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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.

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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

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examined by microscopy using an Axioskop 2 microscope using an oil immersion ×100 objective.

2.7. Statistical analysis

Statistical analyses were performed with the SPSS 15.0 software. The results were expressed as mean ± SEM. Differences between the different groups were evaluated with Student's *t*-test and a *p* value <0.05 was designated as statistically significant.

3. Results

3.1. Irradiation with IPL 800–1200 nm increases fibroblast proliferation

After 48 h irradiation, 1BR3G human skin fibroblasts were observed to proliferate at a fast rate, showing a significant increase of cells in S and G2/M cell cycle phases (Fig. 1). The most significant results were obtained with 2 passes as described in Section 2.

3.2. Irradiation with IPL SA Filter 800–1200 nm increases HA and versican secretion in fibroblast cell line

After culturing for 48 h after irradiation, the levels of HA and versican were firstly determined by Real time PCR. The results show that SA irradiation significantly increased the expression of HA mRNA by 4–5 folds (Fig. 2A). A significant but lower increase was observed for Versican mRNA (Fig. 3A).

Moreover, to study the effect of SA irradiation on the levels of these two matrix protein, the cell lysates and the supernatants were assessed by ELISA. A significant increase of HA in lysed cells and supernatant can be observed (Fig. 2B). The increase is higher in cell lysates. Related to versican protein, a significant increase in lysed cells and supernatant (Fig. 3B) can also be observed. As we observed for HA, versican increase is higher in lysed cells than in supernatant.

3.3. Irradiation with IPL with 800–1200 nm filter does not seem to increase decorin secretion in fibroblasts

Decorin mRNA expression was quantified 48 h after SA irradiation, and a slight but not significant increase was observed (Fig. 4A).

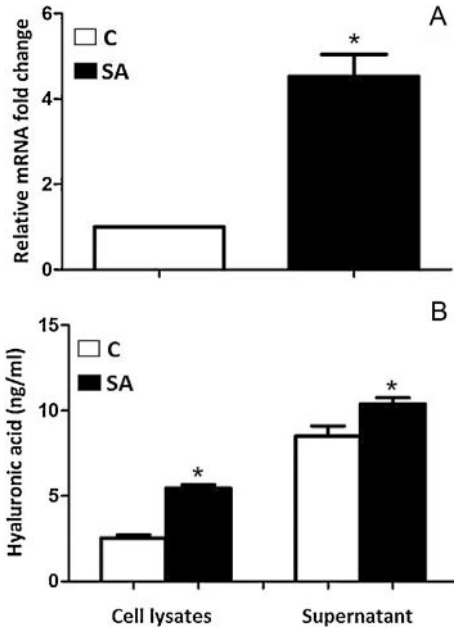

Fig. 2. Effects of IPL SA irradiation on hyaluronic acid in human skin fibroblast. 1BR3G fibroblasts were irradiated by IPL SA as described in Section 2. Cells were harvested 48 h after irradiation and HA mRNA expression was measured by Real-time quantitative RT-PCR (A). Cell lysates and the supernatants were also collected and HA secretion was also measured using ELISA kit (B). Triplicate wells were used and the experiment was repeated three times ($n = 9$). $^{*}p > 0.05$ as compared with the control fibroblast.

Two days after treatment, the levels of decorin protein in cell supernatants and in lysed cells (Fig. 4B) were determined by ELISA. No significant increase in decorin levels can be observed.

3.4. Irradiation with IPL with 800–1200 nm filter increases mRNA for collagen type I, type III and MMP-1

The culture's fibroblasts were treated with IPL radiation at doses described previously and the mRNA expression was measured after culturing irradiated fibroblast for 48 h.

