Key words: Hilterapia®, pulsed Nd:YAG laser, photomechanical effect, tissue repair, extracellular matrix

Relationship between cellular and systemic effects of pulsed Nd:YAG laser.

Cialdai F[1]., Monici M[2].

1) ASAcampus Joint Laboratory, Dept. Clinical Physiopathology, Florence University, Florence, Italy.
2) ASAcampus Joint Laboratory, ASA Res. Div., Dept. Clinical Physiopathology, Florence University, Florence, Italy.

ABSTRACT

Notwithstanding the wide diffusion of lasertherapy in clinics and numerous studies reported in literature, molecular mechanisms of interaction between laser and tissues are not well understood.

The analysis of biological effects induced by laser radiation is rather complicated due to the wide possibility of setting instruments, the variability of applied protocols and the differences in treated tissues.

In this review, we describe our studies on the cellular and molecular mechanisms at the basis of the systemic effects produced by treatment with pulsed Nd:YAG laser, that is known as Hilterapia.

Starting from studies on photothermal effects, the hypothesis is that this type of laser cause an indirect photomechanical effect. The heat produced by transfer of radiation energy to the irradiated volume, diffuse into surrounding tissues, inducing temperature gradients which result in transitory modifications of mechanical-elastic properties of the extracellular microenvironment, thus changing mechanical forces acting on cells.

Considering these studies and knowing the key role of the extracellular matrix, not only as a structural support but also in maintaining tissue homeostasis, our experiments focused on the analysis of extracellular matrix molecules and cytoskeleton behavior, responsible of contact between cell and matrix and considered the best candidate to act as a mechanotrasducer.

The data obtained have shown, in laser-treated cells, an increase in production of ECM molecules, such as aggrecan, collagen I and II, and a reorganization of microtubules and actin microfilaments network. It is well know that similar effects are obtained when cells are subjected to mechanical stress. Our data on absorption of Nd:YAG pulses by matrix components (proteins and polysaccharides) suggest that Nd:YAG pulses principally interact with the extracellular matrix, whose transitory deformation applies a mechanical stress to the cells.

We then focused on the effect of pulsed Nd:YAG on endothelial function and tissue repair processes. In treated endothelial cells and fibroblasts, key elements of angiogenesis and tissue repair, we found overexpression of genes involved in the chemokine-mediated inflammatory pathways. Moreover, the treatment promoted the formation of ordered endothelial monolayers as well as ordered fibronectin fibril assembling. The findings indicate that treatment with Nd:YAG pulses has a stimulatory effect in the acute phase of inflammation and significant effect on the remodelling phase of tissue repair, also considering the important role that fibronectin plays in tissue structure regeneration. Therefore we can support that Hilterapia can efficaciously promote tissue repair processes.

INTRODUCTION

In spite of a wide application in clinics, many studies and a great body of literature, the molecular mechanisms of the interaction between laser and tissues, and the consequent cellular response, are still not completely known. They are object of current and future research in the field of laser biomedical application.

Unfortunately, not always scientifically rigorous studies, a limited knowledge of the molecular and cellular mechanisms underlying the biological effects of laser and, in turn, the systemic effects of laser therapy gave rise to contrasting results, unsupported hypotheses and unconvincing theories.

The studies on laser biological effects are very difficult due to the variety of biological responses that depend on laser source (wavelength, continuous/pulsed mode), operative conditions (fluence, time of exposure, etc..) and biological substrate considered (the body area, the tissue, the cell type etc.). Nevertheless, they are of critical importance for correct clinical applications, to improve instruments and protocols, to increase therapeutical effectiveness.

When the light interacts with a biological tissue a small part of radiation (~3-5%) is specular reflected, the most part propagates within the tissue and it is partially diffused (scattering) and

partially absorbed by molecules which are generically indicated as "chromophores", because they are responsible for tissue colour. Each chromophore absorbs specific wavelengths. Each tissue is characterized by a different qualitative/ quantitative chromophore composition, which determines the response of the tissue itself to different laser radiations (that means different wavelengths). The light energy absorption is necessary to allow any effect at tissue level [1].

