Key words: Photomechanical stress, pulsed Nd:YAG laser, connective tissue, extracellular matrix, Hilterapia®

Effects of pulsed Nd: YAG laser at molecular and cellular level. A study on the basis of Hilterapia®.

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ABSTRACT

Lasers have been widely applied in many different fields of medicine, proving their effectiveness in the treatment of a wide range of diseases. In spite of the great amount of literature, it is difficult to understand the molecular and cellular mechanisms at the basis of the systemic effects induced by laser irradiation because of different kinds of laser used, operative conditions, variety of biological targets and responses.

The application of high power lasers in physiotherapy is quite recent. It is due to the development of instruments which allow the control of photothermal and photomechanical processes so as to obtain therapeutic effects without tissue damage. In particular, pulsed Nd:YAG laser has proved hisversatility and efficacy in the treatment of many different musculo-skeletal diseases and it is believed to have anti-inflammatory, anti-oedema, analgesic and also reparative effects.

The aim of the studies here presented was to contribute in understanding the molecular mechanisms and cellular processes at the basis of the systemic effects produced by pulsed Nd:YAG laser irradiation.

Owing to the lack of chromophores efficiently absorbing Nd:YAG radiation (wavelength 1064 nm) in cells and tissues, we hypothesized that, rather than photochemical processes, aspecific mechanisms probably due to combined photothermal and photomechanical interactions could be responsible for the above mentioned effects of pulsed Nd:YAG laser.

The finding suggestthat cells "sense" pulsed ND:YAG laser irradiation and respond to it through mechanotransduction machinery. We hypothesize that the interaction between tissue and laser radiation alters the mechanics of cell microenvironment, thus acting on the cells as a mechanical stress.

INTRODUCTION

Phototherapy, that is the use of light for the treatment and prevention of diseases, has been widely used from ancient times till now. From the time of the Pharaohs until relatively recent times the source of light was the sun.

The last century saw a rapid evolution in light sources, from inefficient arc lamps to lasers, which are the most advanced kind of light source.

The great advantage of the laser, in comparison with other sources, is the very high intensity and monochromaticity of the emitted radiation and also the possibility to be effectively focalized and coupled to optical fibres.

Lasers have been widely applied in many different fields of medicine, proving their effectiveness in the treatment of a wide range of diseases [1, 2].

In spite of the great amount of literature, the molecular and cellular mechanisms at the basis of the systemic effects induced by laser irradiation are mostly unknown. The studies on this subject are very difficult because of the numerous effects and the variety of biological responses, the kind of laser used, the operative conditions, the biological targets (different areas of the body, different tissues, different cell populations, etc.......). However, they are very important because the increase in knowledge can lead to a higher therapeutic efficacy by improvement of laser sources and treatment protocols.

Depending on interaction time and effective power density, three types of interactions between laser radiation and tissues can be distinguished: photochemical, photothermical and photomechanical[3]. The effects induced by low power lasers, the first to be applied in physiotherapy, are mostly due to photochemical processes. These occur when endogenous or exogenous chromophores introduced in the tissue absorb radiation of suitable wavelegth.

A chromophore molecule which absorbs a photon is converted in an excited state and may subsequently participate in a chemical reaction that leads to the final biological effect [4].

High power lasers have been used at first for tissue ablation and surgery, because they are able to produce important photothermal and photomechanical effects (stress waves) [3]. Their application in fields different from surgery, such as physiotherapy, is quite recent and it has been possible thanks to the development of laser systems with emission modalities which allow the control of photothermal and photomechanical processes, so as to obtain therapeutic effects without tissue damage.

In particular, pulsed Nd:YAG laser has proved his versatility and efficacy in the

