Blue LED causes cell death in human hepatoma by inducing DNA damage

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Research article

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Abstract

Phototherapies, including sunlight, infrared, ultraviolet, visible and laser, parts of which present high curative effect, small invasion, and negligible adverse reactions in cancer treatment. Here we aimed to explore the potential therapeutical effects of blue LED in hepatoma cell and decipher the underlying cellular/molecular mechanisms. We demonstrated that the irradiation of blue LED light in hepatoma cell could lead to cell proliferation reduction along with the cell apoptosis increase. Simultaneously, blue LED irradiation also markedly suppressed the migration and invasion ability of hepatoma cells. Sphere formation analysis further revealed the decreased stemness of hepatoma cell under the treatment of blue LED irradiation. In addition, blue LED irradiation significantly promoted the expression of γ-H2AX, a sensitive molecular marker of DNA damage. Collectively, we demonstrated that blue LED irradiation exhibited anti-tumor effects on liver cancer by inducing DNA damage, representing a potential approach for human hepatoma treatment.

Introduction

Phototherapy is promising medical technique, where sunlight or artificial light including infrared, ultraviolet, visible light and laser, are used to prevent and treat diseases or promote the body’s rehabilitation. Light emitting diode (LED) is being developed as the preferred light source for phototherapy as a result of its’ good economy and none or minimal side effects but various wavelengths. Previous studies have shown that various level of light exposing would cause different biological significance [1–3]. Recent reports uncovered that LED treatment could meaningfully ameliorate lung inflammation, inhibit human gingival fibroblast cell proliferation and facilitate Post-surgical recovery and wound healing [4–7]. Notable among the various species of LED is that blue LED with 430–490 nm wavelengths showed excellent antitumor activity in vitro and in vivo experiments. Blue light could effectively inhibit the tumor progress of skin tumors, melanoma, leukemia, colon cancer mainly depending on the accumulation of intracellular reactive oxygen species (ROS) [8–11]. Consistent with these reports, our previous study demonstrated that blue LED could enhance the anti-tumor effects of arsenic trioxide on human osteosarcoma and cause cell death in colorectal cancer through increasing ROS accumulation and DNA damaged mediated p53 activation [12, 13]. Although blue LED showed powerful anti-tumor effect in the above study, the potential effects of blue LED irradiation in liver cancer remains unclear.

Liver cancer, the second leading cause of cancer mortality in the world, is most prevalent in Asia and Africa, and over forty percent of new cases and deaths happened in China[14–16]. Past research illuminated that the aberrant activation of the Wnt/β-Catenin Signaling, the mTOR pathway, metabolic pathways and liver tumor microenvironment (TME) play an important role on significantly the molecular pathogenesis of liver cancer [17–20]. However, the limitation of current therapies targeting liver cancer points towards the importance of further deciphering the effective treatment of liver cancer. Therefore, how to prolong the survival time and improve prognosis of liver cancer patients is very important.
In current study, we have explored the role of the various doses of blue LED on hepatoma cell, HepG2 and Hep3B. Finally, we found that blue LED could suppress the proliferation, aggregation, migration and invasion of HepG2 and Hep3B and promote the apoptosis of HepG2 and Hep3B. Mechanistically, we further revealed that blue LED treatment could obviously increase the expression of γ-H2AX.

**Material And Methods**

**Cell lines and culture**

Hepatoma carcinoma cell lines Hep3B and HepG2 were cultivated in Dulbecco’s modified Eagle medium (DMEM) (Life Technologies Corporation, California, United States) containing 4,500 mg/L glucose supplemented with 10% fetal bovine serum (FBS). Culture flasks were kept at 37°C in a humid incubator with 5% CO₂.

**LED irradiation**

Cells are irradiation by blue (peaked at 470 nm) light at room temperature for 0 J/cm², 72 J/cm², 144 J/cm², 216 J/cm² and 288 J/cm² respectively.

**Trypan blue dyeing assay**

Preparing 0.4% Trypan blue dye, the adherent cells were digested by trypsin to prepare single cell suspension. The cell suspension was mixed with 0.4% trypan blue solution at 9:1, and the living and dead cells were counted respectively in 3 min. Microscopically, dead cells are stained with a distinctive blue color, while living cells are colorless and transparent. Living cell rate (%) = total number of living cells / (total number of living cells + total number of dead cells) × 100%.

