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Research article

Increased fibroblast proliferation and activity after applying intense pulsed light 800–1200 nm

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ABSTRACT

Background and objectives: Light devices emitting near infrared have been shown to be highly effective for the skin rejuvenation but biochemical and molecular mechanism or optimum dose treatment are not well-known. In our study we try to elucidate why systems emitting near infrared produce skin improvement such as fibroblasts proliferation, increase in gene expression or extracellular matrix (ECM) protein production.

Study design/materials and methods: 1BR3G human skin fibroblasts were used to test the effects of an intense pulsed light device emitting with an 800–1200 nm filter (MiniSilk FT manufactured by Deka®). In our protocol, fibroblasts were irradiated twice successively with a 10 Hz frequency, with a total fluence up to 60 J/cm^2 for 15 s each pass. After incubating for 48 h, fibroblasts were harvested from the culture plates to test cell proliferation by flow cytometer. To determine changes in gene expression (mRNA levels for collagen types I and III and metalloproteinase 1 (MMP-1)) and protein production (hyaluronic acid, versican and decorin) tests were performed after irradiation.

Results: After 48 h irradiation, 1BR3G human skin fibroblasts were observed to proliferate at a fast rate. The study of ECM macromolecules production using ELISA showed an increase of hyaluronic acid and versican production but no changes were observed for decorin. With RT-PCR assays, an increase in mRNA for collagen type I, type III and MMP-1 were observed.

Conclusion: Intense pulsed light emitting near infrared applied in vitro cultured cells increases fibroblasts proliferation and activity, which can be a possible mechanism of action for these devices in aging skin treatment.

1. Background and objectives

The aging of human skin includes intrinsic aging and photoaging, characterized by a thinning epidermis. Collagen fibers appear thickened and fragmented with higher ratio of collagen III to collagen I (Berneburg et al., 2000). Fibers are more loose and straight (Gniadecka et al., 1998) and are decreased. Elastic fibers become structurally and functionally abnormal, with deposit of abnormal elastic fibers, lower degradation and gradual accumulation of solar elastotic material in the upper dermis (Robert et al., 1988). Changes in polysaccharide and proteoglycans of extracellular matrix are reported with abnormal localization and structure (Carrino et al., 2000). Because of the changes in dermal ground substance efficient dermal hydration cannot be maintained (Bernstein and Uitto, 1996). These changes manifest as dry and fragile skin.

Intense pulsed light (IPL) sources are multiwavelength, noncoherent light that typically emit light in the 500–1200 nm range. In order to achieve target selectivity, various cut-off filters are employed, effectively removing lower wavelengths. Pulse duration is adjustable based on the system used, with double or triple pulses for longer exposure times or higher fluences. The therapeutic technology of noninvasive skin rejuvenation of IPL is called photorejuvenation and the technique has been used widely in cosmetic dermatology to improve facial photoaging (Babilas and Szeimies, 2010). The efficacy of intense pulsed light in remodeling the extracellular matrix of aged skin had been proven by an increasing number of clinical trials. Some authors argue that, although the exact mechanism of photorejuvenation following IPL treatments is not completely clear, dermal heating likely results from some absorption by water, as well as propagation of heat from the superficial vasculature (Goldberg, 2008). Stimulation of fibroblast and subsequent neocollagenesis and dermal remodeling, increased epidermal thickness, decreased horny plugs, formation

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of new rete ridges and decreased elastosis have been noted histologically after 6 months following treatment and contribute to clinical improvement (Goldberg, 2000; Hernandez-Perez and Ibiett, 2002). However, its molecular biological mechanism and signaling pathway for treatment is rarely reported.

As IPL devices emit in a wide range, for treating patients with aging skin and non pigmented neither vascular lesions, high cut-off filters are used to obtain wavelengths higher than 800 nm (near infrared). With this wavelength is possible collagen denaturation by heating. Thermal denaturation take place at 63–64 °C, and can occur over a range of temperatures and pulse durations with observable changes in staining and structural collagen fibers (Goldberg, 2008). There are clinical reports of improvement and some histological studies (Clementoni et al., 2011; El-Domyati et al., 2011; Omi, 2012; Kim et al., 2012) but to date, no data are clear on the effects of IPL near infrared on human skin cells and the associated mechanism.

In our study we try to elucidate why systems emitting light near infrared (800–1200 nm) produces skin improvement.

The aim of our study is to determine irradiation effects on:

- Proliferation of fibroblast.
- Gene expression of collagen I, III and metalloproteinase 1 in fibroblasts.
- Synthesis of polysaccharides (PS) and proteoglycans (PG) of extracellular matrix by fibroblast: hyaluronic acid (HA), decorin and versican.

2. Material and methods

2.1. Cell culture

1BR3G human skin fibroblasts were derived from a normal fibroblast 1BR3 (ECACC catalog no. 90011801) transformed with the plasmid pSV3gpt. 1BR3G (ECACC catalog no. 90020507) were kindly provided by Dr. Josep Baullida.

