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Contents

The effects of MLS laser therapy in élite football players affected by muscles injuries: a controlled clinical trial.

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ABSTRACT

Muscle injuries are frequent in élite football players, with a percentage of 30-40% of all injuries. The 22% of total injuries are muscular relapses. The focus of this study was to evaluate how the laser therapy could modify the recovery time in élite football player. The treatments have been performed with a Multiwave Locked System (MLS) laser. The sample group of football players was divided into two groups: the first group has been subjected to the standard rehabilitation program without MLS laser irradiation, the second group has been treated with the new rehabilitation program that included laser therapy.

We compared the average injury's duration in the two groups to establish the efficacy of the MLS laser treatment in accelerating rehabilitation. In spite of a positive trend observed in the laser-treated group, which showed a decrease of the recovery time on the basis of the lesions considered, the difference in comparison with the control group was not statistically significant, also due to the low number of patients considered.

Therefore, the results suggest that laser therapy could be useful to shorten the recovery time after muscle injury, but further studies with a larger number of cases are required to statistically demonstrate the efficacy of the MLS laser therapy.

INTRODUCTION

Soccer is actually the sport most performed in the world [1]. His popularity has lots of financial implications especially in élite soccer. Injuries have a greats influence on team's balance and management, both directly, due to the medical costs, and indirectly, due to a decrease in the team competitiveness caused by the absence of one or several football injured players1. So, each professional football team has increased the amount of medical staff and give them the right tools to optimise their work.

Muscle injuries are frequent in élite football players, with a percentage of 30-40% of all injuries [2,3]. The anatomical region most affected by injuries in soccer is the lower limb [2,3].

The 22% of totals injuries are muscular relapses [4]. These elements underline the importance of primary prevention to reduce incidence of muscular injuries and secondary prevention to reduce incidence of relapses [5,6].

Laser therapy is important to prevent and to treat muscles injuries. In fact, both in in vitro and in clinical studies laser therapy has given large evidence of usefulness to reduce pain [7,8] and inflammation [9,10], to promote reabsorption of oedema [10,11] and wound healing [12]. Moreover, when properly used, laser therapy is devoid of side effects.

However, in spite of a large diffusion of laser therapy, molecular and cellular mechanisms that underlie the observed therapeutic effects are not completely known. There are many studies but often results are conflicting and barely comparable due to the variety of effects and biological response, that depend on the type of laser emission, the operative conditions and biological tissue studied (different body's regions, different tissues, different kind of cells etc...). Frequently, conditions and parameters used in clinical studies cannot be compared with the ones used for in vitro studies. Moreover, laser therapy is at times administrated without a correct evaluation of laser parameters (wavelengths, power, frequency, etc...), status and characteristics of the patient.

The focus of this study is to evaluate how laser therapy could modify the recovery time in élite football players.

MATERIALS AND METHODS

The treatments have been performed with a Multiwave Locked System (MLS) laser (ASA Srl, Vicenza, Italy). It is a high power (average power up to 1.1 W, class IV) IR 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, mean optical power output = 100mW. The frequency of the pulses may be varied in the range 1-2000 Hz.

The second laser diode (808 nm) operates in continuous mode (power 1 W) or in pulsed mode (pulses repetition rate 1-2000 Hz), mean optical power output = 500mW, duty ratio 50% independently of the pulse repetition rate. The two propagation axes are coincident. MLS laser is a device already used for some years in clinics (FDA approved and CE certified instrument) and applied in particular in physical medicine and pain therapy.

We analysed muscular injuries of football players belonging to the A.C.F. Fiorentina youth team. We included in the study all the professional football players of the Team with age from 13 to 19 years, members of the categories Allievi Regionali, Allievi Nazionali and Primavera who have got diagnosis of muscle injury occurred in the period January 2010 - October 2012.

We excluded all Team's football players that used MLS laser therapy for other types of illness (tendinitis, sprain, low back pain, etc...).

We enrolled in the study 32 athletes and divided them in two groups: 18 athletes were treated with standard rehabilitation program (group 1) and 14 athletes were treated with the experimental rehabilitation program (group 2) which included the laser treatment.

Standard rehabilitation program (group 1)

The standard rehabilitation program for muscle injury used by A.C.F. Fiorentina football team consisted of:

- Gym muscular exercises, free body or isotonic machines, that allow the damaged muscle to work in different kinds of muscular contraction (isometric, concentric and eccentric). The muscular exercises should be done under pain threshold (Borg CR10 ≤ 3/10).
- Proprioceptive exercises like bouncer, skimmy and specific exercises on sand.

Organic exercises on cyclette, walking and running on tapis roulant or in soccer field.

- Free body coordinative exercises.
- Static, dynamic or hold-release stretching.
- Finally diat hermy treatment (TECAR®) in capacitive modality.

Experimental rehabilitation program (group 2)

The experimental rehabilitation program had the same contents of standard rehabilitation program with addition of MLS laser therapy. Lasertherapy has been applied as follows:

- For muscle strain (grade of lesion 1°, 1°-2°, 2° and 3°) we used the following parameters: 1500 Hz frequency, 50% of intensity, 10 min exposure, 253,6 J energy delivered by handpiece.
- For contusion and mild strain we used the following parameters: 700 Hz frequency, 50% of intensity, 10 min exposure, 198,3 energy delivered by handpiece.

Laser therapy was administered daily (5 days per week), starting 24-48 h from muscle injury. For treatment, laser was isolated by the other physical therapy's machines and it was staged in a closed little room. Laser therapy was administered in a dedicated room, by means of a scanning automatic arm. Athletes and physiotherapist wore specific protective glasses, provided by ASA srl.

The diagnosis of muscle injury was done by the medical staff of A.C.F. Fiorentina in two steps: immediately on soccer field, based on clinical symptoms reported by football players; then, 24-48 h after the event, muscle injury was confirmed by several clinical tests and diagnostic instrumental tests (Ultrasound or RMN). Immediately after diagnosis of muscle injury, athletes started the rehabilitation program.

The end of the rehabilitation program was fixed on the basis of clinical parameters, like absence of pain at percussion (VAS < 1/10), complete ROM without pain at joint where damaged muscle operates (VAS < 1/10), muscular strength 5/5 (Kendall scale), no pain during rehabilitation exercises (Borg CR 10 ≤ 0,5/10) and a positive psychological attitude of football players towards the return to competitions. Moreover, in many cases the medical staff made an ultrasound control to verify the complete healing of muscular damage.

At the end of the rehabilitative program, athletes started both training with team trainers and secondary prevention program with physiotherapists and trainers.

We analysed the recovery time of each injury, expressed in number of days from the beginnings to the end of rehabilitative program. We also analysed the way of injury (match or training), muscles interested by the lesion and severity of muscle injury.

RESULTS

Graph 1 reports the summary of the patients enrolled in the study. The medical diagnosis and rehabilitation program (group)are reported.