treatment of many different musculo-skeletal diseases and it is believed to have anti-inflammatory, anti-oedema and analgesic effects [5].
In recent studies, even regenerative effects have been revealed. Indeed, it has been proved that treatment with pulsed Nd:YAG laser is effective in accelerating bone formation [6, 7], in inducing neo- chondrogenesis [8, 9] and in promoting cartilage matrix synthesis [10]. In tendon and ligament lesions, pulsed Nd:YAG irradiation has been demonstrated to improve the reparative process and to antagonize the possible fibrotic evolution [11]. In comparison with other laser sources, the advantages of the pulsed Nd:YAG laser consist in high power joined with a wavelength emission (1064 nm) which is weakly absorbed by cell and tissue chromophores, thus resulting in high penetrating capacity and ability to treat deep tissues and structures. Moreover, the photothermal effect can be controlled in terms of patient safety and comfort by modulating pulse intensity and frequency.
Seeing the apparent lack of chromophores efficiently absorbing 1064 nm radiation in cells and tissues, it is quite difficult to ascribe the above reported effects of the pulsed Nd:YAG laser to photochemical processes. It is intriguing enough to hypothesize a less specific mechanism of action, probably due to combined photothermal and photomechanical interactions, producing mechanical stress at cellular level.
Indeed, it is well known that loading/unloading and mechanical stress in general can deeply affect the behaviour of practically all the types of mammalian cells [12]. Endothelial, muscle, bone and cartilage cells, neurons and fibroblasts are very sensitive. These cell populations are involved in inflammation, pain, oedema and tissue repair. Therefore, a basic mechanism acting on them could explain the many different systemic effects produced by pulsed Nd:YAG laser.
The aim of the studies here described was to contribute in understanding the molecular mechanisms and cellular processes at the basis of the systemic effects produced by pulsed Nd:YAG laser irradiation.

MATERIALS AND METHODS

1) Cell Cultures

- Human fetal fibroblasts derived from fetuses 10-12 weeks old were maintained in primary culture in Coon's modified Ham's F12 medium containing 10% fetal calf serum, glutamine 2 mM, penicillin 100 U/ml and 100 µg/ml streptomicin. The cultures were incubated in humidified atmosphere at 37°C and 5% CO_2.
For the experiments here described, cell populations between passages 9 and 13 were used. Cells were seeded in 24 well plates at a density of $7x10^3$ cells/well.
- Human chondrocytes derived from adult hip joint cartilage (Promocell) were cultured in complete chondrocyte growth medium (Promocell), in humidified atmosphere at 37°C and 5% CO_2.
Cell populations between passages 4 and 7 were seeded in 24 well plates at a density of $12x10^3$ cells/well and used for the experiments.
- Human bone marrow cells were obtained from the iliac crest of normal donors marrow aspirates as previously described [13]. Briefly, whole bone marrow was collected and small aliquots were centrifuged for 10 minutes at 900g; the buffy-coat of white blood cells was recovered and plated in 75 cm^2 flasks ($1.6x10^5$ cells/cm^2) in Dulbecco's Minimal Essential Medium (with L-glutamine and 25 mM Hepes) with 50 µg/ml gentamycin, 10% FBS. At the first passage, the morphologically homogeneous population of human mesenchymal stem cells (HMSc) was analyzed for the expression of cell surface molecules using flow cytometry procedures: HMSc, recovered from flasks by trypsin-EDTA treatment and washed in HBSS and 10% FBS, were resuspended in CellWASH buffer (0.1% sodium azide in PBS) with 2% FBS, then incubated with specific conjugated monoclonal antibodies. The ability of HMSc to differentiate along osteogenic, adipogenic and chondrogenic lineages was assayed, as described previously by Pittenger et al. [14]. Cell populations between passages 2 and 4 were used.
Cells were seeded in 24 well plates at a density of $5x10^3$ cells/well.
- Microvascular endothelial cells (postcapillary venular endothelial cells, CVEc) were cultured in DMEM additioned with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 10 % bovine calf serum (BCS, Hyclone, Logan). The cultures were maintained at 37°C and 5% CO_2. Cells between passages 15 and 25, seeded at density $7x10^3$ cells/well, were used in the experiments.

2) Laser Treatment

Subconfluent cells were plated on glass coverslips in 24 multiwell plates and incubated for 24 h in complete medium, 37°C, 5% CO_2, to allow adherence.
In each multiwell plate only 6 wells contained cells. The surrounding wells were filled with black card to avoid light diffusion and reflection. Adherent cell monolayers were directly irradiated for 73 s by a pulsed Nd:YAG laser (ASA S.r.l., Vicenza, Italy), 1064 nm wavelength, 200 µs pulse duration, 10 Hz repetition rate, 458.65 mJ/cm^2 energy fluence. The treatment was repeated for three consecutive days in sterile conditions, under temperature control.
Measurements carried out by a pyroelectric detector indicated that, relatively to the impinging laser energy, 80% was either not absorbed or reflected, 20% was released to the medium and substrate on which the cell monolayer adhered. We did not find any measurable laser absorption by the cell layer alone, as could be expected because generally the cells do not contain endogenous chromophores absorbing at 1064nm, the emission wavelength of Nd:YAG laser.
Controls were prepared and kept in the same conditions used for the treated samples, except for laser irradiation.