When laser radiation propagates through a biological tissue, several effects may be observed. They depend on tissue properties, radiation source characteristics and a combination of both [2,3]. As above explained, the tissue properties significantly affect the amount of light that is absorbed and its propagation length inside the tissue itself [4]. The effects induced in a biological tissue by laser radiation are commonly classified in: photochemical, photothermal and photomechanical, or a combination of them. A suitable choice of laser source and setting parameters, on the basis of the knowledge of tissue characteristics, such as its optical, thermal and mechanical properties, allows to preferentially induce a particular effect, which dominates the others. In biomedicine this means to have an opportunity to select specific applications both for therapeutic and diagnostic purposes.

Photochemical effects take place when the absorbed light induces chemical reactions in the tissue. Photochemical interaction is more selective than photothermal or photomechanical interactions, because it is due to the absorption of radiation by specific chromophores, such as mitochondrial cytochromes involved in the respiratory chain [5].

In non-surgical laser applications, the photochemical effects have been, perhaps, the most studied, also due to the wide diffusion of He-Ne lasers (red wavelengths of the visible spectrum) and phototherapy, which is based on the photochemical interaction between light and a specific chromophore, often an exogenous photosensitizer [6].

Photothermal effects occur when the energy associated with radiation is converted into heat. At tissue level, the results of photothermal interactions depend on the thermal relaxation time of the tissue itself and on the interaction time. Mechanical effects can be considered secondary effects of the photothermal interaction: heating induces mechanical forces which can modify cell morphology and tissue texture. These changes can also lead to tissue damage and even to tissue disruption, this is the case of surgical applications. Till now, photothermal interactions have been studied from the points of view concerning surgical application or laser security, in the former case to obtain tissue ablation, in the latter to avoid undesirable tissue damages. In physiatrics, rehabilitation and sport medicine, peripheral vasodilatation has been considered the principal biological response induced by photothermal interaction. This effect on peripheral circulation is surely important, but recent research has demonstrated that complex biological responses can be induced at molecular and cellular level by photothermal interaction and it is reductive to consider the photothermal effects only in terms of vasodilatation due to an increase in tissue temperature.

A turning point in considering photothermal interactions from a different point of view there has been with the introduction of very short (from femto- to nanoseconds) laser pulses for biotechnological applications (drug delivery, cellular and intracellular surgery) and with the application of pulsed lasers in medical fields other than surgery, such as physiotherapy, physiatrics, etc.....

Moreover, in the last years, also thanks to significant improvements in experimental technologies and analytical techniques, our knowledge on molecular and cellular mechanisms which regulate tissue homeostasis, the interaction between cells and extracellular environment, the tissue repair processes occurring in damaged tissues has been strongly increasing. This opens the way to a better understanding of the molecular and cellular mechanisms underlying the biological response induced by laser-tissue interaction.

Here we review our studies on the cellular and molecular mechanisms at the basis of the systemic effects produced by treatment with pulsed Nd:YAG laser, that is known as Hilterapia.

PULSED ND:YAG LASER AND TISSUE REPAIR: MOLECULAR AND CELLULAR MECHANISMS

Our body is mainly composed by water (70%) and organic compounds (30 %), such as proteins, lipids, sugars, etc. Water highly absorbs infrared radiation (IR), among the organic compounds some chromophores (aminoacids, pyridine coenzymes, haemoglobin, melanin, etc.....) absorb in the UV-visible range. In the near infrared (NI) there is a range of wavelengths (800-1100), known as "therapeutical window" and characterized by low absorption. Therefore, in this interval, scattering strongly affects the propagation of radiation and photons can diffuse far from the point of incidence. The pulsed radiation emitted by Nd:YAG laser (emission wavelength 1064 nm) fits in this "window" and, also thanks to the high power of the pulses, can propagate in depth. Moreover, the duty cycle of pulsed Nd:YAG laser used for Hilterapia allows to obtain photothermal effects which can raise a biological response without tissue denaturation, damage or disruption.

