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Abstract: In recent years, it has become well known that the production of reactive oxygen species (ROS) induced by blue-light irradiation causes adverse effects of photo-aging, such as age-related macular degeneration of the retina. Thus, orange-tinted glasses are used to protect the retina during dental treatment involving blue-light irradiation (e.g., dental resin restorations or tooth bleaching treatments). However, there are few studies examining the effects of blue-light irradiation on oral tissue. For the first time, we report that blue-light irradiation by quartz tungsten halogen lamp (QTH) or light-emitting diode (LED) decreased cell proliferation activity of human gingival fibroblasts (HGFs) in a time-dependent manner (<5 min). Additionally, in a morphological study, the cytotoxic effect was observed in the cell organelles, especially mitochondria. Furthermore, ROS generation induced by the blue-light irradiation was detected in mitochondria of HGFs using fluorimetry. In all analyses, the cytotoxicity was significantly higher after LED irradiation compared with cytotoxicity after QTH irradiation. These results suggest that blue-light irradiation, especially by LED light sources used in dental aesthetic treatment, might have adverse effects on human gingival tissue. Hence, this necessitates the development of new dental aesthetic treatment methods and/or techniques to protect HGFs from blue light irradiation during dental therapy.

Highlights

- Blue light irradiation decreased the cell growth of gingival fibroblasts.
- The cytotoxic effect of blue-light irradiation was observed in mitochondria.
- Reactive oxygen species were induced in mitochondria by blue light irradiation.

Reactive Oxygen Species Production in Mitochondria of Human Gingival Fibroblast Induced by Blue Light Irradiation

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Abbreviations

quartz tungsten halogen: QTH; hydrogen peroxide: H_2O_2; light-emitting diode: LED; reactive oxygen species: ROS; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt: MTS; dihydrofluorescein: DHF; diaminobenzidine: DAB; hydroxyl radicals: $HO^{\bullet}$

Abstract

In recent years, it has become well known that the production of reactive oxygen species (ROS) induced by blue-light irradiation causes adverse effects of photo-aging, such as age-related macular degeneration of the retina. Thus, orange-tinted glasses are used to protect the retina during dental treatment involving blue-light irradiation (e.g., dental resin restorations or tooth bleaching treatments). However, there are few studies examining the effects of blue-light irradiation on oral tissue. For the first time, we report that blue-light irradiation by quartz tungsten halogen lamp (QTH) or light-emitting diode (LED) decreased cell proliferation activity of human gingival fibroblasts (HGFs) in a time-dependent manner (<5 min). Additionally, in a morphological study, the cytotoxic effect was observed in the cell organelles, especially the mitochondria. Furthermore, ROS generation induced by the blue-light irradiation was detected in mitochondria of HGFs using fluorimetry. In all analyses, the cytotoxicity was significantly higher after LED irradiation compared with cytotoxicity after QTH irradiation. These results suggest that blue light irradiation, especially by LED light sources used in dental aesthetic treatment, might have adverse effects on human gingival tissue. Hence, this necessitates the development of new dental aesthetic treatment methods and/or techniques to protect HGFs from blue light irradiation during dental therapy.

Keywords

Blue light, Light-emitting diode, Quartz tungsten halogen, Reactive oxygen species, Human gingival fibroblast, Mitochondria

1. Introduction

Blue light, which possesses the biocidal effect of ultraviolet radiation, has been applied to photo-activated resin composite systems as protection during tooth restoration since the 1980s [1-3]. In this system, a standard quartz tungsten halogen (QTH) lamp (the visible-light source) with an output power of 600 mW/cm^2 (40 s) is typically used. Recently, blue-light has also been used in dental offices to activate bleaching agents such as hydrogen peroxide (H_2O_2) [4]. Hydroxyl radical ($HO^{\bullet}$), which is one of reactive oxygen species (ROS), is generated from H_2O_2 by blue light irradiation. It is well known to be involved in the mechanism of bleaching [5]. During this treatment, the irradiation area is wider and time exposed to blue-light is longer compared with that used in tooth restoration. In the 1990s, the blue light emitting diode (LED) source, which has high brightness and efficiency, was developed [6, 7]. Blue-light irradiation using a LED instead of a QTH source has recently been applied to various dental treatments [8]. Furthermore, LED irradiation with high power output has been used to shorten the treatment time relative to that of conventional dental therapy [9].

