Key words: MLS laser therapy, cell differentiation, myoblasts, extracellular matrix

Effects of MLS laser on myoblast cell line C2C12.

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ABSTRACT

Laser is widely used in many medical fields and its effects are reported by several studies in literature. Very important are the applications in sports medicine, physical medicine and rehabilitation, based on the analgesic, anti-inflammatory and anti-oedema effects of laser therapy, as well as the stimulating action on tissue repair processes. In our study, we analyzed the effects of an advanced laser system, the Multiwave Locked System (MLS), on myoblasts in order to evaluate the effectiveness of this laser in promoting recovery of damaged muscle tissue. The MLS device consists of two synchronized diodes emitting at 808 and 905 nm, respectively. C2C12 murine myoblasts cell line was used as experimental model since it is a widely accepted model in muscle cells behavior studies.

Viability and proliferation was assessed after a single treatment as well as after 4 consecutive treatment (1 treatment/day). No significant changes were observed in viability, while proliferation decreased after 4 treatments. Moreover, we found an increased expression of MyoD, a key factor in myoblasts maturation. Changes in cytoskeleton organization, in particular the networks of actin microfilaments and microtubules, were also observed. Decresed proliferation rate, increased MyoD expression and cytoskeleton rearrangement are consistent with myoblast differentiation.

Finally the expression of molecules involved in the regulation of extracellular matrix (ECM) turnover (collagen I, MMP-2, MMP-9) was analyzed. After 4 treatments, collagen I expression showed a 14% increase while MMP-2 and MMP-9 decreased of 33% and 18%, respectively. These results suggest that MLS treatment could affect ECM turnover shifting the balance toward the production rather than to the degradation.

In conclusion, our findings demonstrate that MLS treatment induces in muscle cells a biological response that could favour muscle cell differentiation and the recovery of diseased muscle tissue. A deeper knowledge of the mechanisms underlying the effects described above and a greater understanding of the changes in the biological response to variations in instrumental parameters setting can lead to concrete improvements in treatment protocols.

INTRODUCTION

Lasers are widely used in biomedicine. Sport medicine, physiatrics and rehabilitation are among the most important fields of application. Here the analgesic, antiinflammatory, anti-oedema and stimulating effects of laser therapy are used to favour tissue repair and function recovery.

According to the literature, many factors can contribute to the stimulating effect.

The moderate vasodilation increases the supply of nutrients and growth factors. For example, it has been demonstrated that low-level laser (LLL) irradiation (Ga-Al-As laser) promotes expression of fibroblast growth factor (FGF) in rat gastrocnemius muscle recovering from disuse muscle atrophy [1]. FGF promotes angiogenesis and lead to fibroblasts activation [2,3] which determines an increase of collagen synthesis, essential for tissue repair and regeneration [4-6]. Neoangiogenesis is crucial for ensuring oxygen and nutritional substances to new tissues and has a very important role in muscle recovery [7,3]

Effects that induce a local increase of nutrients, promote angiogenesis and influence the development of inflammation can strongly affect the healing process and functional recovery of the injured tissues.

Another factor widely recognized as fundamental to the stimulating effect is the red/infrared (IR) laser-induced increase in ATP production in mitochondria [7-9]. After treatment with He-Ne laser, an increase in membrane potential and consequent ATP production have been observed in isolated mitochondria [10]. Moreover, many authors found that red/IR lasers may promote cell proliferation [4,11-13].

All these effects are consistent with the hypothesis that the recovery of injured tissues can be accelerated through the application of suitable laser therapy.

Studies on nerve fibers regeneration [14] showed that reconnection process of nerve cells is accelerated after laser

treatment, leading to the regeneration of insensitive areas [15-17]. Other studies have demonstrated a faster recovery of wound healing [18] and bone fractures [19], as well as a marked reduction in infarct size and myocardial infarct [20].
Many studies report on effects of laser radiation on muscle homeostasis and repair mechanisms in this tissue. In a recent study, using mice as experimental model, the anterior tibial muscle previously damaged by a cryolesion has been exposed to LLLT (GaAlAs Laser, 660 nm). Although a significant reduction in recovery time was not recorded, an increase of collagen IV was found in the treated muscles [21].
Another study on mice demonstrated that He-Ne laser irradiation (632.8 nm), associated with physical exercise, reduced skeletal muscle inflammation, improved the activity of superoxide dismutase and diminished the activity of creatine kinase [22].