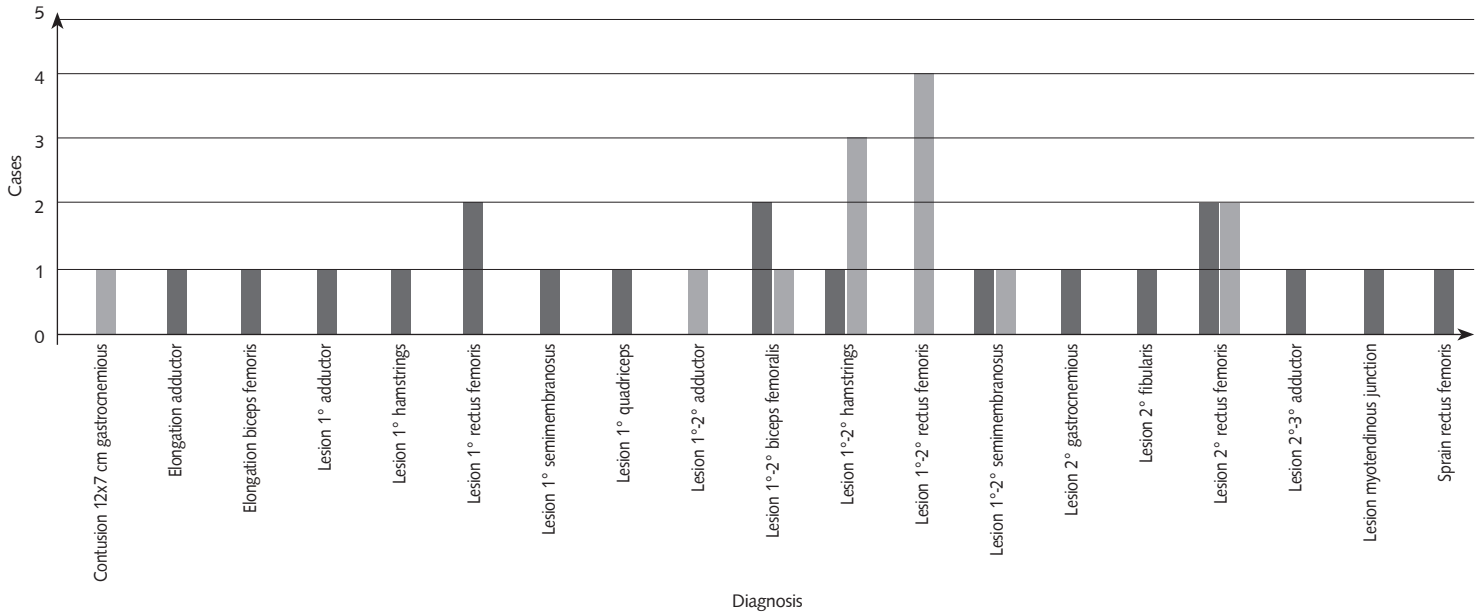

Figure 1: resume of sample divided for medical diagnosis.

Data show a difference in the average time recovery between group 1 and group 2. The group 1 average time recovery is 22,05 days, the group 2 average time recovery results 23,31 days (Graph 2). The difference between the two study groups is not statistically significant (p-value = 0,7085).

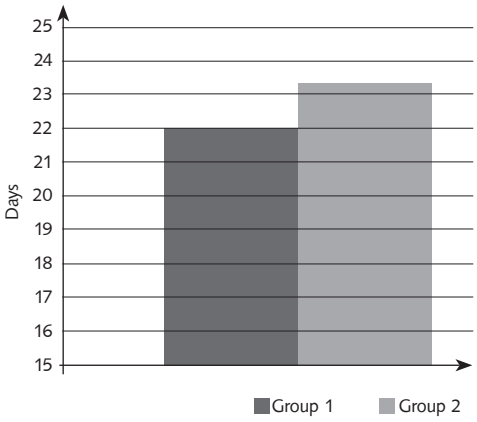

Figure 2: injuries' average duration

In order to analyze the data in detail, the patients were further divided into groups based on the kind of muscular injury and, for each group, the recovery time was calculated (Graph 3). Obviously, under this point of view, we studied only the groups whose patients were present in both the rehabilitation programs; therefore we considered only the groups "lesion 1°-2°" and "lesion 2°".

Into the group "lesion 1°-2°" data show a difference in the average recovery time between group 1 and group 2. The value for group 1 is 26 days, while for group 2 is 23,1 days (Graph 3). However, the difference between the two study groups (p-value = 0,5789)is not significant.

Also considering the group "lesion 2°", data show a difference in the average recovery time between group 1 and group 2. The group 1 average recovery time is 33 days, but drops to 29 days in the group 2 (Graph 3). The difference does not result statistically significant (p-value = 0,7763).

If we analyse the prevalence of muscle groups most affected by injuries, we can see that hamstrings are the most affected by lesions (41%), followed by quadriceps in 38% of cases, adductors 12%, gastrocnemius and soleus 6% and fibular muscles 3% (Graph 4).

The prevalence of injuries divided for type of lesions shows that lesions 1°-2°

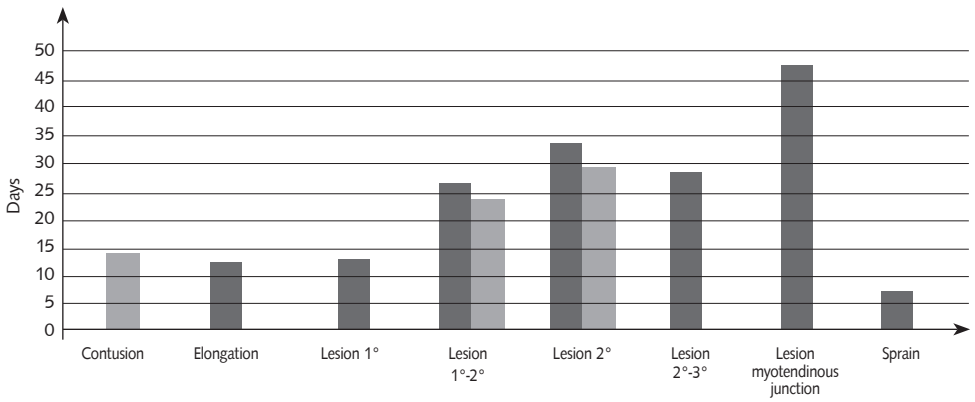

Figure 3: injuries' average duration divided for type of muscle damage

occurred in 44% of cases; followed by lesion 1° and lesion 2°, both observed in 19% of cases; 6% elongation; 3% contusion, mild strain and lesion 2°-3° and lesions of myotendinous junction (Graph 5).
Graph 6 reports the patients in relation to the month and practice performed (match or training) when the injury occurred.
Finally we analyzed the difference between number of injuries verified during training (28% of cases) and number of injuries verified in football matchs (72% of cases). There is a statistically significant difference between the two groups, $p < 0,01$ (Graph 7).