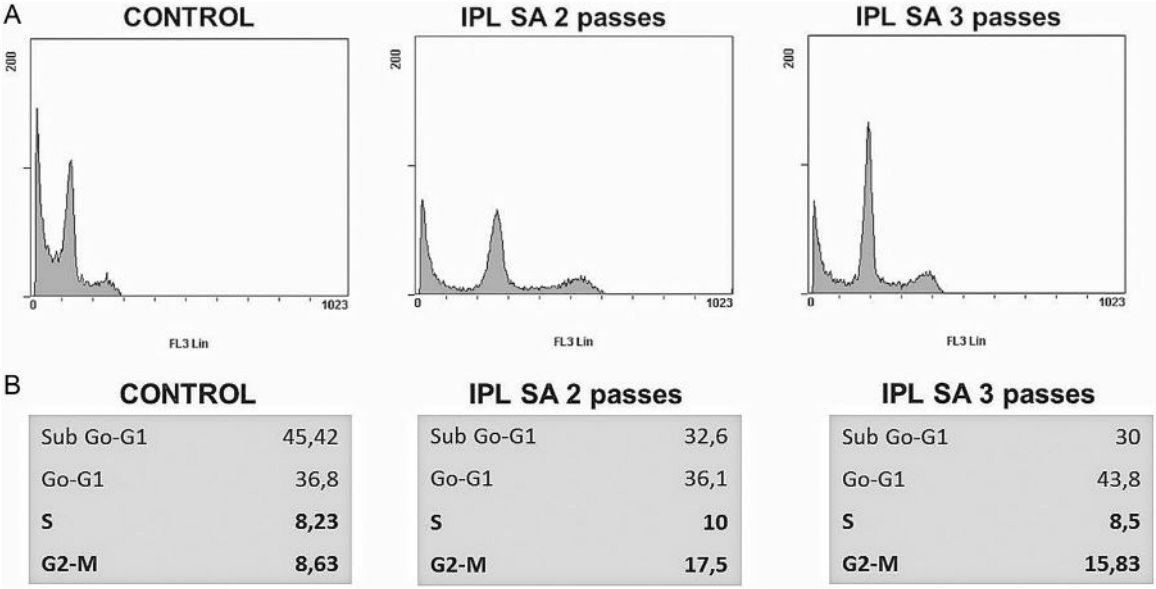

Fig. 1. Effects of IPL SA mode Filter 800–1200 nm irradiation on fibroblasts proliferation. 1BR3G fibroblast cell line was cultured in 10% CFS medium following various passes of IPL SA mode Filter 800–1200 mm irradiation. 48 h after, cells were fixed in 70% ethanol followed by PI stain. (A) Flow cytometry histograms of 1BR3G cells following IPL SA irradiation. (B) Analysis of the subpopulations of cells in cell cycle phases G1, S and G2/M.

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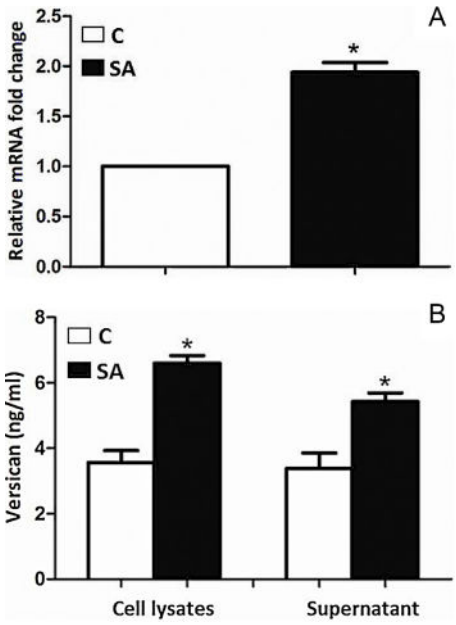

Fig. 3. Effects of IPL SA irradiation on versican in human skin fibroblast. 48 h post-irradiation the expression of versican mRNA was measured by Real-time PCR (A). Intracellular and supernatant versican levels (ng/ml) were also analyzed by ELISA (B). The mean of data corresponding to three independent experiments in triplicate wells. Lines on the top of the bars correspond to SEM. *Statistical evaluations comparing before and after IPL SA irradiation gave $p < 0.05$ by paired T tests.

The mRNA expression levels of procollagen type I (Fig. 5A), procollagen type III (Fig. 5B) and MMP-1 (Fig. 5C) were increased ($p < 0.05$). The most significant changes of mRNA were found for procollagen type III and MMP-1.

To confirm that mRNA increasing of collagen correspond with high intracellular collagen content, we chose Picrosirius red stain method. Picrosirius red is saturated in picric acid solution specifically and consistently stain collagen I and III fibers. Analyses of Picrosirius red stained human fibroblast (Fig. 6) before and after irradiation revealed a significant increase in the amount of fibrosis probably related to intense pulsed light treatment.