Blue light with wavelengths of 400–500 nm is used in oral treatments. This wavelength range of visible light may cause injury to the retina; to the public, this danger is known as a “blue light hazard”. In many studies, it has been reported that the photo-aging effect on the retina, such as during macular degeneration, is induced by oxidative stress due to ROS production via blue light

irradiation [2, 10-12].

Blue light irradiation of teeth is primarily used for bleaching of colored teeth. The general process of dental tooth bleaching is performed as follows: 1) Bleaching agent (25–35% H_2O_2 containing products) is applied to the enamel surface after protection of the soft tissues. 2) To activate the bleaching agent, blue light is irradiated from the outside of oral cavity using light curing unit for 10–15 min [13]. Recently, the use of different light units such as halogen curing lights, LEDs, diode lasers, argon lasers, and plasma arc lamps has been introduced for vital tooth bleaching to achieve better activation of H_2O_2; these techniques have achieved better esthetic results [14]. However, the blue light not only irradiates the target tooth, but also nearby tissue, including gingival tissue. The gingival tissue is commonly protected from H_2O_2 activated during tooth bleaching treatment by coating of the soft tissue with dental resin or petroleum jelly, such as Vaseline, but is not considered to be affected by blue-light irradiation [15-17]. Fibroblasts are among the chief gingival cells. It has been proposed that gingival recession may occur by the inhibition of fibroblast proliferation and the degradation of collagen synthesis due to reactive oxygen species (ROS) [18-20]. ROS may also induce hypersensitivity of the tooth neck by exposing root surfaces associated with gingival recession. It has been reported that blue-light irradiation may induce ROS formation in the mitochondria of mammalian cells [21-24]. In addition, we recently reported the cytotoxicity of dental resin curing source by irradiation with blue-light, which occurs via production of ROS in

tissues of human aortic vascular smooth muscles [24]. However, few studies have focused on the effects of blue-light irradiation on gingival fibroblasts. Therefore, the purpose of this study was to verify the effects of blue light irradiation on gingival fibroblasts using QTH or LED as the light source. In this study, we first demonstrated that proliferation activity of fibroblasts was reduced by QTH or LED blue-light irradiation, and that the first biological target of blue-light irradiation was the mitochondria of gingival fibroblasts.

2. Materials and Methods

2.1 Lighting source

Techno Light KTL-100 (LED) and Techno Light KTS-150 (QTH) were purchased from Kenko Tokina Corporation (Tokyo). Fig. 1 (A) shows the relative spectral emissions of QTH and LED. A blue transmission filter (225S-SPF500, Kenko Tokina Corporation, Tokyo) was used for the LED and QTH units to exclude light of all wavelengths except blue light. The output power of LED or QTH was set to 250 mW/cm^2 at a wavelength of 460 nm using an optical power meter (8230E, ADC Corporation, Tokyo) before each experiment. Fig. 1 (B) shows the wavelength and output power used for the study. To use the lux meter, the total irradiance was calculated according to the Commission Internationale de l'Eclairage (CIE) standard curve in the CIE 1931 color space (ANA-F9, Tokyo Photo-Electric Company Limited, Tokyo) [25].

2.2 Cell culture

Human gingival fibroblasts (HGFs, [26]) that were not immortalized were provided by Tomoko Komatsu (Kanagawa Dental University). The HGFs were grown in fibroblast medium (ScienCell Research Laboratories, Carlsbad, CA) consisting of of 500 ml of basal medium, 10 ml of fetal

bovine serum, 5 ml of fibroblast growth supplements, and 5 ml of penicillin/streptomycin solution. The HGFs were passaged once every 10 days after dispersion using a solution of 0.25% trypsin (Trypsin 250, Wako Pure Chemical Industries Ltd., Osaka, Japan) in Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS(-); pH 7.2) for 5 min at 37°C. The cells were used within 10 passages.

2.3 Cell-proliferation activity assay

The HGFs were seeded into a 96-well plate (Optical bottom black plate, Nalge Nunc International, Rochester, NY) at 16,000 cells per well and then incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The HGFs were then continuously irradiated with LED or QTH light for 0, 1, 3 or 5 min as described previously [24]. After irradiation, the HGFs were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. To prevent interference from irradiation from experiments on other HGFs, a black 96-well plate was used, and non-targeted wells were covered with aluminium foil. The distance of the irradiation source in each experiment was 12.1 mm from the top of the well. The cell viability was assessed using a Cell Titer 96® Aqueous One Solution cell proliferation assay kit (Promega Corporation, Madison, WI). Cell Titer 96® Aqueous One Solution was added to the well at 1:5 ratio. The optical density was read at a

wavelength of 490 nm using an iMark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA) after incubation for 60 min at 37 °C in a humidified atmosphere containing 5% CO_2. Data from the readings were analyzed using Microplate Manager Version 6.0 (Bio-Rad Laboratories Inc., Hercules, CA).

