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ORIGINAL ARTICLE

A preliminary comparison between the effects of red and infrared laser irradiation on viability and proliferation of SHED

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Abstract

The aim of this preliminary study was to compare the effects of different energy densities from red and infrared low-level laser (LLL) on viability and proliferation of stem cells from human exfoliated deciduous teeth (SHED). SHED were irradiated with red laser (R) or infrared laser (IR) set with the following dosimetry: 1.2 J/cm^2 (0.05 J), 2.5 J/cm^2 (0.1 J), 5.0 J/cm^2 (0.2 J), and 7.5 J/cm^2 (0.3 J). Positive (C+) and negative (C−) control groups comprised non-irradiated cells. Data were analyzed by two-way ANOVA followed by Tukey's test ($P < 0.05$). At 24- and 48-h period, group R5.0 showed significantly higher cell viability rates than R1.2 and R2.5. At 48 h, R2.5 also revealed lower proliferation than R5.0. Comparing to the C+ group, R2.5 exhibited lower viability at 72 h, and proliferation at 24 and 48 h. Groups R1.2, IR1.2, and IR5.0 were less viable at 24 h, while R1.2, IR2.5, and IR5.0 revealed lower proliferative capacity at 48 h. Overall, our results showed that LLL can favor viability and proliferation of SHED, especially when cells receive red laser irradiation at 5.0 J/cm^2. Therefore, according to this preliminary investigation, 5 J/cm^2 applied by red LLL induced high rates of cell viability and proliferation, while the same irradiation dose using infrared laser led to negative effects. LLL irradiation with 1.2 and 2.5 J/cm^2 was deleterious to metabolic activity and proliferation of SHED regardless of the type of laser. Further studies are necessary to gain in-depth knowledge about the effects of different wavelengths of LLL on SHED viability and proliferation.

Keywords Stem cells · Cell proliferation · Cell survival · Laser therapy · Low-level light therapy

Introduction

Dentistry has sought to reestablish function and aesthetics of damaged structures from stomatognathic system, through regenerative approaches, since dental materials have a limited life span and often require replacement [1]. Thus, tissue engineering has emerged as an alternative technique aiming to promote repair or to completely replace lost structures, while maintaining vitality. Tissue engineering is based on the principle that undifferentiated cells respond to specific signals that induce proliferation, migration, and differentiation into specialized cell lines [2–8]. Stem cells from human exfoliated deciduous teeth (SHED) are easily and conveniently obtained, and have high proliferative potential [4, 8, 9].

In addition to the scientific progress in the cellular and molecular fields, which allows biological evaluation of different regenerative therapies, technological advances have improved the dental services. Currently, low-level laser (LLL) has become an important tool in the dental armamentarium due to its adjuvant action on the increase of cellular

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metabolism, stimulating epithelialization, vascularization, and collagen synthesis [10, 11]. LLL biostimulation can stimulate or inhibit cellular activities [12], depending on the light parameters as follows: wavelength (λ), power, energy, energy density (fluence), power density (irradiation), time, and laser beam output area [13–16]. Red laser operates at a wavelength between 630 and 680 nm, which is visible to the human eye. Infrared lasers, not visible to the human eye, consist of a wavelength in the range of 750–910 nm [17].

Bringing these scientific advances together, studies seek to find solutions to restore biologically and effectively damaged structures of the stomatognathic system. Since, postnatal stem cells have shown potential for differentiation and neoformation of blood, bone, dental, muscle, and neural tissues [1–9]. Therefore, prior to clinical application of laser therapy to stimulating undifferentiated mesenchymal cells, it is essential to evaluate whether the LLL irradiation parameters cause damage or positively aid the metabolic activity of SHED.

A variety of lasers has been used to regulate unbalanced cellular metabolic activities. A recent systematic review reported that it is necessary to standardize the optimal parameters of LLL, thus improving the effects of this technology [18]. In addition, varied cell lines show different responses after LLL irradiation, generating conflicting results in the literature [19]. For this reason, the ideal combination of irradiation parameters for different needs employing specific cell types must be investigated to establish optimal protocols of biological regeneration of maxillofacial complex structures [20].