3) Loading exposure

Cells were plated on glass coverslips and incubated for 24 h in complete medium, at 37°C and 5% CO_2, to allow adherence. 50 ml centrifuge tubes were partially filled with agarose gel (Merck),

on which the coverslips were placed with the surface perpendicular to the tube axis. To avoid shear stress, the tubes were then completely filled with culture medium.
The exposure to loading was accomplished by hypergravitational stress in a thermostated centrifuge (3-18K, Sigma Zentrifugen). The treatment consisted of 5 periods of 10 min exposure to 10xg (227 rpm) spaced with 10 min recovery periods at 1xg, at 37°C.
Controls were prepared as well, except for loading stimulation.

4) Immunofluorescence Microscopy

At the end of the experiments, the cells were fixed for 5 min in cold acetone, then washed in PBS. After blocking unspecific binding with PBS containing 3% BSA, the cells were incubated overnight at 4°C with the specific anti-human monoclonal antibodies α-tubulin, α and βactin, vimentin, α5β1, αvβ3 integrin, fibronectin, collagen type I and II, aggrecan, Sox 9, Runx 2, PPARγ (Chemicon). The cells were then incubated with anti-mouse IgG conjugated with fluorescein isothiocyanate (IgG-FITC) (Chemicon). Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an epifluorescence microscope (Nikon) at 100x magnification and imaged by a HiRes IV digital CCD camera (DTA).
Image analysis was performed by extracting, for each cell image, the region of interest (ROI) by appropriate software (Image Pro Plus). Then, the mean pixel value (16 bit, gray level) related to the mean fluorescence intensity and therefore to the target expression was calculated.

5) Gene profile analysis

Gene expression profiling was analysed by recovering total RNA from cell cultures: the cells were resuspended in 2X lysis buffer (Applied Biosystems) and then immediately frozen. Total RNA was extracted by using the ABI PRISM® 6100 platform and reverse transcribed using a high-capacity cDNA Archive Kit (Applied Biosystems), random hexamer primers, and the following thermal profile: 25°C for 10 min, 42°C for 1h and 95°C for 5 min. The cDNA was probed with TaqMan assays; results were subsequently analysed within the Panther database platform (www. pantherdb.org).

6) Data analysis

All the experiments were carried out in triplicate.
For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide, and the data were expressed as mean ± SD. Statistical significance was determined using a Student's test.
A p value lower than 0.05 was considered statistically significant.
For gene expression, 1-way ANOVA and Duncan test ($p < 0.001$) were applied.

RESULTS

1) Cytoskeleton

It is well known that cytoskeleton has a key role in the process of mechanotransduction at cellular level [15]. This means that cytoskeleton, together with other cell structures, is responsible for cell "sensitiveness" and, through the reorganization of its network, triggers cell "response" to external forces. Therefore, in order to evaluate whether the effect of pulsed Nd:YAG laser on cells could be attributed, at least in part, to mechanical stress, the expression of the major cytoskeleton proteins, i.e. tubulin, actin and vimentin, has been assessed both in endothelial and connective tissue cells. These proteins are responsible for the assembling of the three structural components of cytoskeleton: microtubules, microfilaments and intermediate filaments, respectively.
After the treatment, significant changes in cytoskeleton network have been observed. In general, all the cell populations studied showed a decrease in tubulin and vimentin expression ranging from 25 % to 35% ($p< 0{,}05$). In addition to the quantitative differences, interesting morphological alterations appeared (Figure 1 and 2).
In comparison with controls (Figure 1A), the microtubules decreased significantly in the peripheral area of the cells (Figures 1B and 1C), while in the perinuclear area the network often became even closer (Figure 1C). Sometimes, in endothelial cells, numerous microtubule organizing centres were found (Figure 1D).