Some authors found an increase in proliferation of muscle satellite cells [1, 23-25]. These cells, usually quiescent, can be activated by factors released by cells of the injured muscle [26-28]. The satellite cells have the function of creating new fibers and replacing the necrotic ones [27].

In the frame of studies aimed at understanding the mechanisms by which laser therapy can promote the repair and functional recovery of skeletal muscle, here we report the results obtained investigating the effect of IR laser radiation on myoblasts.

As for any other radiation source, the main parameters for characterizing laser emission are: power, frequency and wavelength. These ones, together with the features of the irradiated tissues or samples, strongly affect the way the radiation propagates into the tissue/sample and the consequent effects.
In our experiments, we chose as the laser source a Multiwave Locked System (MLS) because we hypothesized that this laser system could be particularly suitable for the treatment of skeletal muscle. In fact the system is characterized by two synchronized emissions with wavelengths 808 and 904, respectively. The two emissions are absorbed by different mitochondrial complexes, therefore the MLS treatment can affect cellular energy metabolism by acting on multiple sites in the respiratory chain at the same time. Radiation with λ = 808nm is absorbed by the cytochrome oxidase (complex IV) which is considered as a principal photoacceptor in mammalian cells [29,30]. It is know that the activation of this mitochondrial enzyme after absorbing a radiation in red/near infrared (IR) promotes the production of ATP [31,32]. The radiation with λ= 905 nm interacts with the complexes I, II, III, IV of the respiratory chain and succinate dehydrogenase [33].

Considering the emission wavelengths and tissue type (muscular tissue) optical properties, it is possible to estimate MLS radiation which is expected to propagate within the tissue a penetration depth of about 10 mm in this kind of tissue; this means that still about 13% of initial power reaches a 20 mm depth. Therefore it is possible to affirm that MLS radiation can interact with deep-located muscle tissue.
Moreover, since our previous data (not yet published) demonstrated that MLS radiation is absorbed by collagen and polysaccharide biogels, which are models of extracellular matrix, we hypothesized that the MLS treatment could also affect cell behaviour by modification of the extracellular microenvironment.

MATERIAL AND METHODS

Cell Cultures

Murine myoblasts have been cultured in Dulbecco's Modified Eagle's Medium supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 10% fetal bovine serum (FBS).
Cells were incubated at 37°C in humidified atmosphere containing 95% air and 5% CO2 in order to maintain a pH value between 7.3 and 7.5. When confluence has been reached, cells have been washed twice with PBS, then treated with a 0,05% trypsin solution and plated on 55 cm2 plates. All the reagents have been purchased from Sigma (Chemical Co St Louis, MO, USA).

MLS Treatment

The laser source was a Multiwave Locked System (MLS) provided by ASA s.r.l. (Arcugnano, Vicenza, Italy). The instrument consists of two assembled laser diodes, with synchronized emissions at 808 and 905 nm, respectively.
The diode with λ = 808nm may emit in continuous mode, with a power P = 1.1W, or pulsed mode with an average power Pa = 0.55W and a maximum frequency of 2000Hz.

The diode λ = 905 nm is characterized by a pulsed emission with a maximum frequency of 2000Hz and an average power Pa = 60mW.
Therefore, the MLS emission can occur in different modes, according to the operator's choice:
Continuous Mode (Continuous Mode Operation, CW): diode with λ = 808 nm, continuous emission and diode with λ = 905 nm, pulsed emission. Pulsed mode (Pulsed Mode operation): diode with λ= 808nm, pulsed emission with pulses repetition frequency f808 (Max value 2000Hz) and diode with λ = 905nm, pulsed emission with pulses repetition frequency f905 = f808.

