Original Paper

Effects of Blue Light Emitting Diode Irradiation On the Proliferation, Apoptosis and Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells

Ye Yuan^b Gege Yan^b Rui Gong^b Lai Zhang^b Tianyi Liu^b Chao Feng^b
Weijie Du^b Ying Wang^b Fan Yang^b Yuan Li^b Shuyuan Guo^c Fengzhi Ding^b
Wenya Ma^b Elina Idiatullina^{b,d} Valentin Pavlov^d Zhenbo Han^b Benzhi Cai^b
Lei Yang^a

^aDepartment of Orthopedics, The First Affiliated Hospital of Harbin Medical University, Harbin,

^bDepartment of Clinical Pharmacy, The Second Affiliated Hospital, and Department of Pharmacology, College of Pharmacy, Harbin Medical University, Harbin, ^cDepartment of Cardiology, the First Affiliated Hospital, Cardiovascular Institute, Harbin Medical University, Harbin, China; ^dCentral Laboratory of Scientific Research, Bashkir State Medical University, Ufa, Russia

Key Words

Bone marrow mesenchymal stem cells • Blue light-emitting diode • Apoptosis • DNA damage • Osteogenic differentiation

Abstract

Background/Aims: Blue light emitting diodes (LEDs) have been proven to affect the growth of several types of cells. The effects of blue LEDs have not been tested on bone marrow-derived mesenchymal stem cells (BMSCs), which are important for cell-based therapy in various medical fields. Therefore, the aim of this study was to determine the effects of blue LED on the proliferation, apoptosis and osteogenic differentiation of BMSCs. **Methods:** BMSCs were irradiated with a blue LED light at 470 nm for 1 min, 5 min, 10 min, 30 min and 60 min or not irradiated. Cell proliferation was measured by performing cell counting and EdU staining assays. Cell apoptosis was detected by TUNEL staining. Osteogenic differentiation was evaluated by ALP and ARS staining. DCFH-DA staining and γ-H2A.X immunostaining were used to measure intracellular levels of ROS production and DNA damage. **Results:** Both cell counting and EdU staining assays showed that cell proliferation of BMSCs was significantly reduced upon blue LED irradiation. Furthermore, treatment of BMSCs with LED irradiation was followed by a remarkable increase in apoptosis, indicating that blue LED light induced toxic effects on BMSCs. Likewise, BMSC osteogenic differentiation was inhibited after exposure to blue LED irradiation. Further, blue LED irradiation was followed by the accumulation of ROS

Y. Yuan and G. Yan equally contributed to this work.

Prof. Lei Yang,
and Benzhi Cai

Department of Orthopedics, The First Affiliated Hospital of Harbin Medical University, Harbin, (China),
Department of Clinical Pharmacy, The Second Affiliated Hospital, Department of Pharmacology,
College of Pharmacy, Harbin Medical University, Harbin, (China) E-Mail yangray83@vip.qq.com

production and DNA damage. **Conclusions:** Taken together, our study demonstrated that blue LED light inhibited cell proliferation, inhibited osteogenic differentiation, and induced apoptosis in BMSCs, which are associated with increased ROS production and DNA damage. These findings may provide important insights for the application of LEDs in future BMSC-based therapies.

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Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal stem cells that can be derived from a wide range of adult tissues and fluids. MSCs are well recognized as having the capacity for self-renewal and differentiation. They can differentiate into cells of the mesodermal lineage, such as adipocytes, osteocytes and chondrocytes, as well as cells of other embryonic lineages [1]. Because of their endogenous role in maintaining stem cell niches, MSCs are involved in organ homeostasis, wound healing, and successful aging [2]. MSCs are emerging as an extremely promising therapeutic agent for tissue regeneration, and they have been broadly used in tissue engineering and cell-based therapies in several medical fields [3-6]. Biophysical stimuli, such as changes in extracellular environmental signals, have been increasingly recognized as promising strategies for affecting the proliferation, migration and differentiation of MSCs [7].