Cells were grown in Modified Eagle's Medium (EMEM) with Earle's Balanced Salt Solution supplemented with 1% Non Essential Amino Acids (NEAA) (Sigma Aldrich, St Louis, MO, USA), 2 mM glutamine, 56 IU/ml penicillin, 56 mg/l streptomycin and 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD). Cells were maintained in 100-mm tissue culture flask in a humidified chamber at 5% CO_2 and 37 °C, and subcultured every 2 to 3 days by trypsin-EDTA (BioWhittaker, Walkersville, MD). For all subsequent experiments, 1BR3G cells were seeded in EMEM medium without phenol red (Lonza, Basel, Switzerland).

2.2. Irradiation

1BR3G human fibroblasts were seeded onto 60-mm culture plates in 4 mL of fresh culture medium without phenol red. After incubation for 1 day at 37 °C in 5% CO_2, the monolayer of subconfluent cells was irradiated with MiniSilk FT manufactured by Deka®: IPL SA mode Filter 800–1200 nm (frequency 10 Hz, emission time 15 s, fluence 60.1 J/cm) twice. Handpiece was moved in a slow motion creating an area about 5 cm × 5 cm which covered the culture plate, performing continuous, linear and flowing movements over the culture without holding the handpiece in one point at 2 cm distance.

2.3. Cell cycle analysis

For cell cycle analysis cells were harvested 48 h after irradiation. After 2× wash with PBS, cells were fixed in 70% ethanol for at least one hour. Fixed cells were treated with RNase for 20 min before addition of 5 μg/ml PI and analyzed by FACScalibur Flow Cytometer (BD Biosciences) using CellQuest software.

2.4. ELISA

The sensitive ELISA method was used to measure the fibroblast GAG protein expression. Confluent cultures of 1BR3G human were harvested with 0.25% trypsin supplemented with 0.02% EDTA. Supernatants were collected after centrifugation at 15,000 rpm for 15 min at 4 °C by refrigerated centrifuge (Eppendorf AG, Germany). Cell lysates were prepared using the freeze/thaw procedure.

Human Hyaluronic Acid (HA) ELISA Kit (Cusabio Biotech Co., Ltd, China), with a detection limit of 0.156 ng/ml, was used to detect HA. Human Versican ELISA Kit (Cusabio Biotech Co., Ltd, China), with a minimum detection limit of 1.56 ng/ml, was used to detect versican. Human Decorin ELISA Kit (Cusabio Biotech. Co., Ltd, China), with a minimum detection limit of 1.56 ng/ml, was used to detect decorin. Briefly, samples were diluted with sample diluent and incubated in microtiter wells coated with antibodies of proteins. After incubation and washing, the biotinylated tracer antibody conjugated with streptavidin-peroxidase was added to the wells. Substrate tetramethylbenzidine (TMB) was added to the wells after a second incubation and washing, and then the oxalic acid was added to stop the enzyme reaction. Absorbance was read on X-Fluor microplate spectrophotometer (Tecan Systems, Inc., USA) at a wavelength of 450 nm.

2.5. Quantification of mRNAs using real-time quantitative RT-PCR

Total RNA was isolated from cells (1×10^6) 48 h after irradiation with the RNeasy kit (Qiagen, Milan, Italy) as described by the manufacturer. First-strand cDNAs were synthesized from 2 μg total RNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), following the recommendations of the manufacturer. As a control for genomic contamination, the same reactions were performed in the absence of reverse transcriptase. All real-time PCR reactions were performed using the ABI Prism 7000 SDS (Applied Biosystems) and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems). FAM labeled primers were distributed by Applied Biosystems and are as follows: hyaluronic acid (HYAL2; Hs01117343_g1), versican (VCAM; Hs00171642_m1), decorin (DCN; Hs00754870_s1), type I collagen (COL1A1; Hs00164004_m1), type III collagen (COL3A1; Hs00943809_m1) and metalloproteinase 1 (MMP1; Hs00899658_m1). Real-time PCR conditions were selected according to the universal conditions recommended by the manufacturer of the instrument. The experiments were carried out in duplicate for each data point. Basic analysis was performed using the SDS 1.9.1 software (Applied Biosystems). In addition, the expression of RNA 18S was used as housekeeping gene (calibrator) to standardize the relative expression of each experimental gene.

2.6. Picro-Sirius red staining

Picro-Sirius red (PSR) was purchased from Abcam (Cambridge, UK). In brief, cells cultured on glass coverslips were fixed in 4% paraformaldehyde at room temperature, carefully washed twice with PBS. Thereafter the nuclei were stained with hematoxylin, followed by a series of PBS washings. Subsequently, the slides were incubated in the staining solution PSR (0.1%) at room temperature for 1 h. The staining solution was removed, and the cells were washed three times with 0.1% acetic acid. For photography, cells on chamber slides were dehydrated and clarified by three changes of 100% ethanol, 5 min each, followed by xylene, three changes, 10 min each, and coverslips were mounted with Permount (Electron Microscopy Sciences, Hatfield, PA, USA). The slides were