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ORIGINAL ARTICLE

Effect of 1064-nm Q-switched Nd:YAG laser on invasiveness and innate immune response in keratinocytes infected with *Candida albicans*

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Abstract
Candida albicans is an opportunistic pathogen commensal in the oral cavity, vagina, and healthy skin. Common therapeutic options for fungal infections are topical or systemic antifungal drugs. Recently, in cutaneous pathologies, lasers and light-based treatments have emerged showing few contraindications and minimal side effects. The Q-switched (Nd-YAG) laser at a wavelength of 1064 nm has been shown to be useful in dermatology, dentistry, and some other medical specialties. It is used to treat onychomycoses, warts, and wounds and in some other treatments. We analyzed the effect of Q-switched (Nd-YAG) laser 1064 nm on human keratinocytes infected with *C. albicans.* In particular, we evaluated the effect of laser on invasiveness of *C. albicans* and on inflammatory and protective response of HaCaT cells infected. The results obtained did not show inhibitory, fungicidal, or fungistatic effects of laser on yeast; in addition, laser did not affect HaCaT vitality. HaCaT cells infected with *C. albicans* and irradiated with laser showed a reduction of invasiveness of TNF-α and IL8 gene expression and an increase of immunomodulatory cytokines such as TGFβ. Furthermore, laser induces a significant over-expression of HSP70B (heat shock protein) and of HBD-2 (Human β defensin-2) in HaCaT infected with *C. albicans*, compared to the untreated control. The use of Q-switched Nd:YAG laser in skin mycosis caused by *C. albicans* reduces yeast invasiveness in keratinocytes, downregulates inflammatory activities, and facilitates cytoprotection and antimicrobial defense. Our results offer a promising therapeutic strategy in the management of skin candidiasis, also in combination with conventional therapies.

Keywords Q-switched Nd:YAG laser · Keratinocytes · *Candida albicans* · Invasiveness

Introduction

Candida albicans is part of the human microbiota that colonizes the oral cavity, gastrointestinal tract, vagina, and skin, but it is also involved in infections. It is particularly common in immunocompromised patients during antibiotic, steroid therapy, poor nutrition, and endocrinopathies, after an implanted medical device or as a consequence of pH variation. *Candida albicans* on the skin is responsible for non-dermatophytic onychomycosis and infections due to burns, wounds, stress, or pregnancy. In fungal infections, the most common therapeutic options are topical or systemic antifungal agents. Topical therapy seldom reaches local therapeutic concentrations. Systemic therapy induces approximately 50% of recovery rates and can damage the liver and the kidney. In addition, it supports the development of bacterial resistance and the colonization of opportunistic pathogens. Recently, in cutaneous pathologies, lasers and light-based treatments have emerged that show few contraindications and minimal side effects. The neodymium-doped yitrium aluminum garnet (Nd:YAG laser) is a laser that issues various wavelengths and can operate in continuous, long-pulsed, Q-switched, or potassium titanyl phosphate (KTP) modes. Q-switched Nd:YAG laser 1064 nm is able to penetrate the tissue more deeply and reduce tissue damage. Several clinical trials have

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been conducted on the effects of Nd:YAG laser in skin pathologies. In particular, the findings of studies evaluating the in vivo effects on onychomycosis, persistent fungal infections of the nail bed and plate, were encouraging. [1–4]. Kolokasidis (2013) treated 131 onychomycosis subjects, among which 19 patients affected by Candida, using Q-switched Nd:YAG 1064/532-nm laser, and obtained positive results [5]. Zhang used a long-pulsed Nd:YAG 1064 nm-laser on 33 patients with onychomycosis with satisfying results [3]. In contrast to these results, Carney et al. observed no mycological or clinical efficacy in the 8 patients who completed the study [6]. In vitro studies on *Trichophyton rubrum*, the most common pathogen responsible for onychomycosis, showed conflicting results. Recent studies reported a mycocidal effect of Nd:YAG laser at 1064 and 532 nm on *Trichophyton rubrum* using a long-pulsed or Q-switched laser [7, 8]. In contrast, Hees et al. did not observe any inhibitory killer effect when applying Q-switched laser at 1064 and 532 nm at 4 and 8 J/cm^2 and long-pulsed laser at 1064 nm at 45 J/ cm^2 on *Trichophyton rubrum* [9]. In onychomycosis caused by *Candida*, Kolodchenko and Bactul (2013) demonstrated that the yeast infection was poorly treatable with 1064-nm Nd:YAG laser [10]. In contrast, Korajev (2010), using long-pulsed Nd:YAG laser, reported good results in the treatment of onychomycosis including those caused by *Candida* [7].