Light emitting diode (LED)-based therapy, a non-invasive biophysical therapy, has been widely and successfully used in clinical practice for several diseases, including arthritic pain [8], osteoarthritis [9], rheumatoid arthritis [10], skin rejuvenation [11], and wound healing [12, 13]. Recent studies have already demonstrated that red LED irradiation has beneficial effects on bone marrow-derived MSCs (BMSCs) proliferation and differentiation. Non-coherent red LED light has been shown to promote BMSC proliferation, but it has failed to induce osteogenic differentiation of BMSCs in normal medium. However, it can enhance osteogenic differentiation and decrease proliferation of BMSCs in osteogenic differentiation medium [14]. In addition, treatments with low-power LED irradiation in the red spectrum (620-660 nm wavelengths) and in the near infrared red (NIR) spectrum (830 nm wavelength) have been reported to promote BMSC proliferation [15].

At wavelengths ranging from 400-500 nm, blue LED light, another type of LEDs, has been shown to induce cell apoptosis and reduce cell proliferation in melanomas [16], B-cell lymphomas [17], skin tumors [18], and skin dermal fibroblasts [19] by regulating autophagy, reactive oxygen species (ROS) and mitochondrial mediated signaling pathways. Additionally, blue light induces photoreceptor apoptosis and causes age-related macular degeneration, also known as retinitis pigmentosa [20]. Moreover, the growth of porphyromonas gingivalis was suppressed by blue LED irradiation, which inhibited the expression of genes associated with DNA replication and cell division [21].

However, no study has tested the effects of blue LEDs on BMSCs. The present study was designed to investigate the effects of blue LED irradiation on BMSC proliferation, apoptosis and osteoblast differentiation, as well as the potential mechanisms of these effects.

Materials and Methods

Culture of bone marrow-derived mesenchymal stem cells

BMSCs (C57BL/6, MUBMX-01001) were purchased from Cyagen (Guang Zhou, China) and cultured in BMSC complete medium (MUBMX-90011, Cyagen) at 37°C in an atmosphere containing 5% CO₂. All experiments were performed on cell cultures at passage 11 or lower.

Blue LED irradiation and cell counting assay

Cells were irradiated one day after plating by using a blue LED (470 nm) at a power density of 20 mW/cm². Cells were irradiated at room temperature for 1 min, 5 min, 10 min, 30 min and 60 min. The cells were

counted using Count Star Easy Cell Analysis (Count Star, Shang Hai, China) 6 hrs after irradiation with blue LED light or no irradiation.

Ethynyl-2-deoxyuridine (EdU) cell proliferation assay

The EdU cell proliferation assay was performed using an EdU Apollo DNA *in vitro* kit (Ribobio, Guang Zhou, China) according to the manufacturer's protocol. Briefly, cells were fixed with 4% paraformaldehyde (m/v) for 30 min and then incubated with 50 μ M EdU at 37°C for 2 hrs. After the cells were permeabilized in 0.5% Triton X-100, they were added to Apollo staining solution and incubated in a shaker for 30 min in the dark. Finally, the cells were incubated with 20 μ g/mL 4',6-diamidino-2-phenylindole (DAPI) for 10 min. The EdU index (%) was the average ratio of the number of EdU-positive cells to total cells in randomly selected areas under a fluorescence microscope (IX73P1F, Olympus Optical, Tokyo, Japan). Cores ≥ 10 cells were included in the analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

The percentage of apoptotic cells was determined using an In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. After the cells were fixed and permeabilized, they were incubated in TUNEL reaction mixture (vial1:vial2=1:9) for 1 hr at 37°C under a humidified atmosphere in the dark. The cells were then incubated with DAPI (20 μ g/mL) for 10 min. The TUNEL index (%) was calculated as the average ratio of the number of TUNEL-positive cells to total cells in randomly selected areas under a fluorescence microscope (Olympus Optical). Cores ≥ 10 cells were included in the analysis.

γ -H2A.X immunostaining

γ -H2A.X immunostaining was used to detect DNA double-strand breaks (DSBs). Cells were fixed using 4% paraformaldehyde (m/v) and permeabilized using a 0.3% Triton X100 solution. The cells were then incubated in PBS supplemented with 0.1% Tween and 2% fetal bovine serum (FBS) (Embriolife®, Vitrocell, Brazil) for 1 hr. Cells positive for γ -H2A.X were detected using mouse anti γ -H2A.X phospho S139 (200 $\times$ diluted, ab26350, Abcam, Cambridge, UK). A secondary incubation step was performed using Alexa Fluor® 594 goat anti-mouse (200 $\times$ diluted, ab150116, Abcam). After a PBS wash, the cells were progressively dehydrated with alcohol, and then, nuclear staining was performed with DAPI (2.5 μ g/mL). Fluorescence signals were visualized under a confocal laser scanning microscope (FV10i, Olympus Optical).