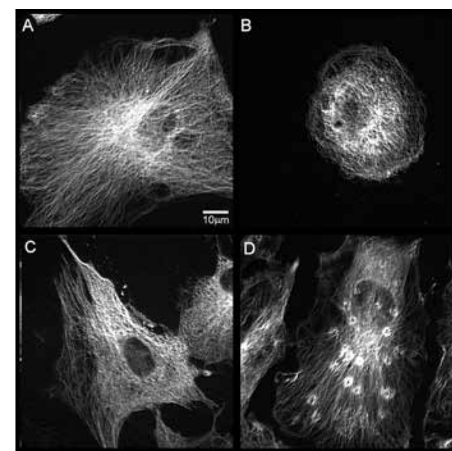

Figure 1: Immunofluorescence Microscopy.
Cells from connective tissues (A, B, C) stained with specific anti-human monoclonal antibodies in order to assay tubulin expression. In comparison with controls (A), the microtubules decrease significantly in the peripheral area of the cells exposed to pulsed Nd:YAG laser (B, C), while in the perinuclear area the network often becomes even closer (C). Numerous microtubule organizing centres can be found (see the endothelial cell in D).

The actin ring, which is a network of actin microfilaments subtending the plasma membrane, was clearly evident in control, non treated cells (Figures 2A and 2B), but quite completely disappeared in the treated ones (Figures 2C and 2D). Transcytoplasmatic stress fibres, long and parallel filaments which crossed the cell from end to end, strongly increased in cells of the connective tissues after the treatment (Figure 2C), while in endothelial cells actin was associated with membrane structures resembling ruffles (Figure 2D). It is known that stress fibres are related to the formation of focal adhesions, connection points between cell cytoskeleton and extracellular matrix components, through membrane proteins known as integrins [16]. Indeed, after laser treatment, changes in distribution of membrane integrins have been observed too (data not shown).

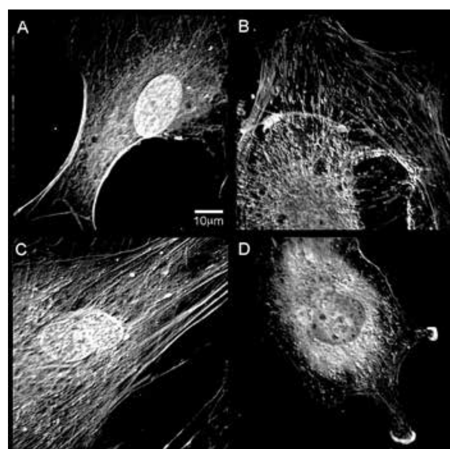

Figure 2: Immunofluorescence Microscopy. Human mesenchymal (A, C) and endothelial cells (B, D) stained with specific anti-human monoclonal antibodies in order to assay actin expression. The actin ring, which is a network of actin microfilaments subtending the plasma membrane, is clearly evident in control, non treated cells (A, B), but quite completely disappears in the treated ones (C, D). Transcytoplasmatic stress fibres strongly increase after treatment with pulsed Nd:YAG laser (C), while in endothelial cells actin is associated with membrane structures resembling ruffles (D).

2) Extracellular Matrix

The extracellular matrix (ECM), that is the extracellular part in animal tissues, provides support and anchorage for cells, regulates intercellular communication and cell's dynamic behavior, modulates the release of growth factors, acts as a compression buffer against mechanical stress [17]. The ECM, which is composed by glycoproteins and glycosaminoglycans, is produced by the cells themselves and its formation is essential for processes like growth, wound healing and tissue repair. The ECM is the defining feature of connective tissues, whose mechanical properties change rapidly, adapting to changes in load [18].

It has been widely demonstrated that mechanical factors regulates the production of ECM [19, 20]. Therefore, part of our study has been devoted to verify whether the PM stress produced by pulsed Nd:YAG laser irradiation was effective in affecting the production of ECM molecules by connective tissue cells. After laser treatment, we analyzed the expression of relevant ECM components in different cell types: collagen II and aggrecan in chondrocytes, collagen I and fibronectin in fibroblasts.

Fibronectin production was assessed also in endothelial cells, because of its importance in reparative processes.

In cells of the connective tissues, the treatment significantly enhanced the expression of the ECM molecules considered: in chondrocytes, collagen II (Figure 3) and aggrecan (Figure 4) increased of about 60% ($p< 0,0001$) and 70% (0,0002), respectively, in comparison with controls; in fibroblasts, collagen I increased of the 25% ($p< 0,01$), while fibronectin increased of the 30% ($p< 0,003$) and significantly changed its distribution: thick and ordered fascicles appeared around the cells, connecting them to the substrate. Similar changes in the distribution of fibronectin fascicles were found in endothelial cells: the randomly distributed network of fascicles observed in control cells was replaced, in treated cells, by fascicles ordered in parallel (Figure 5). Besides, the treatment increased both cell spreading and cell-cell interactions (data not shown).