To verify the role of Nd:YAG laser on the yeasts responsible for cutaneous pathologies, we investigated the effect of Q-switched Nd:YAG laser at 1064 nm on the viability and invasiveness of *C. albicans* in human keratinocytes. Furthermore, in our study, we verified the modulation of inflammatory and immunomodulatory responses after interaction of keratinocytes and *C. albicans* irradiated with Nd:YAG laser at 1064 nm.

Materials and methods

Candida strain

In this study, we use *Candida albicans* (ATCC 53324) obtained from the American Type Culture Collection (Rockville, MD, USA). *C. albicans* was grown in a culture medium prepared according to the manufacture's guidelines (Oxoid, Cambridge, UK). Ten microliters of strain suspension was transferred into 10 ml Sabouraud dextrose and incubated overnight at 30 °C. The cells were then separated from the medium by centrifugation at 2800×*g* for 5 min, washed twice with PBS, and suspended in DMEM for each experiment at a ratio of 1:10 cell/yeast [11].

To evaluate a probable effect of Q-switched Nd:YAG laser on *C. albicans*, we irradiated or not *C. albicans* with 1064-nm Q-switched Nd:YAG laser (Medlite C6 laser, Conbio, USA) at a fluence of 2, 4, 6, and 8 J/cm^2, a pulse width of 5 ns, and a spot size of 4 mm.

Cell culture and treatments

HaCaT cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; VWR), 1% glutamine, and 1% pen-strep (complete medium) at 37 °C in a 5% CO_2 humidified atmosphere. In our experiment, cells were used at 70–80% confluence. HaCaT cells were infected with *C. albicans* and irradiated or not with 1064-nm Q-switched Nd:YAG laser (Medlite C6 laser, Conbio, USA) at a fluence of 2, 4, 6, and 8 J/cm^2, a pulse width of 5 ns, and a spot size of 4 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.

Cell viability measurement

The viability of HaCaT after irradiation with Q-switched Nd:YAG laser was measured by the MTT procedure (Alpha Kit, Biochrom, Berlin, Germany), which is a colorimetric assay for cellular growth and survival using 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazoliumbromide. An MTT dye solution was added to each well and the incubation was continued for 180 min. 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. Following the MTT procedure, the medium was aspirated from each well and replaced with isopropanol to dissolve the formazan crystals formed in viable metabolic active keratinocytes. The content of each well was mixed for 5 min by shaking the plates. The plates were then examined spectrophotometrically at 24, 48, 72 h after irradiation at various fluences (2, 4, 6, 8 J/cm^2) [12]. The viability was calculated by measuring the increase in absorbance at 570 nm and was expressed as a percentage of the control value.

Yeast viability was evaluated by plating several dilutions on Sabouraud dextrose agar and counting the colonies after 72 h at 30 °C, expressed as colony-forming unit (CFU) count.

Values are the mean ± standard deviation (SD) of three replicates in three different experiments.

Invasion assay for *C. albicans*

Human keratinocytes were infected or not with *C. albicans* (1:10) and irradiated or not with Q-switched Nd:YAG laser 1064 nm. After 24 h, to remove non-adherent yeast cells, the keratinocytes were washed three times with PBS. The infected keratinocytes were subsequently treated with 1 ml of DMEM

containing nystatin (Sigma) at a mycocidal concentration (1.6 μg/ml) for 4 h at 37 °C. After this period, the infected cells were treated with trypsin-EDTA for 5 min at 37 °C and lysed by adding 1 ml of cold 0.1% Triton-X100. The cell lysates were diluted in PBS and plated on Sabouraud dextrose agar to identify the viable intracellular yeast by counting the CFU⁄ml after 24 h at 30 °C.

Real-time PCR analysis

Semi-confluent keratinocytes (10^6/well) were infected or not with *C. albicans* and then irradiated or not with Q-switched Nd:YAG laser. After 24 h, total RNA was isolated using the High Pure RNA Isolation Kit (Roche; Milan, Italy). Two hundred nanograms of total cellular RNA was 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 $MgCl_2$, 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.

