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ORIGINAL ARTICLE

Q-switched 1064 nm Nd-Yag nanosecond laser effects on skin barrier function and on molecular rejuvenation markers in keratinocyte-fibroblasts interaction

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Abstract

Skin represents an interface between internal and external environment; it protects human body by regulating the water loss and the maintenance of body temperature, defending against irritant and pathogen agents, and against physical, chemical, and UV damage. It provides to essential physiological functions, such as the important antioxidant defense capacity; its protective/defensive function is performed by a high number of proteins, and shows important functions in maintenance of skin barrier homeostasis. Keratinocytes and fibroblasts play a pivotal role to determine or prevent skin aging in response to intrinsic or extrinsic stimuli, modulating cytokines and several biochemical factors. Non-ablative technologies are playing an increasing role in the management of skin aging, inducing a dermal remodeling without a visible epidermal damage. The objective of this study was to evaluate the effect of Q-switched 1064 Nd-YAG laser (Medlite Conbio C6 Nd-YAG laser, Cynosure USA) in skin barrier function, analyzing the constituents which are strongly altered in aging skin. Particularly, we evaluated the expression of filaggrin, TGase, HSP70, and aquaporins, on HaCaT cells. The expression of proinflammatory cytokines has been investigated too.

As a second step of the study, we analyzed the modulation of the rejuvenation molecular markers on human skin fibroblasts (HDFs) stimulated with keratinocytes conditioned medium (KCM).

Our results demonstrated that Q-switched 1064 nm Nd:YAG laser acts on the skin barrier function, increasing the expression of aquaporins, filaggrin, TGase, and HSP70, modulating the proinflammatory cytokines. In fibroblasts stimulated with keratinocytes conditioned medium (KCM) and irradiated with Q-switched 1064 nm Nd:YAG laser, we can observe a reduction of MMP-1 and an increase in procollagen, collagen type I, and elastin. Our results highlight that Q-switched 1064 nm Nd:YAG laser treatment could represent an effective weapon to fight skin aging.

Keywords Q-switched Nd:YAG laser · Keratinocytes · Photoaging · Collagen

Introduction

Skin aging is the effect of biological clock, influenced by genetic, intrinsic, and extrinsic factors that induce a reduction in the biological activity of cells, a slackening of regenerative processes, and a major sensitivity to environmental factors. Intrinsic factors are time dependent factors, influenced both by genetic background and by a decrease of sex hormone levels, represented by cumulative endogenous damage due to the accumulation of reactive oxygen species (ROS) generated by oxidative cellular metabolism and affecting cellular constituents such as membranes, enzymes, and DNA. Environmental pollution, smoking, alcohol intake, poor nutrition, overeating, ionizing radiation, and particularly exposure to UV radiation are extrinsic factors [1].

The oxidative stress related to the inducing factors reduces production of antioxidants and the function of cytokines and signaling pathways leading to the production of matrix metalloproteinases (MMPs) degrading dermal collagen and elastin [2]. In fact, skin aging is strongly associated with increased levels of matrix metalloproteinases, combined with impaired transforming growth factor (TGF)-β signaling, which may

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reduce old collagen deposition. The metalloproteinases, zinc-dependent endopeptidases, degrade proteins of extracellular matrix (ECM) inducing collagen and elastin fiber degradation, are responsible for skin wrinkles and skin aging. Tissue-specific inhibitors (TIMPs) control the activity of MMPs; the imbalance of MMPs and TIMPs is associated with the breakdown and remodeling of ECM.

Elastin is an essential constituent of various human tissues including the skin, lung, and arteries, providing these elastic tissues stretch and recoil and playing a critical role in supporting and maintaining healthy cells [3, 4]; its degradation is an important effect of the photo-aging, due to poor fibroblast ability to replace damaged fibers.

Several molecules such as cytokines, growth factors, heat shock proteins, antioxidant enzymes, and aquaporins are involved in the tissue damage repair induced by several environmental stressors.

Heat-shock protein 70 (HSP70) is involved in normal wound healing and it is strongly induced by UVB radiation and is responsible for cytoprotection [5]. In chronoaging and photoaging skin, the epidermal immune defense system undergoes significant changes; in fact, Langerhans cells and the level of secreted IL-1 are reduced [6, 7], IL-6, that plays a role in fibroblast proliferation, is decreased [8], and TGF beta1, involved in collagen synthesis, is reduced [9].

Skin is a physical barrier which protects the body from water loss, environmental insults such as pathogens, chemical and physical agents, and from UV radiation. In addition, it provides to essential physiological functions, such as the important antioxidant defense capacity. Its protective/defensive action is performed by a high number of proteins including filaggrin that shows important functions in maintenance of skin barrier homeostasis.

Also transglutaminase (TGase) is highly involved in the increased keratinization and it is used as marker of differentiation; its role is associated mainly with generation of the cross-linked cell envelope and epidermal barrier repair [10].

Aging significantly modifies the epidermal function, in particular, the control of the exchange of substances, especially water and ions, with the external environment. Aquaporins (AQPs) are channels that run along epidermal cell membranes to carry water and small molecules of solute, maintaining water-ion balance of the skin. The expression of AQP3 decreases with the aging of human epidermis, probably related to the development of xerosis, a skin aging marker [11].

In the last years, light and laser sources have been used for skin rejuvenation treatment, in order to stimulate dermal collagen formation, the most abundant protein localized in connective tissue.

Physical treatments such as photodynamic [12], high-energy pulsed CO_2 laser [13], and fractional CO_2 laser therapies are suggested as options for epithelium renewal and keratinocyte proliferation incitement action, to promote new collagen synthesis.