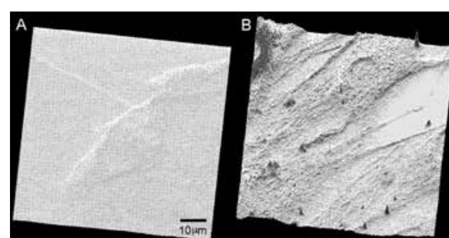

Figure 3: Immunofluorescence Microscopy. Human chondrocytes stained with specific anti-human monoclonal antibodies in order to assay collagen II expression. In comparison with controls (A), the samples treated with pulsed Nd:YAG laser (B) show an increase of about 60% in collagen II expression.

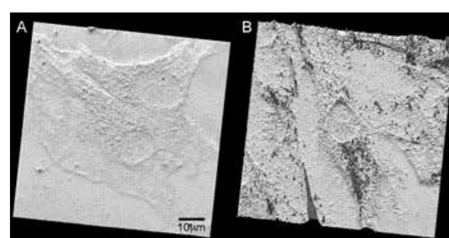

Figure 4: Immunofluorescence Microscopy. Human chondrocytes stained with specific anti-human monoclonal antibodies in order to assay aggrecan expression. In comparison with controls (A), the samples treated with pulsed Nd:YAG laser (B) show an increase of about 70% in aggrecan expression.

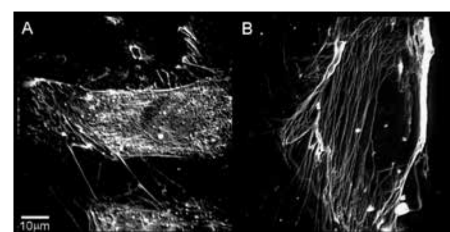

Figure 5: Immunofluorescence Microscopy. Endothelial cells stained with specific anti-human monoclonal antibodies in order to assay fibronectin expression. The randomly distributed network of fibronectin fascicles observed in control cells (A) was replaced, in treated cells (B), by fascicles ordered in parallel.

3) Cell Differentiation

Cellular differentiation is the process by which a pluripotent cell acquires a defined "cell type", this means that the cell becomes specialized in order to perform a specific function, as in the case of a neuron, a blood or a bone cell [21]. During differentiation, both genotypic and phenotypic expression of a cell may change dramatically. A marker of differentiation is a molecule whose expression is particularly intense in a specific type of cell, in one or more differentiation steps. Therefore, the monitoring of one or more differentiation markers in a cell is useful to recognize the type and differentiation degree of the cell itself.

Physical stimuli, in particular the mechanical ones, but also gravitational stress, electromagnetic fields, etc..., are known to affect cell differentiation [22, 23, 24].

In order to assess the effect of pulsed Nd:YAG laser irradiation on cell differentiation, we carried out experiments with HMSc able to differentiate through the osteoblastic, chondrocytic and adipocytic pathways.

After the laser treatment, we analysed HMSc by immunofluorescence microscopy in order to evaluate the expression of Sox 9, Runx 2 and PPARγ, major differentiation markers of osteoblastogenesis, chondrogenesis and adipogenesis, respectively.

The results showed that in control samples all the considered differentiation markers were weakly expressed (Figures 6A, 7A, 8A), as expected in undifferentiated cells. In treated samples we found a significant increase in expression of Sox

9 (Figure 6B) and Runx 2 (Figure 7B), transcription factors which have a key role in the activation of chondrocyte-specific [25] and osteoblast-specific [26] genetic programs, respectively. Transcription factors are proteins that control the transcription of genetic information: Sox 9 and Runx 2 control the expression of genes involved in chondrocyte and osteoblast differentiation. Contrariwise, in the treated cells the expression of the nuclear receptor PPARγ (Figure 8B), which has been identified as a dominant regulator of adipogenesis [27, 28], was even lower than in the control ones.

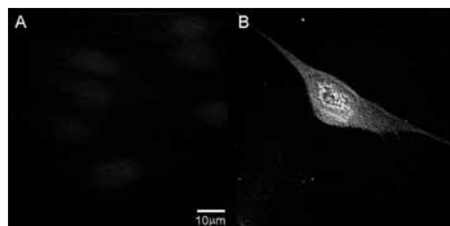

Figure 6: Immunofluorescence Microscopy. HMSc stained with specific anti-human monoclonal antibodies in order to assay Sox 9 expression. The protein is weakly expressed in control cells (A) but strongly increases in cells exposed to pulsed Nd:YAG laser irradiation (B).