Considering the lack of literature on the influence of LLL with different wavelengths on SHED, this preliminary study aimed to compare the effects of different energy densities from red and infrared LLL on viability and proliferation of SHED.

Methods

Cell culture

SHED, isolated through a standard enzymatic digestion protocol and characterized as described by Miura et al. [2], were kindly donated by Prof. Dr. Bruno N Cavalcanti (DDS, MSc, PhD, Institute of Science and Technology, São Paulo State University, São José dos Campos, SP, Brazil) under Institutional Review Board approval (protocol no. 46420). Cells were maintained in MEMα (minimum essential medium Eagle alpha modification, Gibco, Invitrogen, Grand Island, NY, EUA) culture medium, supplemented with 10% fetal bovine serum (FBS) (Fetal Bovine Serum, Certified, Heat-Inactivated, Gibco, Invitrogen) and 1% penicillin and streptomycin (Penicillin-Streptomycin, Gibco, Invitrogen) at 37 °C and 5% CO_2 in a high-humidity incubator, and used between the 10th and 15th passages. The cell culture technique followed the protocol established by Sakai et al. [5].

Groups and irradiation protocol

SHED were seeded in 96-well plates (1×10^4 cells per well) with MEMα supplemented with 10% FBS and allowed to attach overnight. Then, culture medium in all wells was replaced by fresh culture medium supplemented with 1% FBS for 24 h (cellular stress) [16, 21]. Prior to irradiation, all groups received 10% FBS MEMα, except the negative control, in which culture medium was supplemented with 1% FBS.

LLL irradiation protocol determined the groups, according to the wavelength and energy density variation as shown in Table 1. Laser irradiation was performed using a red (660 nm, indium–gallium–aluminum phosphide—InGaAlP) or infrared diode laser (780 nm, gallium–aluminum–arsenide—GaAlAs), 50–60-Hz frequency, and output beam area of 0.03 cm^2 (Twin Flex Evolution, MMOptics® - São Carlos/São Paulo, Brazil), through the bottom of the well (0.03-cm^2 diameter) of plates protected from light [16, 19, 21–24]. Cells were plated in triplicate and the experiment was repeated three times [16, 21, 24]. Cell viability and proliferation assays were performed 24, 48, and 72 h after LLL irradiation [16].

MTT assay

At the end of the experimental periods of incubation, initially, the supernatants were removed and the cells were washed with PBS 1×. Then, SHED maintained with 110 μl of 0.5 mg/ml MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich Co., St. Louis, MO,

Table 1 Groups irradiated with red and infrared laser programmed with different energy densities and control groups

Groups	Wavelength	Energy density	Power	Time	Energy
R1.2	660 nm	1.2 J/cm^2	10 mW	05 s	0.05 J
R2.5	660 nm	2.5 J/cm^2	10 mW	10 s	0.1 J
R5.0	660 nm	5.0 J/cm^2	10 mW	20 s	0.2 J
R7.5	660 nm	7.5 J/cm^2	10 mW	30 s	0.3 J
IR1.2	780 nm	1.2 J/cm^2	10 mW	05 s	0.05 J
IR2.5	780 nm	2.5 J/cm^2	10 mW	10 s	0.1 J
IR5.0	780 nm	5.0 J/cm^2	10 mW	20 s	0.2 J
IR7.5	780 nm	7.5 J/cm^2	10 mW	30 s	0.3 J
C+	Positive control non-irradiated cells 10% FBS MEMα	–	–	–	–
C–	Negative control non-irradiated cells 1% FBS MEMα	–	–	–	–

USA), which blends the cleavage of mitochondrial activity by reducing this salt in formazan (purple) crystals, for 4 h covered without light influence into a high-humidity incubator at 37 °C and 5% CO_2. After, MTT solution was discarded and 200 μl DMSO (dimethyl sulfoxide, Fisher Scientific, Hampton, VA, USA) was added into the wells for 30 min at room temperature to dissolve formazan crystals. Absorbance was read in a microplate reader (Anthos Zenyth 200 RT, Biochrom LTD, Cambridge, UK) at 570 nm [16, 25].