Statistical analysis

Each experiment was performed at least three times. The results are expressed as mean ± standard deviations (SD). The ANOVA test (analysis of variance between groups) was used to analyze the results obtained. The P value was generally evaluated between 0.01 and 0.03, confirming the statistical significance of the results ($P < 0.05$).

Results

Effect of Q-switched Nd:YAG laser treatment on HaCaT and *C. albicans* viability

Q-switched Nd:YAG laser used at 1064 nm did not significantly influence HaCaT vitality at any fluence tested (2, 4, 6, 8 J/cm^2) (Fig. 1A). Q-switched Nd:YAG laser 1064 nm did not modify viability of Candida at any fluence tested as shown in Fig.1B.

Effect of Q-switched Nd:YAG laser on *C. albicans* invasiveness

To determine if Q-switched Nd:YAG laser was able to interfere with yeast invasiveness, HaCaT were infected with *C. albicans* (10:1 yeast to cell ratio) and irradiated with Q-switched Nd:YAG laser at 1064 nm (2, 4, 6, 8 J/cm^2). After 24 h, we evaluated the effect of laser on *C. albicans* invasiveness. The reduction of *C. albicans* invasiveness was observed in a fluence-dependent manner as shown in Table 2. In particular, a major reduction in the number of internalized microorganisms (up to 63%) in respect to control was observed at 8 J/cm^2.

Table 1 Real-time PCR

Gene	Primer sequence	Conditions	Product size (bp)
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-8	5′-ATGACTTCCAAGCTGGCCGTG-3′ 5′-TGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′	1′at 94 °C, 1′ at 56 °C, 1‴ at 72 °C for 33 cycles	297
hBD-2	5′-CCA GCC ATC AGC CAT GAG GGT-3′ 5′-AAC CGG TAG TCG GTA CTC CCA-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

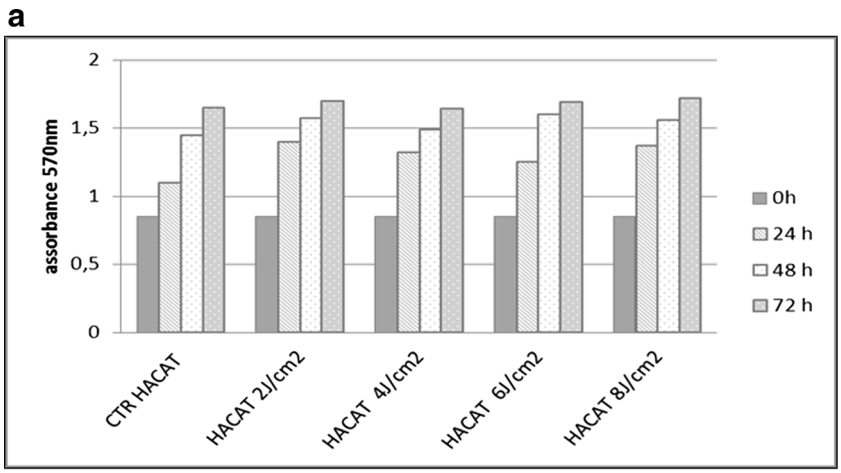

Fig. 1 Cell viability measurement. **a** HaCaT viability after irradiation with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2 measured by the MTT procedure. **b** *Candida albicans* CFU after irradiation with Q-switched Nd:YAG laser at different fluences

b

	Not irradiated	Irradiated 2J/cm^2	Irradiated 4J/cm^2	Irradiated 6J/cm^2	Irradiated 8J/cm^2
Candida albicans	6,23 X 10^8	5,8 X 10^8	6,25 X 10^8	5,68 X 10^8	6,15 X 10^8

Effect of Q-switched Nd:YAG laser on proinflammatory response of HaCaT cells infected with *C. albicans*

To evaluate the ability of Q-switched Nd:YAG laser to modulate, the proinflammatory response of HaCaT cells infected with *C. albicans* was analyzed in the gene expression of the proinflammatory cytokines IL-8 and TNF-α. HaCaT cells did not express basal level of these cytokines. As shown in Fig. 2A and B, HaCaT cells infected with *C. albicans* displayed high levels of TNF-α and IL-8 gene expression. The treatment with Q-switched Nd:YAG laser reduces, after 24 h, gene expression of TNF-α and IL-8 at a fluence-dependent manner; in particular, at a fluence of 8 J/cm^2, a reduction of 68% TNF-α and of 56% IL-8 in respect to control was observed. In contrast, a significant increase in TGF β gene expression (45%, compared to cells infected with Candida and non-irradiated) was observed at a fluence of 8 J/cm^2 (Fig. 2C).