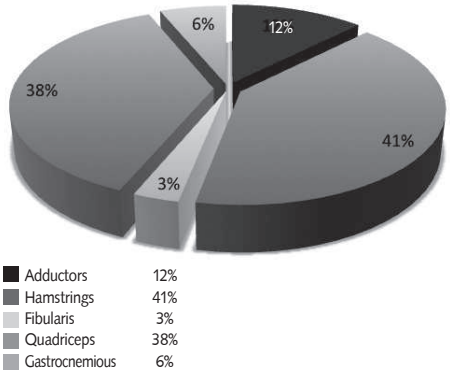

Figure 4: prevalence of injuried muscles

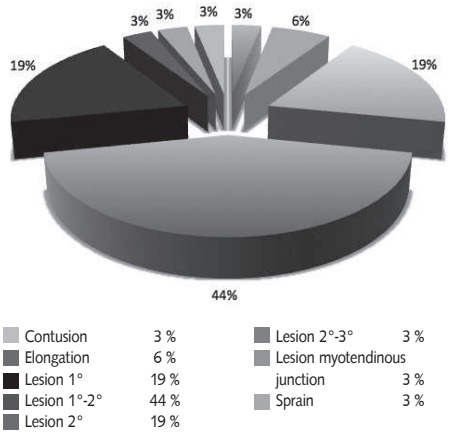

Figure 5:
prevalence of type of muscle damage

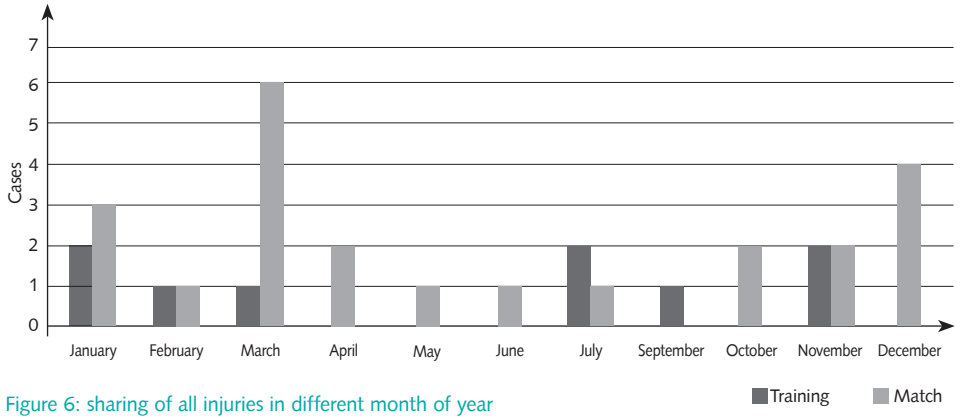

Figure 6: sharing of all injuries in different month of year

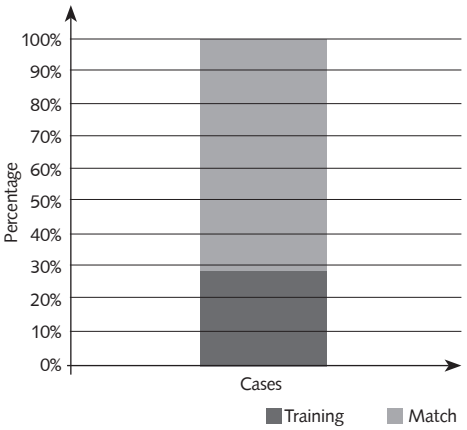

Figure 7: prevalence of injuries occurred in match/training

DISCUSSION

As demonstrated by statistical analysis, the comparison between the average recovery time of groups 1 (control) and that of group 2 (laser treated) does not show significant differences (Graph 2). The average recovery time of group 2 was slightly higher (1 day more), apparently giving the impression that the treatment can delay recovery. A more detailed analysis, which takes into account the kind of muscular injuries (Graph 3), reveals that in group 1 there were many patients with less severe lesions (lesion 1° and mild strain, expected to have a fast recovery) than the injuries affecting patients of group 2. Obviously the lower average recovery time of group 1 strongly depends on the lower seriousness of the lesions
The statistical analysis performed on subgroups of patients more homogeneous regarding to the injury(subgroups with "lesion 1°-2°" and "lesion 2°"), therefore more correct because each group (control and laser treated) had the same kind and degree of muscular injury, shows that both in the subgroup "lesion 1°-2°" and in the subgroup "lesion 2°" the patients treated with laser therapy had a faster recovery in comparison with controls. The application of laser therapy to the subgroups "lesion 1°-2°" and "lesion 2°" decreased the average recovery time of 3 and 4 days, respectively. Statistically, these differences are not significant, due to the low number of patiens studied. However the results suggest that MLS laser therapy improves the recovery from injury.
The muscles most frequently injured are hamstring (41% of our observations) and quadriceps (38% of our observations); this is explained by the acts of run, jump and shot, all very stressful actions for flexor and extensor of lower limb. These data agree with numerous studies reported in literature, for example the study of Hawkins & Fuller [2] and the study of Ekstrand & al [3].
The most frequent kind of injury observed was the lesion 1°-2° (44% of our observations): this is a lesion characterized by intermediate characteristics between 1° and 2° in relation with number of muscle fibres damaged, presence of oedema and hematoma. This classification is described in other scientific reports, for example Costantino C. & Imperio G [13].

The distribution of accidents during the shows that in the coldest months, from October to March, there is an increased risk of injury [14-16]. The peak of injuries has been recorded in March, with 6 of total 32 cases. In this period of the season there are lots of matches, national cup and national league; the high intensity of competitive activity together with the cold climate causes a high risk of injury.
Finally, we also analyzed when muscle injuries which affected our patients occured: 72% of the injuries occurred during football matches and 28% in training sessions. The difference between these two percentages is statistically significant, so we may assert that in football matches there is a higher risk of muscles injury than in training session.

CONCLUSIONS

In spite of a positive trend in cases of "lesion 1°-2°" and "lesion 2°", in which we demonstrated a shorter recovery time for patients who have done the rehabilitation program with laser therapy (group 2), this is not statistically significant. A limit of our study is the low number of patients enrolled, which is largely responsible for the absence of significance from the statistical point of view. However, the results of this pilot study indicate that the application of laser therapy can shorten the recovery time. This is an interesting cue for further studies with a larger number of patients.

Another limit of the study is that laser therapy was always joined with diathermy treatment (Tecar®).

The Tecar therapy was obviously administered also to the control group, then the only variable in the comparison between the two groups (control and laser treated) was laser therapy. However, the association of two physical therapies makes it difficult to isolate the effects of the laser from those of Tecar. It would therefore be necessary to conduct further studies in which the behavior of a group of patients subjected only to laser therapy is analyzed.
In conclusion, the results of this study suggest that laser therapy could be a useful tool to favour muscle repair and shorten the recovery time but further studies are needed to better assess the effectiveness of laser therapy in favouring the recovery of athletes suffering for muscle diseases.

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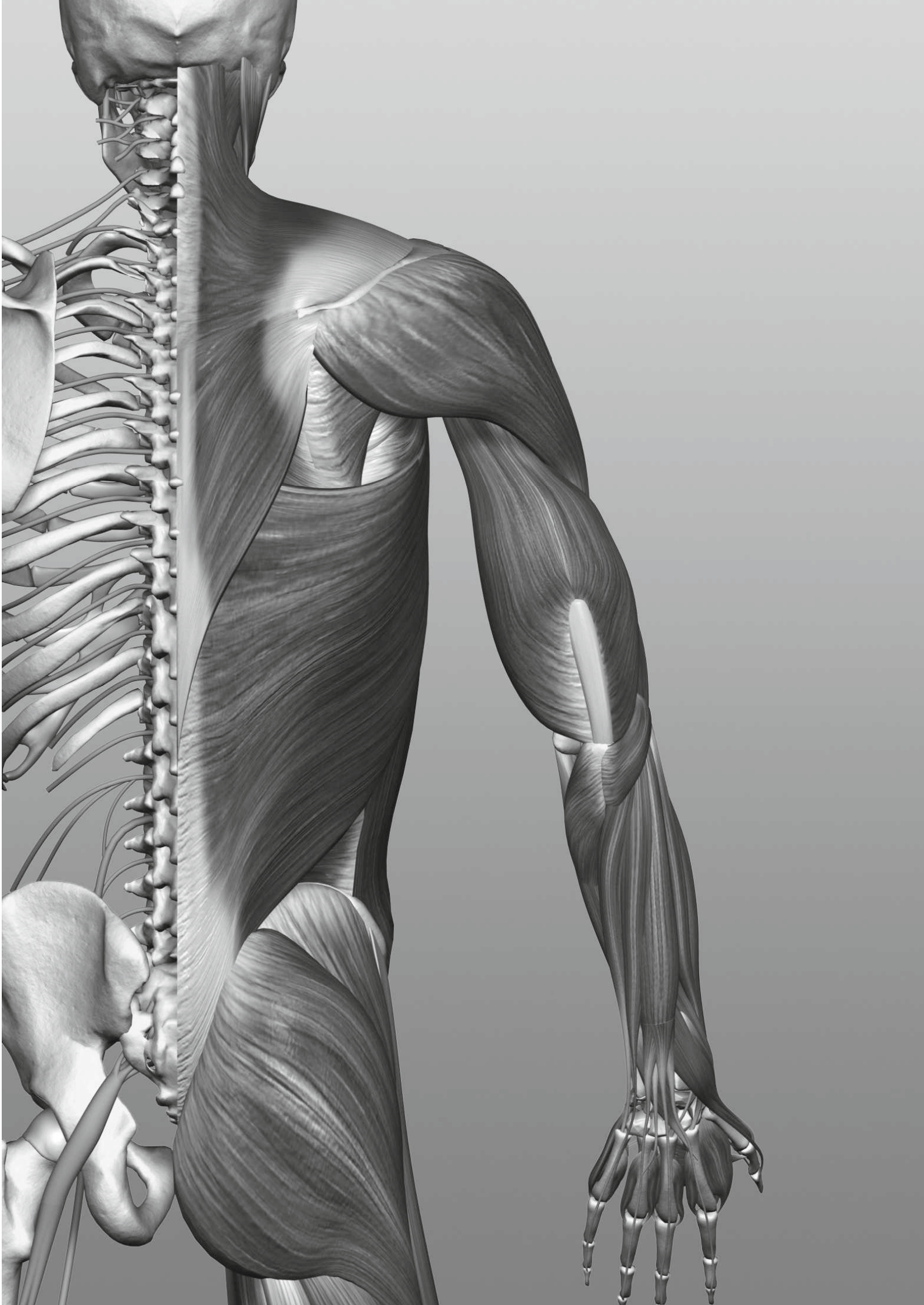

Treatment with laser therapy of cutaneous damages induced by radiotherapy in breast cancer: our institutional experience.