Non-ablative technologies are playing an increasing role in the management of skin aging, introducing a dermal remodeling without a visible epidermal damage. As reported in literature, a number of laser devices and light sources, emitting at various wavelengths, through non ablative mechanisms have been described that effectively improve the appearance of aged skin; Greaves A.S. focused the results of clinical studies about effects of narrow bands of visible light (400–800 nm LED) on photorejuvenation [14]. In a study performed with Q-switched 1064 nmNd:YAG laser, histologic analysis of the laser-treated skin areas showed evidence of dermal remodeling along with epidermal hyperplasia, new collagen formation, an increase in the number of fibroblasts, and angiogenesis [15]. It was demonstrated that Q-switched 532 nm and 1064 nm Nd:YAG laser downregulate the expression of MMP1 and MMP2 enzymes increased in the damaged skin, and upregulate collagen I expression [16]. Goldberg and Schmults, by performing histological studies, proved formation of new collagen and slight fibrosis on the dermis, without damage to the epidermis, after treatment with Q-switched 1064 nm Nd:YAG laser [17, 18]. Recently, Xigun Ye et al. (2012) investigating the effect of Q-Switched 1064 nm Nd:Yag laser on rat skin, showed an increase of procollagen I and III, collagen, and TIMP1 and TIMP2 content and a decrease of MMP2 and MMP3 expression [19]. Gold et al. (2014) using Q-switched 1064 nm Nd:YAG laser in clinical studies on photodamaged skin showed the benefit of the therapy in the treatment of rhymes and photodamage [20].

The aim of this study is to investigate the effect of Q-switched 1064 nm Nd:YAG nanosecond laser on skin barrier function, analyzing the constituents which are strongly altered in aging skin. Particularly, we evaluated the expression of filaggrin, TGase, HSP70, and aquaporins, on HaCaT cells. The expression of proinflammatory cytokines has been investigated too. As a second step of the study, the modulation of the rejuvenation molecular markers on human skin fibroblasts (HDFs) stimulated with keratinocytes conditioned medium (KCM) irradiated for 24 h has been analyzed.

Methods

Cell culture

Human normal epidermal keratinocyte cell line (HaCaT) were cultured in Dulbecco's Minimal Essential Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS,

Table 1 Real-time PCR carried out using sense and antisense primers

Gene	Primers sequence	Conditions	Product size (bp)
AQP3	5′-CTC CAG CAT CCG ACA AGA AGC-3′ 5′-GAG GTC GTA GGC TGT TCT TCG-3′	45″at 94 °C, 45″ at 56 °C, 1″ at 72 °C for 34 cycles	280
TGASE	5′-CCA CGG ATC AGC CAT GAG GGT-3′ 5′-AAC GCC TAG TCG GTA CTC CCA-3	1′ at 94 °C, 1′ at 56 °C, 1″ at 72 °C for 40 cycles	331
HSP70	5′-CTC CAG CAT CCG ACA AGA AGC-3′ 5′-GAG GTC GTA GGC TGT TCT TCG-3′	1′ at 94 °C, 1′ at 63 °C, 1′ at 72 °C for 33 cycles	234
IL-6	5′-TGAACTTCCAAGCTGGCCGTG-3′ 5′-ATGATTCTCAGCCCTCTTCAAAAACTTCTC-3′	1′ at 94 °C, 1′ at 56 °C, 1″ at 72 °C for 33 cycles	297
MMP1	5′-AAC CGG ATC AGC CAT GAG GGT-3′ 5′-CCA GCC TAG TCG GTA CTC CCA-3′	1′ at 94 °C, 1′ at 56 °C, 1″ at 72 °C for 33 cycles	298
Procollagen	5′-CAGAGGGAAGAGTTCCCCAG-3′ 5′-CCTTGGTCTGGTAGGAGACG-3′	1′ at 95 °C, 45″ at 56 °C, 30″ at 72 °C for 33 cycles	370
Collagen	5′-CCA GCC ATC AGC CAT GAG GGT-3′ 5′-AAC CGG TAG TCG GTA CTC CCA-3′	45″ at 95 °C, 45″ at 56 °C, 30″ at 72 °C for 34 cycles	420
Elastin	5′-CCGACTACTACGCCAAGGAATGCAC-3′ 5′-AGGCCGGTTCATGCCATGAGGTGTG-3′	30″ at 95 °C, 45″ at 57 °C, 30″ at 72 °C for 35 cycles	298
IL-1 α	5′-CCA GCC ATC AGC CAT GAG CCA-3′ 5′-AAC CGG TAG TCG GTA CTC GGT-3′	1′ at 94 °C, 1′ at 63 °C, 1′ at 72 °C for 33 cycles	254
TGF-β	5′-CCGACTACTACGCCAAGGAGGTCAC-3′ 5′-AGGCCGGTTCATGCCATGAATGGTG-3′	1′ at 94 °C, 1′ at 60 °C, 2′ at 72 °C for 32 cycles	439
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′	30″ at 95 °C, 1″ at 56 °C, 30″ at 72 °C for 35 cycles	661
TNF-α	5′-CAGAGGGAAGAGTTCCCCAG-3′ 5′- CCTTGGTCTGGTAGGAGACG -3′	30″ at 95 °C, 45″ at 57 °C, 30″ at 72 °C for 35 cycles	324

AQP3 aquaporin 3, *TGASE* transglutaminase, *HSP70* heat shock protein 70, *IL-6* interleukin-6, *MMP1* metalloproteinase 1, *TNFα* tumor necrosis factorα, *IL-1α* interleukin 1α

ATCC n.30-2020), 1% glutamine, and 1% pen-strep (complete medium) at 37 °C in a 5% CO_2-humidified atmosphere.

Human Dermal Fibroblasts (HDF) were grown in Medium 199 and Dulbecco's Minimal Essential Medium (1:4), 0.01 mg/ml hygromicin B (Sigma), fetal bovine serum (FBS, ATCC n.30-2020), at 37 °C in a 5% CO_2-humidified atmosphere. HDF were used between the 5th and 20th passages.

Treatment of HaCaT cells with Q-switched 1064 nm Nd:YAG laser and stimulation of fibroblasts with keratinocyte-conditioned media

5×10^5 HaCaT cells were seeded in multiplates (6-wells, flat bottom) and irradiated with Q-switched 1064 nm Nd:YAG laser (Medlite Conbio C6 Nd-YAG laser, Cynosure USA) following laser parameters (a fluence of 2, 4, 6, and 8 J/cm^2, a pulse width of 5 ns, and a spot size of 6 mm). The optical arm was positioned at a distance of 2.5 cm from the cells and laser irradiation was carried out twice at an interval of 1 s. After stimulation, the samples have been harvested at 6, 12, and 24 h, and total RNA was isolated to determine mRNA level of IL-alpha, TNFalpha, IL-6, aquaporin 3, filaggrin, TGase, and HSP70.