**Ethynyl-2-deoxyuridine (EdU) cell proliferation assay**

Using of EdU Apollo DNA in vitro kit (Ribobio, Guangzhou, China) for dyeing. The cells grew uniformly adherent to the wall at the bottom of the six-well plate, with a density of 2.0 × 10⁵. Using EdU reagent incubation cells after 90 min, with 4% paraformaldehyde fixed cells at 37°C for 15 min, with 0.5% of Triton X-100 after infiltration, will join the rest of the cell cultures of Apollo dyeing liquid dark stain 30 min, and then put cells at 20 µg/mL 4′, 6 ′- amino – 2 - phenyl indole (DAPI) incubation in 20 min, PBS elution 3 times (10 min/time) in the end, under the confocal laser scanning microscope (FV10i) pictures. EdU index (%) is the average ratio of EdU positive cells to total cells in 5 randomly selected regions.

**Cellular wound healing assays**

Cells were plated into 6-well culture plates at the density of 2.5 × 10⁵ cells/mL. When the confluence of cells reached to 70%, dividing three wounds evenly vertically with a 200 µL pipette tip. The cells were rinsed with PBS for twice and then the fresh medium was changed. Cells were kept at 37°C in a humid incubator with 5% CO₂ for 24 hrs. After that, cells were treated with blue LED irradiation. Wound healing
was monitored at 0/6/12/24 hrs with a standard light microscopy (ECLIPSE TS100, Nikon, Japan). The wound area was measured using ImageJ software (National Institutes of Health (NIH), United States).

**Propidium iodide (PI)/Hoechst 33342 staining**

PI/Hoechst 33342 staining was performed using Hoechst 33342/PI Double Staining Kit (Solarbio Science, Beijing China). The cells were evenly grown on the bottom of the culture dish, fixed with 4% paraformaldehyde at 37°C for 15 min, and stained with the dye solution at room temperature and away from light for 30 min. Finally, images of the staining were captured by the confocal laser scanning microscope (FV10i, Olympus, Tokyo, Japan).

**Invasion assays**

A 24 mm Transwell® chambers was used to detect cell invasive abilities according to the manufacturer's protocol. Matrigel was diluted with PBS 1:8 and coated on the upper surface of the membrane at the bottom of the chamber. After 12 hrs of cell starvation, the cells were digested with trypsin and resuspended in serums free medium with cell density of 5 × 10^4 cells/mL, 200 µL of cell suspension was taken to the upper compartment, and 300 µL of 20% serum DMEM medium was added to the lower compartment, with blue light irradiation of 0 J/cm^2^ and 144 J/cm^2^). After 24 hrs, cells migrated through the membrane were stained with 0.1% crystal violet (Beyotime Biotechnology, China) for 15 min and counted using a light microscopy (ECLIPSE TS100, Nikon).

**Spheroid formation assay**

A total of 1000 hepatoma cells were plated in ultralow attachment plates. The cells were cultured for 10 days in DMEM/F12 medium (Invitrogen, Shanghai, China) supplemented with 4 mg/mL insulin (Sigma, Shanghai, China), B27 (1:50, GIBCO, Shanghai, China), 20 ng/mL EGF (Sigma, Shanghai, China) and 20 ng/mL basic FGF (Sigma, Shanghai, China). Liver cancer cells in good condition were digested with trypsin, centrifuged, washed twice with PBS, and resuspended with the prepared stem cell culture medium. The cells were cultured in six-well plates with ultra-low adsorption cells for 10 days, during which the cells were irradiation with blue light 0 J/cm^2^ and 144 J/cm^2^). Finally, counted using a light microscopy (ECLIPSE TS100, Nikon).

**Immunofluorescence experiment**

After cells irradiation by blue light of 0 J/cm^2^ and 144 J/cm^2^, were fixed with 4% paraformaldehyde for 15 min at 37°C, followed by permeability of 0.1% Triton X-100 at room temperature for 15 min, and sealed with goat serum albumin for 60 min. The cells were incubated with primary antibody at 4°C overnight, washed with PBST for three times, then incubated with secondary antibody at room temperature in dark for 1 h, and stained with DAPI in dark for 20 min. under the confocal laser scanning microscope (FV10i) pictures. It was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The following primary antibodies and dilutions were used for immunofluorescence microscopy experiments: γ-H2AX (Abcam, ab26350). The following secondary antibody mouse IgG (Abcam. ab150113) were used.
Western blotting

After cells irradiation by blue light of 0 J/cm² and 144 J/cm², the protein was extracted with RIPA buffer (Beyotime Biotechnology). Protein fractions were collected by centrifugation at 13,500 rpm for 15 min, and then supernatants were heated with SDS buffer at 100°C for 4 min. Proteins separated by 10% SDS-PAGE and transferred to PVDF membrane. The blots were then blocked with 5% non-fat milk powder in PBST buffer for 1 h, and incubated at 4 °C overnight with the appropriate primary antibodies at appropriate dilutions. After washing, the bolts were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The following primary antibodies was used: γ-H2AX (Abcam, ab26350). The following secondary antibodies were used: β-actin (Absin, abs830031).