Human β defensin-2 was upregulated after infection of HaCaT cells with *C. albicans* treated with Q-switched Nd:YAG laser

To demonstrate whether Q-switched Nd:YAG laser was able to upregulate the expression of the HBD2 gene, HaCaT cells were infected with *C. albicans* treated or not with Q-switched Nd:YAG laser. As shown in Fig. 3, HaCaT cells infected with *Candida* for 24 h showed an upregulation of the HBD2 expression compared to the control cells. HaCaT infected with yeast treated with Q-switched Nd:YAG laser showed an important increase of the HBD-2 gene expression of 76% at a fluence of 8 J/cm^2 compared to cells infected with *Candida* and non-irradiated (Fig. 3).

Induction of HSP70B gene expression in HaCaT cells infected with *C. albicans* treated with Q-switched Nd:YAG

To analyze the cytoprotective response of HaCaT cells infected with *C. albicans* irradiated with Q-switched Nd:YAG laser at 1064 nm (2, 4, 6, 8 J/cm^2), we analyzed the heat shock protein (HSP) 70B gene expression. As shown in Fig. 4, after infection with *Candida*, HaCaT cells displayed an increment of Hsp70B gene expression, compared to non-infected cells. After irradiation with laser at 1064 nm, an increase (until 42%) of the Hsp70B gene expression was observed, in respect to non-irradiated control at a maximal fluence of 8 J/cm^2 (Figs. 5 and 6).

Table 2 Effect of Q-switched Nd:YAG laser on *Candida albicans* invasiveness

	Non-irradiated	Irradiated 2 J/cm^2	Irradiated 4 J/cm^2	Irradiated 6 J/cm^2	Irradiated 8 J/cm^2
Candida albicans	7.15 × 10^8	7.6 × 10^8	6.15 × 10^8	5.66 × 10^8	2.65 × 10^8