In a second step, confluent HDF in multiplates (6-wells, flat bottom) have been stimulated with keratinocyte-conditioned medium (KCM) taken after 24 h after irradiation. KCM is the medium (DMEM) used to culture HaCaT during the irradiation. After stimulation, samples were taken at 6, 12, and 24 h to determine mRNA levels of MMP1, TGF-Beta, elastin, procollagen, and collagen type I. HaCaT non irradiated and HDF stimulated with KCM of keratinocytes non irradiated have been used as negative controls.

MTT proliferation assay

Cell viability of HaCaT after irradiation with Q-switched 1064 nm Nd:YAG laser and of HDF, treated or not with KCM was measured by the MTT assay (Alpha Kit, Biochrom, Berlin, Germany), a colorimetric assay for cellular growth and survival using 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazoliumbromide. A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells, and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation, or activation. Ten

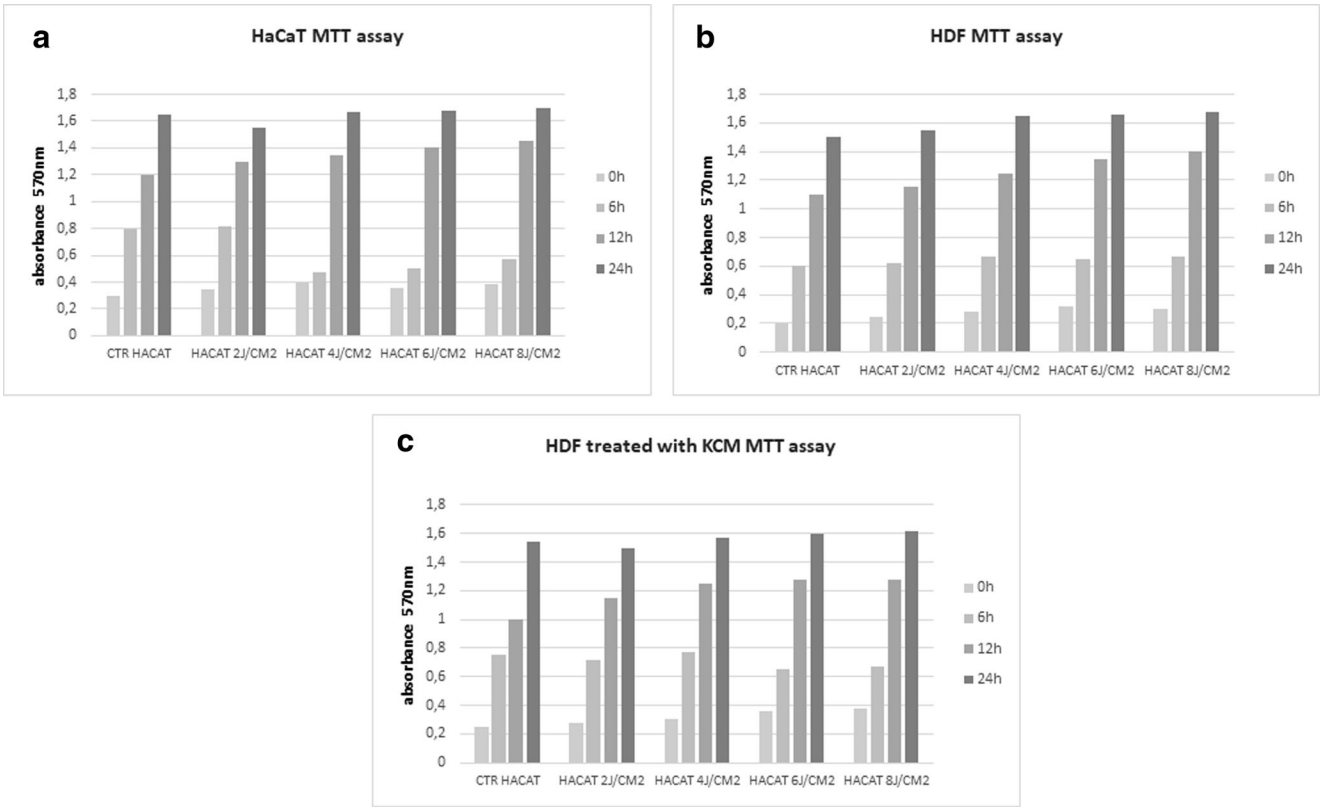

Fig. 1 Cell viability measurement. HaCaT (**a**), HDF (**b**), and HDF, after treatment with keratinocyte-conditioned medium (**c**), viability after irradiation with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2 at 6, 12, and 24 h after irradiation measured by the MTT assay

microliters of MTT labelling reagent (Sigma-Aldrich, Milan, Italy) was added to each well (final concentration 0.5 mg/ml). After 4 h, 100 μl of the solubilization solution was added to each well and plates were incubated overnight. Spectrophotometric absorbance was measured using a microplate (ELISA) reader at wavelength of 570 nm [21]. The viability has been expressed as a percentage of increase in absorbance of samples vs negative control.

Real-time PCR analysis

Total RNA was isolated from HaCaT and HDF cells using the High Pure RNA Isolation Kit (Roche; Milan, Italy). Two hundred nanograms of total cellular RNA were reverse-transcribed (Expand Reverse Transcriptase, Roche; Milan, Italy) into complementary DNA (cDNA) using random hexamer primers (Random hexamers, Roche; Milan, Italy) at 42 °C for 45 min, according to the manufacturer's instructions. Real-time PCR was carried out with the LC Fast Start DNA Master SYBR Green kit (Light Cycler 2.0 Instrument, Roche; Milan, Italy) using 2 ml of cDNA, corresponding to 10 ng of total RNA in a 20-ml final volume, 3 mM MgCl2, and 0.5 mM sense and antisense primers (Table 1). A melting curve was made at the end of each amplification to ensure the absence of non-specific reaction products. The accuracy of mRNA quantification depends on the linearity and efficiency of the PCR amplification. Both parameters were assessed using standard curves generated by increasing amounts of cDNA. Quantification is based on the threshold-cycle values, which are measured in the early stage of the exponential phase of the reaction, and on normalization to the internal standard curve obtained with the housekeeping b-actin gene to avoid discrepancies in input RNA or in the reverse transcription efficiency. The PCR products were examined on 1.4% agarose gel.