In order to analyse photothermal effects and subsequent mechanical stress in biotissues, the interaction time and thermal relaxation of the tissue should be considered. If the laser radiation is delivered to the tissue in a very short time, in comparison with the thermal relaxation, the resulting heating is

confined in the volume of tissue in which laser light is absorbed and may induce direct photomechanical effects, generally with consequent tissue damage or disruption. For biological tissues, a thermal relaxation time of about 1 µs is reported [2]. The duration of Nd:YAG pulses used in our experiments and also in clinical applications (Hilterapia) is about 200 µs, therefore it cannot induce direct photomechanical effect.

When pulse duration is longer than the relaxation time, as in the case we are considering, heat can diffuse in the surrounding tissues, inducing temperature gradients with predominant photothermal modifications and subsequent transitory mechanical deformations of the biological micro-environment. This kind of photomechanical stress can be considered a secondary effect of the heating and therefore defined "indirect photomechanical effect" [7].

The three-dimensional architecture of a biological tissue can be considered as a network of polymeric fibers, the extracellular matrix (ECM), in which cells are embedded. ECM is essentially constituted by polysaccharides and proteins. Collagens have a key role in ECM [8]: each tissue shows a different, often specific, qualitative/quantitative collagen content which strongly affects tissue properties and function.

For a long time, only structural functions have been ascribed to the ECM, merely considered the medium surrounding the cells. More recently, it has been demonstrated that ECM has a key role in tissue homeostasis and, beyond the structural functions, ECM has many other functions which concern the regulation of cell behaviour. Living cells are hard-wired by a continuous series of protein filaments and tubules, autoassembled to form a network, the cytoskeleton (CSK), which is anchored both to nuclear- and plasma-membrane. The anchoring points of CSK to the plasma membrane are constituted by clusters of membrane proteins, known as integrins, which on the inner side bind the CSK and on the outer side bind ECM fibers or the membrane integrins of neighbouring cells, thus originating a continuous network, which constitutes the weft of the tissues [9]. In the tissues, the ECM undergoes continuous remodelling, in particular during development, angiogenesis, wound healing and all the tissue repair processes. The ECM acts as a reservoir of biochemical factors, i.e. cell growth factors, and most important, provides to the cells geometrical, topographical and mechanical constraints, which are relevant signals in the regulation of cell behaviour [10]. The ECM is produced by cells, with qualitative and quantitative characteristics which strongly depend on the signals the cells receive from the microenvironment. Therefore, through ECM remodelling, tissue properties continuously change and adapt in order to maintain homeostasis and optimize function [11].

DISCUSSION

In our earlier studies on the effects induced by pulsed Nd:YAG laser radiation in connective tissue cells (fibroblasts, chondrocyites and human mesenchymal stem cells (hMSC) able to differentiate through the osteoblastic and chondrocytic pathways) we found that, in comparison with non treated controls, the cells exposed to the treatment increased the production of ECM molecules, such as collagen I (+ 30%), collagen II (+ 90%), aggrecan (+ 70%) [12,13]. Moreover, particularly in hMSCs, we observed a strong increase in expression of genes involved in differentiation of cells belonging to specialized connective tissues (bone and cartilage), while the expression of genes involved in adipogenesis slightly decreased [14] (Fig. 1).