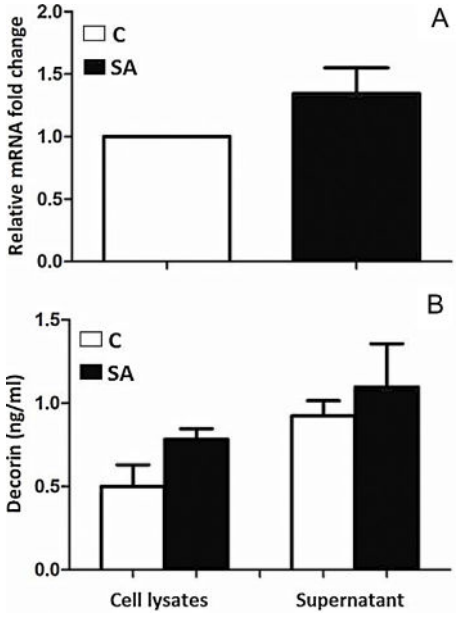

Fig. 4. Effects of IPL SA irradiation on decorin in human skin fibroblast. Two days after IPL SA irradiation. Decorin mRNA level was detected by Real-time PCR (A). Levels of decorin (ng/ml) in 1BR3G fibroblast were assessed by ELISA (B) using both cell lysates and supernatant. Data represent mean ± SEM for three independent experiments performed in triplicate. No significant induce of decorin production was found after IPL SA exposure.

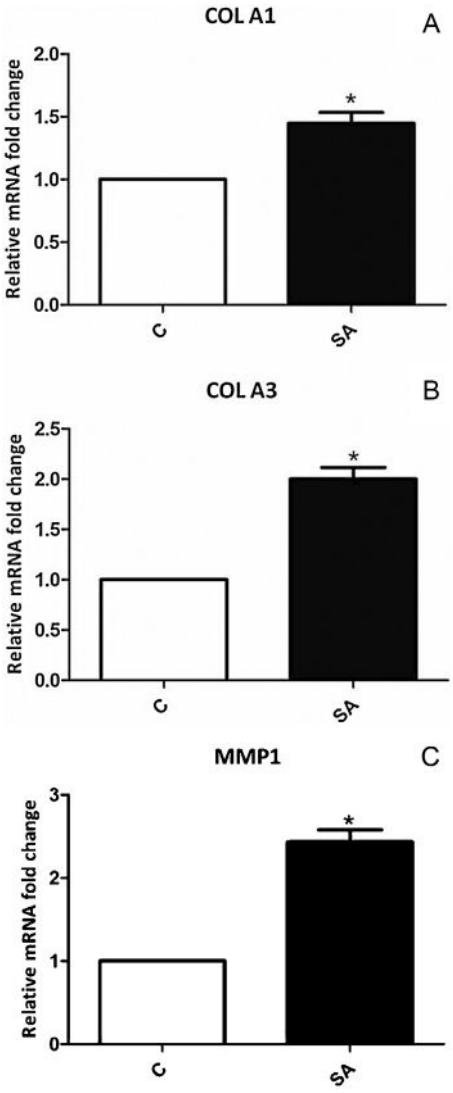

Fig. 5. Effects of IPL SA irradiation on collagen type I (COL A1) (A), collagen type III (COL A3) (B) and MMP-1 (C) expression. Real-time RT-PCR analysis of the mRNA expression was carried out 48 h post-irradiation. The data represent the mean ± SEM from triplicate determinations of at least three independent experiments for each case. *A significant difference compared with the control in all genes were found after IPL SA irradiation ($p < 0.05$).

4. Discussion

Intense pulsed light (IPL) is used for the improvement of the visual appearance of photodamaged skin. IPL is a laser-like device that uses a flash lamp to produce a non-coherent pulsed light with variable pulse durations and intervals. This therapy, also called photorejuvenation, is mainly used in the treatment of certain skin diseases, including photoaging and telangiectasias, among others (Prieto et al., 2005; Scattone et al., 2012). Our research is focused on human skin fibroblasts cell cultures to elucidate how systems emitting light near infrared (800–1200 nm) could produce dermal changes in gene expression and ECM proteins and contribute to photorejuvenation.

The therapeutic effect of IPL is associated with appropriated energy density, although the specific molecular mechanism is still