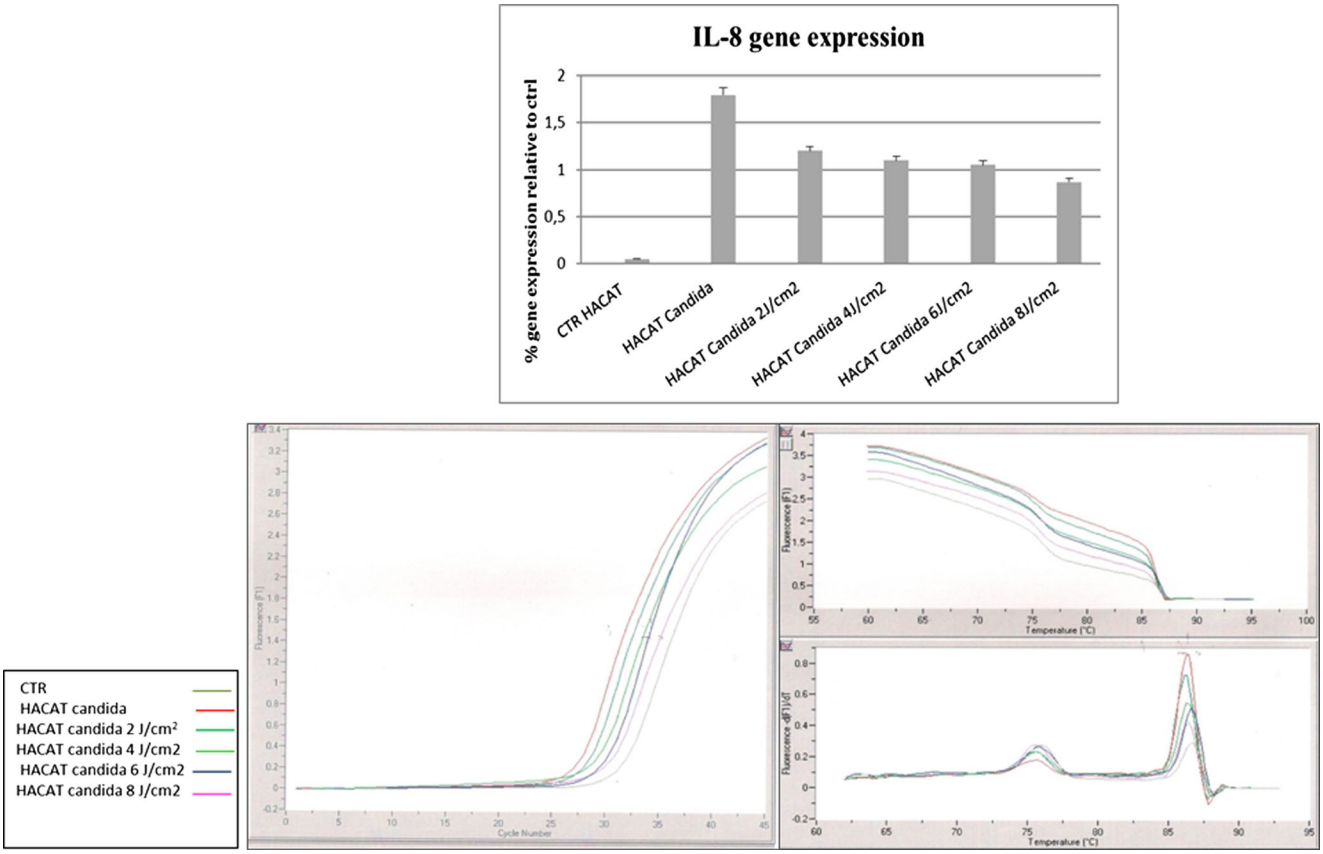

Fig. 2 Real-time PCR analysis using specific primers for cytokines. Relative IL-8 gene expression from HaCaT infected or not with *C. albicans* and irradiated with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2. Standard curves were used for the quantification of IL-8 gene expression. Melting curves were performed for all reactions. The diagrams contain the simultaneous amplification of gene-specific PCR fragments in serial dilution as detected by laser scanning

Discussion

In the control of fungal infections, the conventional therapies require long and expensive treatments, associated with the emergence of resistance and side effects. Q-switched Nd:YAG laser was the first non-ablative laser used in human pathologies. In our study, Q-switched Nd:YAG laser did not affect keratinocyte viability or proliferation. Poon et al. have reported that the high resistance of keratinocytes is probably due to a dispersion of energy by a scattering action of keratin in the peripheral cytoplasm [13]. *Candida* strain was treated with 1064-nm Q-switched Nd:YAG laser with 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 results obtained did not show any inhibitory, fungicidal, or fungistatic effects on *C. albicans*. We evaluated the invasive capability of *C. albicans* treated with laser in human keratinocytes. The invasiveness of *C. albicans* was downregulated in a laser fluence-dependent manner. At 8 J/cm^2, we observed a major decrease, up to 63% compared to the control. This finding suggests that treatment with laser might reduce the incidence of systemic mycosis that may develop in immunocompromised patients.

In addition, we verified the biological effects on the yeast/keratinocyte interaction after treatment with laser. Innate immunity plays a key role in Candida infection. Reduced local immunity is an important factor contributing to the development of fungal cutaneous disease. Chemical and physical barriers such as the skin epithelial structures and antimicrobial products have an important role in antimicrobial innate defense [14–17]. Interaction with microorganism surface structures activates cellular signal transduction, transcription factors, and production of inflammatory, immunomodulatory markers and antimicrobial peptides [18–20]. Among the antimicrobial peptides, the most important are B-defensins (HBDs), cysteine-rich peptides with an antimicrobial activity against Gram-positive and Gram-negative pathogens and yeasts. In particular, upregulation of HBD2 expression in epithelial cells plays an antimicrobial role against *Candida*. Our results show that *C. albicans* induces in keratinocytes an activation of immunomodulatory and proinflammatory cytokines, as demonstrated in our previous studies [19]. In our model, HaCaT infected with *C. albicans* and irradiated

