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In vitro study on the safety of near infrared laser therapy in its potential application as postmastectomy lymphedema treatment

Francesca Cialdai [a], Ida Landini [b], Sergio Capaccioli [c], Stefania Nobili [b], Enrico Mini [d], Matteo Lulli [c], Monica Monici [a,*]

[a] ASAcampus Joint Laboratory, ASA Research Division, Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale Pieraccini 6, I-50139 Florence, Italy
[b] Section of Clinical Pharmacology and Oncology, Department of Health Sciences, University of Florence, Viale Pieraccini 6, I-50139 Florence, Italy
[c] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale Morgagni 50, I-50134 Florence, Italy
[d] Section of Internal Medicine, Department of Experimental and Clinical Medicine, University of Florence, Largo Brambilla 3, I-50134 Florence, Italy

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ABSTRACT

Clinical studies demonstrated the effectiveness of laser therapy in the management of postmastectomy lymphedema, a discomforting disease that can arise after surgery/radiotherapy and gets progressively worse and chronic. However, safety issues restrict the possibility to treat cancer patients with laser therapy, since the effects of laser radiation on cancer cell behavior are not completely known and the possibility of activating postmastectomy residual cancer cells must be considered.

This paper reports the results of an *in vitro* study aimed to investigate the effect of a class IV, dual-wavelength (808 nm and 905 nm), NIR laser system on the behavior of two human breast adenocarcinoma cell lines (namely, MCF7 and MDA-MB361 cell lines), using human dermal fibroblasts as normal control. Cell viability, proliferation, apoptosis, cell cycle and ability to form colonies were analyzed in order to perform a cell-based safety testing of the laser treatment in view of its potential application in the management of postmastectomy lymphedema. The results showed that, limited to the laser source, treatment conditions and experimental models used, laser radiation did not significantly affect the behavior of human breast adenocarcinoma cells, including their clonogenic efficiency. Although these results do not show any significant laser-induced modification of cancer cell behavior, further studies are needed to assess the possibility of safely applying NIR laser therapy for the management of postmastectomy lymphedema.

1. Introduction

Postmastectomy lymphedema is a discomforting disease [1] whose incidence is approximately 5% after surgery and can increase to 30% following radiotherapy.

Lymphedema is a chronic condition that progressively worsens. It is characterized by increased protein content, excess of intra- and extracellular fluids in the tissues, surplus deposition of fibrous tissue and chronic inflammation, which result in swelling and deformity of the upper limb, accompanied by a brawny edema. The symptoms are: limb heaviness, weakness, pain, restricted shoulder mobility, burning and elevated skin temperature. Lymphedema has a serious adverse impact on quality of life of the patients and can lead to psychological morbidity [2].

Standard treatments for lymphedema include pressotherapy, compression bandaging, manual lymphatic drainage, exercise and skin care. These treatments, generally applied in combination with each other to form a multifaceted intervention known as Complete Decongestive Therapy (CDT), are expensive, time-consuming, require qualified medical professionals, are poorly accepted by patients and have limited effectiveness [3–5].

The majority of alternative methods for the management of lymphedema falls into the category of the Physical Agent Modalities (PAMs), which can be classified as thermical, mechanical or electromagnetic [5]. In a recent review aimed at evaluating the

Abbreviations: NIR, Near-Infrared; CDT, Complete Decongestive Therapy; PAMs, Physical Agent Modalities; MLS, Multiwave Locked System; PI, Propidium Iodide; ROS, Reactive Oxygen Species; LLLT, Low Level Laser Therapy.

* Corresponding author.
E-mail addresses: francesca.cialdai@unifi.it (F. Cialdai), ida.landini@unifi.it (I. Landini), sergio.capaccioli@unifi.it (S. Capaccioli), stefania.nobili@unifi.it (S. Nobili), enrico.mini@unifi.it (E. Mini), matteo.lulli@unifi.it (M. Lulli), monica.monici@unifi.it (M. Monici).

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effectiveness of the PAMs, the authors concluded that the current body of published literature lacks sufficient quality to provide the evidence to support the majority of alternative treatment modalities to enhance current treatment protocols. However, some studies on the application of laser therapy to the management of postmastectomy lymphedema met the criteria to be defined "likely to be effective", although the sample size was limited and further research is needed to support a recommendation for practice [5].

Indeed, double-blind, placebo-controlled trials [6–8] have shown that Low Level Laser Therapy (LLLT) can be effective in improving postmastectomy lymphedema symptoms. In these studies, the treatments consisted in irradiating (λ = 904 nm, energy density 1.5 J/cm^2) from 10 to 17 points distributed in the diseased area, with a number of sessions that ranged from 9 to 36. The results showed a significant reduction in the volume of the affected arm [6,7] as well as extracellular fluids and tissue hardness [6], improved shoulder mobility and grip strength [7]. The improvement observed in treated patients, compared to controls, remained significant at 2–3 months after treatment [6,7].

Kozanoglu et al. [8] compared the long-term efficacy of pneumatic compression and LLLT in the management of postmastectomy lymphedema. They found that both treatments were effective in reducing the volume of the limb at the end of the treatment cycle, at 3 and 6 months, but the improvement was greater in the group of patients treated with LLLT. At 12 months, the beneficial effects (decrease of swelling and pain) were still significant only in this group.

A previous study with different treatment modality [9], in which a dual wavelength (632 nm and 904 nm) scanning laser was used and higher energy densities (2–4 J/cm^2) were applied, had already highlighted the effectiveness of laser therapy in significantly reducing both the extracellular fluid and total volume of the affected arm, inducing fibrosis softening and improving heaviness, aching and tightness. Two years follow up showed that volume reduction and tonometry persisted over time [10].

Other studies, carried out using different NIR laser sources (single and double wavelength, wavelengths ranging from 808 nm to 905 nm) and energy densities similar to those applied in the studies reported above, further confirmed that laser irradiation induced an improvement in lymphedema [11–15].

Therefore, laser therapy has been proposed as an alternative treatment for the management of postmastectomy lymphedema.

Indeed, the effectiveness of laser therapy in the treatment of edema resulting from various causes (trauma, inflammation, surgery, etc.) is known for a long time and has been confirmed by recent studies [7,16–19].

The mechanisms underlying the anti-edema effects of laser therapy are not completely known, but it has been reported that laser radiation may act on microcirculation [20] and lymphatic vessels [10], affects the regulation of inflammation [21], the behavior of fibroblasts [22,23] as well as the production and assembly of extracellular matrix molecules [24,25].

Although there are indications in favor of the application of laser therapy in the treatment of postmastectomy lymphedema and the development of advanced laser systems can further improve the effectiveness of the treatments, safety still remains an open issue. In fact, despite the observation that laser therapy has already been widely used to treat complications developing in cancer patients after surgical tumour resection, chemo- and radiotherapy [26], the long-term safety has been poorly studied. The above clinical studies on laser application in postmastectomy lymphedema did not report the occurrence of adverse effects related to laser exposure during the follow-up period, generally $\leqslant$12 months, but only two studies reported data related to a period $\geqslant$2 years and only one of them reported recurrence and survival at 5 and 10 years [9,15].