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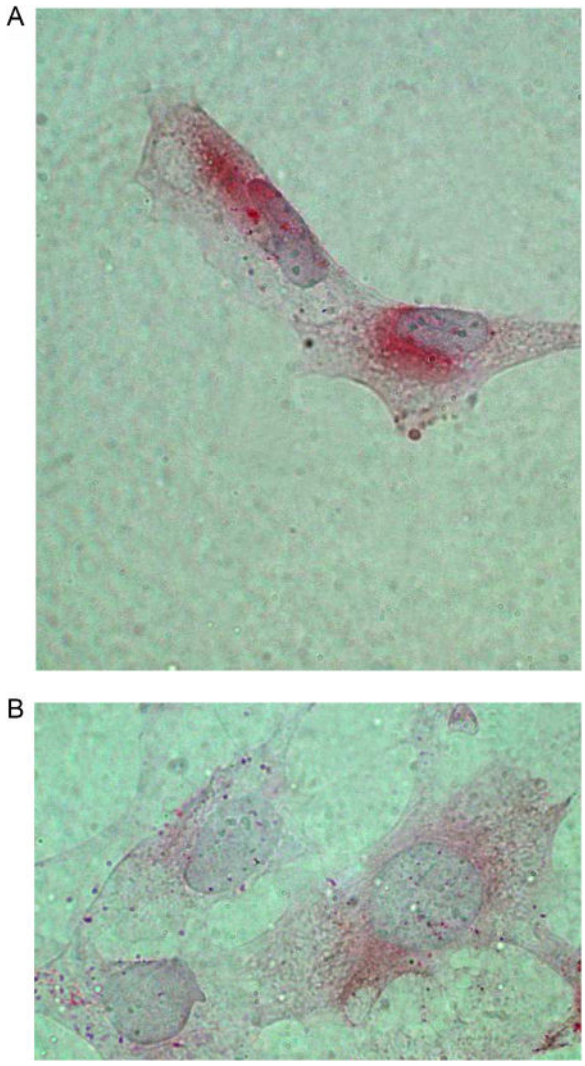

Fig. 6. 1BR3G fibroblasts Picrosirius red staining after SA irradiation. Human fibroblasts were cultured on glass coverslips, and fixed and stained with PSR (0.1%) as described in Section 2. The nuclei were previously stained with hematoxylin. Control (A) and SA irradiated 1BR3G (B) images showed the highest collagen content after fibroblast irradiation.

unknown (Wong et al., 2009; Wang et al., 2011). Our study showed that SA Filter 800–1200 nm using a 60.1 J/cm^2 energy density double-pulsed, induces a significant skin fibroblast proliferation. These results are in agreement with previous infrared laser studies where an increase in the number of fibroblasts is associated with clinical improvement in wrinkle appearance. The precise mechanism responsible for increasing fibroblasts is unknown. Fibroblasts cells exhibit specific splicing program independently of their tissue of origin (Mallinjoud et al., 2014). Regarding cell proliferation, some fibroblast growth factors (FGF) have been described in tumoral processes (Feng et al., 2014) and in hypertrophic and keloid scars (Tiede et al., 2008). The binding of FGF and heparan sulfate proteoglycan (HSPG) to the extracellular ligand domain of FGFR induces receptor dimerization, activation and autophosphorylation of multiple tyrosine residues in the cytoplasmic domain of the receptor molecule. A variety of signaling proteins are phosphorylated in response to FGF stimulation leading to stimulation of intracellular signaling pathways that control cell proliferation, cell differentiation, cell migration, cell survival and cell shape (Eswarakumar et al., 2005). Our study does not focus on mechanism for increasing fibroblasts, so any tests have been performed searching it.

Mechanical properties of the dermis are determined principally by the ECM. Age-related changes in dermal ECM are expected to be involved in age-related changes in the mechanical properties of skin (Carrino et al., 2011).

The dermis is a tissue that contains an extensive ECM. Collagen is the predominant fibrous protein of the ECM, moreover the dermis is mainly composed of types I and III collagens, which are responsible for the skin elasticity and integrity. Talwar et al. (1995) reported that procollagen type I and III levels are decreased in photoaging and/or aged skin. Moreover, controlled thermal skin injury has been shown not only to increase type I and III procollagen mRNA expression levels but also to change the structure and length of collagen (Bernstein et al., 1996). Our study demonstrated that IPL SA Filter 800–1200 nm treatment promote the production of collagen I and III, which may have some association with IPL by direct stimulation and/or photothermolysis which is in accordance with other authors (Chang et al., 2012). In this way infrared laser IPL increases the substantial production and rearrangement of collagens in the dermis and it could make the skin more elastic and rejuvenated.

The remodeling of skin collagen involves not only the proliferation of new collagen fibers but also the degradation of denatured collagens. Metalloproteinases (MMP) are endopeptidases that perform a degradative function, generally targeting the extracellular matrix (Perez-Garcia, 2004). MMP1 is called collagenase and its main substrate are collagen type III, I, II, VII and X (Li et al., 2014). Our study showed that the expression of MMP1 was increased after infrared laser irradiation (Lu et al., 2010). Although it is well established that MMP expression was increased in damaged skin (Ohnishi et al., 2000; Jansen et al., 2007), some authors reported that the IPL management had no impact on MMP secretion levels in fibroblasts (Wu et al., 2012). Recent studies demonstrated that the significant differences in the expression of MMP (down and up-regulation) may be related to the laser parameters such as wavelength and fluence (Dang et al., 2010; Huang et al., 2011). Moreover, these authors speculate that it could be an overlooked mechanism of skin rejuvenation, in which increased MMP1 will be implicated, contributing to the degradation of senescent collagens.