Measurement of reactive oxygen species (ROS) production

Intracellular levels of ROS production were measured using a Reactive Oxygen Species Assay Kit (S0033, Beyotime Biotechnology, Shang Hai, China) according to the manufacturer's instructions. After washing three times with PBS, cells were incubated with 10 μ M DCFH-DA probes for 30 min at 37°C. Following the removal of the superfluous DCFH-DA probes that did not penetrate into BMSCs, the cells were fixed in 4% paraformaldehyde (m/v) for 30 min and stained with DAPI (20 μ g/mL) for 10 min. The cells were imaged with a fluorescence microscope (Olympus Optical). Cores ≥ 10 cells were included in the analysis.

Alkaline phosphatase (ALP) assays

Osteogenic differentiation of BMSCs was performed using BMSC osteogenic differentiation medium (MUBMX-90021, Caygen) according to the manufacturer's protocol. ALP enzyme activity was used to detect matrix mineralization deposition using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Biotechnology). On the 7th day of osteogenic differentiation, the medium was discarded, and the cells were washed with PBS. BMSCs were then fixed with 4% paraformaldehyde (m/v) for 30 min. Following fixation, the cells were stained with BCIP/NBT solution for 30 min and then washed with distilled water. Then, the stained cells were photographed under a standard light microscope (ECLIPSE TS100, Nikon, Japan).

Alizarin Red S (ARS) staining

On the 7th day of osteogenic differentiation, the cells were fixed, washed with PBS, and then stained with 40 mM Alizarin Red S (Cyagen) for 5 min. Photos were taken randomly using standard light microscopy (ECLIPSE TS100, Nikon).

Fig. 1. The effects of blue LED irradiation on BMSC growth. (A) Detection map of blue LED output intensity: the peak wavelength is at 470 nm. Following blue LED irradiation, BMSC number was determined using a cell counting assay (B) The panel shows total cell number following irradiation by blue LED light. * $P < 0.05$; ** $P < 0.01$. (C) Representative images taken under a microscope (magnification $\times 40$).

Statistical analysis

All assays were repeated at least three times, and values are given as the mean $\pm$ SEM. Statistical analysis was performed using the Mann-Whitney U test (GraphPad Software Inc., San Diego, CA). P values < 0.05 were considered statistically significant.

Results

Inhibition of BMSC growth upon blue LED irradiation

We used spectroscopic analysis to identify the maximal light emission of the blue LED, which, at a 470 nm wavelength, was approximately 1,500 absorbance units, as shown in Fig. 1A. To examine the effect of blue LED irradiation on BMSC growth, we exposed the BMSCs to blue LED light for 1 min, 5 min, 10 min, 30 min and 60 min and subsequently evaluated proliferation using cell counting assays. No obvious differences were observed between the cells treated with short-term irradiation for 1 min and the corresponding non-irradiated control cells. However, cell growth began to significantly decrease starting at treatments for 5 min (Fig. 1B). Obvious shrinkage and rounding of the BMSCs were observed upon treatment with blue LED irradiation for 30 min and 60 min (Fig. 1C).

To further identify whether blue LED irradiation has a toxic effect on cell proliferation, EdU staining was employed to determine the percentages of proliferative cells after irradiation for up to 60 min. Compared to the control groups, decreased percentages of EdU⁺ cells were observed for cells irradiated with blue LED light for 10 min, 30 min and 60 min (Fig. 2). The percentages of EdU⁺ cells were reduced from approximately 30% to 19.8%, 5.8% and 2.4% respectively, indicating that blue LED irradiation significantly inhibits BMSC proliferation.

Fig. 2. Anti-proliferative effects of blue LED exposure on BMSCs. An ethynyl-2-deoxyuridine (EdU) staining assay was performed to assess BMSC proliferation following exposure to blue LED irradiation at different time points. (A) Representative immunofluorescence staining of BMSCs. DAPI (blue), EdU (red) and merged images were shown. Photos of the selected areas were taken randomly under fluorescence microscopy. Scale bar: 20 μ m. (B) The panel shows the percentages of EdU-positive cells. * $P < 0.05$; *** $P < 0.001$.