On the other hand, the impact of laser radiation on cancer cell behavior is partly unknown and the abundant literature presents controversial results. The main question is whether or not laser irradiation increases proliferative rate and invasiveness of tumor cells, which would constitute an adverse effect of laser radiation that could be potentially harmfull in oncologic patients.

Therefore, it is desirable to increase the limited safety information in order to develop guidelines for laser application in postmastectomy lymphedema.

Although molecular mechanisms induced by laser radiation are not completely understood, they involve mitochondrial photo-acceptors and production of Reactive Oxygen Species (ROS) [27–30]. Furthermore, laser radiation might affect cell cycle progression, protein synthesis, cell energy metabolism, proliferation and apoptosis by modulation of specific kinases and phosphatases [21,28,31].

The effect of laser radiation on tumor cell growth, cell cycle progression and apoptosis has been widely studied and compared to the effect produced on non-neoplastic cells by the use of many different sources (generally low power lasers), wavelengths (mostly red and NIR radiation), energy doses (from tenths to tens of J/cm^2) and treatment modes. These studies produced a large amount of data, sometimes conflicting and difficult to compare, because they were obtained under different conditions, but still very important in the common goal of helping to define systematically guidelines for safely use laser sources when oncologic patients are treated. Some of these results are reported in Table 1 and show that the effects differ not only by changing the treatment conditions but also by applying very similar treatment conditions in different cell populations

The heterogeneity of results suggests:

(1) the need to further increase the current knowledge in this field;
(2) the need of assessing suitable experimental models on the safety of the laser sources and treatment parameters before application in oncologic patients.

In the last decade the application for therapeutic purposes of multi-wavelength sources and high-power lasers has become widespread. In rehabilitation, physical- and sports medicine, these devices are effectively used to decrease inflammation, promote resorption of edema and hematoma as well as stimulate tissue repair. They have been already used to treat secondary disorders in cancer patients [32], but studies on the effects of these emissions on tumor cells are limited.

The aim of the present study was to evaluate the *in vitro* safety (cell-based safety testing) [33] of a high power (Class IV), dual-wavelength, NIR laser system in view of its potential application in the management of postmastectomy lymphedema. After exposure to laser radiation, the behavior of human breast adenocarcinoma cells (MCF-7 and MDA-MB361 cell lines, both characterized by epithelial like morphology) was studied in terms of viability, proliferation, cell cycle progression, apoptosis and cloning efficiency. Human diploid fibroblasts were used as a control of non-cancerous cells.

2. Materials and methods

2.1. Cell cultures

Dermal diploid fibroblasts (a kind gift from Dr. Mocali A., Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy) were derived from 2-mm punch biopsies taken up from the upper arm of female healthy donors with patient informed permission. Human breast adenocarcinoma cell

Table 1
Selection of *in vitro* studies regarding the effects of laser radiation on normal and neoplastic cells.

Author/years	Cell species	Laser parameters	Cellular effects
Monici et al., (2013) [21]	Murine myoblasts (C2C12 skeletal muscle cell line)	IR laser with two synchronized sources (laser diodes 808 nm and 905 nm). One treatment a day, for 3 consecutive days; frequency: 1500 Hz; energy delivered: 68 J; irradiation time: 8 min	= Cell viability ↓ Cell proliferation ↑ Expression of early marker of differentiation (MyoD) Changes of cell morphology and cytoskeletal architecture leading to the formation of tube-like structures ↑ Numerous ATP-binding proteins and proteins involved in the regulation of muscle metabolism, as PP1 (proteomic analysis)
Frigo et al., (2010) [22]	–Primary fibroblast cell culture from human keloids –3T3 cell line	GaAlAs 660-nm laser (mean output: 50 mW, spot size 2 mm^2, power density 2.5 W/cm^2) irradiation times: 60 or 420 s; fluences:150 or 1050 J/cm^2; energy delivered: 3 or 21 J ; 3 exposure in subsequent days	Primary fibroblast cell culture from human keloids with 3 J: ↑ Proliferation (trypan blue exclusion test) Primary fibroblast cell culture from human keloids and 3T3 with 21 J: ↓ Cell in proliferative phase of the cell cycle (Propidium iodide staining flow cytometry data)
Schartinger et al. (2012) [23]	–Human bronchial epithelial cells (BEAS-2B) –Human gingival fibroblasts –Oral squamous cell carcinoma cells (SCC-25)	GaAlAs-diode laser 660 nm. Power output 350 mW. Irradiation time 15 min; 3 exposure in subsequent days	Human gingival fibroblasts: ↑ Proliferation Non-neoplastic epithelial cells and in SCC-25 oral carcinoma cells: ↓ Proliferation A proapoptotic effect of laser therapy was observed in SCC-25 cells
Huang et al., (2011) [27]	ASTC-a-1 cells, HeLa cells, human hepatocellular liver carcinoma (HepG2) cells, and African green monkey SV-40-transformed kidney fibroblast (COS-7)	The cells in the selected area were irradiated for 10 min with HF-LPLI (High fluence low power laser irradiation) 632.8 nm, 120 J/cm^2. The power intensity was maintained at 0.2 W/cm^2	HF-LPLI could cause Akt/GSK3b signaling pathway inactivation through ROS generation The inactivation of the Akt/GSK3b pathway was crucial for cell apoptosis induced by HF-LPLI GSK3b promotes Bax activation through down-regulation of Mcl-1
Gao et al., (2006) [28]	ASTC-a-1 cells	LPLI (Low power laser irradiation) He–Ne laser (632.8 nm, 5 mw) at fluence of 0.8 J/cm^2 High fluence LPLI He–Ne laser (632.8 nm, 40 mw) at fluence of 60 J/cm^2	LPLI ↑ Proliferation ↑ Activation of PKCs (protein kinase Cs) High fluence LPLI ↓ Cell viability ↑ Apoptosis ↓ PKCs activity
Wu et al., (2008) [29]	ASTC-a-1 cells	High fluence LPLI He–Ne laser (633 nm, 40 mw) at fluence of 120 J/cm^2, 10 min. The power intensity was maintained at 0.2 W/cm^2	↑ Apoptosis ↑ ROS ROS causes onset of mitochondrial permeability transition which in turn causes cytochrome c release ↑ Bax activation
Shefer et al., (2003) [31]	i28 mouse myogenic cells	Cells were irradiated through a grid composed of 1.8 × 1.8 mm squares, for 3 s per square, with a He–Ne laser (632.8 nm, 4.5 mW; 1.8-mm beam diameter)	↑ de novo protein synthesis (by modulating the activity of key enzymes that regulate capped mRNAs translation)
Kreisler et al. (2002) [39]	Human gingival fibroblasts (HGF)	809-nm semiconductor laser (power output 10 mW) used in the cw-mode. Time of exposure varied between 75 and 300 s. Energy fluences of 1.96–7.84 J/cm^2 1, 2 or 3 exposures	↑ Proliferation after 24 h of irradiation ↓ Proliferation after 48 and 72 h of irradiation in energy-dependent manner
Kreisler et al. (2003) [40]	Epithelial tumor cells derived from human laryngeal carcinoma	CW GaAlAs-diode laser 809 nm delivered by a 600 μm optic fibre. Power output 10 mW. Irradiation time 75, 150 and 300 s (1.96, 3.92 and 7.84 J/cm^2, respectively) single exposure	↑ Proliferation in comparison with the controls Differences highly significant on the first to third days after irradiation ($p < 0.001$)