Results

Blue LED irradiation inhibits cell viability and promotes apoptosis in HCC cells

To investigate whether irradiations of blue LED could affect HCC cell growth, the morphologic changes of HCC cells were observed. Firstly, we use two hepatoma cell lines, HepG2 and Hep3B, irradiated with different doses of blue LEDs of 72 J/cm², 144 J/cm², 216 J/cm² and 288 J/cm², to examine the changes in cell viability. As exhibited in Fig. 1A, with the higher light intensity, the more terrible cell morphology would be observed. It is manifested as a decrease in viable cells, as well as an increase in the ratio of dead cells to dead cells under the treatment of blue LED irradiation (Fig. 1B).

Furthermore, EdU staining assay was used to assess the effects of blue LED irradiation on cell proliferation in HepG2 and Hep3B cells with or without blue LED irradiation. Compared with non-irradiated control cells, significant suppression of the proliferation potential of HepG2 and Hep3B cells was also observed in blue LED irradiation group (Fig. 2A and 2B). PI/Hoechst assay was performed to assured cell apoptosis. The 144 J/cm² group showed a significant increase of PI positive cells, indicating that LED blue light irradiation has a role in promoting apoptosis in hepatoma cells (Fig. 3A and 3B).

LED blue irradiation inhibits the migration and invasion of hepatocellular carcinoma cells

In addition, wound healing and Trans-well assays were then employed to evaluate whether cell invasion and migration were affected by blue LED irradiation in HCC cells. As shown in Fig. 4A and 4B, the wound healing assay revealed that the size of the wounds in the control group decreased over time compared to the 144 J/cm² LED blue light irradiated group in 24, 48 and 72 hrs, and the speed of wound healing could be used to measure cell invasion. Compared with the non-irradiated control group, wound closure was significantly inhibited in the irradiated group. The cells were then further evaluated for changes in their ability of migration, and crystal violet is a basic dye that can bind to DNA in the nucleus, thereby
producing nuclear staining. The cell migrated through matrigel-coated membranes were markedly inhibited in blue LED treated groups compared with control groups (Fig. 4C). Consistently, the absorbance value of crystal violet at 570 nm reflects the cell invasion capacity (Fig. 4D).

**Blue LED irradiation inhibits stem cell potential**

Cancer stem cells play an important role in tumor survival, proliferation, metastasis and recurrence. Essentially, cancer stem cells maintain the vitality of tumor cell populations through self-renewal and unlimited proliferation; the motility and migration ability of cancer stem cells in turn make the metastasis of tumor cells possible. Therefore, cancer stem cells often exist in the hypoxic niche, vascular niche, invasion niche, can be in a dormant state for a long time and have a variety of drug resistance molecules but not sensitive to the external physical and chemical factors that kill tumor cells, resulting in tumor recurrence, migration, resulting in difficult to cure. It can be seen that cancer stem cells are a group of cells with both self-renewal ability and invasion and migration ability, while the current experiments can only unilaterally prove the self-renewal ability or invasion and migration ability of cancer stem cells. The sphere-forming test of tumor cells (sphere-forming size and number) is the gold standard for measuring the stemness of tumor cells. Therefore next, we evaluated the effect of LED blue irradiation on the functional properties of cells by studying their ability to form colonies and spheres after they were irradiated with blue light. Obviously, all HCC cell lines in the 144 J/cm² LED blue irradiation group had significantly reduced colony-forming and sphere-forming abilities. In contrast, the control group was not affected (Fig. 5).

**Blue LED irradiation exhibited anti-tumor effects on liver cancer by inducing DNA damage**

Our study has fully demonstrated that blue LED irradiation inhibits hepatoma cell viability, as well as proliferation, migration, invasion, and stem cell potential. Next, we try to clarify the potential regulatory mechanisms of blue light irradiation-induced cell death.