Because it is known that mechanical stress stimulates the production of ECM molecules, favours the osteogenesis and chondrogenesis and inhibits adipogenesis, in order to better understand the processes occurring in laser-treated cells, we repeated our experiments comparing three types of samples: non treated cells, cells exposed to pulsed Nd:YAG laser, cells exposed to loading. The results showed that the exposure to 73 sec of irradiation by pulsed Nd:YAG laser (1064 nm wavelength, 200 µs pulse duration, 10 Hz repetition rate, 458.65 mJ/cm^2 energy fluence) for 3 consecutive days produced the same effects of exposure (5 cycles of 10 min each) to 10xg in terms of increase in production of ECM molecules and changes in expression of genes involved in cell differentiation [13,14]. These findings induced us to hypothesize that cells recognize pulsed Nd:YAG irradiation as a mechanical stress. In order to verify this hypothesis, we analysed CSK of laser-treated cells, because it is widely known that CSK is sensitive to mechanical stress and it is considered the best candidate to act as a mechanotransducer [15]. As expected, in laser-treated cells, we found that CSK underwent a reorganization both in microtubules and actin microfilament network. Moreover, the distribution of integrins, which anchor the CSK to the cell membrane, changed accordingly [12, 13].

On the basis of our results and considering that: i) at the wavelength emission of Nd:YAG laser tissue absorption is quite low, in particular by the cellular component; ii) when IR radiation is absorbed, the energy transferred from photons to chromophores (absorbing molecules) generally causes vibrational phenomena; iii) it has been already demonstrated that photothermal effects produced by laser pulses >1µs can induce modifications in the architecture of collagen and other macromolecular networks [16]; we advanced the hypothesis that Nd:YAG pulses principally interact with ECM, producing local temperature gradients and thus inducing transitory photothermal deformations of the extracellular microenvironment which, in turn, applies a mechanical stress to the cells through the integrin clusters at the anchoring points.

In order to verify the absorption of Nd:YAG laser pulses by different tissue components and biological substrates, we measured the attenuation of laser radiation passing through cell layers, cell culture media, artificial models of ECM constituted by biocompatible hydrogels of polysaccharides and proteic biomatrices. It is noticeable that polysaccharides and proteins are the principal components of ECM and their artificial matrices are already used in tissue engineering and to favour tissue repair.

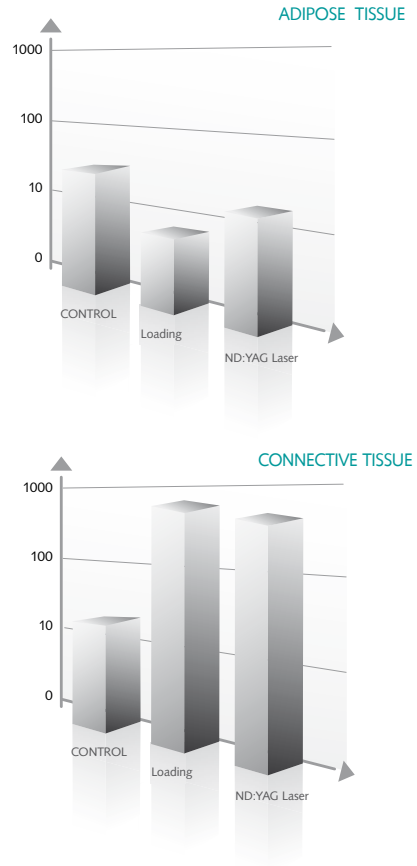


Fig. 1 is reported in arbitrary units the gene expression of pathways involved in differentiation of adipose tissue cells and connective tissue cells. Both loading and treatment with pulsed Nd:YAG laser induce a slight decrease in expression of genes involved in adipogenesis and a very strong increase in expression of genes involved in differentiation of cells belonging to connective tissues. It is noteworthy that connective tissues have mechanical and antigravitational functions.