Fig. 3. Pro-apoptotic effects of blue LED irradiation on BMSCs. The percentages of apoptotic BMSCs upon irradiation by blue LED light for up to 60 min were measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. (A) Representative immunofluorescence staining of DAPI (blue), TUNEL (green) and merged images. Photos were taken randomly using fluorescence microscopy. Scale bar: 20 μ m. (B) The panel shows the percentages of TUNEL positive cells. * $P < 0.05$; ** $P < 0.01$.

Induction of BMSC apoptosis by blue LED irradiation

To investigate the effects of blue LED irradiation on cell apoptosis, we performed TUNEL staining in BMSCs. As shown in Fig. 3, no obvious differences were observed between the control groups and cells treated with blue LED irradiation for less than 5 min. However, the percentages of TUNEL⁺ cells were significantly higher following treatment with blue LED irradiation for 10 min, 30 min and 60 min. The average percentage of apoptotic cells was increased to a maximum of 31%. These data suggest that blue LED irradiation has toxic effects on BMSCs.

Osteogenic differentiation of BMSC was inhibited by blue LED irradiation

BMSCs, non-hematopoietic stromal cells, have the ability to differentiate into osteoblasts [3]. Thus, we evaluated alkaline phosphatase (ALP) activity, an early marker of osteogenesis, at day 7 during osteogenic differentiation of BMSCs. Lower ALP activity was observed in blue LED irradiated groups compared to the non-irradiated control group (Fig. 4A). Alizarin Red S (ARS) staining revealed that the calcium deposits on the 7th day of BMSC osteogenic differentiation were significantly reduced following treatment with blue LED irradiation (Fig. 4B).

Fig. 4. Inhibitory effects of blue LED irradiation on BMSC osteogenic differentiation. ALP staining was used to detect ALP activity, and ARS staining was used to detect the formation of calcium nodules. BMSCs were irradiated by blue LED light at a power density of 20 mW/cm² for 10 min every day during osteogenic differentiation.

Representative cell images of (A) ALP staining and (B) ARS staining show the status of osteogenic differentiation on the 7th day for BMSCs irradiated with blue LED light and not irradiated.

Increased intracellular reactive oxygen species (ROS) levels in BMSCs after blue LED irradiation

Intracellular ROS production plays important roles in the proliferation and apoptosis of various cell types [22]. It has been reported that ROS production regulates aging, senescence, and osteogenic differentiation in mesenchymal stem cells [23, 24]. To further evaluate the effects of blue LED irradiation on ROS production, we performed DCF-DA staining in blue LED treated and untreated BMSCs. As shown in Fig. 5A, we found that blue LED treatment induced nuclear ROS production in BMSCs following irradiation for at least 10 min. After long-term irradiation for 30 min and 60 min, elevated ROS production was observed in both the nucleus and the cytoplasm. Following irradiation for 10 min, 30 min and 60 min, the percentages of ROS⁺ cells significantly increased by 33.0%, 42.4% and 53.4%, respectively (Fig. 5B). These findings indicate that blue LED irradiation results in a significant increase in intracellular ROS generation by BMSCs.

Blue LED irradiation leads to DNA damage in BMSCs

The phosphorylation status of H2A.X (γ -H2A.X), a marker for DNA damage, has been shown to indicate whether cells have repaired damaged DNA, resulting in cell survival or apoptosis. [25] Thus, in this study, we investigated whether blue LED irradiation causes DNA damage in BMSCs using γ -H2A.X immunostaining. The percentages of γ -H2A.X positive cells were increased significantly in cells treated with blue LED irradiation for

Fig. 5. Effects of blue LED light on the generation of reactive oxygen species (ROS) in BMSCs. The peroxide-sensitive fluorescent probe DCFH-DA was used to measure intracellular levels of ROS in BMSCs after blue LED irradiation. (A) Representative immunofluorescence staining of BMSCs. DAPI (blue), ROS (green) and merged images were shown. Photos were taken randomly. Scale bar: 20 μ m. (B) The panel shows the percentages of ROS-positive cells. **P<0.01.

5 min, 10 min, 30 min and 60 min (Fig. 6). After irradiation for 60 min, we observed a marked increase in DNA damaged in 23.4% of BMSCs. Together, these results indicate that blue LED irradiation results in increased DNA damage in BMSCs.