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Table 1 (*continued*)

Author/years	Cell species	Laser parameters	Cellular effects
Vinck et al., (2003) [41]	Fibroblasts were obtained from 8-days old chicken embryos	–Laser GaAlAs 830 nm power output ranging from 1 to 400 mW and a frequency range from 0 to 1500 Hz Treatment consisted of 5 s irradiation at a power output of 40 mW resulting in a radiant exposure of 1 J/cm^2. 3 exposures –Light emitting diode device 570 nm (power range 10–0.2 mW) 660 nm (power range 80–15 mW) 950 nm (power range 160–80 mW) The infrared and the red beam delivered radiant exposures of 0.53 J/cm^2 and the green beam emitted 0.1 J/cm^2, corresponding to exposure-times of respectively 1 min, 2 min or 3 min and a respective power output of 160 mW, 80 mW or 10 mW, 3 exposures	↑ Proliferation in comparison with the controls 570 nm Proliferation >660 nm, 950 nm and 830 nm laser light 660 Proliferation >950 nm and 830 nm laser light
Pereira et al., (2002) [42]	NIH-3T3 fibroblasts	Ga-As diode laser (904 nm; 120 mW) energy densities varying from 3 to 5 J/cm^2 over a period of 1–6 days	3, 4 J/cm^2 ↑ Proliferation = procollagen synthesis 5 J/cm^2 = proliferation
Taniguchi et al., (2009) [43]	HIG-82 rabbit synovial fibroblasts	Low level laser therapy (660 nm); 40 mW/cm^2 Irradiation time: 2 minfluence of 4.8 J/cm^2	↑ Proliferation (this effect was reduced by cAMP, Cyclic adenosine monophosphate)
Hawkins et al., (2007) [44]	Normal or wounded human skin fibroblast	Helium–neon (632.8 nm), diode (830 nm) and Nd:YAG (1064 nm) laser;one exposure of 5 J/cm^2 or 16 J/cm^2 on day 1 and again on day 4. Power density 2.07, 5.95, 12.73 mW/cm^2 respectively	Wounded fibroblasts with 632.8 nm, 5 J/cm^2: ↑ ATP viability after 1 h ↓ caspase 3/7 activity after 24 h ↑ proliferation
Moore et al., (2005) [45]	Endothelial cells and fibroblasts isolated and characterized from the aorta and skin of two adult male C3H mice	KTP-pumped tunable dye laser (Series 600 XP, LaserScope, Inc., San Jose, CA) (625, 635, 645, 655, 675 nm) 810-nm diode laser (Diomed Ltd., Cambridge, UK) For all wavelengths tested, power density 5 mW/cm^2 energy density of 10 J/cm^2	Red light ↑ Proliferation (especially 655, 675 nm) 810 nm ↓ Proliferation (fibroblasts)
Pinhero et al., (2002) [46]	H.Ep.2 cells (laryngeal cancer cells)	5-mW diode lasers; 635 and 670-nm; beam cross section approximately 1 mm) at local light doses between 0.04 and 4.8.10(4) Jm(−2)	↑ Proliferation with 670-nm laser light applied at doses between 0.04 and 4.8.10 (4) Jm(−2)
Sroka et al., (1999) [47]	Skeletal myotubes (C2), normal urothelial cells (HCV29), human squamous carcinoma cells of the gingival mucosa (ZMK1), urothelial carcinoma cells (J82), glioblastoma cells (U373MG), and breast adenocarcinoma cells (MCF7)	Different sources (410, 488, 630, 635, 640, 805, and 1064 nm and broad band white light) The irradiation varied between 0 J/cm^2 and 20 J/cm^2. In the case of C2 and ZMK1 cells, the dependency of the irradiance was investigated using the parameters 50 mW/cm^2 and 150 mW/cm^2. HCV29 and J82 cells were illuminated with irradiances of 10 mW/cm^2 and 100 mW/cm^2	Mitotic rate: ↑ J82, HCV29 with 410, 635 and 805 nm ↓ C2 with 635 nm Max MR : J82, HCV29, C2 with 4 and 8 J/cm^2 Min MR: J82, HCV29, C2 with 20 J/cm^2 Min MR: ↓ MCF7, U373MG, and ZMK1 with increasing J/cm^2 ↓ All cell lines with 20 J/cm^2
Powell et al., (2010) [48]	Human breast adenocarcinoma (MCF-7), human breast ductal carcinoma with melanomic genotypic traits (MDA-MB-435S), immortalized human mammary epithelial (SVCT and Bre80hTERT) cell lines	3 different sources: 780 nm continuos (50 mW) 830 nm continuos (30 mW) 904 nm pulsed (90 mW) 0.5, 1, 2, 3, 4, 10, 12 J/cm^2 for 780 nm 0.5, 1, 2, 3, 4, 10, 15 J/cm^2 for 830 and 904 nm 1, 2 or 3 exposures	Although certain doses of laser increased MCF-7 cell proliferation, multiple exposures had either no effect or showed negative dose response relationships No sign of malignant transformation of cells by laser phototherapy was detected under the conditions applied