2.4 Electron microscopic analysis

The HGFs were seeded into a 35 mm dish (TC Dish, Nalge Nunc International, Rochester, NY) at 400,000 cells per dish, and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The HGFs were then continuously irradiated with LED or QTH light for 0 or 5 min. After irradiation, the HGFs were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. The distance of the irradiation source in each experiment was 12.1 mm, which was unified with the experimental conditions for the cell-proliferation activity assay. The HGFs were collected and then fixed with 2.5% glutaraldehyde for 1 h. After postfixing with 1% osmium tetroxide for 1 h, the cells were dehydrated and then embedded in Quetol 651 (Nissin EM, Tokyo, Japan). After embedding, ultrathin sections were cut and mounted on copper grids. Sections were stained with uranyl acetate and lead citrate for 1 min, then examined under an electron microscope (JEM 1220; JEOL, Tokyo, Japan).

2.5 Oxidative stress assay

Intracellular levels of ROS production were measured using CellROX® green reagent (Promega Corporation, Madison, WI). The HGFs were then irradiated with LED or QTH for 0 or 5 min after adding 20 μM CellROX® green reagent to the culture medium. To prevent interference from irradiation from experiments on other HGFs, a black 96-well plate was used, and non-targeted wells were covered with aluminium foil. The distance of the irradiation source in each experiment was 12.1 mm from the top of the well. After irradiation, the fluorescence intensity was measured with a SPECTRAFluor fluorescence microplate reader (Tecan, Männedorf, Switzerland) at an excitation wavelength of 485 nm and emission wavelength of 535 nm after incubation at 37 °C for 1 h in a humidified atmosphere containing 5% CO_2. Measurements were analyzed using LS-PLATE Manager 2001 software (Wako Pure Chemical Industries, Osaka, Japan).

2.6 Statistical analysis

The results are expressed as mean ± standard deviation. Tukey's multiple comparison tests were used for statistical analysis. A P value of less than 0.05 was considered to be statistically

significant.

3. Results

3.1 Comparison of cell-proliferation activity

We examined the influence of blue-light irradiation under an LED or QTH light source on fibroblast proliferation activity (Fig. 2). The fibroblasts seeded in the 96-well plate were irradiated with LED or QTH for 0, 1, 3, or 5 min. Groups irradiated for 3 and 5 min under LED and QTH light sources had significantly decreased proliferative activities compared with the non-irradiated control. Further, blue LED irradiation for 5 min significantly decreased proliferative activity to a greater extent compared with that induced by QTH irradiation for 5 min.

3.2 Morphological examination by transmission electron microscopy

Fig. 3 shows that most of the mitochondria in the LED-irradiated group were already damaged, with unclear cristae, vacuoles, and swelling after 24 h. Some of the mitochondria in the QTH-irradiated group were damaged, and most mitochondria were not damaged in the control group. Furthermore, no damage was observed in cell organelles other than mitochondria in all groups.

3.3 Intracellular levels of ROS production

We examined intracellular ROS production induced by blue-light irradiation with LED or QTH (Fig. 4). The fibroblasts seeded in the 96-well plate were irradiated with an LED or QTH light source for 0 or 5 min after addition of CellROX ® Green Reagent to the each well. The level of intracellular ROS production in the LED- or QTH-irradiated groups were significantly increased compared with the non-irradiated control group. Furthermore, the intracellular level of ROS production in the LED-irradiated group was also significantly increased compared with that in the QTH-irradiated group.