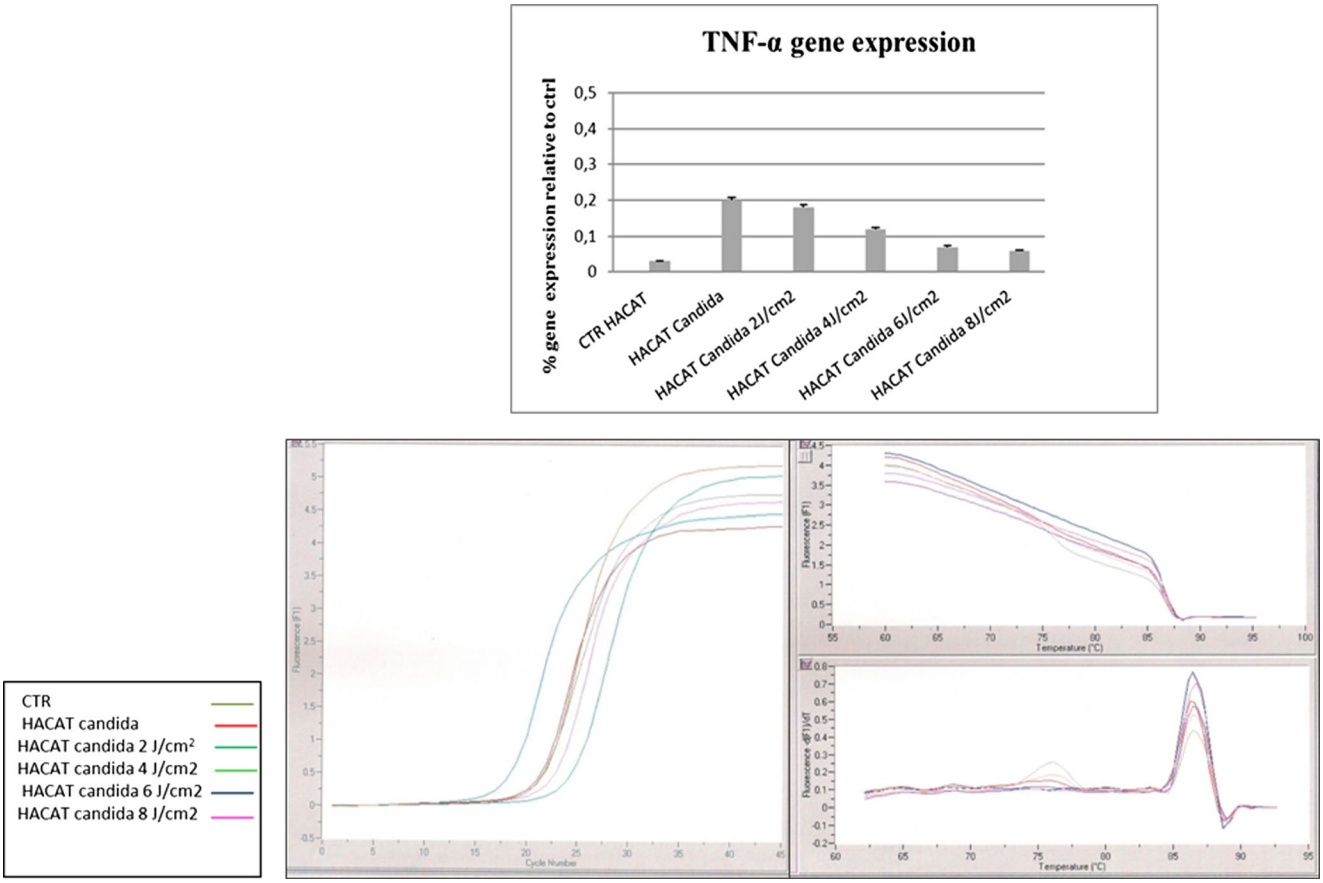

Fig. 3 Real-time PCR analysis using specific primers for cytokines. Relative TNFα gene expression from HaCaT infected or not with *C. albicans* and irradiated with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2. Standard curves were used for the quantification of TNFα gene expression. Melting curves were performed for all reactions. The diagrams contain the simultaneous amplification of gene-specific PCR fragments in serial dilution as detected by laser scanning