Ben-Dov et al., (1999) [49]	Rat satellite cells purified from hind-leg muscles of 3-week-old Charles River rats Pmi28 mouse satellite cells (cell line)	He-Ne laser (632.8 nm, 4.5 mW; 1.8 mm beam diameter)	3 s of exposition: Primary culture ↑ Cell cycle regulatory proteins Pmi28 cell line ↑ PCNA (proliferating cell nuclear antigen) In primary culture cell proliferation was dependent on the rat's age: ↑ 3 weeks of age = 6 weeks of age ↑ MHC Myosin heavy chain
Fukuhura et al., (2006) [50]	Rat calvarial cells	Ga-Al-As diode laser, 905 nm 1.25, 3.75, and 6.25 J/cm^2 (150, 450, and 750 s, respectively)	3.75 J/cm^2 Irradiation (effect disappeared at 24 h and 48 h after irradiation)Arrest of the cell cycle 12 h after ↑ Bone formation 1.25 and 6.25 J/cm^2 = Cell cycle progression
Crisan et al., (2013) [51]	Human fibroblast skin	830 nm, 980 nm and 2.940 nm Frequency 50 Hz, energy density 5.5 J/cm^2, output power 1 W, power density 0.5 W/cm^2 Irradiation time 110 s	830 and 980 nm ↑ Mitochondrial activity = Apoptosis 2.940 nm ↓ Mitochondrial activity ↑ Apoptosis and necrosis
Sperandio et al., (2013) [53]	Dysplastic oral keratinocytes – DOK cell line and oral squamous cell carcinoma cell lines SCC9 and SCC25	GaAlAs – 660 nm or 780 nm, 40 mW, 2.05, 3.07 or 6.15 J/cm^2	DOK ↑ Cell viability SCC9 (24 h 48 h) 780 nm ↑ cell viability 660 nm ↓ cell viability SCC25 (24 h 48 h) ↑ Cell viability DOK ↑ Specific proteins related to cancer invasion and progression, such as pAkt, Hsp90, pS6 ser240/244 and Cyclin D1 SCC9 780 nm 24 and 72 h ↑ Expression of pAkt SCC25 660 nm and 780 nm; 48 h 780 nm; 72 h ↑ Expression of pAkt
Gomes Herquez et al., (2014) [54]	SCC25 human tongue squamous cell carcinoma	InGaAlP laser 660 nm, 30 mW 0.5 J/cm^2 (L0.5) and irradiance of 0.03 W/cm^2 for 16 s (0.48 J) 1.0 J/cm^2 (L1.0) and irradiance of 0.03 W/cm^2 for 33 s (0.99 J)	1.0 J/cm^2 ↑ Proliferation ↑ Cyclin D1 ↑ Nuclear β-catenin ↓ E-cadherin ↑ MMP-9 ↑ Invasion potential
Marchesini et al., (1989) [56]	Human tumor cell lines HT29 (colon carcinoma) MCF-7 (breast carcinoma) M14 and JR1 (malignant melanomas)	Argon and argon-dye laser (488–515 nm; 630 ± 5 nm; 645 ± 5 nm; 640 ± 5 nm) Radiant exposures 4.2–150 kJ/m^2 at irradiances ranging from 35–500 W/m^2	41 experiments performed: 5 showed a significant statistical increase in number of colonies, 3 showed a decrease. The trend of most data was toward an increase in colony formation

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Table 1 (*continued*)

Author/years	Cell species	Laser parameters	Cellular effects
Al-Watban and Andres (2012) [57]	*Normal cells* Chinese hamster ovary (CHO) & Mouse embryonic fibroblasts (CCL-226 and 3T3) & Human skin fibroblasts (HSF) *Neoplastic cells* Murine fibrosarcoma (RIF-1) & Mouse mammary adenocarcinoma (EMT-6)	A 35-mW HeNe (632.8-nm) laser and power density of 1.25 mW/cm^2 Irradiation times of 16, 32, 48, 64, 80, 96, 112, 128, 144, and 160 s for three consecutive days to deliver cumulative doses of 60, 120, 180, 240, 300, 360, 420, 480, 540, and 600 mJ/cm^2	Colony forming unit: ↑ 180 mJ/cm^2 ↓ 420–600 mJ/cm^2

lines MCF-7 and MDA-MB361 (ATCC, USA) were a kind gift of Dr. Normanno N. (Cell Biology and Preclinical Models Unit, INT-Fondazione Pascale, Naples, Italy).

Cells were routinely cultured in growing medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 100 mg ml^{-1} streptomycin, 100 U ml^{-1} penicillin, 2 mM glutamine and 10% fetal bovine serum (FBS). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

2.2. Laser treatment

Laser source and treatment modality have been previously described [21]. Briefly, the treatments have been performed with a Multiwave Locked System laser (MLS laser, ASA Srl, Vicenza, Italy). It is a commercially available laser source built in compliance with EC/EU rules, which received FDA approval and is widely used in clinics. MLS laser is a class IV, NIR laser with two synchronized sources (laser diodes). The two modules have different wavelengths, peak power and emission mode. The first one is a pulsed laser diode, emitting at 905 nm, with 25 W peak optical power; each pulse is composed of a pulse train (100 ns single pulse width, 90 kHz maximum frequency). The frequency of the pulse trains may be varied in the range 1–2000 Hz, thus varying the average power delivered to the tissue. The second laser diode (808 nm) may operate in continuous (power 1 W) or frequenced (repetition rate 1–2000 Hz) mode, 500 mW mean optical power output, duty ratio 50% independently of the repetition rate. The two laser beams work simultaneously, synchronously and the propagation axes are coincident.

24 h before starting treatment, cells were seeded in the central 8 wells of a 24-multiwell plate (5×10^3 cells/well). For irradiation, the plate without cover was placed inside a holder designed to allow a controlled scan treatment and ensure its reproducibility in all the experiments. The plate was aligned with the handpiece, sliding in an overlying track, at a distance of 2 cm from the bottom of the wells, so that the spot size of the area irradiated by the two superimposed laser beams, impinging perpendicular to the sample surface, had the same dimension of two adjacent wells (26 mm). A single experienced operator performed all the treatments in scan mode. This method allowed a better homogeneity of the energy distribution over the treatment area compared to the point mode, moreover this application modality is often used in clinical applications on patients when a relatively large part of the body needs to be treated, as in the case of edema. The scan of the samples was performed manually, by moving the handpiece with a speed of 5.6 cm/s, under the guide of a metronome. Each scan of 8 wells lasted ~2 s and scans were performed continuously until the end of the treatment time. A treatment of 10 min, with 1500 Hz frequency, duty ratio 50%, energy density 9 J/cm^2, irradiance 15 mW/cm^2 was repeated once a day, for 3 consecutive days in sterile conditions. The 3 treatments were carried out 24 h away from each other. The parameters chosen were very close to those commonly applied in clinical practice when sources of the same type as that used in this study are used to treat edema.

The transmission of the radiation through the bottom of the empty well was $\geqslant$98% for both the wavelengths. The reflection on the surfaces of the well was negligible. The absorption of radiation by the culture medium was $\leqslant$2% for both the wavelengths. The increase in temperature after irradiation was $\leqslant$0.3°C.

The treated samples were compared with controls maintained in the same conditions, except for the exposure to laser radiation.

2.3. Cell viability and proliferation

In order to obtain a highly reliable evaluation of the effect of MLS laser treatment on the proliferation rate of cancer cells, we

used the ADAM-MC automated cell counter (Digital Bio, NanoEnTek Inc, Korea), which is based on the staining of mammalian cell DNA with the fluorescent dye Propidium Iodide (PI).

Viability and proliferation were assessed 24 h after a single treatment and 24 h after the third treatment. Cells were detached with trypsin/EDTA and resuspended. Equal volumes (50 μl) of cell suspension were stained with AccuStain solutions T (Propidium Iodide/lysis solution) and N (Propidium Iodide/PBS) according to the manufacturer's instructions.