When frequency changes, the emission features allow the average power of the 905nm diode emission to change, while the average power of the 808nm diode emission does not change. In fact, when the frequency changes the 808nm diode emission duration changes in proportion, in this way the average power remains the same. It is the temporal distribution of the released energy which changes. With the

same emission time (and spot sizes), the whole energy (808nm + 905nm) changes when the set frequency changes.
For our experiments, cells have been plated on slides Ø of 13mm (5000 cells per slide) previously sterilized and put in multiwell (plates of 24 wells) to carry out the treatment. Each plate has been put in a holder which allowed an easy scanning of the samples. Each scanning lasted 20s. The treatment was repeated once a day for 4 consecutive days in sterile conditions. The treated samples have been compared with controls maintained in the same conditions, except for the exposure to MLS laser device.
The following treatment parameters have been applied: 8 min exposure to 1500Hz emission frequency. To calculate the energy given to each sample during a single treatment (E) it has been considered the following relation:

$$E = P_t \cdot (t_t / n) \quad (1)$$

where n is the number of samples (8 in our experiment), t_t is the treatment time, P_t is the average power, estimated on the slide surface (132 mm^2), equal to the sum of the two laser sources contribution (P_t ~ 200mW). Entering the data in the formula (1), we obtain E ~ 12.0 J.

Cell viability
Cell viability after exposure to MLS was determined by a Trypan Blue assay. The dye is capable of selectively penetrate into dead cells. After treatment, cells are washed and detached with trypsin/EDTA for a few minutes. Then cells are centrifuged and resuspended in a solution of PBS and Trypan Blue (dilution factor: 2) and counted, after 5 min of incubation, using Neubeuer emocytometer.

Immunofluorescence
After treatment the cells were fixed in cold acetone for 5 minutes and then washed with PBS without Ca and Mg. After blocking unspecific binding with PBS containing 3% bovine serum albumin (BSA), cells were incubated overnight at 4°C with the specific antibodies: anti-α actin, anti-collagen I, anti-α tubulin and anti-vimentin antibodies (Chemicon Int, Temecula, CA), anti-Myo D antibody (Santa Cruz Biotechnology, Heidelberg, Germany), anti-MMP-2 and anti-MMP-9 antibodies (Abcam, Cambridge, UK). The cells were then incubated with the FITC (fluorescein isothiocyanate) conjugated specific secondary antibodies (specifically: anti-mouse IgG for tubulin and Myo D antibodies, anti-rabbit IgG for collagen I and MMP-2 antibodies, anti-mouse IgM for vimentin antibody and anti-goat for MMP-9 antibody) (Chemicon Int, Temecula, CA). Cells incubated with anti-α actin antibody did not need incubation with the secondary antibody since a mouse anti-actin Alexa Fluor® 488 conjugated was used. Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an inverted epifluorescence microscope (Eclipse TE2000-E, Nikon, Italy) with oil immersion objective (CSI S fluor 100x, N.A. = 1.3) at 100x magnification and imaged by a HiRes IV digital CCD camera (DTA, Italy). Fluorescence excitation has been achieved by selecting the 365nm emission line of a mercury vapor lamp (HBO 100W, Osram). About 30 cells from different fields have been imaged for each slide.

Image processing
The image processing has been performed by using a specific program written in the LabVIEW language (National Istruments). By first obtaining a binarized image, in which pixels corresponding to cells and those corresponding to the background have been given the value of 1 and 0 respectively, the program is able to distinguish the cell signal from the background; as a second step, it calculates the average cell intensity by applying the binarized images to the original gray-scale ones. It is then possible to compare the average fluorescence intensity of a first images set (control samples) with the intensity of a second one (treated samples).

Data Processing
The experiment has been made three times to confirm the results. For each slide 30 images have been acquired and selected in a random way. The fluorescence intensity of each field (analyzed with previously described method) has been expressed as the average pixel intensity corresponding to the visualized cells. Intensities corresponding to the 30 acquired fields have been further mediated to give a final value, whose error has been calculated as Standard Deviation (SD). The statistical significance has been determined using the T-Student's test (chosing $p<0.05$).