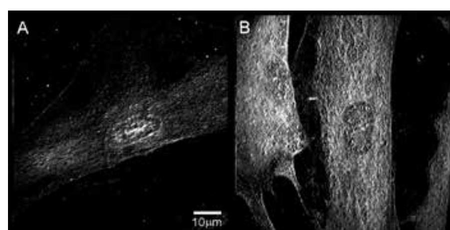

Figure 7: Immunofluorescence Microscopy. HMSc stained with specific anti-human monoclonal antibodies in order to assay Runx 2 expression. The protein is weakly expressed in control cells (A) but strongly increases in cells exposed to pulsed Nd:YAG laser irradiation (B).

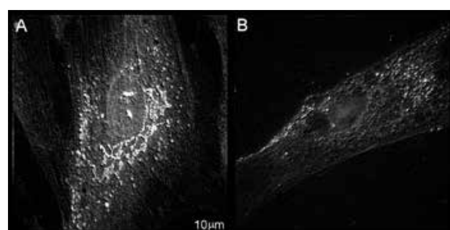

Figure 8: Immunofluorescence Microscopy. HMSc stained with specific anti-human monoclonal antibodies in order to assay PPAR expression. The protein is weakly expressed in control cells (A) and furtherly decreases in cells exposed to pulsed Nd:YAG laser irradiation (B).

4) Genetic expression

In order to verify whether the changes in phenotypic expression of connective tissue cells induced by pulsed Nd:YAG laser had a match at genotypic level, we analysed gene expression profile in connective tissue cells exposed to the laser irradiation. Moreover, we compared the gene expression profile induced by pulsed Nd:YAG laser with the one induced by loading, because important studies demonstrated that alterations in the cellular force balance due to mechanical stress can influence gene expression in the nucleus [29]. Gene expression profiling revealed that in laser-treated cells many important pathways were overexpressed (Figure 9). Among them, the "general transcription regulation pathway" and the "cytoskeletal regulation by Rho GTPase pathway", in agreement with the changes observed at phenotypic level in protein expression and cytoskeleton organization.

Considering the expression of single genes, we found a significant upregulation of the genes MEN1, NF1 and GLI1 ($p<0.05$), which are involved in the commitment of multipotential mesenchymal stem cells into the osteoblast lineage [30], in bone development and remodelling [31], in the control of chondrocyte and osteoblast differentiation [32], respectively. This result is in agreement with the increase in Sox 9 and Runx 2 expression found at phenotypic level. Contrariwise, the gene encoding for PPARγ resulted strongly downregulated, in agreement with the observed decrease in protein expression.