Crystal violet assay

To stain cell deoxyribonucleic acid (DNA), in the determined periods, cells were washed with PBS 1×. Next, methanol solution (methyl alcohol 99.8%, Sigma-Aldrich Co., St. Louis, MO, USA) was added for 10 min. After cell fixation, 200 μl crystal violet (CV) solution was maintained in the wells for 3 min followed by PBS 1× washing twice. Thereafter, sodium citrate (0.05 mol/l) was added to the wells for 10 min to absorbance reading in a microplate reader (Anthos Zenyth 200 RT, Biochrom LTD, Cambridge, UK) at 540 nm [16, 25].

Sulforhodamine B assay

In each experimental period, cells were fixed by 10% trichloroacetic acid solution, and incubated for 1 h at 4 °C. After washing in running water for 5–6 times and outdoor drying, cell protein was stained by 0.4% sulforhodamine B (SRB) solution in 1% acetic acid and incubated at room temperature for 30 min. Then, excess SRB was removed by 1% acetic acid washing and remaining SRB was solubilized in 10 mM Trisbase unbuffered for 1 h at room temperature. The absorbance was determined in a microplate reader (Anthos Zenyth 200 RT, Biochrom LTD, Cambridge, UK) with a 565-nm filter [5].

Statistical analysis

Data were submitted to two-way ANOVA followed by Tukey's test and analyzed through "R Statistical Software." Data were expressed as means and standard deviation (± SD) through graphics. Statistical significance was set at 5% ($p < 0.05$).

Results

The viability and cell proliferation of the groups were compared in relation to irradiation with different energy densities of red laser and infrared laser, as well as between the irradiation with the same energy density in the different types of laser. All the data described below showed statistically significant difference.

At 24 h, groups R1.2, IR1.2, IR5.0, and C− showed lower viability than C+. Groups R1.2 and C− also exhibited lower mitochondrial activity rates than R5.0 and R7.5. In the comparison between red and infrared lasers employing the same energy density, group IR5.0 showed lower viability than R5.0. At 48 h, all groups presented significantly higher absorbance values than C−, except groups R1.2 and IR7.5. In addition, cell viability in R1.2 was significantly lower than that in the other groups irradiated with red laser and C+. Group IR7.5 revealed lower viability than IR2.5 and IR5.0. Comparing the different laser types, cells from groups R1.2 and R2.5 were less viable than the corresponding energy density groups applied with infrared laser. At 72 h, C− showed lower viability than C+ and all irradiated groups, except R2.5. Group R2.5 was also less viable than C+. Groups R1.2 and R2.5 remained less viable than groups IR1.2 and IR2.5 by MTT assay (Fig. 1).

Through CV assay, group C− presented lower viability than C+, IR2.5, R5.0, and R7.5, while R2.5 was less viable than R5.0. Groups IR5.0 and IR7.5 revealed lower absorbance than the corresponding groups irradiated with red laser at 24 h. At 48 h, the control groups and all groups irradiated with red laser were less viable than R5.0. There was no difference in cell viability after irradiation with different energy densities of infrared laser. However, group IR5.0 remained less viable than R5.0. At 72 h, group C− was less viable than all irradiated groups, except R2.5 and IR5.0. There was no significant difference between the same energy densities applied with different types of lasers (Fig. 2).

By SRB assay, groups IR5.0 and C+ presented respectively higher proliferation than C− and R2.5, at 24 h after irradiation. In this period, the different types of lasers did not influence proliferation rate when emitting the same energy densities. At 48 h, C− exhibited lower proliferation than C+, IR7.5, R5.0, and R7.5 groups. Cells from such red laser groups proliferated more rapidly than R2.5. R7.5 group also showed greater proliferation than R1.2. Groups R1.2, R2.5, IR2.5, and IR5.0 had lower proliferation rates than C+. Cell proliferation was lower in R2.5 than IR2.5. At 72 h, group IR1.2 showed lower proliferation than IR7.5. C− revealed reduced proliferative capacity in comparison with the other groups. Group R5.0 exhibited lower proliferation than IR5.0 (Fig. 3).