4. Discussion

The effect of blue-light irradiation on biological systems has been reported in the literature. Most of these reports relate to temperature changes resultant from QTH or LED irradiation of biological systems [27-33]. Unfortunately, there are currently few studies examining blue-light photochemistry in dentistry. Moreover, although blue LED has recently become the mainstream source for blue-light in dental therapy, there are few reports on the biological photochemistry of LED [30, 34]. In our previous study, we reported the effect of blue-light irradiation of a dental halogen curing source on vascular smooth muscle [24]. Our study was the first to report that stress induced by ROS production via photochemical effects of blue-light irradiation, rather than the elevated temperature, primarily targets vascular smooth muscle. In this study, we first demonstrated that blue-light irradiation with a QTH or LED light source decreased cell proliferation activity in a time-dependent manner. In addition, cell proliferation was significantly inhibited by blue LED irradiation for 5 min, compared with inhibition due to irradiation by a QTH light source. In our previous studies, proliferation of human vascular smooth muscle cells was suppressed by more than 80% upon QTH light irradiation for 5 min [24]. Therefore, we set 5 min as the maximum for the subsequent experimental irradiation time instead of 10–15 min in clinical practice. This cell proliferation assay is based on bioreduction of the substrate, [3-(4,5-dimethylthazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner

salt] (MTS), into brown formazan product by reduced nicotinamide adenine dinucleotide phosphate or nicotinamide adenine dinucleotide phosphate via dehydrogenases in metabolically active cells. The quantity of formazan product, as measured by the absorbance at 490 nm, is directly proportional to the number of living cells in the culture, and is used as an index to express cell proliferation or cytotoxicity.

On the basis of the above findings, we further examined morphological changes to the cells under blue-light irradiation with LED or QTH light sources. Using transmission electron microscopy, we found that only mitochondria in the gingival fibroblasts were damaged by blue LED or QTH irradiation for 5 min, the same organelles that indicated significant inhibition of cell proliferation activity (Fig. 3). In particular, morphological cytotoxicity (such as unclear cristae, vacuoles, and swelling) in mitochondria due to blue light LED irradiation was recognized clearly. The general irradiation time of blue light is less than 1 min during dental restoration. However, it is more than 5 min during tooth bleaching treatment. Therefore, the results of this study suggest that blue light irradiation of more than 5 min in tooth bleaching might enhance the cytotoxic effect of ROS on oral soft tissue. In addition, the biological effects of blue light irradiation are initiated by photoreduction of flavins and/or flavin-containing oxidases within peroxisomes and mitochondria (and probably cytoplasmic sites as well), resulting in H_2O_2 production. Endogenous peroxidase and oxidase enzymes subsequently mediate the conversion of H_2O_2 to water coupled with oxidation of

dihydrofluorescein (DHF) and 3,3′-diaminobenzidine (DAB) to fluorescein and polyDAB. Catalase, which can also oxidize DAB to polyDAB, converts H_2O_2 to water and dioxygen. The latter competes with oxidation of DHF derivatives. When the level of H_2O_2 exceeds enzymatic activities, it diffuses into the cytoplasm, where additional dye reduction occurs. H_2O_2 and its metabolite, the $HO^{\bullet}$, may result in toxicity through genetic mutation, inhibition of glycolysis and DNA synthesis, and lipid peroxidation [22]. Therefore, our results suggest that ROS production of HGFs due to blue-light irradiation by LED and QTH light sources can damage mitochondria, which are the first-target organelles.

Irradiation is reported to promote several routes of ROS generation in the cells of organisms. These routes always produce other radicals in response to other non-radicals, a process known as a chain reaction [35-37]. For this reaction, preventive antioxidants, such as peroxidase, function primarily by suppressing the production of ROS in biological systems. An imbalance between ROS production and antioxidant defense, which may arise from deficiencies of antioxidants (such as glutathione, ascorbic acid, or α-tocopherol) and/or from the increased formation of ROS, may result in oxidative stress [38]. The production of ROS was evaluated using fluorescent CellROX® green reagent, which binds to DNA upon oxidation, thereby staining nuclei and mitochondria green [39]. Following this analytical method, we demonstrated that blue-light irradiation using LED or QTH light source for 5 min increased ROS production in the HGFs (Fig. 4). Therefore, this result suggests

that blue-light irradiation with LED or QTH increased ROS production, and the level of ROS production is due to an imbalance of the intracellular antioxidant defense mechanism in the nucleus and/or the mitochondria in the gingival fibroblasts.