Western blot analysis

About 1 × 10^6 HDF were treated or not with KCM by HaCaT treated with Q-switched Nd:YAG laser. The cells were scraped with 1 mL phosphate buffered saline (PBS), and the cell pellet was homogenized with 300 μL ice-cold buffer (50 mM HEPES at pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1.5 mM MgCl2, and 5 mM EGTA) supplemented with 20 mM sodium pyrophosphate, 40 μg/mL aprotinin, 4 mM PMSF, 10 mM sodium orthovanadate, and 25 mM NaF. Total extracts were cleared by centrifugation for 30 min at 4 °C at 10,000 rpm and assayed for the protein content by Bradford's method. Fifty micrograms of protein from each cell lysates were separated by a 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes; the filters were stained with 10% Ponceau S solution for 2 min to verify

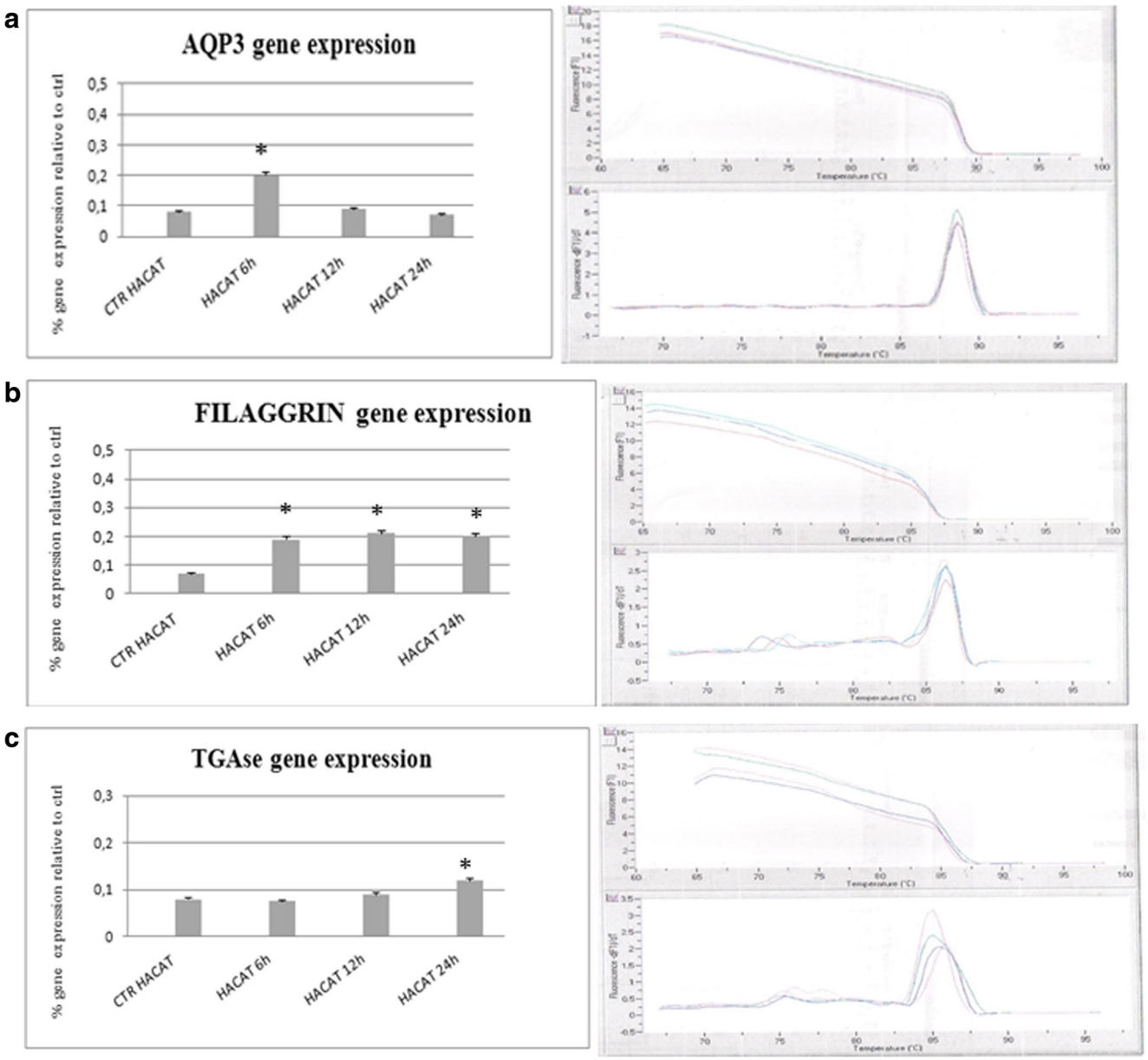

Fig. 2 Real-time PCR analysis. **a** Relative AQP3 gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence 8 J/cm^2 (left) and **b** melting curve performed for all reactions (right). Relative filaggrin gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence 8 J/cm^2 (left) and (**c**) melting curve performed for all reactions (right). Relative TGAse gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence 8 J/cm^2 (left) and melting curve performed for all reactions (right)

equal loading and transfer efficiency. Blots were blocked overnight with 5% nonfat dry milk, then incubated with anti procollagen (sc-8782) (10 μg/mL; Santa Cruz Biotechnology), 1 μg/mL in TBS (150 mM NaCl, 20 mM Tris–HCl at pH 8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filter was incubated with 1:2500 peroxidase conjugated 1:2500 anti-mouse immunoglobulin (Santa Cruz) for 1 h at 22 °C. It was extensively washed and finally analyzed using the enhanced chemiluminescence system (Amersham, Little Chalfont, UK). Protein loading was checked by reprobing the membranes with α-tubulin, in order to show that protein levels were not changed.