Discussion

In this study, we investigated the effects of blue LED irradiation (with maximal light emission at 470 nm in wavelength) on BMSCs. The results of this study demonstrated that (1) blue LED irradiation inhibited cell growth, proliferation, and osteogenic differentiation, as well as induced cell apoptosis in BMSCs; (2) the underlying mechanisms of these effects were associated with the accumulation of ROS and increased DNA damage in BMSCs.

Previous studies have indicated that blue LED irradiation inhibits cellular growth and induces apoptosis in several cell types both *in vitro* and *in vivo*. [17, 21, 26, 27] These studies are consistent with what we found in BMSCs. However, the underlying mechanisms by which blue LED inhibits cell growth are still unclear. It has been previously shown that oxidative damage is induced by increased ROS generation, leading to the inhibition of cell proliferation and the induction of cell apoptosis, mainly through PKC inactivation and caspase-3 activation. [28] Additionally, increased ROS production could result in increased DNA damage. [29] Previous studies have also shown that DNA damage stimulates ROS production through the H2A.X-Nox1/Rac1 signaling pathway. [30] In our study, we found that blue LED irradiation increased ROS production and induced DNA damage in BMSCs. Interestingly we observed that DNA damage began to occur in BMSCs starting 5 min after irradiation, which was earlier than the induction of cell apoptosis and inhibition of cell proliferation. Thus, we assumed that DNA damage and intracellular ROS production might be direct or indirect mechanisms for the inhibitory effects of blue LED light on BMSCs.

It has been demonstrated that some specific proteins [31-33], miRNAs [34, 35] and molecules [36-38] are involved in the regulation of osteogenic differentiation in BMSCs. These findings suggested that osteogenic differentiation is a complicated multifactorial process. We observed that blue LED irradiation possessed an inhibitory effect on the osteogenic differentiation of BMSCs. This inhibition effect may due to interactive regulatory actions among these specific proteins, miRNAs and molecules. In addition, the cell growth inhibition and cellular injury resulting from blue LED irradiation could also partially lead to impaired BMSC differentiation.

Fig. 6. Effects of blue LED light on DNA damage in BMSCs. γ -H2A.X immunostaining followed by confocal microscopy was used to determine the status of DNA damage in BMSCs irradiated by blue LED light. (A) Representative immunofluorescence staining of BMSCs. DAPI (blue), γ -H2A.X (red) and merged images were shown. Photos were taken randomly. Scale bar: 20 μ m. (B) The panel shows the percentages of γ -H2A.X positive cells. Arrows indicate specific staining of γ -H2A.X in BMSCs. * $P < 0.05$; ** $P < 0.01$.

As we know, BMSC transplantation has been used widely for medical therapy in animal studies. This technique has been used to repair articular cartilage defects [39], regenerate injured rat uterus [40], enhance diabetic wound healing [41], and repair chronic liver fibrosis or cirrhosis [42]. It is therefore necessary to culture and induce differentiation of BMSCs *in vitro* before transplantation *in vivo*. However, during the period of *in vitro* culture and differentiation, it is impossible to avoid exposing BMSCs to blue light irradiation emitted by natural light. This blue light negatively affects the transdifferentiation capability of BMSCs and may produce adverse effects on these cells. Thus, our data suggest an important caveat for the further use of BMSCs *in vitro*.

Although several studies have investigated the effects of LEDs with various wavelengths, such as 620 nm [14], 630 nm [43], and 660 nm [44] red light and 830 nm [15] near infrared light, on BMSCs, the available colors represent a broad range of wavelengths, which may produce different effects. Thus, it is necessary to further elucidate the effects of LEDs with different wavelengths and to determine the molecular mechanisms of these effects. Additionally, it will also be interesting to determine whether irradiation with a combination of different LED colors will have synergistic effects with medical treatments or drugs.

Conclusion

We have for the first time demonstrated that blue LED irradiation has toxic effects on BMSCs, characterized by the inhibition of cellular proliferation and osteogenic differentiation and the induction of cell apoptosis. These adverse effects may also be a result of increased ROS production and DNA damage. The results of this study will help us understand the mechanistic effects of blue LED irradiation and provide a reminder for the utility of BMSCs and medical phototherapy *in vitro*.

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Disclosure Statement

The authors indicate no potential conflicts of interest.

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