AccuStain Solution T permeabilizes plasma membrane, stains nucleus and allows measurements of total cell number, while AccuStain Solution N exclusively stains non-viable cells.

By a disposable microchip, the samples were placed into the instrument for measuring.

PI fluorescence was excited by a 532 nm laser source, automatically focused onto the cell suspensions contained into the disposable microchip. The fluorescence emission at 605 nm was collected and analyzed by a CCD camera. Viable cells were calculated by difference between total and dead cells. The provided data represented the average of counts performed on 22 different frames acquired randomly on each sample. Therefore, the system is highly reliable.

2.4. FACS analysis of cell cycle

The kinetic evaluation of cell cycle progression requires the synchronization of cells. Therefore, the effect of laser treatment on cell cycle was analyzed on synchronized fibroblasts, MCF-7 and MDA-MB361 cells. Cell cycle synchronization was obtained by a non-pharmacological method, based on serum depletion associated with cell-to-cell contact inhibition aimed to induce cell quiescence [34]. In brief, cells were seeded in 6-well plates and grown to confluence. Following substitution of serum-rich medium (10% FCS) with a serum-poor medium (0.5% FCS), cells were incubated in high density conditions for 48 h. Subsequently, cells were released from the G1/S arrest by replating them in the central 8 wells of 24-multiwell plates at 60% confluence and re-addition of serum-rich medium. At 6 h after replating, laser treatment was performed. 24 h after a single laser treatment, cells were harvested. For flow cytometry analysis, cells were trypsinized, washed twice with PBS and incubated for 30 min at 4°C in a cytoplasmic-lysis buffer (0.1% w/v trisodium citrate, 0.1% NP-40) containing 10 μg/mL of PI. The cell cycle was analyzed using a FACS Canto flow cytometer (Becton & Dickinson, Franklin Lakes, NJ, USA).

2.5. Evaluation of apoptosis

24 h after 1 day and 3 days laser treatments, apoptotic cells were scored by flow cytometry with GUAVA Personal Cell Analysis System and the Guava Nexin Assay (GUAVA Technologies, Hayward, CA), which uses Annexin V-PE to detect phosphatidyl serine on the external membrane of apoptotic cells. The cell-impermeant dye 7-aminoactinomycin D is included in the kit as an indicator of membrane structural integrity in order to assess the Annexin V- reactive cells into the early and late stages of apoptosis. Indeed, 7-aminoactinomycin D is excluded from living, healthy cells and early apoptotic cells but permeates late-stage apoptotic and dead cells. The assay was performed according to the manufacturer's instructions.

2.6. Clonogenic cell survival assay

The clonogenic cell survival assay assesses the ability of a cell to proliferate indefinitely, a characteristic that clinically could facilitate tumor recurrences [35].

The effect of laser irradiation on the colony forming efficiency of primary human fibroblasts and human breast carcinoma MCF-7 and MDA-MB361 cells was evaluated according to the method of Sobrero and Bertino [36].

Briefly, exponentially proliferating cells were placed onto 24-multiwell plates in DMEM medium with 10% fetal bovine serum at a density of $1x10^2$ cells/dish in order to allow attachment of single cells and the growth of a single colony. Cultures were incubated at 37°C and 5% CO_2 in a humidified incubator. After 24 h, when the cells were attached to the bottom of the dishes but not yet divided, the laser treatment was performed as described above and repeated for 3 consecutive days. Control samples were handled alike, except laser treatment. Treated and untreated samples were observed for up to 21 days, depending on the proliferation rate of the individual cell line. Colonies were fixed in methanol and acetic acid (3:1 vol/vol), stained with 1% orcein in 50% acetic acid, and counted with an automated colony counter (Oxford Optronix).

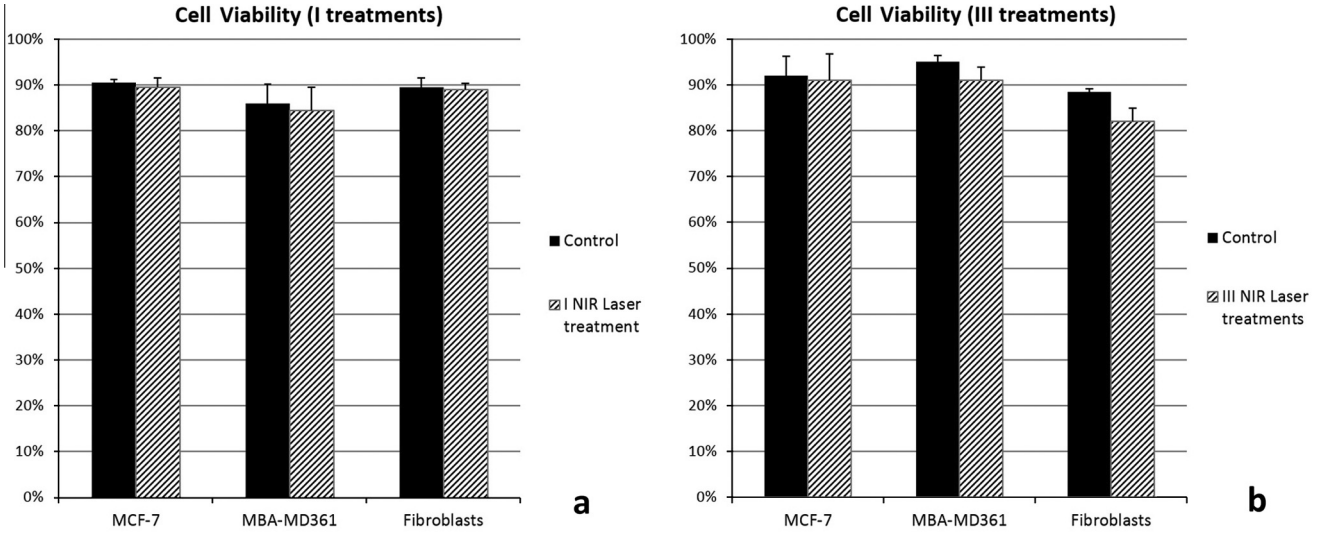

Fig. 1. MCF-7, MBA-MD361 and fibroblast viability assessed 24 h after a single laser treatment (a) and 24 h after the third laser treatment (b). Data were obtained by ADAM-MC automated cell counter.

The colony forming efficiency was determined as the percentage of colonies compared with the number of cells seeded for each cell line. In order to evaluate the effect of laser treatment on the colony forming efficiency of the different cell populations, data were expressed as the percentage of colonies in laser treated cultures compared with control cultures.

2.7. Statistical analysis

Data are presented as means ± S.D. Three independent experiments were performed in triplicate. Unpaired Student's *t*-test was performed to evaluate pair wise differences, with a $p < 0.05$ being considered significant.