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ABSTRACT

Background and aims.
Patients treated with radiotherapy on the entire breast may present an acute, subacute or chronic cutaneous damage of the healthy tissues involved in the radiation fields. The aim of the present study is the assessment, through a controlled clinical study, of the effectiveness of Low Level Laser Therapy in reducing pain and inflammation and in stimulating skin healing of radiotherapy ulcerations.

Material and methods.
From February 2009 to March 2010, 100 patients affected by breast cancer have been recruited, with an average age of 47 years. 47patients were treated with laser with an interval of 3-4 days between applications on the inflammation or ulcerations area meanwhile the rest 53 patients were treated with lenitive creams. All enrolled patients were subjected chemotherapy with various schemes combinated or not with hormonal treatment. We evaluated the cutaneous acute toxicity according to the RTOG scale either during radiotherapy and during follow-up (3 months after radiation treatment).

Results.
All patients completed the radiotherapy; 60% of patients presented G0-G1 cutaneous toxicity, 28% have developed G2 cutaneous toxicity, 12% have developed G3 toxicity; no patient presented G4 toxicity. Analysis of the data revealed a shorter time for the healing of the cutaneous toxicity after topical treatment with LLLT compared to the patients that had no LLLT treatment.

Conclusions.
This clinical trial showed that low level laser therapy was effective in stimulating wound healing and pain reduction, and strongly suggest that its application could be useful in treating radiotherapy (actinic) induced ulcerations. Further analysis on a larger number of patients is necessary for definitive results but our data as far indicates huge effect of the LLL treatment by decreasing the healing time of skin toxicity.

INTRODUCTION

External beam radiotherapy alone or in association with surgery and/or chemotherapy represents an integrating and irreplaceable part in the treatment of the breast cancer. In the last 30 years, technological improvements and greater precision in the delivery and in the dose distribution of radiotherapy have reduced the incidence of radio-induced complications [1]. However, a minimal part of patients may present an acute, subacute or chronic cutaneous damage of the healthy tissues involved in the radiation fields.

The treatment of acute effects on the skin and on the mucosae (cutaneous erythema, edema, pigmentation and/or mucositis) [2,3] is important. Despite topical treatments (creams, pastes or sprays) that are used on the radio-treated surfaces both during the radiation treatment, lasers provide low-energy stimulation of tissues that results in increased cellular activity during wound healing [4,5]. Lasers provide low-energy stimulation of tissues that results in increased cellular activity during wound healing [4,5]. Wound healing has three phases: first, a substrate is laid down, second, cells proliferate, and third, there is remodelling of tissue. The functions being stimulated

include both collagen production and angiogenesis [6,7]. So, the data published so far suggests the laser biostimulation produces its primary effect during the cell proliferation phase of the healing process, but also in the preliminary inflammation phase and in the final phases of tissue maturation [8,9].
At cellular level, it has been demonstrated that mitochondria are receptive to monochromatic near-infrared laser light which probably increases the respiratory metabolism of certain cells [10-12] with the enhancement of ATP production and the increase of the mitochondrial inner membrane potential.

Given the photobiological nature of low-power laser effects [13,14], some molecule (photoacceptor) must first absorb the light used for the irradiation and then, after promotion of electronically excited states, primary molecular events from these states can lead to a measurable biological effect at the cellular level. In 1988 [15] it was suggested that the mechanism of interaction between Laser and cell substrates was based on the absorption of monochromatic visible and near infrared radiation by components of the mitochondrial respiratory chain. Absorption and promotion of electronically excited states cause changes in redox properties of these molecules and the acceleration of electron transfer (primary reactions). Primary reactions in mitochondria of eukaryotic cells were supposed to be followed by a cascade of secondary reactions (photosignal transduction and amplification chain or cellular signalling) occurring in cell cytoplasm, membrane, and nucleus [15]. In 1995, an analysis of five action spectra suggested that the primary photoacceptor for the red-NIR range in mammalian cell is a mixed valence form of cytochrome c oxidase [16]. Further signalling pathways which follow IR Laser interaction with the cytochrome c oxidase have been recently discovered [11].
The results of various studies [17-19] gave finally the demonstration, through direct observation, that the suggested mechanism [15] of low power laser therapy at the cellular level is based on the increase of oxidative metabolism in mitochondria, which is caused by electronic excitation of components of the respiratory chain (e.g., cytochrome c oxidase). This causes an increase in the ATP production, the increase of the mitochondrial inner membrane potential, and the shift from a catabolic to an anabolic condition, i.e the recovery of the energetic homeostasis of the cell.
Other processes, that depend strictly on the availability of ATP, are prompted by Low Level Laser Therapy: fibroblast proliferation [20], DNA synthesis [21], attachment and synthesis of collagen and protocollagen growth factor production (including keratinocyte growth factor [KGF], transforming growth factor [TGF], and platelet-derived growth factor [PDGF], macrophage stimulation, lymphocyte stimulation (activation and ability to bind pathogens), and a greater rate of extra cellular matrix production have been reported with laser light treatment (for example the fostering of the formation of type I and type III protocollagen specific pools of mRNA) [22-30].

Furthermore there is a positive effect of laser treatment on well-known aspects of inflammation such as mast cell proliferation and degranulation [31].
At the clinical level, the final results of this cascade of events prompted by the application of low level laser therapy are the acceleration of the healing time and the increase in the biomechanical indices of tissue healing. Animal studies on the enhancement in wound healing prompted by low power density laser light have been performed in toads, mice, rats, guinea pigs, and swine [32-35].
Human studies showed that low power laser emissions were able to stimulate epithelialization during wound closure and healing skin grafts (see ref. 36 for a thorough meta-analysis on the clinical studies), together with a significant pain reduction.
The applications of low level laser therapy to counteract the side effects of chemotherapy and radiotherapy have been used, up to the present time, for the prevention and treatment of oral mucositis. Laser therapy was shown to significantly reduce the incidence and the severity of mucositis in chemotherapy, as far as both pain and healing are concerned [37,38].
This clinical trial showed low level laser therapy was effective in stimulating wound healing and pain reduction, and strongly suggest that its application could be useful in treating radiotherapy (actinic) induced ulcerations.

MATERIALS AND METHODS

From February 2009 to March 2010, in the Radiation Oncology Department of Padua, 100 patients affected by breast cancer were recruited. Of the 100 patients recruited, 47 patients who developed any grade of toxicity were treated with LLLT application twice on the week with an interval of 3-4 days between the applications.