Statistical analysis

All experiments were performed three times and the measurements taken in triplicate, unless otherwise indicated. The results are expressed as means ± standard deviations (SD). Outliers were excluded from the analysis where appropriate. The ANOVA test (analysis of variance between groups) was

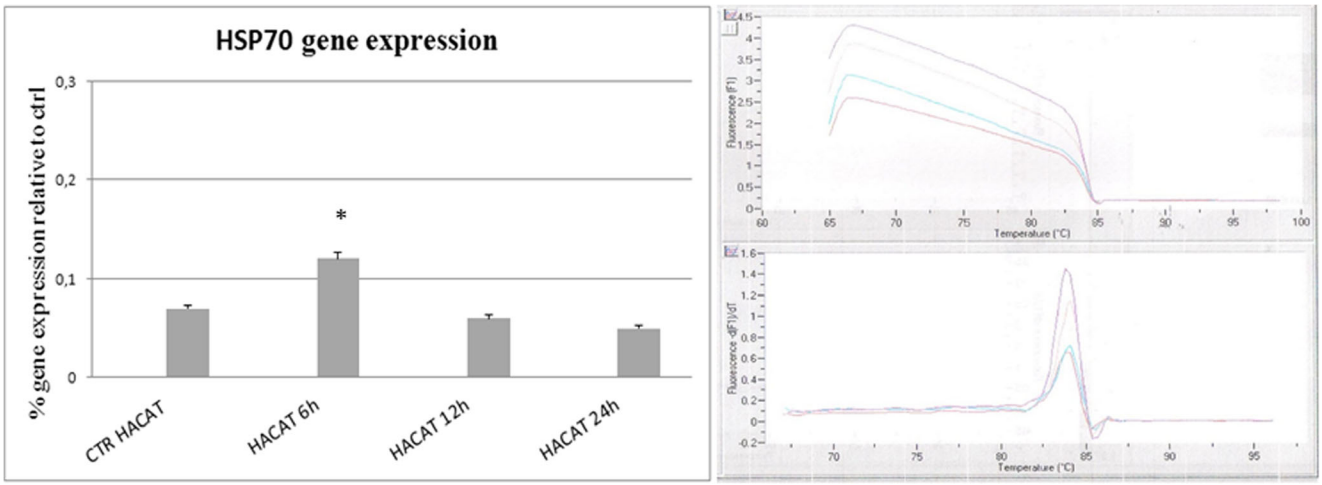

Fig. 3 Real-time PCR analysis. Relative HSP70 gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence 8 J/cm^2 (left) and melting curve performed for all reactions (right)

used to analyze the results obtained. Student's *t* test was used to determine statistical differences between the means, and $p \leq 0.05$ was considered a significant difference.

Results

Cell viability assay

Q-switched Nd:YAG laser used at 1064 nm at 2, 4, 6, and 8 J/cm^2 did not significantly influence HaCaT and HDF morphology and viability as shown in Fig. 1a, b as demonstrated by MTT assay after 6, 12, and 24 h after irradiation. In addition, keratinocyte-conditioned medium did not affect on HDF cell viability (Fig. 1c) at all times tested.

Induction of AQP3, Filaggrin, TGase, and HSP70B gene expression in irradiated HaCaT cells

To determine the potential effect of Q-switched 1064 nm Nd:YAG laser on skin barrier, we first examined gene expression of AQP3 at 6, 12, and 24 h after irradiation. As shown in Fig. 2, it was observed:

- an increase in AQP3 gene expression of 52% after 6 h respect not irradiated cell, while after 12 and 24 h levels of AQP3 are similar to control (Fig. 2a).
- an increase in Filaggrin gene expression of about 50% at all times tested compared to control; precisely 48% at 6 h, 52% at 12 h, and 51% after 24 h post irradiation (Fig. 2b).
- after 24 h, TGase gene expression increased until 38% compared to control, while its value is overlapped to control at 6 and 12 h after irradiation (Fig. 2c).

To analyze the cytoprotective response of irradiated HaCaT cells, we analyzed the heat-shock protein 70B gene expression, at 6, 12, and 24 h after laser irradiation. As shown in Fig. 3, an increased expression of Hsp70B, up to 41%, after 6, 12, and 24 h post irradiation has been observed, compared to control cell.

Pro-inflammatory cytokines expression in irradiated HaCaT cells

Q-switched 1064 nm Nd:YAG laser induced pro-inflammatory cytokines gene expression (IL-6, IL-1α, and TNF-α) in irradiated HaCaT. Particularly, after 24 h of irradiation, have been observed an enhancement of IL-6 gene expression until 56% (Fig. 4a), an increase of IL-1 α of 35% (Fig.4b), and an increase of TNF-α gene expression of 44% (Fig.4c). At 6 and 12 h, the levels of proinflammatory cytokines were overlapped to control.

MMP1, TGF-β, procollagen, collagen type I, and elastin gene expression from human dermal fibroblast (HDF) stimulated with irradiated keratinocyte-conditioned media (KCM).

HDF were stimulated with KCM (taken 24 h after HaCaT irradiation with laser) for 6, 12, and 24 h. HDF stimulated with supernatant of untreated keratinocytes has been used as negative control. HDF, stimulated with KCM after irradiation, showed a significant reduction of MMP1 gene expression after 6, 12, and 24 h of treatment (40%, 36%, and 32% respectively, compared to control) (Fig.5).

In addition, TGF β gene expression showed an increase of 38% compared to that obtained in control cells after 24 h of