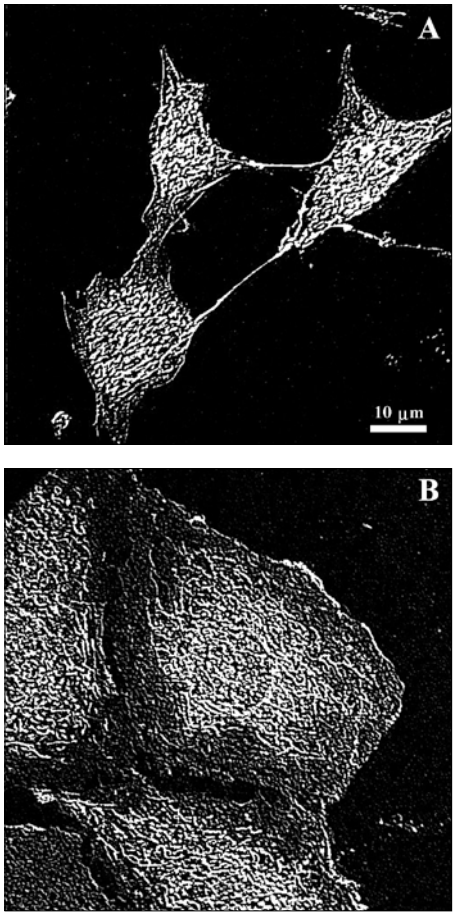
The measurements, carried out by a pyroelectric detector, indicated that, relatively to the impinging laser energy, there was not measurable radiation absorption by cell monolayers; in the case of culture cells, laser radiation passing through the culture medium and the substrate on which the cells adhered was attenuated of about 20%; the attenuation was up to 25% and 30% in polysaccharide and proteic matrices (thickness 3 mm, water content from 50% to 70%), respectively. These data proved that Nd:YAG pulses are absorbed by components of the extracellular environment, thus supporting our hypothesis.

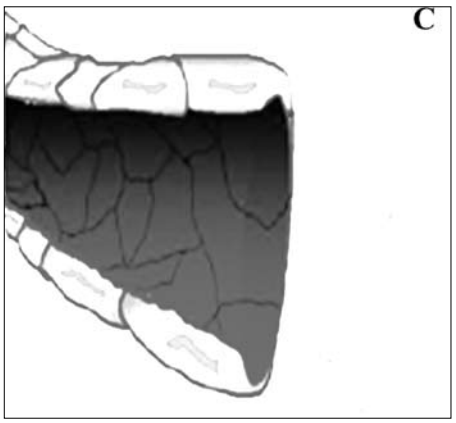
Further steps in our studies have been made investigating the effects of pulsed Nd:YAG laser on endothelial function and tissue repair processes.

A proper circulation and endothelial function is needed for tissue homeostasis. When a tissue is damaged, neoangiogenesis is critical for repair processes, because new vessels provide nutrients to support the cells responsible for tissue remodelling and facilitate the clearance of debris [17]. Altered endothelial function is strongly related to inflammation and oedema [18]. Because laser is often applied in clinics for treating both inflammation and oedema, we studied the effect of pulsed Nd:YAG laser on endothelial cells, which form the inner layer of the vessel wall. We found that Nd:YAG treatment induced cell spreading and favoured the formation of an ordered endothelial monolayer, while non treated cells, remained randomly distributed [19] (Fig. 2). This effect could be of consequence in improving endothelial function and promoting neoangiogenesis.