Although collagen is the major ECM molecule of the dermis, other molecular components are present contributing to the overall mechanical properties of skin. Among the non-collagenous components of the dermis there are proteoglycans (PG) and glycosaminoglycan conjugated proteins (GAG), that are important constituents of human skin connective tissue and essential for maintaining mechanical strength of the skin. We carried out the detection of some of these ECM components: hyaluronic acid, versican and decorin using sensitive method such as real-time RT-PCR.

Our results showed a hyaluronic acid and versican increased expression after IPL 800–1200 nm treatment. These observations are in agreement with previous data that demonstrate a significant higher proportion of versican and hyaluronic acid in fetal skin than adult skin (Knudson and Knudson, 1993; Sorrell et al., 1999; Jung et al., 2012). These matrix components are thought to support the active cellular proliferation, migration and differentiation events required for skin growth and development (Knott et al., 2009).

Hyaluronic acid is the most abundant PG in dermis, synthetized in the plasma membrane of dermal fibroblast and secreted to the extracellular space with functions as ground substance to fill space in (ECM). In aged skin HA is decreased in dermis and epidermis (Oh et al., 2011).

Versican is a large chondroitin sulfate proteoglycan distributed with elastic fibers in the human dermis (Bernstein and Uitto, 1996; Hesselstrand et al., 2002) which can be found expressed not only in the dermis but also in the basal layer of the epidermis, hair follicles and sweat glands (Zimmermann and Ruoslahti, 1989; Hasegawa et al., 2007). Versican also binds to HA via its N-terminal region

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and is the mayor HA-binding molecule (Bode-Lesniewska et al., 1996) and can impart viscous properties to cutaneous microfibrils (Hasegawa et al., 2007). Versican has also been found in highly proliferative tissues, such as tumors (Ricciardelli et al., 2009; Carrino et al., 2011) and have differences in the amount and disaccharide composition from tumors and normal dermis, that correlates with the differences between fetal skin versican and adult skin versican (Carrino et al., 2000). Strong versican expression has been seen mostly in dermal and stromal components around the tumors than in the tumor cells in selected tumors developed on sun-exposed areas. This observation was more evident in malignant than in benign tumors. Basal cell carcinomas are positive for versican in 80% (Kunisada et al., 2011).

Decorin is an interstitial PG abundant in the dermal ECM (Merle et al., 1999; Velez-DelValle et al., 2011). It binds to multiple collagen types, mostly type I collagen and other proteins such as transforming growing factor beta (Reed and Iozzo, 2002) and modulates its activity (Yamaguchi et al., 1990). In addition to this, evidence suggests that binding of decorin in the same region as the MMP-1 cleavage site, inhibits collagen cleavage by MMP-1 (Geng et al., 2006; Stuart et al., 2011). It is produced primarily by dermal fibroblast and is localized in dermal extracellular matrix (Li et al., 2013a). Increase of decorin expression has been reported in a variety of fibrotic conditions including systemic sclerosis (Kuroda and Shinkai, 1997).

The role of decorin in aged skin is discussed. Some authors have reported differences in decorin distribution (Ito et al., 2001), increased levels after UVA radiation (Kawashima et al., 2006), decreased levels after solar ultraviolet irradiation (Gambichler et al., 2007; Li et al., 2013a), or decreased levels in aged skin in vivo (Oh et al., 2011). In the present study, no significant changes in decorin expression have been found and this observation is in agreement with previous data that demonstrate human skin decorin minimal age-related differences (Carrino et al., 2011) and changes in decorin molecular size in aged skin which is significantly smaller than in young skin (Li et al., 2013b).

So, clearly new experiments are necessary to elucidate the role of the decorin in dermis.

5. Conclusion

Irradiation with IPL SA Filter 800–1200 nm increases fibroblast proliferation, mRNA procollagen type I, III and MPP-1.

Irradiation with IPL SA Filter 800–1200 nm increases some extracellular matrix proteins such as HA and versican but no significant increasing of decorin has observed after irradiation.

IPL with 800–1200 nm treatment produces changes in vitro which can explain clinical results of aging improvement when is applied for antiaging treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aanat.2014.11.005.

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