with laser showed a downregulation of TNF-α and IL8 expression and an increase of immunomodulatory cytokines such as TGFβ. Growth factors and cytokines, in particular TNF-α, trigger reactive oxygen species responsible for cellular damage. Downregulation of TNF-α expression limits damage due to free radicals and favors tissue healing. The over-expression of TGF beta plays a role in cytokine proinflammatory reduction. Other mechanisms of keratinocyte defense are activated by laser treatment; HSP70 has a role in cytoprotection against the effects of stress and trauma associated with reduced inflammation [21, 22] and cell proliferation. Q-switched Nd:YAG laser induces a significant over-expression of HSP70B in keratinocytes, compared to the untreated control. In addition, the increase of HBD2 in HaCaT infected with *C. albicans* treated with laser has a role in antimicrobial response for its chemotactic activities in adaptive immunity and wound healing, favoring skin defense [14]. In summary, the use of Q-switched Nd:YAG laser in skin mycosis caused by *C. albicans* reduces yeast adhesion and invasiveness in keratinocytes, downregulates inflammatory activities, and facilitates cytoprotection and tissue healing. It has been reported that adherence to a host serves as an initial and critical step in the establishment of yeast as a commensal or as a pathogen. In particular, biochemical studies have revealed the involvement of specific interactions between cell-surface lectins and mannose yeast residues are involved in the killing of *Candida* and are responsible for the initial phase of anti-*Candida* activity [23]. We might hypothesize that irradiation with Q-switched Nd:YAG laser modifying cell yeast surface inhibits the adhesion of yeast and consequent modulation of invasiveness. Q-switched Nd:YAG laser, also in combination with conventional therapies, could be used for treatment of skin candidiasis reducing treatment times, pharmacological doses, antifungal resistance, and side effects. This constitutes the first study to report on the relationship between Q-switched Nd:YAG laser irradiation and modulation of