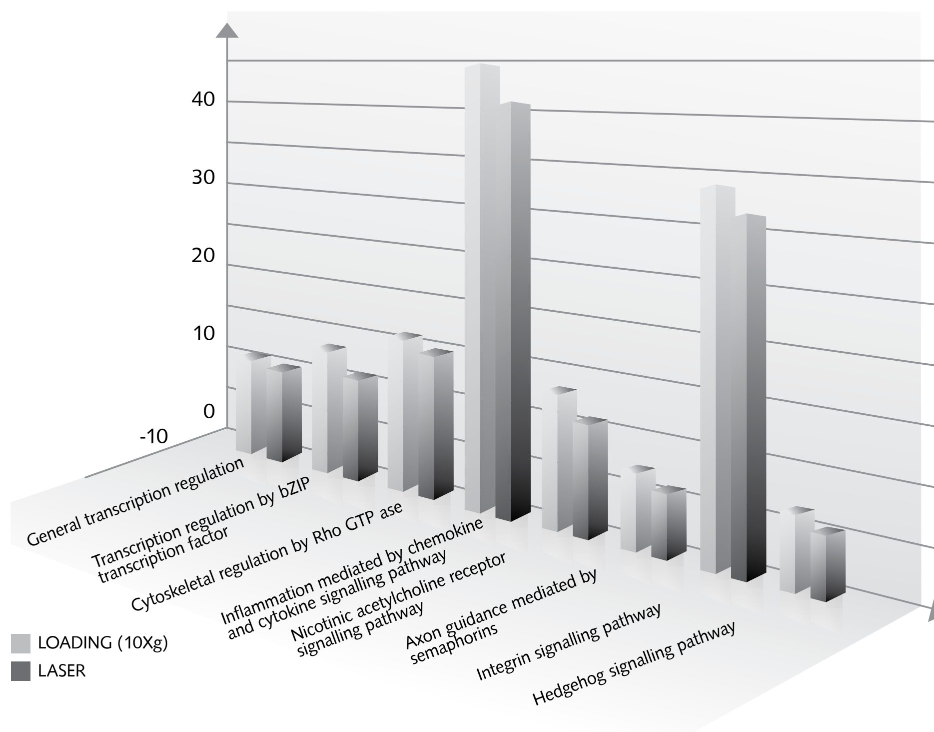

Figure 9: Gene expression profiling reveals that in laser-treated cells many important pathways are overexpressed in comparison with controls.

Interestingly, we obtained similar results in cells exposed to overload (Figure 9).

DISCUSSION

The results clearly demonstrate that the treatment with pulsed Nd:YAG laser affected cytoskeleton organization in all the cell types (endothelial, mesenchymal, connective tissue cells) which have been investigated in our experiments. The rearrangement concerned both the distribution of the three major cytoskeleton components (microtubules,

microfilaments, intermediate filaments) and the expression intensity of the proteins responsible for their assembling, tubulin, actin and vimentin, respectively. Besides the changes in cytoskeleton network, differences in distribution of membrane integrins were found. These findings are consistent with the hypothesis that pulsed Nd:YAG laser radiation induces mechanical stress on cells, likely changing the mechanics of the cell microenvironment. Indeed, very similar effects have been found in the same cell types exposed to loading [33], which is a kind of mechanical stress normally occurring in physiological conditions. Mechanical stress is ever present in the cellular environment, whether through external forces that are applied to tissues or endogenous forces that are generated within the cytoskeleton. Over the past decade, in vitro studies have demonstrated that cells may sense mechanical stress through changes in the balance of forces that are transmitted across transmembrane adhesion receptors (integrins) which link the cytoskeleton to the ECM and to other cells. These changes, in turn, alter the ECM mechanics, cytoskeletal organization, cell shape and nuclear scaffold [34]. Actually, the more advanced research deals with possible mechanisms by which applied forces, cell-generated forces and changes in substrate mechanics can exert changes in cell function through common mechanotransduction machinery [35].

Mechanotransduction, that is the process of translating external forces acting on a cell into a biological response, involves the ECM, integrins, calcium channels, guanosine triphosphatases (GTPases), adenylate cyclase, phospholipase C (PLC) and mitogen-activated protein kinases (MAPKs), all of which play important roles in early signalling triggered by mechanical stress. Cytoskeleton has a central role in mechanotransduction, orchestrating multiple signal pathways [15]. Moreover, it has been discovered that many of the signalling molecules that are turned on by integrins are not floating around in membrane lipid bilayer; rather, they are immobilized on the cytoskeleton structures and specifically concentrated within the anchoring sites. This suggests that the mechanism of signal integration is based on the spatial organization of signaling molecules associated with cytoskeleton elements within the anchoring sites. [36, 37]. For example, it has been demonstrated that mechanical stress enhances the proliferation and metabolic activity of osteoblast-like cells through the essential role of microtubules and microfilaments, respectively [38].

Further support to the hypothesis that cells sense pulsed Nd:YAG laser irradiation as a mechanical stress, and respond to it by activation of mechanotransduction machinery, was supplied from the results of our investigation on the effect of laser treatment on the production of ECM.

The exposure to pulsed Nd:YAG laser stimulated the synthesis of ECM molecules in connective tissue cells. In particular, enhanced expression of collagen II and aggrecan was observed in chondrocytes, while collagen I and fibronectin increased in fibroblasts. These findings are in agreement with many reports of other authors describing fibroblast and chondrocyte activation and control of ECM production by different kinds of mechanical stress [39, 40, 41]. Moreover, in a previous study, we demonstrated that the effects of pulsed Nd:YAG laser on ECM production by connective tissue cells are very similar to the ones induced by loading [33], which is a physiological stimulus because connective tissues have antigravitational function.