The device used was a Diode laser with a wavelength of 980 nm and red (visible), 5W peak emission,4J/cm2 energy with

GRADE 0	GRADE 1	GRADE 2	GRADE 3	GRADE 4
No changes	Light and/or painless erythema Epilation Desquamation Dryness	Sensitive and/or intense erythema Desquamation Partial sweating Moderate edema	Desquamation Widespread sweating Marked edema	Ulceration Hemorrhages Necrosis

Table 1 - RTOG scale used

an application area of 225 - 400 cm2 . We used a 5000 Hz frequency. The treatment time depended by the skin extend of the wound.

The application mode consisted of point action in order to accelerate local cell stimulation for healing.

The remaining 53 patients were treated with daily application of lenitive skin creams. The topical treatment of irradiated skin began the first day of radiotherapy and lasted until 3 months after the end of radiation treatment. Patients had to repeat the application of the cream every day (2-3 times/day).

Radiotherapy was delivered with a 3D conformational technique, and the total dose was 60 Gy in 30 fractions (2 Gy/die). All patients were treated with tangential beams using 6 Mv photons both for whole breast therapy and electron bean for tumor bed boost. From the beginning of the treatment, every week each patient was submitted to skin examination to evaluate cutaneous toxicity [39]. The evaluation was carried out using the RTOG scale [40](table 1). Cutaneous toxicity caused by radiations was estimated also during the follow-up, which was conducted approximately after 2-3 months of the end radiation treatment in all patients. All the patients who reported a G3 skin toxicity were treated locally with steroid products.

RESULTS

Patients enrolled in our study and treated with external radiotherapy for breast cancer were 100. 47 of them were treated with LLLT twice at week with an interval of 3-4 days between applications. The average number of sessions of LLLT was 4, so the time of treatment was 15 days. 23 (49%) of these patients had G1 cutaneous toxicity,18 (38%) G2 and 6 (13%) G3.

As the treatment with LLLT proceeded we continued to evaluate the patients skin toxicity according to the RTOG toxicity scale.

Patients with G1 toxicity treated with LLLT had an average mean time of healing of 9-10 days. Those with G2 toxicity an average mean time of healing of 15-18 days and 4 of these patients still had a G1 toxicity after 2- 3 months of follow up. In the G3 skin toxicity group the average mean time of healing after LLLT treatment was about 25-30 days with a 50% of patients that still had G1 toxicity after 3 months of follow up. Three months after the end of radiotherapy, at the first follow-up visit, only 15% of the radiotherapy-treated patients(all groups) still showed G1 cutaneous toxicity.

We compared the previous LLLT treated group of patients with another group treated with external radiotherapy for breast cancer that had only daily cutaneous applications of lenitive creams for the healing of cutaneous toxicity.37 (74%) patients of these group had G1 cutaneous toxicity,10 (20%) G2 and 6 G3.All patients who manifested G2 toxicity stopped the first topical treatment and were treated with cortisone creams [41], which determined a reduction in toxicity grade in 70% of the cases. The mean average time of complete healing in the G1 group was 18-20 days, in the G2 group 30 days and in the G3 group about 50 days. Patients who manifested G3 cutaneous toxicity were treated with cortisone and healing creams. Three months after the end of radiotherapy, at the first follow-up visit, 29% of the radiotherapy-treated patients still showed G1 cutaneous toxicity. Our results are summarized on table 2

DISCUSSION

The breast cutaneous damage induced by radiation treatment on patients affected by breast cancer have been often evaluated. Some studies tried to evaluate the best topical treatment and the correlation between systemic therapy and skin radio-induced damage [42].

Macmillan et al. [39] added to the knowledge on the risk factors for skin

Grade of toxicity	Number of patients	Patients treated with LLLT	Patients treated with lenitive creams	Average total time of treatment with LLLT	Average total time of treatment with LLLT
G1	60	23	37	9 days	20 days
G2	28	18	10	18 days	30 days
G3	12	6	6	30 days	50 days

Table 2 - Patients and results.

breakdown. These include concurrent chemotherapy, the use of a bolus, and smoking. Porock and Kristjanson [43] noted that a lot of the current research on radiation-induced skin reactions has focused on patients with breast cancer.
There are many factors that probably influence the appearance of side effects on irradiated breasts. Bentzen et al. [40] found increased acute skin toxicity when patients received chemotherapy. Anthracyclines, paclitaxel and docetaxel are involved with growing possibility in skin side effects [44,45].

Turessonand Notter [46] found the peak acute reaction to be correlated with age, menopausal status, bilateral treatment and the type of radiation. The reasons for such variability in risk factors for acute skin reactions are not clear but could be related to differences in the study population or the small number of patients analyzed in the actual trial.

In our study 97 patients treated with external radiotherapy for breast cancer in our department 47 of them were treated with LLLT with an interval of 3-4 days between applications with mean time of treatment of about 23 days. 23 (49%) of these patients had G1 cutaneous toxicity, 18 (38%) G2 and 6 (13%) G3.The average number of applications of LLLT was 7.
As the treatment with LLLT proceeded we continued to evaluate the patients skin toxicity according to the RTOG toxicity scale (table1).

Patients with G1 toxicity treated with LLL had an average mean time of healing of about 9-10 days. Those with G2 toxicity an average mean time of healing of about 18-20 days and 4 of these patients still had a G1 toxicity after 2- 3 months of follow up. In the G3 skin toxicity group the average mean time of healing after LLLT treatment was about 30-40 days with a 50% of patients that still had G1 toxicity after 3 months of follow up. Three months after the end of radiotherapy, at the first follow-up visit, only 15% of the radiotherapy-treated patients(all groups) still showed G1 cutaneous toxicity (table 2).

We compared the previous LLLT treated group of patients with another group treated with external radiotherapy for breast cancer that had only daily cutanous applications of lenitive creams for the healing of cutanous toxicity.

We found that comparing the two groups we had a decrease of 50% of the mean average time of healing (10 vs 20 days) in rhe G1 patients,a 21% of decrease in the G2 group and a 25% of decrease in the G3 one.

Further analysis on a larger number of patients is necessary for definitive results but our data as far indicates huge effect of the LLLT treatment by decreasing the healing time of skin toxicity.

CONCLUSIONS

Today there is growing interest in the treatment of cutaneous side effects of radiotherapy. Particularly women treated for breast cancer ask us not only the clinical resolution of their oncologic story but also a satisfactory esthetic condition. Patients are also concerned about the most effective and faster way of decreasing the side effects of the radiotherapy.
In our study we confirmed the capacity of the LLLT treatment to decrease the time of skin toxicity induced by radiation therapy on patients treated in our institute for breast cancer. Further analysis on a larger number of patients is necessary for definitive results.

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High Intensity Laser Versus Low Intensity Laser Therapy in Management of Postmenopausal Osteoporosis.

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ABSTRACT

Background:
It is estimated that 30%-50% of women will suffer an osteoporotic fracture in their lifetime. Laser therapy has a positive effect on bone regeneration and healing that is dependent on the characteristics of the light itself (eg, intensity and wavelength).

Objective:
The aim of the present study was to compare the possible effect of High Intensity Laser Therapy (HILT) versus Low Level Laser Therapy (LLLT) on bone mineral density (BMD) of lumbar vertebrae in postmenopausal women with osteoporosis.

Methods:
Thirty postmenopausal osteoporotic women participated in the study and were randomly divided in two groups. Group I consisted of 15 women receiving HILT, Group II consisted of 15 women receiving LLLT. Both groups have been exposed to three sessions of treatment per week for six successive weeks. Bone Mineral Density (BMD) of lumbar spine (L1.-5) was measured by Dual X-ray absorptiometry (DXA). Evaluation of lumbar BMD was performed before and after the end of the six weeks of treatment.

Results:
Comparing mean values before and after treatment, the BMD measures showed that both groups had a statistically significant improvement after laser therapy. Comparing the two groups, the improvement showed by BMD was higher in Group I (HILT) than in Group II (LLLT). The difference between the two groups was statistically significant ($P > 0.05$)

Conclusion:
Laser can be an effective method for the management of osteoporosis and improvement of BMD in postmenopausal women. On the basis of the findings of this study, HILT results more effective than LLLT.