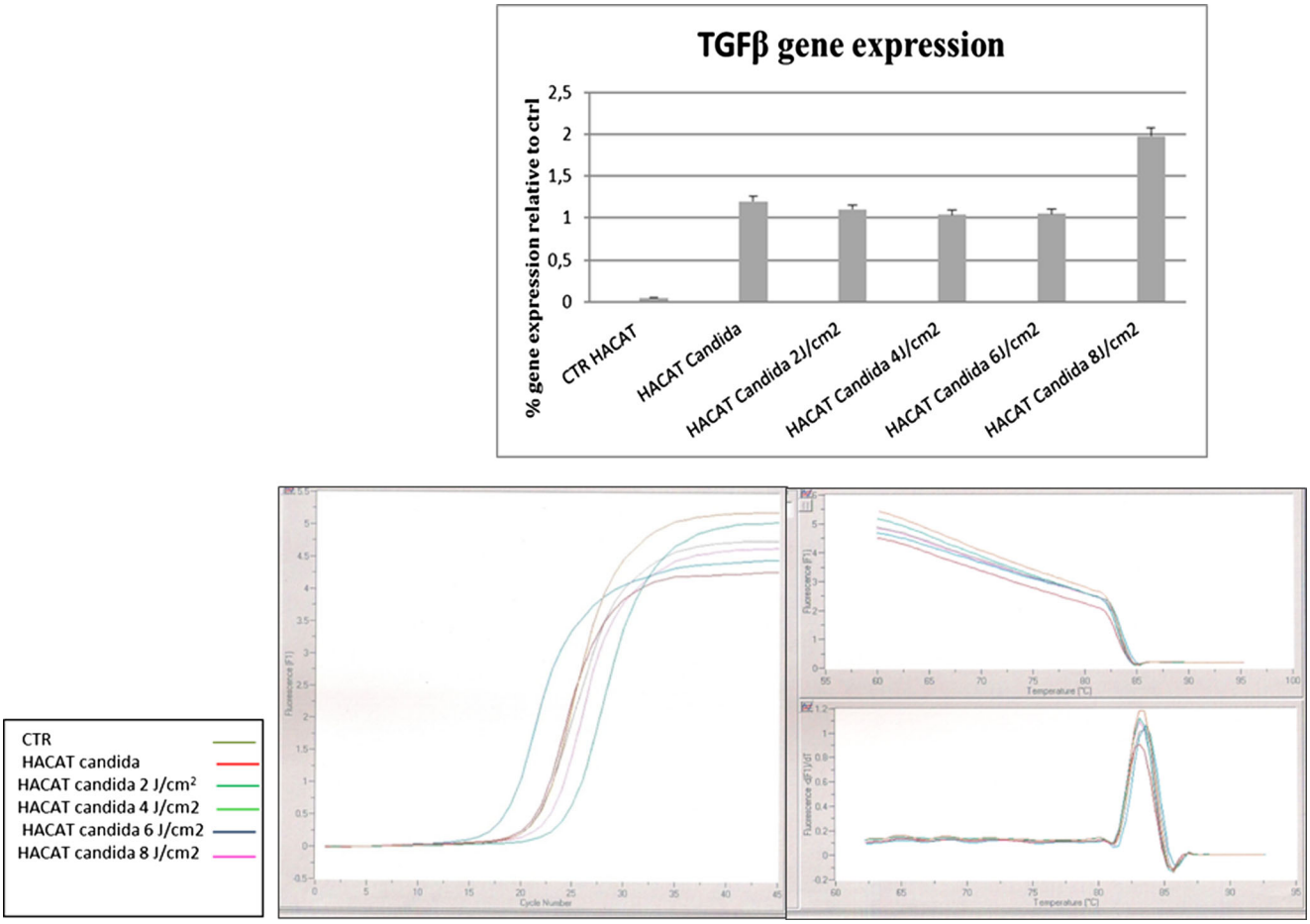

Fig. 4 Real-time PCR analysis using specific primers for TGFβ. Relative TGFβ gene expression from HaCaT infected or not with *C. albicans* and irradiated with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2. Standard curves were used for the quantification of TGFβ gene expression. Melting curves were performed for all reactions. The diagrams contain the simultaneous amplification of gene-specific PCR fragments in serial dilution as detected by laser scanning

C. albicans infectiveness on human keratinocytes. Future studies are necessary to elucidate possible modifications of the *Candida* surface caused by the laser and to extend the current finding to in vivo systems and further define the signaling system responsible for these observations.

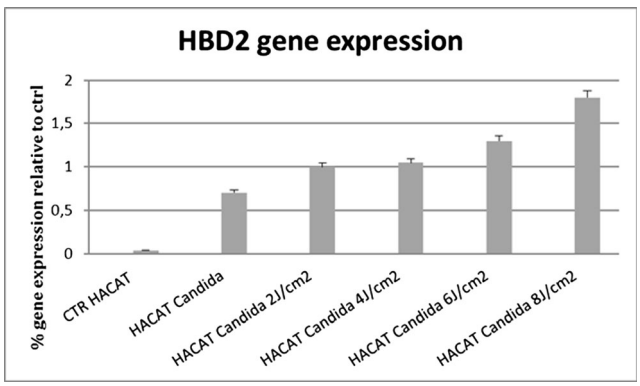

Fig. 5 Real-time PCR analysis using specific primers for HBD-2 mRNA expression. Relative mRNA expression from HaCaT infected or not with *C. albicans* and irradiated with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2

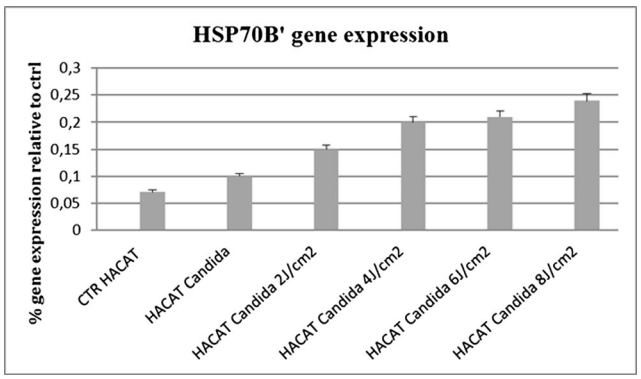

Fig. 6 Real-time PCR analysis using specific primers for HSP70B mRNA expression. Relative mRNA expression from HaCaT infected or not with *C. albicans* and irradiated with Q-switched Nd:YAG laser 1064 nm at fluences of 2, 4, 6, and 8 J/cm^2

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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 participants included in the study.

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