Discussion

The determination of appropriate energy densities is fundamental to achieve the desirable effect. Laser parameters employed in the present study were based on previous works [26, 27]. Our results showed that LLL can favor viability and proliferation of SHED, especially when cells receive red laser irradiation at 5.0 J/cm^2. In addition to the applied doses, the efficacy of LLL therapy relies on the absorption of light by the target tissue. Lasers with different wavelengths have been used in the field of dentistry in the

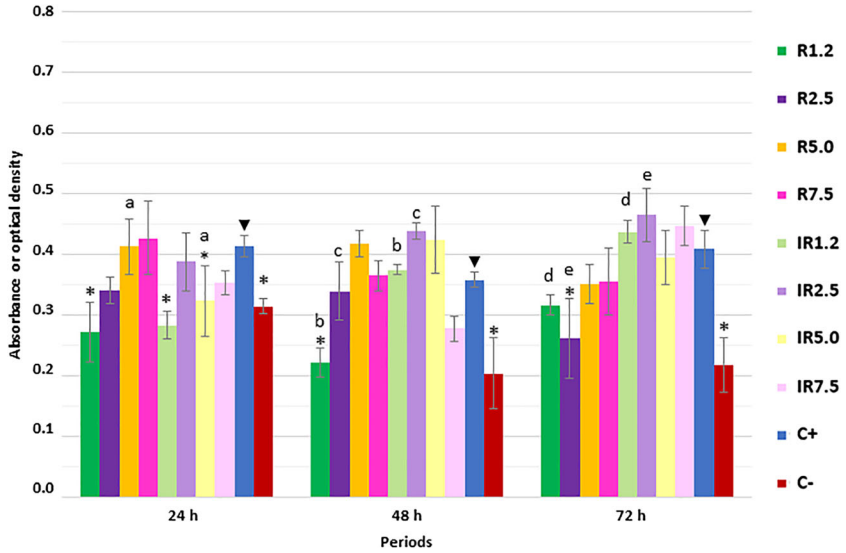

Fig. 1 Cell viability analysis by MTT assay at 24, 48, and 72 h after irradiation with red laser (R1.2–R7.5 J/cm^2) and infrared laser (IR1.2–IR7.5 J/cm^2). Positive (C+) and negative (C−) control groups were non-irradiated cells kept in medium with 1 and 10% FBS, respectively. Same letters (a–e) indicate statistically significant difference between the irradiation with the same energy density in the different types of laser. Different symbols (asterisk and black down-pointing triangle) indicate statistically significant differences relative to C+ and the other groups (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$; the bars refer to standard deviation ± SD)

range of red (λ 630 to 750 nm) or infrared ($\lambda > 750$ nm) [14, 17, 28]. The ideal wavelength depends on the specificity of the photoreceptor molecules [28, 29]. The highest effective penetration in the tissue occurs within the optical window, in which there are no tissue-specific absorption molecules to these wavelengths. It allows the passage and scattering of red and infrared rays to structures such as cytochromes of mitochondria (red laser) or cell membranes (infrared laser), since the main tissue chromophores have a high absorption rate at wavelengths less than 600 nm, and above 900 nm, the absorption of light is greater by water [17].

Red lasers are indicated for superficial modulation of the tissue, and low doses 0.01–10 J/cm^2 tend to be sufficient, whereas, for effects in deeper structures, infrared lasers are usually employed at higher doses in the range of 10–50 J/cm^2 [17]. Considering that in this study the monolayer cell culture technique was used, it is suggested that this point