INTRODUCTION

Osteoporosis has been defined as a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk [1,2].

Osteoporosis and fractures related to bone fragility represent a serious and global public health problem. Currently, it is estimated that 30%-50% of women and 15%-30% of men will suffer an osteoporotic fracture in their lifetime. It is a silent "epidemic" that has become a major health hazard in recent years, afflicting over 2000 million people worldwide [3].

There are two types of osteoporosis: type I, due to a decrease in cumulating estrogens, which affects trabecular bone (especially vertebral bone) and affects females more than males, in a ratio of 6:1; type II, senile osteoporosis, which is age related and occurs in cortical and trabecular bone, affects females and males in a ratio of 2:1 [4]. One in three women over the age of 50 years will develop the disease during their lifetime, with a loss of 20% bone mass in 5 to 7 years following the menopause [5].

A sharp decrease in ovarian estrogen production is the predominant cause of rapid, hormone-related bone loss during the first decade after menopause, as a result of higher bone turnover, an imbalance between bone formation and resorption with net bone loss [6].
The mechanism by which estrogens protect bone mass appears to be an indirect one, since there are no known estrogen receptors in bone. Most likely, at an earlier age estrogens control the rate of bone absorption by the effect on parathyroid hormone; once estrogen levels are diminished, resorption occurs at a much faster rate [7].

Low bone mass can only be diagnosed by measuring bone mineral density (BMD) by various techniques, of which the gold standard is DEXA (Dual energy X-ray Absorptiometry). BMD assessment confirms diagnosis, detects disease in asymptomatic state, predicts chances of future fractures, and is also useful for monitoring response to therapy [8, 9].
A World Health Organization working group proposed that osteoporosis should be diagnosed in epidemiologic studies when bone mineral density is 2.5 standard deviations (SDs) or more below the mean for healthy young adult women at the spine, hip, or wrist (corresponding to a T-score of ≤ -2.5). For every 1 standard deviation below the mean, the fracture risk roughly doubles [10, 11].

The acronym 'laser' means 'light amplification by stimulated emis¬sion of radiation'. Lasers are electromagnetic wave amplifiers which can produce pencil-like beams of electromagnetic waves with special properties. The earliest medical lasers, developed in the 1960s and 1970s, were relatively high powered and utilized the concentration of energy in a tiny, pencil-like beam for tissue destruction and coagulation. Some beneficial effects were noted in sites adjacent to the coagulated tissue, at which low energy had been applied. This led to the therapeutic use of low-energy lasers [12]. Low level laser therapy (LLLT) takes place at low radiation intensities, with an output up to 500 mw, which have been reported to have stimulatory, anti-inflammatory and analgesic effects [13-14].

Laser alters the cellular functions and affects the mitochondrial respiratory chain by increasing the activity of certain enzymes such as cytochrome oxidase and adenosine triphosphatase [15]. It also increases DNA synthesis, collagen and pro-collagen production, and may increase the cell proliferation or alter locomotory characteristics of cells [16].
Low energy laser irradiation has positive effects on bone fracture healing. The mechanisms by which low-energy laser irradiations affect bone healing is still not clear [16-17]. In studies on animals, He-Ne laser accelerated the deposition of bone matrix and increased vascularization, altered the osteoblast and osteoclast cell populations, enhanced fracture healing [18] and improved bone regeneration [19]. Also, it was found that LLLT can accelerate bone formation by increasing osteoblastic activity [20], vascularization [21], organization of collagen fibers, and ATP levels [22].

The introduction of High Intensity Laser Therapy (HILT) in the field of physical therapy is relatively recent. High power pulsed Nd:YAG laser works with high peak power and is able to reach deep tissues, such as deep joints, that are difficult to reach for classical lasers [23] . The use of pulsed Nd:YAG laser has spread for pain therapy with excellent results [24]. Studies exist which describe the anti-inflammatory, anti-oedeme and antalgic effects of Nd:YAG laser, thus justifying its use in the therapy of pain [25, 26].
To our knowledge, no studies up to date have been conducted on possible effects of HILT on BMD of lumbar vertebrae in postmenopausal women with osteoporosis. The aim of the present study was to compare the possible effect of HILT and LLLT on BMD of lumbar vertebrae in postmenopausal women with osteoporosis.

MATERIALS AND METHODS

Patients:

Thirty postmenopausal women were recruited from Kaser El-aini Hospital and Ain Shams Hospital, Cairo –Egypt. DEXA was used to diagnose osteoporosis in lumbar vertebrae with no evidence of vertebral compression fractures.
We enrolled in the study patients with age ranging from 51 to 60 years (to avoid inclusion of older patients with multiple medical problems) with no history of cancer, renal disease, gastrectomy, metabolic bone disease or any condition (such as a neurogenic, myopathic or connective tissue disorder) that could cause secondary osteoporosis. The women did not intake any drug associated with accelerated bone loss (steroids) or any drug affecting bone metabolism (estrogen, calcium, vitamin D). The body mass index did not exceed 30 Kg/m2. The patients did not smok and led sedentary life style without participation at any exercise training during this study. They had natural menopause at least 1 year before entry into the study with no history of ovariectomy. All women were given a full explanation of the treatment protocol and a written informed consent form giving agreement to participation and publication of results was signed by the patients and the study was approved by the Departmental Council and the

Ethics Committee of the Faculty of Physical Therapy, Cairo University.
Subjects were randomly assigned to two groups: Group (I) consisted of 15 subjects with BMD in lumbar vertebrae below normal level (osteoporosis); they were treated with HILT. Also Group (II) consisted of 15 subjects with BMD in lumbar vertebrae below normal level (osteoporosis), but they were exposed to LLLT. Randomization was performed simply by asking the patients to choose a piece of paper on which A or B letter was written. (A) Corresponded to Group I (HILT) while (B) corresponded to Group II, which received LLLT.

INSTRUMENTATION:

(I) Dual Energy x-ray Absorptiometry (DEXA)

(Model QDR-1000W, Hologic, Inc., Waltham, MA) was used for the qualitative assessment of BMD in the vertebral bodies of the lumbar spine for both groups. DEXA performs an imaging test that measures bone density (the amount of bone mineral contained in a certain volume of bone) by passing x-rays with two different energy levels through the bone. It is used to diagnose osteoporosis (decrease in bone mass and density). It is also called bone mineral density scan (BMD scan).

(2) High Intensity Laser Therapy (HILT):

An Hilterapia system HIRO 3.0 (ASA, Vicenza, Italy) was used to deliver high intensity laser therapy. The source was a Nd:YAG laser with pulsed emission (1064 nm), very high peak power (up to 3 KW), high energy content (up to 350 mJ per pulse), high levels of fluence (energy density) (360-1780 mJ\ cm2), short pulse duration (< 120 µs), low frequency (10-30 Hz), duty cycle of about 0.1%. It has been recognized and approved by the FDA (Food and Drug Administration, USA) in 2004.

(3) Low Level Laser Therapy (LLLT):

Was performed with a LEVELASER M300D equipped with the optional version made of an He-Ne and IR laser, minimum power 22/35 mW. So, the emissions used for the treatment were continuous red and pulsed infrared light with wavelengths of 632.8 and 904 nm, respectively.

PROCEDURES:

A. Evaluation:

A screening test including careful history taking and gynecological examination was conducted for each subject before entry in this study. After that, BMD of lumbar spine (L1.-5) was measured by DEXA densitometry. Evaluation of lumbar BMD was performed before and after the end of six weeks of treatment.