Interestingly, we observed that pulsed Nd:YAG laser radiation affected also fibronectin distribution and the assembling of fibronectin fibrils. In fact, in treated fibroblasts, thick bundles of fibronectin fibrils appeared. The same effect was observed by other authors in fibroblasts cultured in hypergravity, that is in loading conditions [42]. In treated endothelial cells, a highly organized array of fibrils replaced the randomly distributed network showed by control samples. Fibronectin, which is synthesized by all the cell kinds (tumor cells excluded), is particularly important in ECM organization: it is able to bind collagen and cell membrane, thus connecting ECM macromolecules each other and with cell surface. It is involved in cell growth and differentiation, cell adhesion and migration. In particular, it mediates cell adhesion preventing cell migration [43]. The effectiveness of pulsed Nd:YAG laser in controlling ECM production and arrangement of fibronectin molecules could be of consequence in wound healing, tissue repair and regeneration. On the other hand, our very preliminar experiments (not yet published data) showed that in unloading conditions, which are known to delay wound healing, both fibroblasts and endothelial cells produce an impressive quantity of fibronectin. It forms an intricate network which catches cells, thus increasing their adhesion and hampering migration. In particular, the fibronectin rearrangement induced by pulsed Nd:YAG laser could affect adhesion, spreading and migration of endothelial cells and, consequently, angiogenesis and endothelium permeability. These effects on endothelial cell function could be at the basis of the irradiation-mediated improvement in tissue repair and oedema resolution.

The efficacy of pulsed Nd:YAG laser in favouring tissue repair processes could be partly due to its ability in inducing specific cell differentiation patterns. In fact, our experiments demonstrate that pulsed Nd:YAG laser irradiation can also affect cell differentiation, which is one of the three major genetic programs in cells, together with growth and apoptosis.

Indeed, laser treatment was effective in inducing specific differentiation patterns in HMSc, without the cooperation of added biochemical factors. In comparison with controls, we found in treated HMSc a significant increase in expression of Sox 9 and Runx 2, major markers of chondrogenesis and osteoblastogenesis, respectively, while the expression of PPARγ, specific marker of adipogenesis, decreased. We demonstrated that the

same differentiation patterns were induced in HMSc by loading, while unloading enhanced the expression of PPARγ, and depressed that of Sox 9 and Runx 2 [44]. The close likeness between the effects of pulsed Nd:YAG laser and loading on HMSc differentiation together with the fact that laser treatment promoted chondrogenesis and osteoblastogenesis, maturation processes of cells belonging to tissues with antigravitational function, strengthen the hypothesis that cell response to the stimulation by pulsed Nd:YAG laser occurs via mechanotransduction machinery.
Finally, our experiments demonstrated that pulsed Nd:YAG laser can affect gene expression. A close relationship between phenotypic and genotypic expression in connective tissue cells exposed to pulsed Nd:YAG laser irradiation has been found. In fact, in comparison with controls, important pathways involved in cytoskeleton regulation and integrin signalling, as well as genes involved in chondrogenesis and osteoblastogenesis resulted overexpressed. Moreover, once again the changes produced in gene expression profile by pulsed Nd:YAG laser resulted nearly the ones produced by loading.

CONCLUSIONS

In conclusion, the results of our experiments demonstrate that pulsed Nd:YAG laser may deeply affect cell behaviour.
The exposure to laser radiation induces:

- reorganization of cytoskeleton network, observed in all the cell populations studied;
- increase in production of ECM by cells of connective tissues, which could be of consequence in tissue repair and regeneration;
- regulation of fibronectin production/ distribution and fibril arrangement, which may strongly affect endothelial cell function;
- induction of specific differentiation patterns in HMSc, which undertake maturation pathways typical of the specialized cellular elements belonging to tissues with antigravitational function;
- changes in gene expression profile, in agreement with the changed phenotypic expression.

These effects, ocurring at molecular and cellular level, are consistent with the systemic ones observed in patients treated with pulsed Nd:YAG laser, therefore they can be regarded as the cellular response at the basis of Hilterapia®.
Moreover, the findings show that the irradiation with pulsed Nd:YAG laser induced in cells of the connective tissues as well as in HSMc and endothelial cells a biological response very close to the one produced by loading. Contrariwise, unloading conditions often induce opposite effects. The findings suggest that cells "sense" pulsed ND:YAG laser irradiation as a mechanical stress and respond to it through mechanotransduction machinery. We hypothesize that the interaction tissue- laser radiation and consequent energy release, alters the mechanics of cell microenvironment thus acting on the cell as a mechanical stress.

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