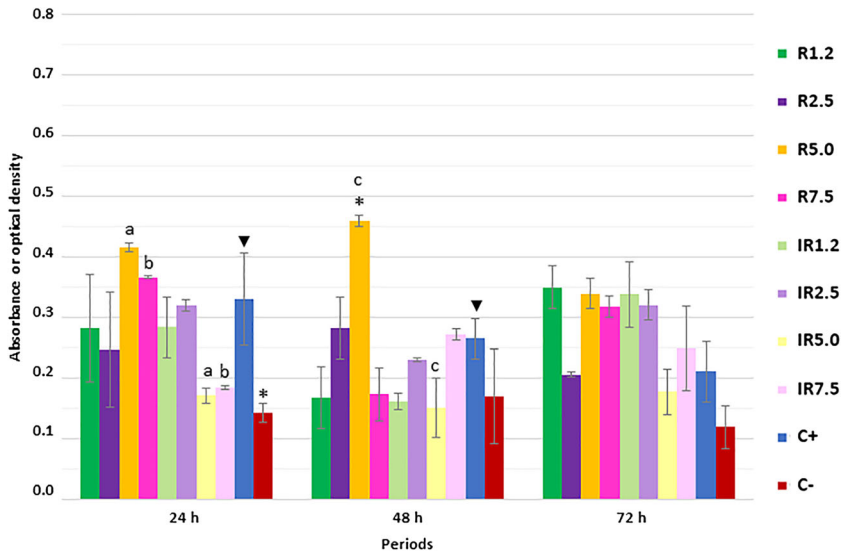

Fig. 2 Cell viability analysis by CV assay at 24, 48, and 72 h after irradiation with red laser (R1.2–R7.5 J/cm^2) and infrared laser (IR1.2–IR7.5 J/cm^2). Positive (C+) and negative (C−) control groups were non-irradiated cells kept in medium with 1 and 10% FBS, respectively. Same letters (a–c) indicate statistically significant difference between the irradiation with the same energy density in the different types of laser. Different symbols (asterisk and black down-pointing triangle) indicate statistically significant differences relative to C+ and the other groups (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$; the bars refer to standard deviation ± SD)

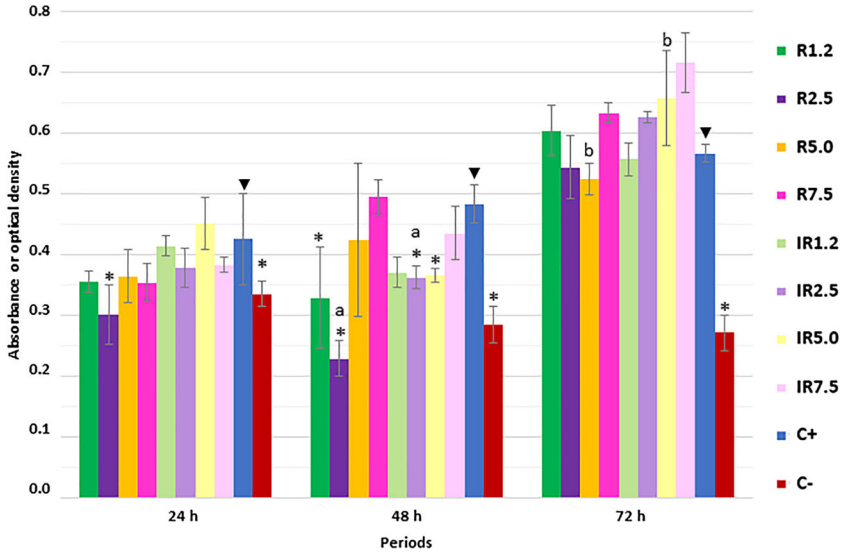

Fig. 3 Cell proliferation analysis by SRB assay at 24, 48, and 72 h after irradiation with red laser (R1.2–R7.5 J/cm^2) and infrared laser (IR1.2–IR7.5 J/cm^2). Positive (C+) and negative (C−) control groups were non-irradiated cells kept in medium with 1 and 10% FBS, respectively. Same letters (a, b) indicate statistically significant difference between the irradiation with the same energy density in the different types of laser. Different symbols (asterisk and black down-pointing triangle) indicates statistically significant differences relative to C+ and the other groups (by two-way ANOVA and Tukey's post hoc test, with $p < 0.05$; the bars refer to standard deviation ± SD)

justifies the greater biostimulatory and inhibitory effects for SHED irradiated with red laser.

Some studies have compared the action of red and infrared lasers on fibroblasts [13, 21] and myoblast cell line [20]. Few studies evaluated the effects of LLL therapy on SHED, most of which evaluated red laser irradiation [23, 26, 27, 30, 31]. Diniz, Matos, and Marques [32] investigated the influence of infrared laser irradiation on viability of SHED cultured with components of dentin adhesives. To the best of our knowledge, the investigation of the red and infrared laser effects on photobiomodulation of SHED remains unexplored.