3. Results

3.1. Cell viability and proliferation

We evaluated the effect of NIR laser treatment on cell viability and proliferation rate of human primary fibroblasts and two different human breast adenocarcinoma cell lines, namely MDA-MB361 and MCF-7. The analysis of cell viability carried out 24 h after a single laser treatment did not show significant differences between control and treated samples in any of the cell types considered (Fig. 1a). Similarly, the analysis of samples exposed to three laser treatments showed no significant changes in comparison with controls (Fig. 1b).

Cell counts performed 24 h after a single treatment showed a slight decrease in MCF-7 cells ($p = 0.29$) and a slight increase in fibroblasts ($p = 0.30$), in comparison to their associated controls (Fig. 2a). Assaying the samples 24 h after the third treatment, we observed a slight decrease in both MCF-7 ($p = 0.20$) and MBA-MD361 cells ($p = 0.25$), in comparison to untreated controls (Fig. 2b). The changes were not statistically significant ($p > 0.05$). Therefore, we concluded that laser treatment had no significant effects on the proliferation rate of the cell types considered.

3.2. Cell cycle analysis

To investigate if laser treatment could perturb the cell cycle, flow cytometry analysis of the cellular DNA content was performed. The percentages of cells in the different phases of the cell cycle were determined 24 h after a single laser treatment. The results (Table 2) did not show significant differences between treated samples and related controls in any cell type considered. Therefore, the exposure to laser radiation did not affect cell cycle progression.

Table 2
Cell cycle analysis. Analysis of cell cycle in unsynchronized, synchronized, post-release untreated and post-release laser-treated human fibroblasts, MCF-7 and MDA-MB361 cells. Data represent means ± SD of three independent experiments.

		G1 (%)	S (%)	G2/M (%)
MCF-7	Unsynchronized	66.4 ± 0.2	24.3 ± 0.3	9.3 ± 0.2
	Synchronized	89.3 ± 0.8	7.6 ± 0.5	3.1 ± 0.3
	Untreated control	46.9 ± 0.3	46.3 ± 0.5	6.8 ± 0.2
	Treated	48.3 ± 1.4	45.5 ± 0.9	6.2 ± 0.4
MDA-MB361	Unsynchronized	69.4 ± 0.6	28.9 ± 0.9	1.7 ± 0.3
	Synchronized	74.9 ± 0.2	22.7 ± 0.4	2.4 ± 0.6
	Untreated control	62.7 ± 0.3	29.3 ± 0.2	8.0 ± 0.2
	Treated	62.9 ± 0.2	28.8 ± 0.2	8.3 ± 0.2
Fibroblasts	Unsynchronized	65.7 ± 2.2	29.5 ± 3.4	4.8 ± 1.2
	Synchronized	82.1 ± 0.7	5.0 ± 1.4	12.9 ± 0.7
	Untreated control	67.9 ± 1.7	18.2 ± 2.9	13.9 ± 1.1
	Treated	68.1 ± 1.6	17.2 ± 1.7	14.7 ± 1.3

3.3. Evaluation of apoptosis

The possibility that laser treatment could modify the apoptotic threshold has been evaluated by flow cytometry with Annexin V-PE assay. As shown in Fig. 3, in comparison to untreated controls, neither a single nor three laser treatments modified the percentage of living, early apoptotic and late apoptotic cells in any cell population considered.

3.4. Clonogenic cell survival assay

The impact of laser treatment on the ability of tumor cells and fibroblasts to form colonies was determined. In untreated samples, primary human fibroblasts and MDA-MB361 cells were unable to form colonies, while MCF-7 cells were able to form colonies.

In this kind of assay, the possibility of not having colony formation represents an expression of the different colony-forming

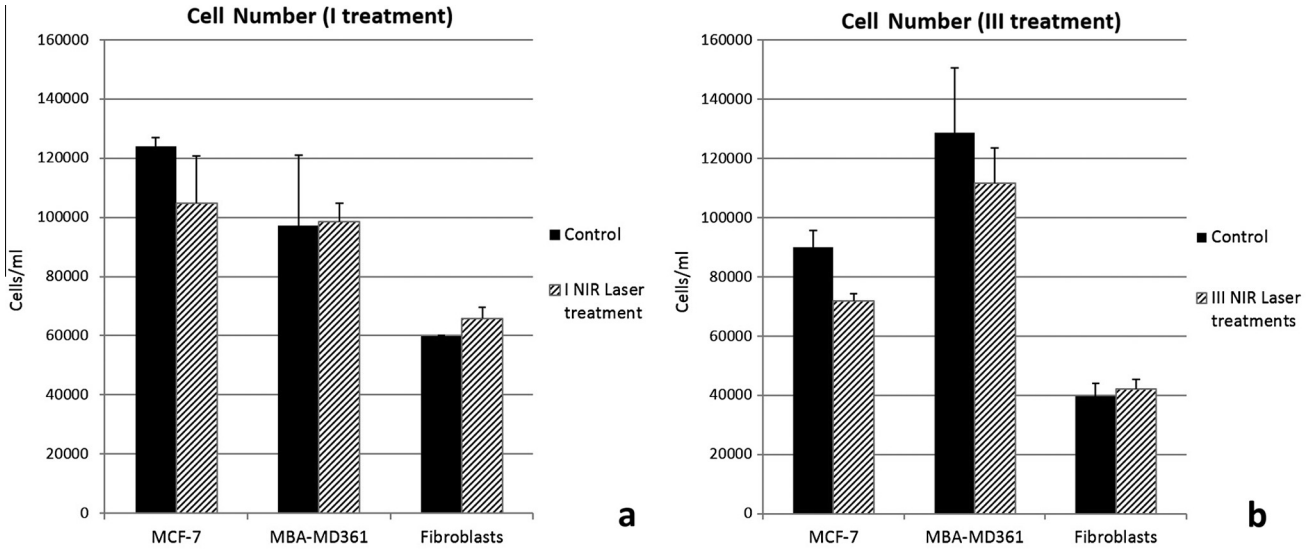

Fig. 2. MCF-7, MBA-MD361 and fibroblast proliferation assessed 24 h after a single laser treatment (a) and 24 h after the third laser treatment (b). Data were obtained by ADAM-MC automated cell counter.