Tissue repair is a complex series of events, which has great importance for

Fig. 2





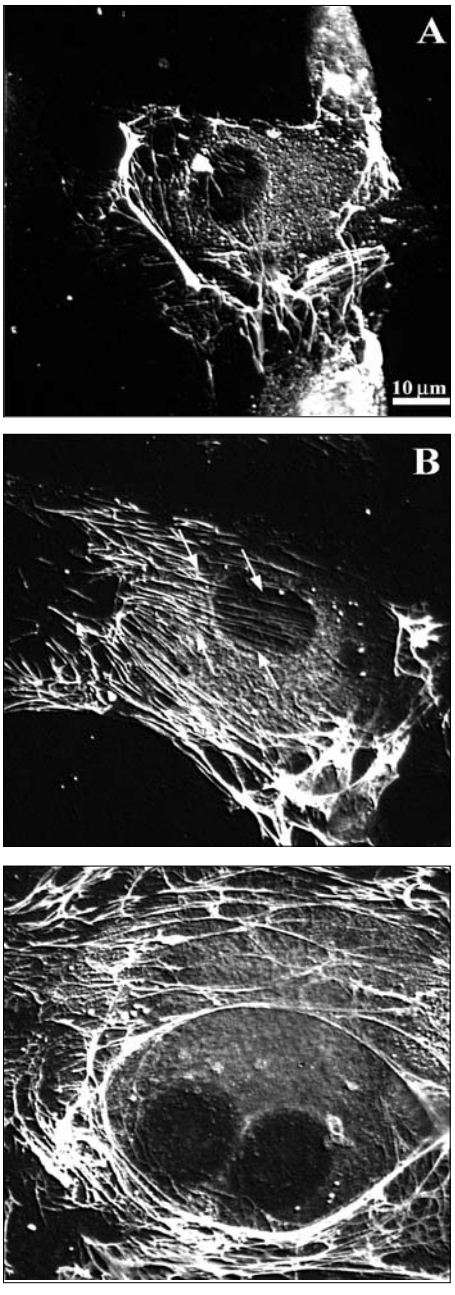
Figs. 2A and **2B** show endothelial cell cultures treated and non-treated (control) with pulsed Nd:YAG laser, respectively. The treatment favours cell spreading and the formation of an ordered monolayer while non-treated cells are randomly distributed. The drawing in **Fig. 2C** schematically represents the formation of the endothelial monolayer in a new vessel.

organism survival. Following tissue injury, an inflammatory phase occurs, which is needed to trigger repair by recruitment and activation of white blood cells, fibroblasts and endothelial cells in the damaged area. During repair, fibroblasts and endothelial cells synergetically act for sequentially remodelling a series of ECMs of increasing complexity and order. In this process of tissue structure restoration fibronectin (FN) has a key importance. FN is produced by cells, in particular fibroblasts and endothelial cells, and secreted outside. Then it autoassembles to form a network of insoluble fibrils which become part of the ECM. FN strongly affects ECM composition, because its fibrils constitute a template for collagen assembling. Moreover, FN connects ECM components each other and with cell surface, thus contributing to the regulation of many cell functions, as growth and differentiation, adhesion and migration [20]., which are involved in tissue repair.

We carried out experiments aimed at discovering and understanding whether pulsed Nd:YAG laser treatment could affect tissue repair, in particular FN production and assembly. The results demonstrated that the treatment increased fortyfold the expression of genes involved in the chemokine-mediated inflammation pathway, which has an important role in triggering the remodelling phase of repair by attracting fibroblasts and endothelial cells in the damaged area. Moreover, in treated fibroblasts and endothelial cells a slight increase in FN production was observed. Interestingly, the

treatment induced the formation of very ordered arrays of FN fibrils [12,19] (Fig. 3). Therefore, pulsed Nd:YAG irradiation affected both FN production and assembly. These data suggest that Hilterapy could have significant effects on the remodelling phase of tissue repair.

Fig. 3



Figs. 3 Fibronectin expression revealed by immunofluorescence microscopy in non-treated (control, 3A) and treated (3B and 3C) endothelial cells. In treated cells, the formation of parallel fibronectin fibrils can be observed (see arrows).

Summarizing, pulsed Nd:YAG laser treatment seems to have a stimulating effect in the acute phase of inflammation and to favour a proper assembling of FN fibrils, which is very important for the formation of a functional tissue.

In conclusion, our results demonstrate that the effects of Nd:YAG pulses on culture cells are very similar to those induced by mechanical stress, thus supporting our hypothesis that Nd:YAG pulses produce in the tissue photothermally-induced transitory mechanical deformation of the extracellular microenvironment that is conveyed at cellular level through the ECM-integrins-CSK network.

The findings that Nd:YAG pulses favour ECM production and proper fibronectin assembling, differentiation of connective tissue cells, formation of ordered endothelial monolayers indicates that pulsed Nd:YAG laser treatment can efficaciously promote tissue repair processes.

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