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Raman Scattering Spectra Characterize the Influence of LED Irradiation on Human Skin Fibroblasts

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Abstract. LED phototherapy has been widely applied in clinical practice for treating the skin-related diseases such as acne, pimples, burn and etc.. However, the underlying mechanism for the therapeutic effects involved in bioprocess was still unclear. Although quite a few researches proposed various potential hypotheses through strict experiments, the methods they performed with like morphological observation and immunohistochemistry seemed subjective and cumbersome operations. Raman scattering, a technique to parse molecular information by providing fingerprint spectra at the molecular level, could be a better alternative method for its high efficient and usability. Therefore, we tried to study the influences of LED light irradiation on human skin fibroblasts by comparing the Raman spectra from the cells under irradiation with three lights at different wavelengths: red (λ=625±5 nm), yellow (λ=590±5 nm) and blue (λ=465±5 nm). Results indicated red and yellow light at 625 nm and 590 nm might promote the anabolism including the synthesis of bio-macromolecules such as proteins and DNAs, while blue light at 465 nm might tend to make the macromolecules catabolized into small molecules. Raman scattering could be applied as a high efficient technique for cell researches.

Keywords: LED, phototherapy, Raman scattering, human skin fibroblast

1. Introduction

It has been well documented that the low-level laser therapy (LLLT) affects various biological process both *in vivo* and *in vitro* [1-3]. Effects including cellular proliferation, growth factor release, biosynthesis such as collagens and ATPs genesis could be induced by low-energy laser irradiation significantly at the cellular level[4]. Pulsed ruby laser irradiation was reported to accelerate mouse fibroblasts growing *in vitro* condition with a 5 times increase in contrary to control group at irradiation level of 10 J/cm2 in 694.3 nm wavelength, which inferred the ruby laser may stimulate mitotic activity [5]. Collagen production exceed controls by 30~50 % in a study of ruby laser irradiating on Wister rats with incisions on dorsum, showing the enhancement of collagens synthesis under ruby laser [6]. Another investigation reported that He-Ne laser at wavelength of 632.8 nm could increase ATP level in cells cultivated *in vitro*, suggesting the laser's positive influence on ATP generating [7]. Besides, numerous researches involved with lasers or light influence on bioprocess in other visible wavelength were reported in recent years [8-10].

However, the underlying mechanism of how visible laser or light interact with these bioprocess remains unclear. A variety of relative potential hypotheses were proposed for interpreting the photo-induced mitogenic effects of low intensity laser radiation, including mitochondrial enzymes being heating locally

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[11], flavins and cytochromes taking in light to affecting process of electron transfer in the respiratory chain of mitochondria [12], intracellular calcium increasing by photo-activation [13] and etc..

Anyhow it have drew more attentions that how to benefit from this bio-effect brought by low energy laser in clinical rather than the significance itself. Accordingly, lots of researches focusing on the application of phototherapy began to spring up in medical field. A recent study reported that the 780-nm laser noninvasive phototherapy was effective for patients with long-term peripheral nerve injury and could progressively improve nerve function[14]. For another case, Q-switched Nd:YAG laser with wavelength of 1604 nm was used in treatment of atrophic facial scars of acne vulgaris and achieved a marked therapeutic effect[15].

The LED therapy, a new technique emerging in recent years, is becoming more and more popular in clinical therapy, especially in skin-related diseases treatment, for its mild irradiation, low cost, portable, and easy to use compared with laser. Blue and red light combination LED phototherapy was proved to be an effective, non-painful and safe treatment for mild to moderately severe acne vulgaris[8]. According a randomized double-blinded clinical study in treatment for patients with facial wrinkles, both of 830 and 633 nm LED phototherapy could significantly reduce the wrinkles and increase skin elasticity without no adverse effects, which might be an ideal approach for skin rejuvenation[16]. Such a widespread application trend of LED phototherapy in clinical medical treatment certainly brought about lots of new research subjects, one of the most intrinsic questions was how LED light interacts with skin cells. The study of the potential mechanism under LED phototherapy seemed to be more essential and urgent in that case. Generally, conventional methods in cell research involved in light-induced-altering are morphological observation, immunohistochemistry, real-time RT-PCR, and etc.. However their disadvantages of low efficient and cumbersome operation are obviously and hence a kind of new technique with high sensitivity and easy operability is expected.

Raman scattering, a kind of inelastic light scattering process, was developed to be a high efficient technology for substance detection and analysis in last several decades since its being proposed first time by Raman C.V. in 1928[17]. Raman spectra resolved information about molecular vibration, rotation, crystals structure and phase transition from various substances by which the overall composition and relative quantification of the molecular constituents could be detected and analyzed at the molecular level.