B) Treatment:

All subjects in this study were exposed to three sessions per week for six successive weeks. The treatment procedure was explained to all subjects. Skin was cleaned with alcohol. During the irradiation, the position of the subjects was the same for both groups (prone lying position with a pillow under her abdomen). The eyes of both patient and operator were protected by goggles at all times so that laser ray could never reach eyes. Laser was irradiated to the lumbar vertebrae (L1-5) using the following laser parameters:

Group I - patients received HILT (Nd:YAG), with pulsed emission (1064 nm), very high peak power (up to 3 kW), elevated energy content (up to 350 mJ), high levels of fluence (energy density) (360-1780 mJ /cm2), brief duration (< 120 µs), low frequency (10-30 Hz), Duty Cycle of about 0.1%. The delivery technique for this group was scanning with total energy of 4000 joule. HILT was delivered in two different phases: initial phase and terminal phase. In the initial phase, three sub-phases of fast manual scan (10 cm scanned in about 1.5 seconds) were performed to lumbar region with increasing fluences (710 -910 -1530 mJ/cm2) and decreasing frequencies (30-20-15 Hz), a total energy of 2000 joules reached the lumbar region. The final phase consisted of 3 sub-phases of slow scanning (10 cm scanned in about 3 seconds) with increasing fluences (710-910-1530 mJ/cm2) and decreasing frequencies (30-20-15 Hz), a total energy of 2000 joules reached the lumbar region. Scans were longitudinal or transversal to the anatomical structure to be treated, ideally following a straight lines path [27].

Group II – patients were irradiated by LLLT to the lumbar vertebrae (L1-5). The characteristics of the laser beam included: He-Ne and IR lasers with wavelengths 632.8 and 904 nm, respectively; frequency of 3000 Hz; power output 25 mW; beam diameter 1.5 mm. The delivery technique for this group was automatic scanning with energy density of 4 J/cm2. Laser scan over the lumbar region by adjusting the laser scanned area with amplitude-frequency adjustments of horizontal and vertical scanning. The laser-head position was servo-controlled by two motors and could be turned vertically within a range of 110°. The laser emission was vertical starting from the lower part of the head; laser beam was punctiform and could perform horizontal or vertical scanning within a 30° range (±15°). The laser unit automatically calculated the duration of the therapy on the basis of the treated area and the energy density to be transferred.

OUTCOME MEASURE

BMD was collected at lumbar spine using DEXA for both groups pre-treatment and at the end of treatment after six weeks.

Groups	Pre treatment		Post treatment		t value	P value
	mean	SD	mean creams	SD		
HILT group I	-3.2	0.25355	-1.0667	0.67788	11.117	< 0.0001[a]
LLLT group II	-3.1333	0.22887	-2.5667	0.49522	3.697	< 0.002[a]
Mean Difference	-0.06667		1.5			
t value	-0.756		6.92			
P value	0.456[b]		< 0.0001[a]			

SD: Standard Deviation a: Significant b: Non significant
Table I - BMD Mean values pre and post treatment and mean differences in both the groups under study.

DATA ANALYSIS

The data were analyzed using paired t-test to compare the values found pre and post treatment into each group. Independent t-test was used to compare between the two groups at pre and post treatment. The level of significance was set at 0.05 for all tests.

RESULTS

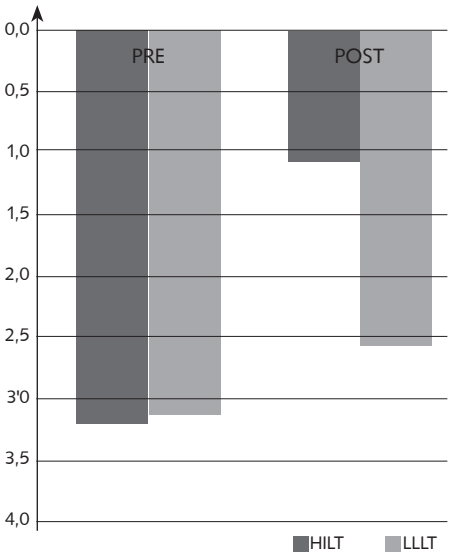

Figure 1:
Comparison between pre and post treatment BMD mean values in both the study groups

As shown in table I and figure 1, before treatment the mean value found analyzing the measures of BMD performed on patients belonging to the Group I (HILT) was – 3.2 ± 0.25, while in the Group II (LLLT) the mean value of BMD was – 3.1333 ± 0.22. By comparing Group I and Group II, the statistical analysis did not reveal any significant difference, indicating that patients enrolled in the study were homogeneously distributed in the two groups. Immediately after the end of the treatment, the mean value of BMD found in patients belonging to the Group I (HILT) was – 1.06 ± 0.6. Compared to the pretreatment value, it revealed a highly significant (P>0.0001) improvement in BMD in response to HILT

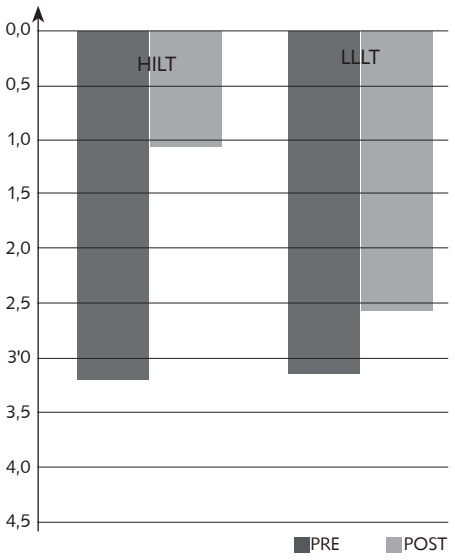

Figure 2:
Comparison between both HILT and LLLT groups in pre & post treatment of BMD.

(table I, figure 2). Also group II showed a statistically significant increase in BMD after LLLT, with a mean value of – 2.5 ± 0.4 (table I, figure 2).

The comparison between the two groups as regards the extent of improvement in BMD observed after the laser therapies clearly pointed out that the increase in BMD induced by HILT was significantly higher than that produced by LLLT (t value: 6.92 and p <0.0001; see table I and figure).

DISCUSSION

The study we have described in this paper had a dual purpose: to evaluate the effectiveness of laser therapy in the treatment of osteoporosis and to compare the effects obtained with two different laser therapies, the former performed with a low level laser emission (LLLT), the latter performed with a pulsed high intensity Nd:YAG laser (HILT).

It has been suggested that LLLT may influence the healing process by affecting various physiological functions and processes such as blood flow, lymphatic flow, inflammation, cellular proliferation and differentiation [21].

Our study show that there was a significant difference between the pre and post treatment mean values of BMD

in patients treated with LLLT. These results are in accordance with the data of Ninomiya et al. [28], who reported that low energy laser irradiation has positive effects on bone fracture healing and therefore may stimulate bone formation. It was found that LLLT reduced the healing time following implant placement and improved bone regeneration, which is a very complex physiological process influenced by a series of biomechanical, biochemical and hormonal factors [19].
Researchers studied bone healing after laser irradiation using histological, histochemical and radiographic measures. These studies have shown conflicting results, because some observed an acceleration of fracture healing [29] while others reported delayed fracture healing after low-level laser irradiation [30]. In recent years, the studies performed by Kandra et al. [31] demonstrated that LLLT stimulates the bone implant interaction.

The histomorphomeric analysis of the treated groups demonstrated a higher bone to implant contact than the control groups [19, 31]. Renno et al. [27] investigated the effects of LLLT (infrared, 830 nm) on the bone properties and bone strength of rat femur after ovariectomy. Laser irradiation was initiated 1 day after the operation and was performed three times a week, for 2 months. Femora were submitted to a biomechanical test and physical properties evaluation.

The results indicated that LLLT was able to prevent bone loss in rats [27]. Khandra et al., [32] demonstrated that LLLT has the ability to stimulate the attachment and proliferation of human osteoblasts like cells cultured on titanium implant material indicating that LLLT could modulate the activity of cells surrounding implant material [32]. Márquezet et al., [33] assessed with histological analysis the effect of laser modulation on the repair of surgical defects on the femur of rats filled with lyophilized bovine bone. The results showed that there was histological evidence of improved collagen fiber deposition at early stages of the healing and increased amount of well-organized bone trabeculae at the end of the experimental period on irradiated animals [33].