RESULTS
The aim of this study was to evaluate the effects of MLS treatment on muscle cells and to identify mechanisms possibly involved in the stimulation of tissue repair. For our experiments, we used a murine myoblasts cell line (C2C12) widely accepted as a model in muscle cells behavior studies. In particular, the research focused on cell viability and proliferation, organization of cell cytoskeleton, expression of MyoD, an early marker of muscle differentiation, and proteins involved in the extracellular matrix turnover (collagen I, MMP2, MMP9).

Viability and proliferation
In order to verify the effect of the exposure to MLS emission on cell viability and proliferation, Trypan blue assays were carried out 24 h after the first treatment and 24 h after the fourth treatment.
As shown in Fig.1, in both cases, no significant differences were observed between treated samples and controls as regards cell viability, which resulted higher than 97.5% in all the samples.
Cell proliferation did not change significantly after the first treatment, but showed a decrease of the 25% after four treatments (Fig.2)

Cytoskeleton
The cytoskeleton is an important structure for the cell since it allows both movement and shape modifications and

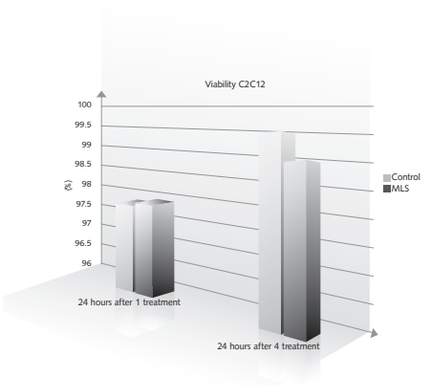

Fig.1. C2C12 Cell viability assessed 24 h after MLS treatment and 24 h after the fourth MLS treatments. (Control vs. MLS). Data were obtained by Trypan Blue assay.

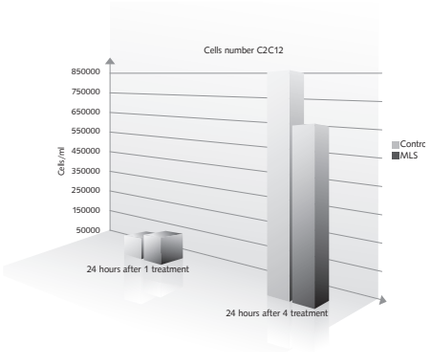

Fig. 2. C2C12 Cell proliferation assessed 24 h after MLS treatment and 24 h after the fourth MLS treatment. (Control vs. MLS). Data were obtained by Trypan Blue assay.

has an important role in intracellular transport and signalling. The cytoskeleton is mainly composed of three elements: actin microfilaments, microtubules and intermediate filaments made of tubulin and vimentin, respectively.

The distribution of actin, tubulin and vimentin in myoblasts exposed to MLS treatments was studied by immunofluorescence microscopy and image processing.

Actin is modified by mechanical stimulation, in particular by physical stimulation. It can be used as a sensitivity marker of the cells when exposed to physical factors [34]. Moreover, it is considered an important marker for muscle cells differentiation [35].

As shown in Fig.3 (a,b), after MLS treatments, actin expression decreased by about 13% and cleary changed the organization of the microfilament network. The microfilaments appeared more concentrated in perinuclear area. The treated samples showed also changes

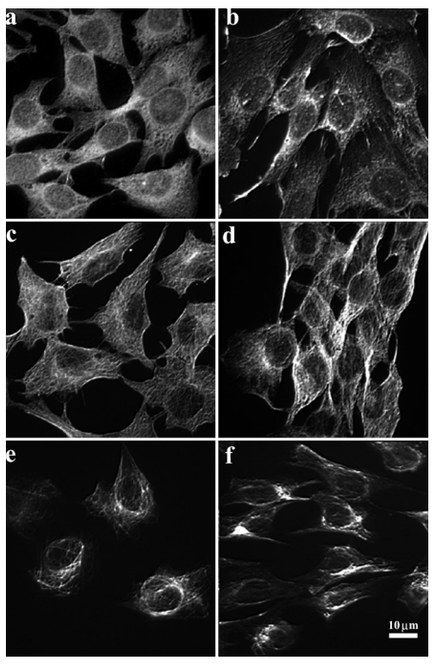

Fig. 3. Expression of cytoskeleton components assessed by immunofluorescence microscopy. Actin expression in control (a) and cells exposed to MLS treatment (b). Tubulin expression in control (c) and cells exposed to MLS treatment (d). Vimentin expression in control (e) and cells exposed to MLS treatment (f).