During the normal growth of eukaryotic cells, DNA can be damaged due to various external and internal factors, such as X-rays, environmental pollutants and reactive oxygen species (ROS) substances. Among these DNA damages, DNA double strand breaks (DSBs) are considered to be the most serious type of damage because they affect the double helix structure of DNA. DSBs can activate the DNA damage response (DDR) mechanism of cells, thereby rapidly phosphorylating histone H2AX (phosphorylated H2AX histones are called γ-H2AX). Subsequently, γ-H2AX accumulates at double-stranded breaks to form foci formed by the accumulation of large amounts of γ-H2AX. The number of γ-H2AX foci in cells can be used to evaluate DNA double-strand breaks, which can then be used to evaluate the mutagenic ability of genotoxic factors. Immunofluorescence is currently the most important method for studying γ-H2AX. If H2AX was phosphorylated and formed foci in the cells, γ-H2AX was significantly increased after fluorescence irradiation (Fig. 6A and 6B). Consistently, western blotting results showed that the total γ-
H2AX content in HepG2 and Hep3B cells was significantly increased after LED blue light irradiation, which means that blue light irradiation severely damaged the DNA of hepatoma cells.

**Discussion**

In this study, we for the first time discovered that blue LED irradiation presented anti-tumor effect in liver cancer which are strongly supported by the promotion of cell apoptosis and the inhibition of cell proliferation and cell migration and cell invasion. By the time, blue LED irradiation could also significantly destroy the stemness characteristic of human liver cancer stem-like cells HepG2 and Hep3B. Furthermore, we identified that DNA damage maker γ-H2AX also dramatically downregulated under blue LED treatment. This study demonstrated that the antitumor ability of blue LED irradiation for liver cancer were modulated by DNA damage, which provides new approach and strategy for the clinical treatment of human liver cancer.

Phototherapy has successfully achieved a good therapeutic effect for many diseases, such as spinal cord injury [21], acne [22], vitiligo [23] etc. Our previous studies have explored the anti-tumor function of blue LED in human osteosarcoma through the regulation of ROS production and DNA damage [24]. Besides, we also confirmed that combined treatment of blue LED irradiation and arsenic trioxide in human osteosarcoma played coordinated roles, improves and strengthens anti-tumor effect of arsenic trioxide [25]. However, the role of blue LED irradiation in human liver cancer still remain unclear. Thus, we detected cell viability and apoptosis of liver cancer cells HepG2 and Hep3B under blue LED irradiation at 0 J/cm$^2$ and 144 J/cm$^2$ respectively. Results of those analysis indicated that blue LED irradiation exhibited powerful lethal action to human liver cancer cells. Cancer metastasis is a multi-step process including cell migration and invasion, which represents the advanced stage of malignancy. Consisting with our results, recent works have carried out that blue LED could inhibit cell migration and invasion of colon cancer cells and fibrosarcoma cells [26].

Cancer stem cells (CSCs) are a small part of cells within tumors, which is supposed to be cancer-initiating cells and has characteristic of self-renewal, differentiation, and tumorigenicity [27]. Liver cancer has been reported to be derived from liver stem cells presenting in adult liver tissue with endogenous or exogenous liver origin. Sphere-formation analysis is a common method used to identify the properties of CSCs in vitro. In the present study, blue LED irradiation strongly destructed the liver cancer cell capacity of generating liver tumor nodules.

DNA damage is a permanent change in DNA nucleotide sequence that occurs during replication and results in changes in genetic characteristics. DNA damage triggers a series of cellular responses and stimulates cell cycle checkpoints [28]. Cell cycle checkpoints can repair the damage before it interferes with the DNA replication machinery, which will cause cell death once it fails. Core histone protein H2AX (termed γ-H2AX) persistence is a persuasive molecular marker of DNA damage. Consistently, immunofluorescence staining results significant shown that blue LED irradiation increased the expression of γ-H2AX in HepG2 and Hep3B cells, which represents the accumulation of DNA damage.
In conclusion, blue LED irradiation lead to inhibition of cell proliferation, migration, invasion, and induction of cell death via inducing DNA damage in liver cancer cells. Therefore, our study will provide a better understanding of blue LED irradiation on liver cancer therapy.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and material**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

M.Y.H., G.H.L., X.Q.H., Y.W., H.L., Q.W., G.G.Y., R.G., G.X.L., T.L., B.Z.C. and L.Q.L. performed research; Y.Y., G.G.Y., M.Y.H., H.L. analyzed data; Y.Y., M.Y.H., G.H.L., X.Q.H., Y.W. designed the study and wrote the manuscript.