In consideration of the advantages mentioned above, Raman scattering technology was applied to characterize the chemical altering of human skin fibroblast under irradiation of LED light, in order to investigate the influences brought by red, yellow and blue three colors light in perspective of Raman spectra. This paper explored to characterize the chemical changes in cell and LED irradiation related researches using Raman scattering.

2. Materials & Methods

2.1. Cell culture and preparation

The human skin fibroblasts (HSF) were gifted from Shanghai iCell Bioscience Inc. All cells were kept in flasks with conventional sterile cell-culture medium (84% DMEM, 15% fetal bovine serum, 1% double-antibiotic solution of 100 U/ml penicillin and 100 μg/ml streptomycin) under condition of 37 ℃ and 5% CO2. After a number of population doublings, when overspread the flask bottom to the plating density of about 80%, the cells were digested with 0.25% trypsin and fall from the inner surface of flask bottom. The dissociated cells were then transferred into 12-well cell-culture plate and kept in the same condition for 4 days, in order to prepare for the next LED irradiation assay.

2.2. LED device design and irradiation treatment

The LED device was designed with three wavelength LEDs arrays, including red, blue and yellow groups, installed on the same plate with partial overlapping. Each array consisted of 21 LEDs (3×7 array), of which every LED provided about 1 watt light power in total to all directions. According to the measurement of light intensity with an optical power meter, cells were exposed to light irradiation of approximately 3 J/cm^2. LED device was placed over the cell-culture plates with a distance of 15 cm above so that the cells

could be irradiated with a corresponding wavelength in a vertical direction. The red, blue and yellow group cells were irradiated under red, blue and yellow color LED light for 1 hour respectively while the control group didn't receive any irradiation (Table 1), and all the assays proceeded in dark environment without any other light irradiation.

2.3. SERS measurement

Cell culture fluids in all plates were discarded and the cells attaching on the bottom surface were rinsed with sterile phosphate buffered saline (PBS) solution for 3 times to remove the cell-culture fluid. All operations above were performed in sterile ultraclean workbench. A Raman microscope (LabRAM HR Evolution, Horiba, JP) equipped with a 633 nm He-Ne laser device was employed to excite and collect the

Raman spectra, in which the initial parameters were configured as follows: resolution of 1 cm^{-1}, acquisition time of 10 s and scan range from 400 to 1800 cm^{-1}. Every time before measuring, the system was calibrated by 520 cm^{-1} band from silicon reference sample. The laser was focused on the surface of the cells treated by silver colloid via a L50× microscope objective (N.A. 0.5). Each well was measured for 3 parallel spectra from different spots to reduce the random error.

2.4. Data processing and analysis

All raw Raman spectra acquisitions were performed with LabSpec software (Horiba, Japan). The baselines of raw data were corrected by Subase V2.10 software (self-developed, HyStudio, Shanghai University, China) with integration of a Vancouver Raman Algorithm based on fifth-order polynomial fitting method[18] to remove the fluorescence backgrounds. The mean spectral curves plotting and standard errors calculating of each group were performed by Origin 8.0 software (OriginLab, USA).

Table 1. Distribution of groups according to the irradiation condition and protocol

Group	Irradiation Condition	Irradiation Protocol
CTR	Control	No irradiation
RED	Red light	λ625±5 nm, 1h
YELLOW	Yellow light	λ590±5 nm, 1h
BLUE	Blue light	λ465±5 nm, 1h

3. Results & Discussion

Raw data of Raman spectra from CTR group (no irradiation), RED group (λ=625±5 nm irradiation for 1 h), YELLOW group (λ=590±5 nm irradiation for 1 h) and BLUE group (λ=465±5 nm irradiation for 1 h) were acquired under the same condition and their signal baselines were corrected to remove the potential fluorescence backgrounds. In order to lessen the random errors, all parallel and repeated measurement data were averaged to obtain the mean spectra. The distinct and tangible heaves in spectral curves (higher than 200 a.u. and narrower than 100 cm^{-1}) were found out and extracted to be characteristic peaks so that all peaks could be grouped together as a specific distribution pattern to abstractly represent the spectrum for the sample. Since the Raman peaks come from the corresponding chemical components in cells (Table 2), the changes of characteristic peaks, increase or decrease, may reveal their quantitative alteration. Despite no obvious changes were observed in the cells morphology (Fig. 1), the Raman spectra and peaks distribution patterns showed some differences (Fig. 2 A and B). Accordingly, in this way, the influence of LED light irradiation on human skin fibroblasts could be parsed by comparing the peak distribution patterns before and after irradiation for each group.