in the cell morphology, which resulted elongated, when compared with control samples. From a quantitative point of view, the expression of tubulin, which is the main constituent of microtubules, did not change following laser treatment. However, as observed in the case of actin, a different organization of the microtubule network has been observed: in fact, in control cells microtubules were organized radially while in treated cells appeared randomly distributed. See Fig.3 (c,d).

We did not find any significant effect of the treatment on vimentin, the protein which form the intermediate filaments [Fig.3 (e,f)].

Extracellular matrix

The extracellular matrix (ECM) is the non-cellular component of a tissue. It has many functions depending on the composition. For example, it acts as support and anchorage for cells and is a reservoir of growth factors. Cells bind to ECM via membrane proteins called integrins. Through these molecular "bridges", ECM

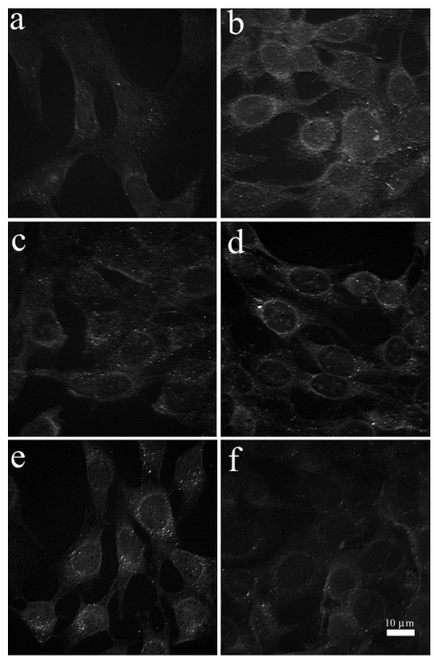

Fig. 4. Expression of extracellular matrix components assessed by immunofluorescence microscopy. Collagen I expression in control (a) and cells exposed to MLS treatment (b). MMP-2 expression in control (c) and cells exposed to MLS treatment (d). MMP-9 expression in control (e) and cells exposed to MLS treatment (f).

deformations can transmit mechanical stresses to the cells and affect cytoskeleton organization; in the same way cells can induce changes in ECM [36,37].

The ECM turnover is a key factor in the repair process of traumatized muscle.

The main ECM protein is collagen, which forms very dense fibres. Different types of collagen are present in the various tissues. Collagen I is the most abundant in the human body. It can be found in tendon, muscle, endomysial fibrils, the organic part of the bone tissue [38,39] and in the scar tissue. After exposure to MLS, myoblast cultures showed a moderate (14%) but significant increase ($p < 0,025$) in collagen I expression. Fig.4 (a,b)

The homeostasis of the ECM is also regulated by proteins belonging to metalloprotease family (MMP), which are involved in ECM degradation and repair during normal physiological processes [40,41]. These proteins are also involved in pathological conditions like arthritis [42]. In myoblast cultures treated with MLS we analyzed the expression of

matrix metalproteinase-2 (MMP-2) and matrix metalproteinase-9 (MMP-9), which degrade collagen IV, one of the most abundant types of collagen in skeletal muscle. In comparison with control samples we found a decrease of expression of 33% and 18% respectively Fig. 4 (c,d and e,f).

Differentiation markers

As above described, the data of our experiment revealed a decrease in proliferation but no significant changes in viability. Since this means that the MLS treatment does not induce cell damage, we hypothesized that the reduction in the growth rate could be due to the triggering of a differentiation process. Therefore, we analysed in the treated cells the expression of the differentiation marker MyoD. The differentiation markers are molecules which are expressed when cells pass from proliferation to maturation. Each tissue has its own differentiation markers. MyoD, an early marker of myogenesis, belongs to a protein family known as myogenic regulatory factors (MRFs). The main MyoD function is removing cells from cellular cycle and blocking proliferation. It is mainly expressed in muscle cells, where it has an important function in regulating muscle differentiation [43,44]. Our results demonstrate that MLS treatment induced an increase of the 26% in MyoD expression (Fig. 5).