In this study, SHED irradiated with red laser at energy density of 5 J/cm^2 showed higher viability rates in all evaluated periods. This dose produced more stimulatory effect than the irradiation with 1.2 J/cm^2 at 24 and 48 h by MTT assay. Similarly, Souza et al. [27] revealed lower viability of SHED irradiated with red laser at 1.2 J/cm^2 at 24 h by MTT assay. However, in this period, Fernandes et al. [26] showed that the density of 1.2 J/cm^2 stimulated SHED viability, despite the agreement with the present study, that 5.0 J/cm^2 enhances the metabolic activity of this cell type. Moura-Netto et al. [30] also demonstrated that red laser at 5 J/cm^2 induced high rates of SHED viability at 48 h. By CV assay, this parameter still stimulated significantly the viability of SHED compared to the other groups at 48 h.

At 48 h of the SRB assay, the energy densities of 1.2 and 2.5 J/cm^2 promoted lower cell proliferation than the positive control, regardless of the laser applied. In disagreement with Fernandes et al. [26], which showed greater proliferation in SHED irradiated by red laser programmed with these energy densities in this period. In the present study, SHED irradiated with red laser at 2.5 J/cm^2 still proliferated less than those irradiated with 5 J/cm^2.

The influence of the same energy density applied by different wavelengths revealed varied results, but consistent with the biomodulation, stimulating or inhibiting cellular responses. Thus, 5 J/cm^2 applied with red laser stimulated greater viability than with infrared at 24 h by MTT assay, and at 24 and 48 h by CV assay. SHED irradiated with red laser at 1.2 and 2.5 J/cm^2 were less viable than cells submitted to infrared laser by MTT assay at 24 and 48 h. The energy density of 2.5 J/cm^2 induced less cell proliferation when applied with red laser than infrared at 48 h by SRB assay.

Knowing that irradiation with certain wavelengths differs in their photochemical and photophysical properties, the theory of Smith [28] arose to complement the theory proposed by Karu [29], who proposed that the absorbed red light promotes photochemical changes in the receptors of the mitochondria, accelerating the cellular metabolic activity. Smith [28] stated that infrared light initiates metabolic events through photophysical effects directly into the cell membrane, certainly by the activation of calcium channels. Despite different effects, both lasers aim to biomodulate cellular activities. Therefore, the biological response after cell irradiation results from photochemical and photophysical changes produced by the absorption of non-electromagnetic radiation. In visible light, molecules absorb the photon, occurring electron migration to a more energetic orbit and release of energy upon return to the ground state. The absorption of the radiation in the infrared spectrum causes rotations and molecular vibrations [28]. In 1991, Smith [28] assumed that at the beginning of the twenty-first century, there would be sufficient information about the therapeutic effects of phototherapy on the cells, and the best wavelength would be determined for specific applications. However, much remains to be investigated.

Due to the wide range of LLL parameters and methodologies employed, the reproducibility of laboratory studies to confirm the published responses is fundamental to strengthen the scientific evidence on cellular photobiomodulation [33]. Each specific type of cell shows particular behavior concerning its metabolic response after cell stimulation [16]. The main limitation of this preliminary study was the lack of initial response parameters for comparison between the effects of different wavelengths of LLL on SHED; thus, many questions still need to be elucidated. Therefore, thorough studies about cell cycle and the influence of LLL mechanism of action on signaling pathways of photoreceptors and activation of ion channels must be performed using controlled methodology to fully clarify the effects of certain LLL parameters on SHED viability and proliferation.

Conclusions

According to the results of this preliminary investigation, 5 J/cm^2 applied by red LLL induced high rates of cell viability and proliferation, while the same irradiation dose using infrared laser led to negative effects. LLL irradiation with 1.2 and 2.5 J/cm^2 was deleterious to metabolic activity and proliferation of SHED regardless of the type of laser, and the reduction of viability and cell proliferation rates was higher after the application of these doses with red laser. Further studies are necessary to gain in-depth knowledge about the effects of different wavelengths of LLL on SHED viability and proliferation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the Institutional Review Board regarding ethical aspects (protocol no. 46420), and the procedures were performed according to the Helsinki Declaration.

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