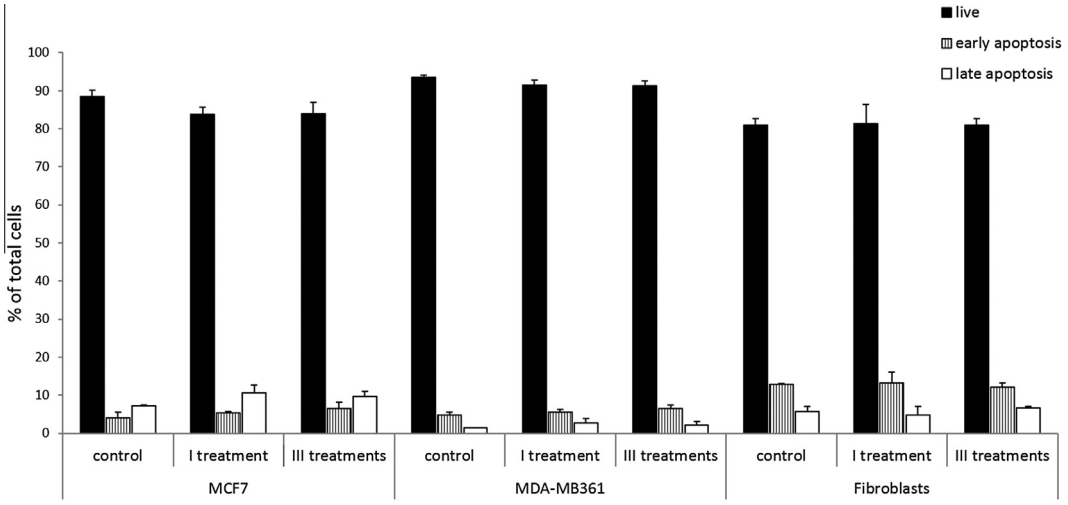

Fig. 3. Number of living, early and late apoptotic human fibroblasts, MCF-7 and MDA-MB361 cells exposed to one or three laser treatments. Bar graphs show the compiled mean values ± SD of three independent experiments.

Table 3
Clonogenic cell survival assay. Colony-forming ability of cultured fibroblasts, MCF-7 and MDA-MB361 human breast adenocarcinoma cells after three laser treatments.

Cell type	Colony formation (% of control)	
	Untreated controls	Treated samples
Fibroblasts	0	0
MCF-7	19	18
MDA-MB361	0	0

ability of different cell populations. In literature it is reported that normal diploid fibroblasts derived from primary culture exhibit a limited proliferative potential *in vitro*, depending on the age of the donor and the biopsy site [37]; MDA-MB361 and MCF-7 cells have low and higher ability to form colonies, respectively [38].

The aim of the test was to verify whether or not laser treatment induced an increase in the colony-forming ability.

In treated samples, primary human fibroblasts and MDA-MB361 cells remained unable to form colonies. The percentage of colonies formed by MCF-7 cells did not differ from that of untreated controls (Table 3). Therefore, laser treatments did not affect the cloning efficiency of the cell types considered.

4. Discussion

Although laser sources emitting red and NIR radiation have been widely and safely used in sports medicine, physical medicine and rehabilitation to promote fast resorption of edema and hematoma and decrease inflammation [7,16–19], the treatment of postmastectomy lymphedema requires studies aimed at understanding if laser irradiation can affect the behavior of tumor cells. In fact, in oncologic patients, tumor cell proliferation would constitute an adverse and potentially harmfull effect of the therapy. The effects of red and NIR laser radiation on tumor cell growth have been widely studied, but conclusive results have not been achieved. Moreover, most of the studies have been carried out using low power sources and wavelengths ranging from 630 nm to 830 nm. The novelty of the present study is that it provides information on the *in vitro* behavior of breast adenocarcinoma cells irradiated by a high power, dual wavelength (808 nm + 905 nm), NIR laser with simultaneous and synchronous emissions. In recent years, the use of high power, multi-wavelength sources has spread in clinics, but the studies on their biological effects are still relatively few. It is also worth noting that one of the two wavelengths applied in this study is 905 nm, very close to the most applied wavelength (904 nm) in clinical trials on the laser treatment of postmastectomy lymphedema.

The results of the study showed that viability and proliferation rate of fibroblasts, MCF-7 and MDA-MB361 cancer cells exposed to laser treatment did not change significantly, in comparison to the related untreated controls.

Many studies focused on viability and proliferation of fibroblasts exposed to red and NIR laser radiation, with different results [22,23,39–44]. The prevailing evidence indicates that both red and NIR laser radiation, under certain treatment conditions, can increase fibroblast proliferation. The response to irradiation depends on wavelength [41,44,45], energy dose or fluence [22,44], mode of treatment [22,39,44], culture conditions and physiological state of the cells (e.g. activated/not activated) [22,44]. In most of the studies above, wavelengths in between 570 nm and 780 nm were used. Therefore, the present study, where a source with double emission at 808 nm and 905 nm has been used, may not be compared with them. Irradiating with a wavelength of 809 nm and fluences <8 J/cm^2, Kreisler et al. [39] found an increase in proliferation 24 h after treatment, in contrast with the results presented here, which, however, were obtained with higher fluence. Moore et al. [45], using 810 nm wavelength and 10 J/cm^2 fluence, found a decrease in proliferation, but the analysis was performed 72 h after treatment. It is noteworthy that in studies where an increase in proliferation was found, regardless to the wavelength used, the fluence was <8 J/cm^2 [22,23,39,41,43,45].

When considering the effect of red and NIR laser radiation on cancer cells, the results reported in literature are even less consistent than those on fibroblasts. In this case, it is even more difficult to compare the different studies, since not only different sources and treatment parameters have been used but also the experimental models utilized are extremely varied, coming from different types of tumors. Schartinger et al. [23] observed that 660 nm laser radiation (power densities from 0.39 to 63.7 mW/cm^2) induced apoptosis in the human oral carcinoma SCC-25 cell line, while Pinheiro et al. [46] found a higher proliferation rate in laryngeal cancer cells (HEp-2 cells) exposed to 670 nm laser radiation (0.04–4.8 J/cm^2). Sroka et al. [47], analyzing the effects of different wavelengths and energy densities on several normal and cancer cell lines, found that different cell populations responded differently (in terms of mitotic rate changes) to the various

combinations of parameters tested. In general, proliferation increased with energy density between 4 and 8 J/cm^2, while it decreased at higher fluences. Consistent with the results of the present study, they found that the proliferation of irradiated (805 nm) MCF7 cells did not increase, on the contrary the mitotic rate slightly decreased with increasing fluence. More recently, Powell et al. [48] investigated the effect of different laser emissions (780, 830, 904 nm) and energy densities (from 0.5 to 15 J/cm^2) on the proliferation rate of human breast carcinoma (MCF-7), human melanoma (MDA-MB435S) and immortalized human mammary epithelial (SVCT and Bre80hTERT) cell lines. Laser irradiation did not change the proliferation rate of the MDA-MB435S melanoma cells nor that of Bre80hTERT cells while significantly increased the proliferation of SVCT cells, which were responsive to all the wavelengths assayed. Although certain treatment conditions (combination of wavelength and energy density) increased MCF-7 cell proliferation after a single exposure, multiple exposures had either no effect or showed negative dose response relationships. In particular, 904 nm emission induced an increase in MCF-7 proliferation after a single treatment and a decrease after three treatments. Therefore, the results of the present study agree with those obtained by Powell and coauthors [48] on MCF-7 exposed to three treatments, but disagree as regards the effect of a single treatment, which resulted statistically not significant, as previously reported by Sroka et al. [47]. The discordant results might be due to the different laser sources and energy densities applied.