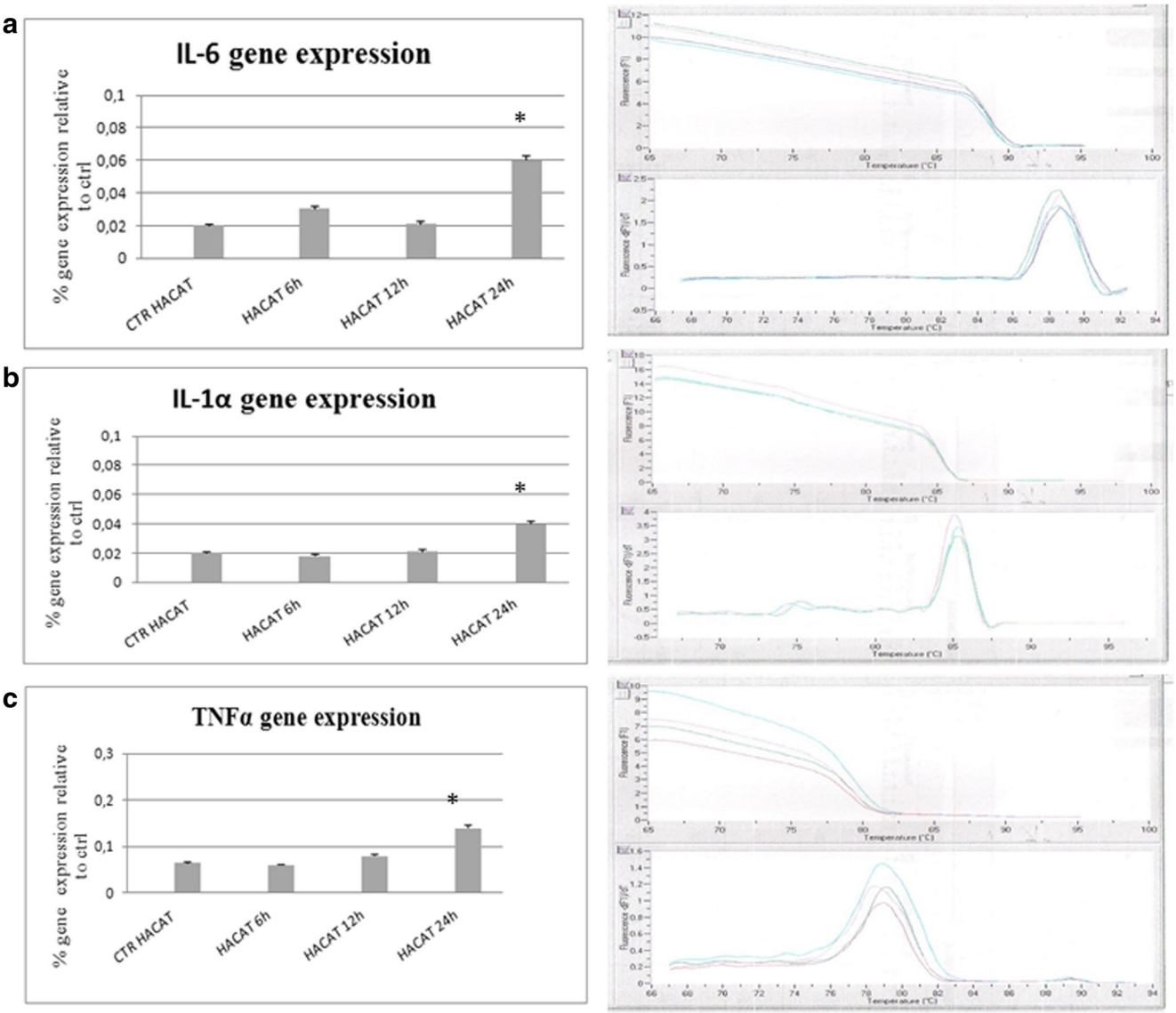

Fig. 4 Real-time PCR analysis using specific primers for cytokines. **a** Relative IL-6 gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and **b** melting curve performed for all reactions (right). Relative IL-1α gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and **c** melting curve performed for all reactions (right). Relative TNF-α gene expression from HaCaT irradiated with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and melting curve performed for all reactions (right)

treatment, while after 6 and 12 h its level appeared overlapped to control (Fig.6).

In same experimental model, the effect of KCM on gene expression of procollagen, collagen type I, and elastin have been analyzed. As showed in Fig.7a, has been observed an enhancement of procollagen until 57% at 12 h, and of 37% after 24 h of incubation with KCM. In addition, an increase of collagen type I gene expression until 46% at 24 h of incubation has been noted (Fig. 7b). Also a marked increase of about 61% of elastin gene expression after 24 h of treatment with KCM has been obtained in treated cells compared to control (Fig. 7c). The results of procollagen were confirmed by Western blot analysis as shown in Fig. 8.

Discussion

Non-ablative Q-switched 1064 nm Nd:YAG laser is characterized by very short pulse width, so, higher energy doses can reach the higher dermis layers without damaging the epidermis [22], so, it can be a good candidate among the dermatological strategy to fight skin aging. Skin aging is a part of a natural human "aging mosaic" in different organs, tissues, and

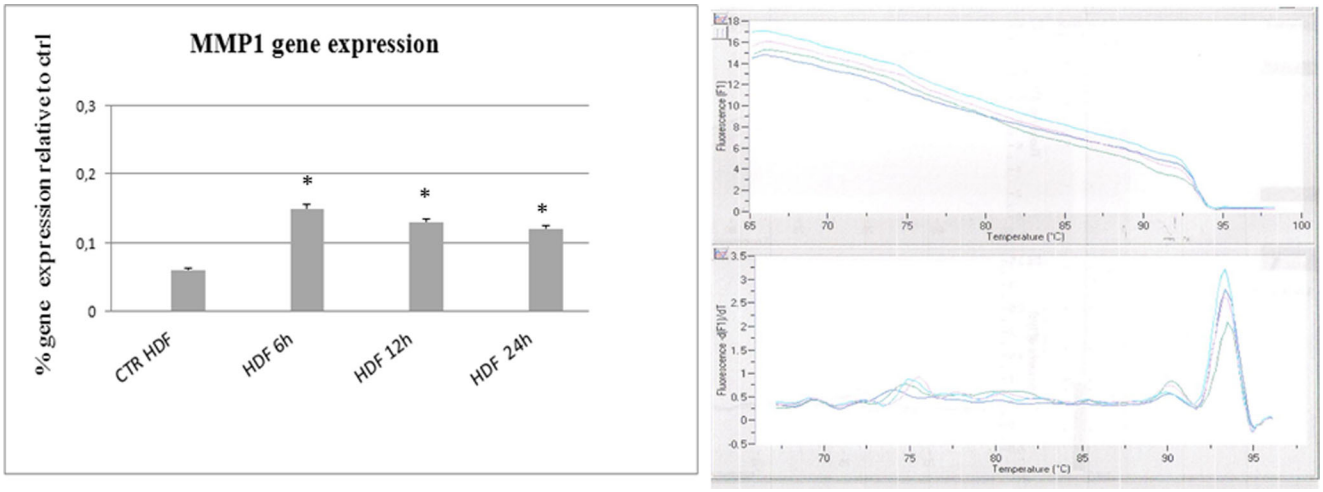

Fig. 5 Real-time PCR analysis. Relative MMP1 gene expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and melting curve performed for all reactions (right)

cells. While the aging signs of internal organs are masked from the ambient "eyes," the skin provides first obvious marks of the passing time.