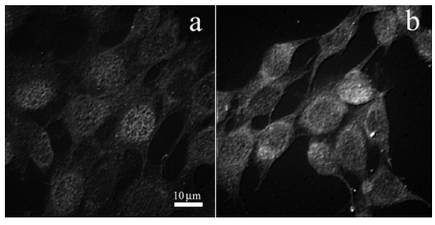

Fig. 5. MyoD expression assessed by immunofluorescence microscopy. Control (a) and cells exposed to MLS treatments (b).

DISCUSSION

The analysis of the data obtained by our experiments shows that the exposure to MLS treatment, even if repeated over time, did not produce significant changes in cells viability, which never fell below 97.5%. The proliferation decreased moderately, but significantly, after 4 treatments.

In literature there are many studies concerning the effect of laser radiation on cell viability. The results are often controversial and depends on laser type and experimental models used. However our results are in accordance with those reported by Ferreira et al. in a study on the effect of red/IR lasers on C2C12 cells, the same as our experimental model [45]. Recent studies carried out on different cell types showed that proliferation increased after exposure to wavelengths ≤ 780 nm, while it decreased by irradiation at 810 nm [12,46].

Since the unchanged cell viability demonstrated the absence of acute cell damage, the slower rate of growth induced us to hypothesize that MLS treatment could promote muscle cell differentiation. This hypothesis was indeed confirmed by the increase in MyoD that we found in treated myoblasts. As above explained, MyoD is an early marker of myoblast differentiation and plays a key role in the maturation of muscle cells [47].

The analysis of cytoskeleton organization, made through immunofluorescence microscopy, has shown that MLS treatment induced a considerable reshape both in microtubules distribution and in the network of actin microfilaments.

These data are in agreement with results we obtained previously in chondrocytes and fibroblasts exposed to IR laser treatment [48] and also with the studies of Ricci et al [49], where changes in organization of actin filaments and stress fibers formation in endothelial cells of rabbit aorta (REAC) subjected to LLLT are described.

It is well know that important changes of the cytoskeleton can be inducted by physical stimulation and laser radiation is not an exception. These changes can determine important effects on cells behavior, since microtubules have a primary function in regulating distribution and positions of intracellular organelles and actin is involved in cell shape determination, and regulates the adherence/migration processes [50]. Moreover, in muscle cells, actin has a very important and significant function. Finally, the transition from proliferation to differentiation, such as that observed after MLS treatments, involves changes in cell morphology and therefore in cytoskeleton organization.

Indeed, it has been demonstrated that substances like phospholipase D induce myogenic differentiation through a remodeling of actin cytoskeleton [51].

MLS treated samples showed also changes in expression of molecules which have important functions in reshaping the ECM. Collagen I expression increased, in agreement with what other authors have found recently in tissues exposed to GaAlAs laser (λ = 808nm) [52].

On the contrary, the expression of MMP-2 and MMP-9, involved both in migration and myoblasts differentiation [53], diminished. The moderate increase in collagen and reduction in MMP-2 and MMP-9 could affect myoblasts migration and ECM formation.

In conclusion, the results we obtained on cell viability and proliferation, structural changes of the cytoskeleton, MyoD, collagen I, MMP-2 and MMP-9 expression demonstrate that MLS treatment does not affect myoblast viability but can affect migration, differentiation and production of ECM molecules.

These results indicate that MLS treatment is able to induce, in muscle cells, a biological response that can affect muscle function. This response is consistent with therapeutic effects observed at systemic level and suggest that MLS therapy could be effective in treating muscle diseases by direct action on myoblast behaviour. Additional studies to further understand the molecular mechanisms underlying the observed effects are needed, since a better understanding of mechanisms and biological responses evoked by use of different instrumental parameters can lead to significant improvements in therapeutic protocols.

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