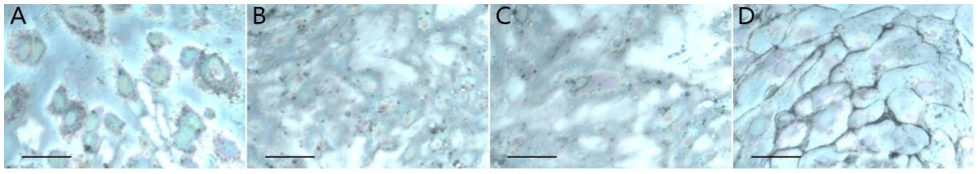

Fig. 1: Microphotography images for human skin fibroblasts (HSF) after LED irradiation and before Raman spectral measurement. (A) Control group HSF without any irradiation. (B) RED group HSF after red light (λ=625±5 nm) irradiation for 1 h. (C) YELLOW group HSF after yellow light (λ=590±5 nm) irradiation for 1 h. (D) BLUE group HSF after blue light (λ=465±5 nm) irradiation for 1 h. Scale bar = 20 μm.

Raman spectra from human skin fibroblasts seemed to be similar in trends and shapes in the experimental groups (Fig. 2 A and B), indicating the contents kept the same no matter which color light irradiation they underwent. Main chemical contents revealed by Raman spectra fell into two categories, the macromolecules which played role in metabolism including proteins (peaks at 621, 1155 and 1602 cm^{-1}), collagens (peak at 1032 cm^{-1}), DNA (peak at 788 cm^{-1}) and lipids (peak at 1449 cm^{-1}) as well as the small molecules which were the components to synthesis the macromolecules including phenylalanine (peak at 1001 cm^{-1}), nucleotides (peaks at 1182 and 1585 cm^{-1}) and phosphates (peak at 1203 cm^{-1}). Therefore, the influences of LED light at different wavelengths on bioprocess in human skin fibroblasts could be inferred by analyzing the changes of characteristic peaks distribution patterns for each group.

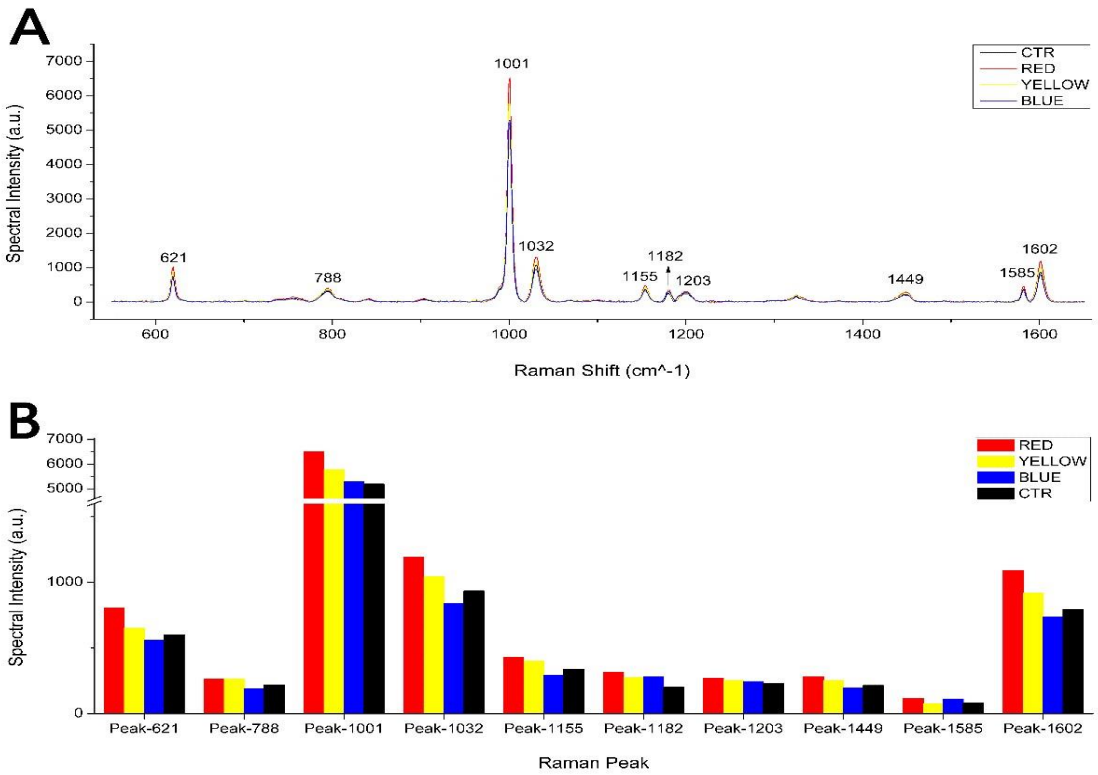

Fig. 2: Raman spectra reveal the changes of human skin fibroblasts induced by different wavelength LED light irradiation. (A) The post-baseline-correction Raman spectra of human skin fibroblasts under red light irradiation (λ=625±5 nm, red solid line), yellow light irradiation (λ=590±5 nm, yellow solid line), blue light irradiation (λ=465±5 nm, blue solid line) and no light irradiation (black solid line). (B) Characteristic peaks distribution patterns abstracted from Raman spectrum of each group.