Although irradiation by both LED and QTH light sources was calibrated to provide the same output power for 5 min as that of 460 nm light, LED irradiation resulted in higher cell cytotoxicity in all examinations of this study. However, the blue filter transmitted wavelengths shorter than 500 nm, as shown in Fig. 1. In addition, by comparing the wavelengths of LED or QTH irradiation, we found that LED has higher output power in the short wavelength range, whereas QTH has the higher output power in the longer wavelength range. Thus, LED light, which is richer in short-wavelength iradiation, induced higher cell cytotoxicity, despite adjustments for same output power by the same filter. In particular, blue light irradiation by the LED light source exerted cytotoxicity by excitation of flavins in the mitochondria; flavins have excitation maxima at 450 nm [40]. Therefore, excitation produced more ROS in HGF mitochondria compared with levels produced by irradiation using the QTH light source. Thus, intracellular ROS production due to light of short wavelengths, of which blue LED light contains in greater amounts, might lead to greater cell cytotoxicity. In addition, the output power of light that we used in the present study was 250 mW/cm^2 at 460 nm. The total irradiances of QTH and LED were 514.5 and 372.3 mW/cm^2, respectively. These levels of output power are low compared with that of blue light used in clinical practice [8]. Therefore, blue light

irradiation using LED light source with higher output power in may induce additional cytotoxicity of ROS such as H_2O_2 on soft tissues.

In conclusion, we demonstrated that LED and QTH blue light, which are used in daily dental treatments, are rich in short-wavelength irradiation and may generate ROS such as H_2O_2 and $HO^{\bullet}$ in gingival fibroblasts. Furthermore, mitochondria may initiate cytotoxicity in these fibroblasts. Consequently, our findings suggest that ROS production may be significant during dental treatments such as dental bleaching, which uses blue LED or QTH light irradiation. However, a dental rubber dam is commonly used to protect gums from agents in dental treatment such as H_2O_2. Therefore, using black, orange, or both as colors for the rubber dam to absorb at 460 nm might protect from damage to gingival tissues in clinical practice.

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Figure captions

Fig. 1.

(A) Relative spectral emission curve obtained using the LED light source and QTH light source, and the transmittance of the 22.5S-SPF filter. The LED curve is represented by dotted lines with long dashes, the QTH curve is a dashed line, and the 22.5S-SPF curve is a continuous line. (B) The output power curves of the LED light source and QTH light source. The LED curve is represented by dotted lines with long dashes, and the QTH curve is a dashed line.

Fig. 2.

Irradiation-time-dependent proliferation activity of HGFs. The data were analyzed by MTS assay and normalized to 100% of the control group (0 min irradiation). The data are expressed as mean ± SD (n = 4,5). *$p < 0.05$ implies significant difference *vs* control. $^{\dagger}p < 0.05$ implies significant difference *vs* QTH light exposure for 5 min. Experimental conditions are described in the Materials and Methods.

Fig. 3.

Electron micrographs of HGFs. (A) Effect of 5 min irradiation on HGFs. (B) Effect of 5 min irradiation on mitochondrial ultrastructure. N, nucleus; white arrows, typical normal mitochondria; black arrows, and typical mitochondria damaged by blue-light irradiation. The scale bar represents 1 μm. Experimental conditions are described in the Materials and Methods.

Fig. 4.

Intracellular levels of ROS production in HGFs after 5 min irradiation. The data were analyzed by fluorescence intensity and normalized to 100% of the control group (0 min irradiation). The data are expressed as mean ± SD (n = 3). $*p < 0.05$ implies significantly difference *vs* control. $^{\dagger}p < 0.05$ implies significant difference *vs* QTH. Experimental conditions are described in the Materials and Methods.

Figure(s)