Skin aging is a complex biological process influenced by combination of endogenous (genetics, cellular metabolism, hormone, and metabolic processes) and exogenous (chronic light exposure, pollution, ionizing radiation, chemicals, toxins) factors. These factors lead together to cumulative structural and physiological alterations and progressive changes in each skin layer as well as changes in skin appearance. Keratinocytes and fibroblast play an important role to determine or prevent skin aging in response to intrinsic or extrinsic stimuli, modulating cytokines, and several factors that are able to maintain the skin homeostasis.

In the first step of this study, we evaluated the laser effects on HaCaT cells through the analysis of pro inflammatory cytokines and several factors such as aquaporins and filaggrin, that play a key role in skin hydration and integrity and are involved in skin appearance, metabolism, mechanical properties, and barrier function; also, the expression of TGase was investigated.

Laser exposure performed at different fluences and for different times showed no alteration of morphology or proliferation of the keratinocytes.

Among the aquagliceroporins, AQP3 is present in the epidermal basal layer [23, 24]. Water transport mediated by AQP3, favors the migration of keratinocytes, while the transport of glycerol favors elasticity, hydration, and restoration of the skin barrier. AQP3-deficient mice exhibit reduced of skin hydration and reduced elasticity [25].

Our study showed, for the first time, an increase (until a maximal of 52%) in the expression of AQP3 in Q-switched 1064 nm Nd:YAG laser-treated keratinocytes compared to untreated cells at the same time of incubation.

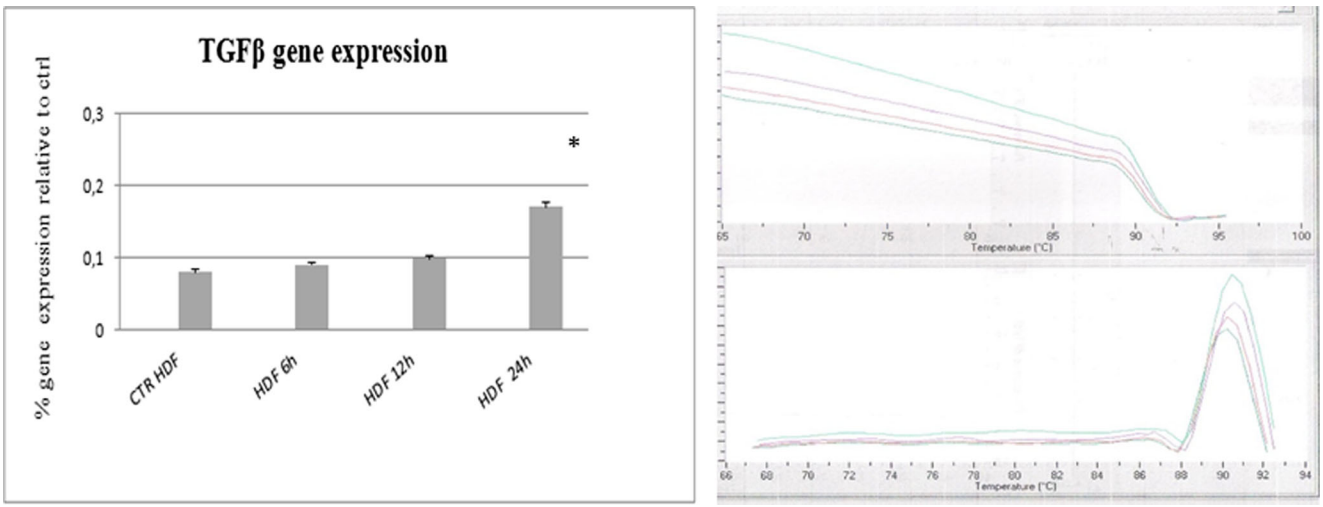

Fig. 6 Real-time PCR analysis. Relative TGFβ gene expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and melting curve performed for all reactions (right)

