Oshemkova SA, Vorobjev IA. Blue light inhibits mitosis in tissue culture cells. Biosci Rep 1998;18:215-24.

Gritsch K, Ponsonnet L, Schembri C, Farge P, Pourreyron L, Grosgogeat B. Biological behaviour of buccal cells exposedto blue light. Mat Sci Eng C-Bio S 2008;28:805-10.

Hwang IY, Son YO, Kim JH, Jeon YM, Kim JG, Lee CB, Park JS, Lee JC. Plasma-arc generated light inhibits proliferation

and induces apoptosis of human gingival fibroblasts in a dose-dependent manner. Dent Mater. 2008;24:1036-42.
Joiner A. The bleaching of teeth: A review of the literature. J Dent. 2006;34:412-9.
Kashima-Tanaka M, Tsujimoto Y, Kawamoto K, Senda N, Ito K, Yamazak M. Generation of free radicals and/or active oxygen by light or laser irradiation of hydrogen peroxide or sodium hypochloride. J Endodont. 2003;29:141-3.
Kim JW, Lee KS, Nahm JH, Kang YG. Effects of compressive stress on the expression of M-CSF, IL-1β, RANKL and OPG mRNA in periodontal ligament cells. Korean J Orthod. 2009;39:248-56.
Kook SH, Son YO, Choe Y, Kim JH, Jeon YM, Heo JS, Kim JG, Lee JC. Mechanical force augments the anti-osteoclastogenic potential of human gingival fibroblasts *in vitro*. J Periodont Res. 2009;44:402-10.
Lee YH, Jung JE, Lee JC, Moon HJ, Lee NH, Jhee EJ, Yi HK. The enhancement of apoptosis by combined with proteasome inhibitor and DNA synthetic inhibitor in oral cancer. Int J Oral Biol. 2008;33:25-31.
Lewis JB, Wataha JC, Messer RL, Caughman GB, Yamamoto T, Hsu SD. Blue light differentially alters cellular redox properties. J Biomed Mater Res Part B Appl Biomater. 2005;72:223-9.
Noda M, Wataha JC, Kaga M, Lockwood PE, Volkmann KR, Sano H. Components of dentinal adhesives modulate heat shock protein 72 expression in heat-stressed THP-1 human monocytes at sublethal concentrations. J Dent Res. 2002;81:265-9.
Pflaum M, Kiebassa C, Garmyn M, Epe B. Oxidative DNA damage induced by visible light in mammalian cells: extend, inhibition by antioxidants, and genotoxic effects. Mutat Res. 1998;408:137-46.
Rohanizadeh R, LeGeros RZ, Fan DA, Daculsi JG. Ultrastructural properties of laser-irradiated and heat-irradiated dentin. J Dent Res. 1999;78:1829-35.
Sun G. The role of lasers in cosmetic dentistry. Dent Clin N Am. 2000;44:831-49.
Wataha JC, Lockwood PE, Lewis JB, Rueggeberg FA, Messer RL. Biological effects of blue light from dental curing units. Dent Mater. 2004;20:150-7.
Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. Mol Vis. 1999;5:32.
Zach L, Cohen G. Pulp response to externally applied heat. Oral Surg Oral Med Oral Pathol. 1965;19:515-30.
Zigman S. Lens UVA photobiology. J Ocular Pharmacol. 2000;16:161-5.