Figure 1

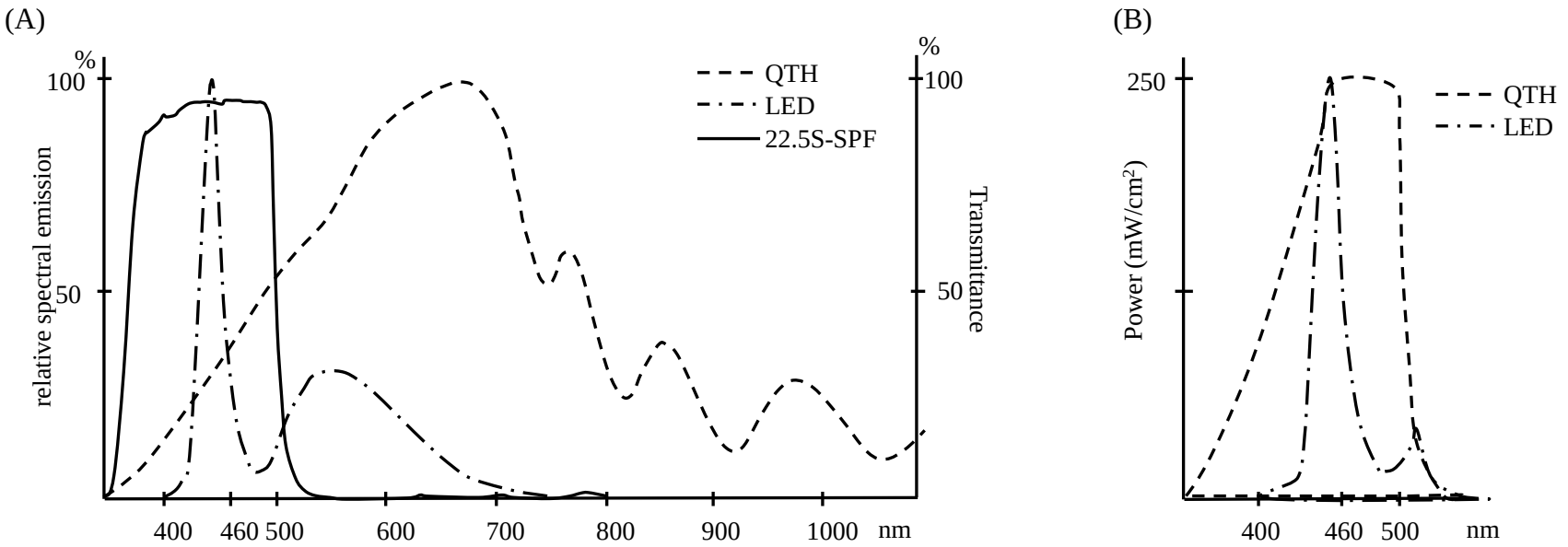


Figure 2

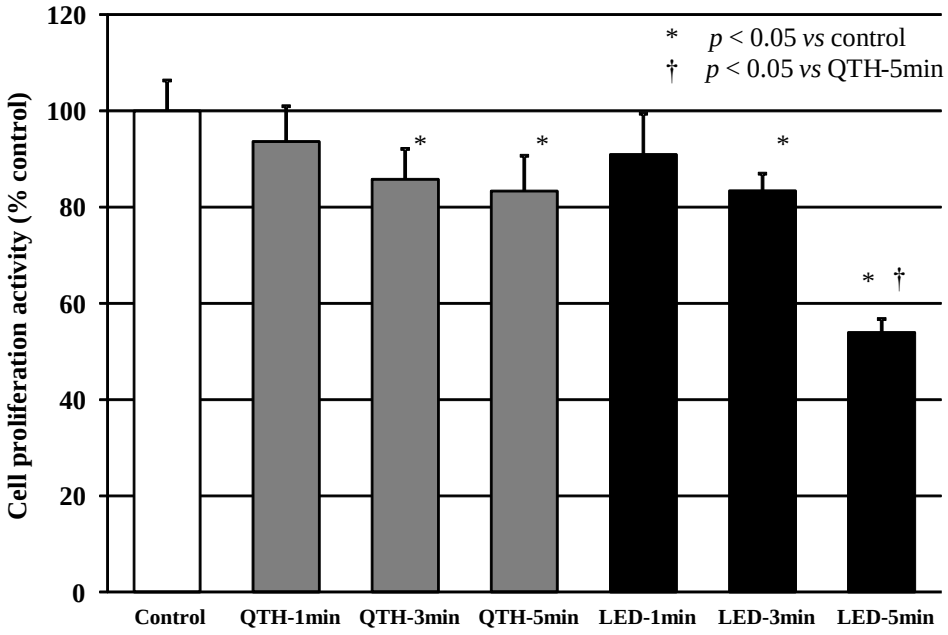

120
100
80
60
40
20
0
Cell proliferation activity (% control)
* p < 0.05 vs control
† p < 0.05 vs QTH-5min
*
*
*
* †
Control
QTH-1min
QTH-3min
QTH-5min
LED-1min
LED-3min
LED-5min