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**Figures**
Blue LED exposure reduces viability of hepatoma cells. Hep3B cells and HepG2 cells were treated with blue LED lights for 0 J/cm², 72 J/cm², 144 J/cm², 216 J/cm² and 288 J/cm² respectively. (Bar: 100 μm) A The representative images from hepatoma cells irradiated with blue LED lights. (B) The number of live cells tested by Trypan blue exclusion. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 1

Blue LED exposure reduces viability of hepatoma cells. Hep3B cells and HepG2 cells were treated with blue LED lights for 0 J/cm², 72 J/cm², 144 J/cm², 216 J/cm² and 288 J/cm² respectively. (Bar: 100 μm) A. The representative images from hepatoma cells irradiated with blue LED lights. (B) The number of live cells tested by Trypan blue exclusion. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 2

Blue LED exposure inhibits proliferation of hepatoma cells. Hep3B cells and HepG2 cells were irradiated with blue LED for 0 J/cm² and 144 J/cm² respectively. (Bar: 100 μm) (A and B) The proliferation of hepatoma cells determined by EdU staining. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 2

Blue LED exposure inhibits proliferation of hepatoma cells. Hep3B cells and HepG2 cells were irradiated with blue LED for 0 J/cm² and 144 J/cm² respectively. (Bar: 100 μm) (A and B) The proliferation of hepatoma cells determined by EdU staining. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 3

Blue LED lights promotes apoptosis in hepatocarcinoma cells. Hep3B cells and HepG2 cells were exposed to blue LED for 0 J/cm² and 144 J/cm² respectively. (A and B) The apoptosis in hepatocarinoma cells detected by Hoechst/PI staining after blue LED lights exposure. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 3

Blue LED lights promotes apoptosis in hepatocarcinoma cells. Hep3B cells and HepG2 cells were exposed to blue LED for 0 J/cm2 and 144 J/cm2 respectively. (A and B) The apoptosis in hepatocarcinoma cells detected by Hoechst/PI staining after blue LED lights exposure. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Blue LED lights impede migration and invasion in hepatocarcinoma cells. Hep3B cells and HepG2 cells with blue LED exposure for 0 J/cm² and 144 J/cm² respectively. (A and B) The migration of hepatocarcinoma cells assessed by Wound healing assay (A), (Bar: 200 μm) The ratio of wound healing in migrated cells (B). (C and D) The representative images from hepatoma cells stained with crystal violet (C), and the invasiness reflected by the absorbance value at 570 nm (D). Data are expressed as the mean ± SEM. ***P < 0.001.
Figure 4

Blue LED lights impede migration and invasion in hepatocarcinoma cells. Hep3B cells and HepG2 cells with blue LED exposure for 0 J/cm² and 144 J/cm² respectively. (A and B) The migration of hepatocarcinoma cells assessed by Wound healing assay (A), (Bar: 200 μm) The ratio of wound healing in migrated cells (B). (C and D) The representative images from hepatoma cells stained with crystal violet (C), and the invasiveness reflected by the absorbance value at 570 nm (D). Data are expressed as the mean ± SEM. ***P < 0.001.
Figure 5

Blue LED lights reduces the potential of stem cells in liver cancer. Tumor sphere formation of hepatoma cells after blue LED lights irradiation for 0 J/cm² and 144 J/cm² respectively.
Figure 5

Blue LED lights reduces the potential of stem cells in liver cancer. Tumor sphere formation of hepatoma cells after blue LED lights irradiation for 0 J/cm² and 144 J/cm² respectively.
Figure 6

Blue LED exposure increases γ-H2AX expression in liver cancer cells. Hep3B cells and HepG2 cells with blue LED irradiation for 0 J/cm² and 144 J/cm² respectively. (A and B) Representative images from γ-H2AX immunostained liver cancer cells. (Bar: 100 μm) (C and D) The protein levels of γ-H2AX after exposure of blue LED lights. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.
Figure 6

Blue LED exposure increases γ-H2AX expression in liver cancer cells. Hep3B cells and HepG2 cells with blue LED irradiation for 0 J/cm² and 144 J/cm² respectively. (A and B) Representative images from γ-H2AX immunostained liver cancer cells. (Bar: 100 μm) (C and D) The protein levels of γ-H2AX after exposure of blue LED lights. Data are expressed as the mean ± SEM. **P < 0.01; ***P < 0.001.