Consistent with the data obtained on cell viability and proliferation, we found that laser treatment did not alter significantly cell cycle progression nor apoptotic threshold of fibroblasts, MDA-MB361 and MCF-7 cells.

Papers which concern the effects of red and NIR laser radiation on cell cycle are relatively few and heterogeneous, both with regard to the experimental protocols used and the results obtained. Several studies have been carried out using He-Ne laser sources (632 nm) and energy densities of 1 J/cm^2 or less. In these conditions, which are not comparable with those used in the present study, an activation of early cell cycle regulatory genes have been observed by some authors [31,49], but the effect seemed to depend on the type of cells and age of the animals from which the cells were derived [31]. More recently, Fukuhara et al. [50] reported that NIR laser radiation (Ga-Al-As diode laser, 905 nm) could induce the arrest of the cell cycle in rat calvarial cells monitored 12 h after irradiation, but the effect was observed only with energy density 3.75 J/cm^2 and, however, it disappeared after 24 h and 48 h. The treatment conditions used by Fukurara et al. [50] are more comparable with the ones used in the present study and also the results are in agreement, but they were obtained on different experimental models.

Numerous papers also reported different apoptotic responses to laser treatments.

Crisan et al. [51], evaluating the influence of three IR laser emissions on human skin fibroblasts, demonstrated that Short Wavelength Infra Red (SWIR) radiation (2.940 nm) caused inhibition of mitochondrial activity and significant cell necrosis and apoptosis, while NIR emissions at 830 nm and 980 nm induced mitochondrial activity and, in agreement with the findings here presented, did not affect apoptosis.

Conversely, in an *in vivo* study on tissue repair process, Rocha Júnior et al. [52] revealed an increase in the number of apoptotic fibroblasts in rat wound tissue treated with 870 nm Ga-As laser irradiation (3.8 J/cm^2), compared to controls. The discrepancy of results could be due to the different wavelength, energy density and experimental model (activated fibroblasts).

The impact of laser treatment on cancer cell apoptosis remains to be understood and the results in literature are discordant. Studying the behavior of the same model (SCC-25 oral cancer cells exposed to 660 nm laser radiation), but using different energy densities and exposure modalities (see Table 1), Schartinger et al. [23] reported that laser treatment induced apoptosis and did not exhibit tumor-promoting effect while Sperandio et al. [53] and Gomes Henriques et al. [54] reported that, depending on energy density, it could induce or not apoptosis and decrease or increase proliferation. Also studies performed by Huang et al. [27] on the human lung adenocarcinoma cell line ASTC-a-1 demonstrated that red (632 nm) laser radiation could induce proliferation and inhibit apoptosis or vice versa, depending on the fluence used. For fluences exceeding 60 J/cm^2, 632.8 nm laser irradiation has been demonstrated to commit human lung adenocarcinoma cells (ASTC-a-1) to apoptosis by activation of caspase 3 [55], in turn induced by the activation of ROS and mitochondrial permeability transition [29].

A comparison between our results and those of other authors who studied the effects of laser radiation on apoptosis and cell cycle is very difficult, because experimental models and wavelengths used for treatments are often very different. However, the complex of results reported in literature indicates that the impact of laser radiation on cancer cell apoptosis, cell cycle progression and cell proliferation can be quite different depending on the wavelength, energy density, mode of treatment and the type of treated cells. The prevailing evidence indicates that certain treatment conditions, that is some red and NIR wavelengths administered at low energy density (generally 0.5–5 J/cm^2) may affect cell cycle progression and promote cell proliferation. The effect is dose-dependent and generally disappears with increasing energy density, up to inhibition of proliferation and induction of apoptosis at fluences >25 J/cm^2. The findings here presented are consistent with this scenario.

Laser irradiation did not change the cloning efficiency of fibroblasts nor that of the two cancer cell populations, proving that the treatment did not promote tumor cell clonogenicity (Tab. III). In the treated samples the percentage of colonies formed by the MCF-7 cells remained substantially unchanged in comparison with controls, as well the MDA-MB361 cells remained unable to form colonies and their clonogenicity was not promoted by treatment.

Very few studies investigated the effect of laser radiation on the cloning efficiency of tumor cells [56,57], with controversial results. They applied wavelengths $\leqslant$645 nm, a wide range of energy density, different cell populations, different numbers of cells/dish in the colony forming assay and different study design. The very different parameters (e.g. wavelength, doses) used, do not allow a direct comparison between these studies and the current study. Marchesini et al. [56] found a trend toward an increase in colony formation in samples exposed to some of the treatment conditions investigated, but the results were not conclusive. Al-Watban and Andres [57], irradiating a series of murine cell lines with very low biomodulatory energy density, found that the optimum dose to increase the colony-forming efficiency was 180 mJ/cm^2 and energy density $\geqslant$420 mJ/cm^2 had an inhibiting effect.

To the best of our knowledge, the effect of NIR wavelengths on the clonogenic efficiency of tumor cells has never been reported before.

In conclusion, the results of the present study showed that irradiation by a NIR, dual wavelength (808 nm and 905 nm), high power laser did not affect the behavior of human dermal fibroblasts and breast adenocarcinoma cell lines in terms of proliferation, cell cycle progression, apoptosis and cloning efficiency.

These results are consistent with the possibility of safely applying the source and treatment parameters used in the present study for the management of postmastectomy lymphedema, because the treatment did not induce an increase in tumor cell growth. *In vitro* cultures of tumor cells may not represent the complexity of the biological environment *in vivo*, however, *in vitro* studies are

increasingly applied as early strategies to safety assessments and for addressing potential causes of adverse effects.

Moreover, the fact that the treatment did not activate the proliferation of fibroblasts might be a further advantage in treating lymphedema, where generally an increase in fibroblast proliferation and scar deposition in the tissues is consistent with the mechanism producing the disease [58].

The effectiveness of the NIR, dual wavelength emission in reducing inflammation, proved also by *in vitro* studies [21], could further inhibit the activation of fibroblasts.

Even if the data seem to exclude the possibility that the tested treatment protocol activates postmastectomy residual cancer cells, long-term effects have not yet been assessed (on going research) and cannot be excluded. Therefore, it is necessary to continue with research.

From the body of data in the literature it is also evident that the studies performed so far do not allow general conclusions, but rather show that the impact of laser radiation on the behavior of cancer cells strongly depends on type of cell and the treatment parameters used, first of all the wavelength, but also energy density and mode of administration.

Therefore, the use of laser therapy on oncologic patients requires extreme caution and further studies: each peculiar application needs a careful selection of the laser source and a rigorous evaluation of its effects on appropriate cellular and animal models.

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