Figure 3

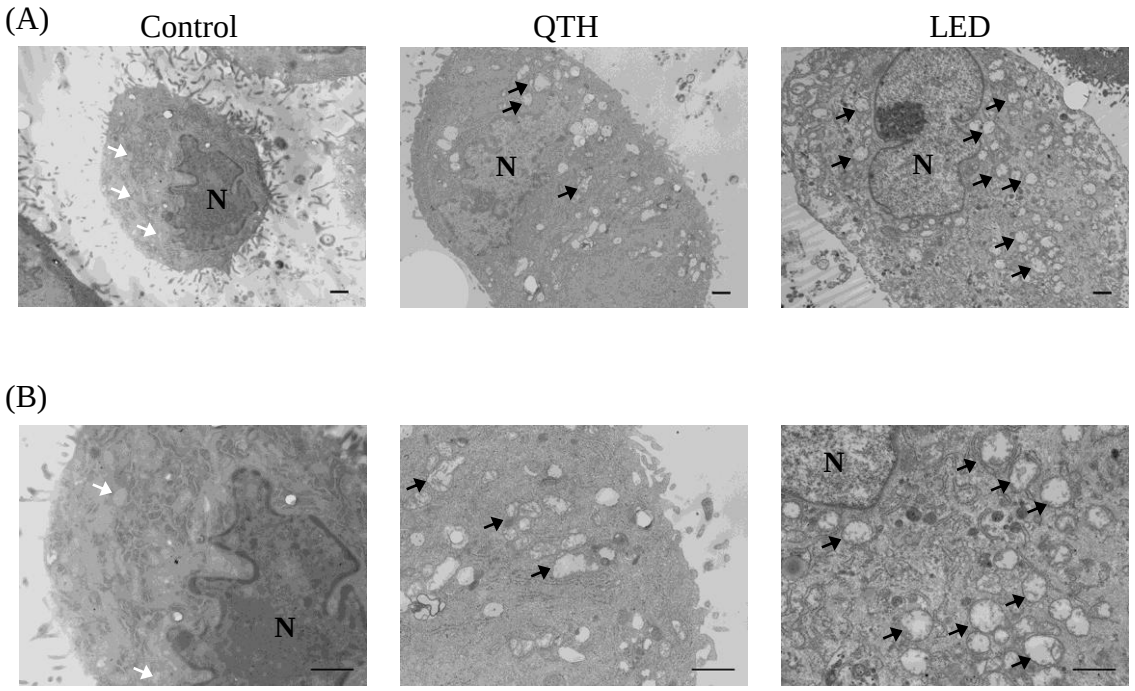
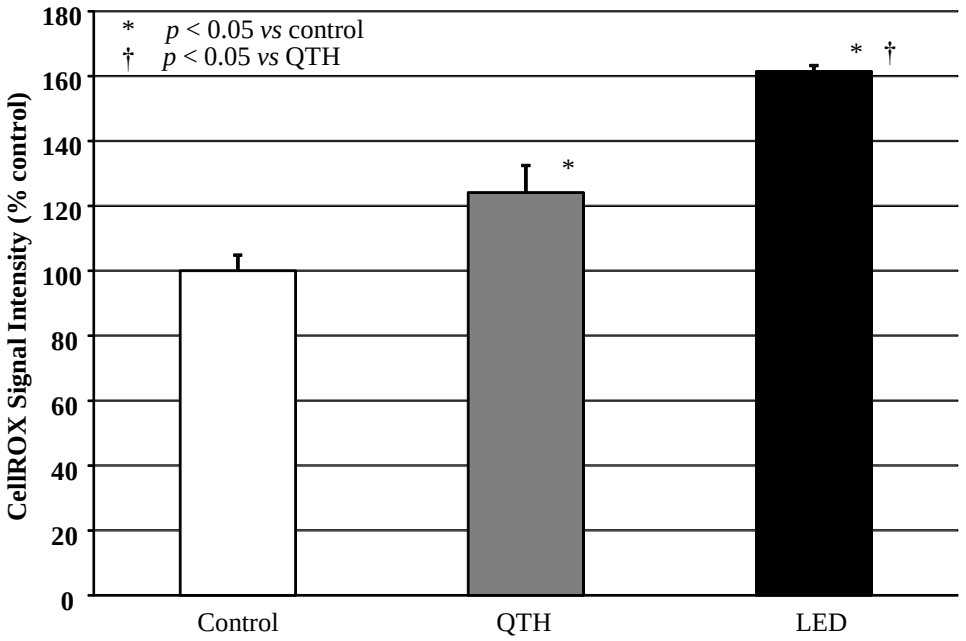


Figure 4


* p < 0.05 vs control
† p < 0.05 vs QTH
CellROX Signal Intensity (% control)
180
160
140
120
100
80
60
40
20
0
*
* †
Control
QTH
LED