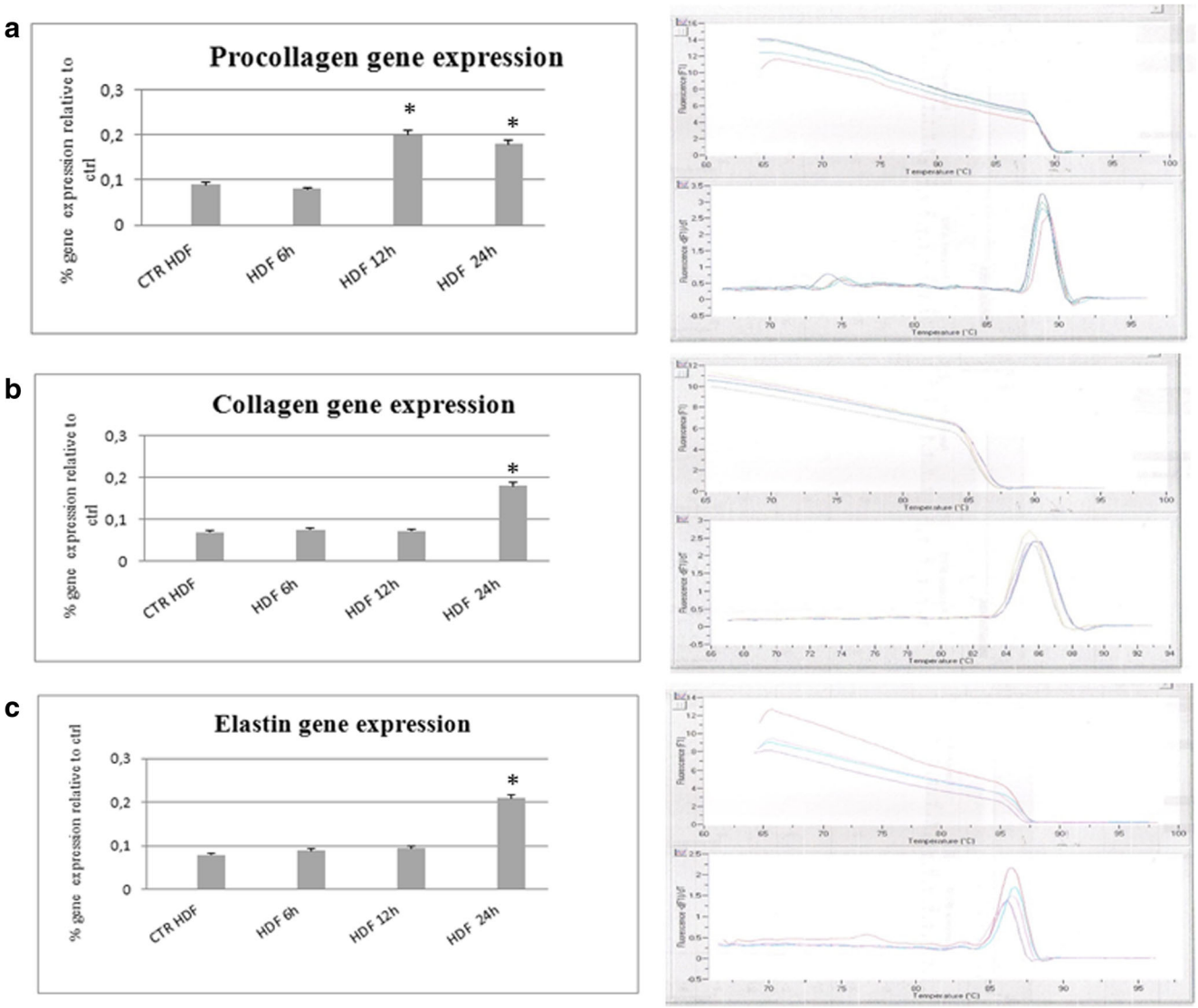

Fig. 7 Real-time PCR analysis. **a** Relative procollagen gene expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and **b** melting curve performed for all reactions (right). Relative collagen type 1 gene expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and **c** melting curve performed for all reactions (right). Relative Elastin gene expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 (left) and melting curve performed for all reactions (right)

Also filaggrin and TGase play an important role in skin barrier function. Particularly, TGase mediates cross-linking of several structural proteins and lipids of skin barrier and is involved in the terminal stages of normal keratinocyte differentiation [26].

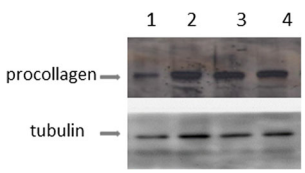

Fig. 8 Western blot analysis. Procollagen protein expression from HDF, stimulated with KCM after irradiation with Q-switched Nd:YAG laser 1064 nm at fluence of 8 J/cm^2 normalized with α tubulin

The environmental stressor may produce disease conditions in skin associated mainly to protein damage. To restore cellular homeostasis, some mechanisms of cellular defense might be activated by inducing the expression of various genes, including the heat-shock proteins (HSPs). They have a pivotal role in the cytoprotection and repair of cells and tissues. One potential mechanism of protection is the ability of HSP to inhibit genetic expression of proinflammatory cytokines. In our previous studies, we have demonstrated that HaCaT cells infected with *Candida albicans* and irradiate with Q-switched Nd:YAG laser induces a significant over-

expression of HSP70B in keratinocytes, compared to the untreated control [27].

The increase in filaggrin, TGase, and HSP70, founded in our study demonstrates that this laser treatment could favor the recovery of structural proteins damaged during chrono and photoaging [28]. To promote skin rejuvenation, growth factors and cytokines, often have been included in several topical cosmetic products, for their ability to induce collagen synthesis, but due to their molecular weight they hardly penetrate the stratum corneum [29].

Here, we obtained, after 24 h of laser treatment, an increased expression of TNF α, IL-6, and IL1α in keratinocytes. This result demonstrates that laser treatment could be more effective than cosmetic preparation containing cytokines, in fact endogenous laser induced cytokines could penetrate the basal membrane and act on the fibroblasts underlying layer. As demonstrated by Kondo et al., cytokines and growth factors released by keratinocytes can cross a basal membrane that separates keratinocytes by fibroblasts into a two-chamber co-culture [30].

The second step of our study has been focused on the keratinocytes/fibroblasts interaction. In our experimental model, confluent HDF have been stimulated with keratinocyte-conditioned medium, taken after 24 h after irradiation. Fibroblasts synthesize for 80–85% collagen type 1 and for 10–15% collagen type III. Photoaging and chronological aging are mainly characterized by the reduction of collagen, glucosaminoglycan, and elastin. Studies on remodeling of ECMs in photoaging by Rock et al. showed that collagen fragments clotted by metalloproteinases inhibit the synthesis of hyaluronic acid in fibroblasts treated with UVB. The authors conclude that this effect may be responsible for the formation of wrinkles and dryness.

This is the first study that demonstrates that Q-switched 1064 nm Nd:YAG is able to increase type 1 collagen as well as procollagen, elastin, and TGF-beta in human fibroblasts KCM treated, while it reduces MMP1. MMP1 is the primary responsible for ECM degradation. IL-1, IL-6, and TNF alpha are modulators of MMP1 and MMP3 produced by fibroblasts.

After 6, 12, and 24 h MMP1 levels are reduced of 40%, 36%, and 32% respectively compared to untreated controls, while TGF β gene expression is increased of 38% respect control after 24 h of treatment. TGF β is main immunomodulatory cytokine that suppresses inflammation and promotes proliferation and new collagen synthesis. On the basis of the results obtained in vitro could be speculate, that in vivo cytokines and/or growth factors released by keratinocytes treated with Q-switched 1064 nm Nd:YAG laser may cross the basal membrane lead to a reduction of MMP-1 and an increase in procollagen, collagen type I, and elastin in fibroblasts. Further studies, with blocking antibodies or with receptor antagonist of cytokines to be added at KCM before of HDF treatment, will be needed to clarify which cytokines and/or growth factors released in KCM are responsible for the observed effects.

Despite our enthusiasm for results obtained with in vitro system, we must never lose sight of in vivo situation because in vitro finding is limited and in vitro-in vivo correlation is a correlation hard to evaluate if we want consider the complexity of an integrate organism. With an in vitro system, we have obtained results that suggest as the Q-switched 1064 nm Nd:YAG laser treatment could represent an effective weapon to fight skin aging. It acts on the skin barrier function, increasing the expression of aquaporins, filaggrin, TGase, and HSP70, modulating the proinflammatory cytokines, and inducing the restoration of matrix skin proteins. In addition, its non-invasive nature, the low complication profile, the ability to reach the higher dermis layer and its minimal downtime, increase its safety and beneficial results.

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Compliance with ethical standards

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

Informed consent Informed consent was obtained from all individual authors included in the study.

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