According to the altering of peaks distribution patterns after LED light irradiation at three different wavelengths, all peaks in RED and YELLOW group are higher than that in control group (Fig. 3 A and B), indicating that both of red and yellow LED light at 625 nm and 590 nm are able to promote the cell anabolism: boost in the proteins, collagens, DNAs and lipids synthesis as well as the phenylalanine and nucleotides absorption. However, in BLUE group, peaks of macromolecules at 621, 788, 1032, 1155, 1449 and 1602 cm^{-1} were lower than that in control group (Fig. 3 A) while peaks at 1001, 1182, 1203 and 1585 cm^{-1} behaved conversely (Fig. 3 B), which suggested that blue LED light at 465 nm may enhance the catabolism in cells so that the macromolecules being broken down into small molecules made the results reversed. In addition, red light at 625 nm performed better than yellow light at 590 nm on anabolism promotion. Together with blue light's inhibitory effects, it seems that long wavelength light tended to effect bioprocess positively and the longer the wavelength is, the better the effect will be, while short wavelength light effect negatively to the contrary.

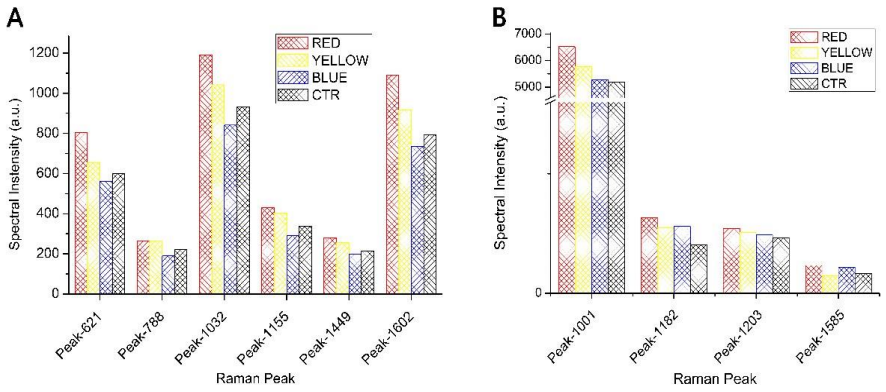

Fig. 3: The influences of LED light at different wavelengths with the bioprocess of human skin fibroblasts (HSF) inferred by the characteristic peaks distribution patterns. (A) Characteristic peaks for macromolecules. (B) Characteristic peaks for small molecules. Red (λ=625±5 nm) and yellow (λ=590±5 nm) light irradiation may promote the anabolism in HSF such as the synthesis of proteins (peak-621, peak-1155 and peak-1602), collagens (peak-1032), DNAs (peak-788) and lipids (peak-1449) as well as the absorption of phenylalanine (peak-1001), nucleotides (peak-1182 and peak-1585) and phosphates (peak-1203), while blue light ($_{\lambda}$=465±5 nm) irradiation facilitates the catabolism of macromolecular degradation, which are embodied in the macromolecules peaks reduction (being disintegrated) and small molecules peaks increase (macromolecules being disintegrated into these components).

Table 2: Tentative Raman spectral peak assignments for HSF in this study

Peaks (cm^{-1})	Major assignments	Macro-/micro- molecules
621	C-C twisting mode of phenylalanine (proteins)[19-21]	Protein
788	C_5' -O-P-O-C_3' phosphodiester bands in DNA[21]	DNA
1001	Symmetric ring breathing mode of phenylalanine[22]	Amino acid
1032	CH_2CH_3 bending modes of collagen &phospholipids[18]	Collagen
1155	C-C (&C-N) stretching of proteins[19, 20]	Protein
1182	Cytosine, guanine, adenine[19]	Nucleotide
1203	Nucleic acids and phosphates[23]	Phosphate
1449	C-H vibration (lipids)[21]	Lipid
1585	C=C olefinic stretch (protein assignment)[24]	Protein
1602	δ(C=C), phenylalanine (protein assignment)[25]	Protein

4. Conclusion

The influences of LED light irradiation with different wavelengths were studied using Raman scattering on human skin fibroblasts: red and yellow light at 625 nm and 590 nm might promote the anabolism including the synthesis of bio-macromolecules such as proteins and DNAs, while blue light at 465 nm might tend to make the macromolecules catabolized into small molecules. Results show the influence of LED light irradiation on human skin fibroblasts could be analyzed by Raman scattering with high efficient.

5. Acknowledgement

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