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 to obtain therapeutic effects without tissue damage. In particular, pulsed Nd: YAG laser has proved its versatility and efficacy in the treatment of many different musculoskeletal diseases and it is believed to have anti-inflammatory, anti-edema, analgesic and also reparative effects. The interaction between tissue and laser radiation can alter the mechanics of cell micro-environment, thus acting on the cells as a mechanical stress [34].
The results of the present study show that there was a very significant difference between the mean values of BMD pre- and post-treatment in patients exposed to HILT.

Moreover, although an improvement in BMD has been observed both in Group I (HILT) and in Group II (LLLT), the improvement induced by HILT was significantly higher than that induced by LLLT. This could be due to the characteristics of the source used in the HILT, which emits very short pulses that can reach deeper tissue during the treatment.

In conclusion the results indicate that laser therapy is an effective method for the management of osteoporosis in postmenopausal women and HILT is more effective than LLLT in improving BMD.

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Effects of PEMFs-ELFs (Pulsed Electromagnetic Fields-Extremely Low Frequencies) on Morfology and Differentiation of C2C12 Mouse Myoblast Cell Line.

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ABSTRACT

An increasing number of reports shows the ability of ELF-PEMFs to change the behavior of cells and evoke biological responses. Therefore, the interest on the use of ELF-PEMFs in clinics is increasing and new fields of application are explored, in addition to the well-established application in the treatment of bone diseases. However, our understanding of the cellular and molecular mechanisms that underlie the clinical observations is still lacking. A better knowledge is required to improve the clinical applications and treatment parameters.

The aim of this study was to analyze ELF-PEMFs (50Hz, 2mT) effect on a myoblast model (C2C12 cell line) through morphological and molecular assays. This cell line is a well-characterized model to study muscle cell differentiation and tissue repair. To assess the effect of treatment time on the biological response we used two different time of stimulation: 15 minutes (short-treatment) and 3 hours (long-treatment). The samples were analyzed immediately after the treatment and 24 h, 72 h, 6 days later. Viability and proliferation were assessed by MTT assay. Morphology and cytoskeleton organization were analyzed by immunofluorescence microscopy. The effect of ELF-PEMFs on myoblast differentiation was investigated by analyzing the expression of markers typically expressed during myogenesis: MyoD, myogenin and MHC.

After both the treatments we found a weak decrease in proliferation but no effects on cell viability. The network of microfilaments and microtubules changed, especially after 3 h exposure to ELF-PEMFs. The expression of myogenesis markers increased and the translocation of the transcription factor MyoD to the myoblasts nucleus was observed. In conclusion the results showed that ELF-PEMFs are able to induce in the myoblast model a biological response consisting in cytoskeleton remodelling and increase in expression of myogenesis markers. The effect depended on the exposure time.

INTRODUCTION

Electromagnetic fields and magnetotherapy are commonly used in physical medicine. Generally, the EMFs applied in clinics are pulsed (PEMFs), with frequency lower than 100 Hz and field intensity ranging from μT to a few tens of mT, therefore they are classified as Extremely Low Frequency (ELF) PEMFs. Recent studies have shown that ELF-PEMFs can change cell behavior and activation by affecting biochemical and biophysical processes. The molecular and cellular mechanisms underlying the effects of ELF-PEMFs are not completely understood, but recently many progresses have been made: physical processes at the atomic and molecular level are at the basis of the biological response evoked by ELF-PEMFs, since they can affect chemical bonds, dipole orientation, charge diffusion, receptor clustering, etc.....[1].

In living tissues endogenous EMFs are generated by physiological activities, for example, muscle vibrations induce mechanical strains in bone tissue and low frequency EMFs are generated both during postural muscle activity and walking, 5-30 Hz and <100 Hz respectively [2]. Muscle contraction has an important role in maintaining bone mass, since bone cells are sensitive to EMFs in the 15-30 Hz range

[3]. Thus ELF-PEMFs stimulation has been used successfully to treat a wide range of bone disorders, such as rheumatologic diseases [4] and osteoporosis, and accelerate the healing of fractures [5]. Moreover ELF-PEMFs are widely used in clinics for beneficial effects due to analgesic action, anti-oedematous activity, vasodilation and anti-inflammatory action [1]. Magnetotherapy provides a safe, non invasive and easy method to directly treat the site of injury, thus the use of this tool of physical medicine is increasing, either as single therapy or in combination with other physical devices or drugs.

It has been shown by several studies that ELF-PEMF are able to treat musculoskeletal disorders and muscle hypotrophy with faster and better results than traditional methods [6].

At cellular level, it has been demonstrated that ELF-PEMF affect plasma membrane permeability by modifying ion-channel structure and kinetics [7] and altering the concentration of intracellular ions, mainly calcium [1]. Moreover, it has been demonstrated that ELF-PEMFs affect cellular functions, such as cell proliferation and differentiation [8]. ELF-PEMFs modulate critical cellular pathways and levels of transcription of genes related to apoptosis, cell cycle, control-related proteins [9], cytochrome P450 and inducible nitric oxide synthase enzyme activity [10].

The results of preliminary studies performed in our laboratory demonstrated that ELF-PEMFs are able to trigger differentiation in SHSY5Y cells, a neuroblast model [11]. Moreover, we found that ELF-PEMFs can induce cytoskeleton rearrangement in myoblastic cells [12].The aim of this study was double: to confirm previous findings on the effects of ELF-PEMFs on myoblasts and analyze how ELF-PEMFs may affect the transcription of factors which regulate myogenesis and their intracellular distribution.

MATERIAL AND METHODS

Cell Culture

Murine myoblasts (C2C12 skeletal muscle cell line, American Type Culture Collection, Manassas, VA, USA) were routinely cultured in growing medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 µg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 10% fetal bovine serum (FBS). Cells were incubated at 37°C and 5% CO2. All the reagents for cell cultures have been purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell viability

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

MTT assay

Cell proliferation after exposure to ELF-PEMs was determined by MTT assay, following the method of Spinner [13]. Cells were cultured in 96-well plates and treated with PEMF-ELF (2 mT-50 Hz) for 15 min or 3 h. MTT assays were performed immediately, 24h, 72h and 6 days after the exposure. After the designated time period, 10 µl MTT solution was added to each well. After 4 h incubation, the supernatant was discarded and DMSO (100 µl) was added. Once the blue crystals were dissolved, the optical density (OD) was measured at the test wavelength of 595 nm and the reference wavelength of 630 nm using a plate microreader (Victor III, Perkin Elmer, VA, USA). O.D. (Optical Density) was calculated as the difference between the absorbance at the reference wavelength and the test wavelength. The percent viability was calculated as (O.D. of PEMFs-ELFs-treated sample/ control O.D.) X100.(n=3, mean ± SD).

Immunofluorescence microscopy

Control and treated samples were fixed for 5 min in cold acetone, then washed in phosphatase buffered saline (PBS). After blocking unspecific binding with PBS containing 3% bovine serum albumin, cells were incubated overnight with the specific antibodies: anti- actin, anti-tubulin and anti-MyoD. The cells were then incubated with the fluorescein isothiocyanate (FITC) conjugated specific secondary antibody (anti-mouse IgG). Cells incubated with the anti- actin antibody did not need incubation with the secondary antibody since a mouse anti actin Alexa Fluor® 488 conjugated was used. All antibodies were purchased from Chemicon Int, (Temecula, CA). Negative controls were obtained by omitting the primary antibodies. Samples were evaluated by an epifluorescence microscope (Nikon, Florence, Italy) at 100X magnification and imaged by a HiRes IV digital CCD camera (DTA, Pisa, Italy).

Exposure to ELF-PEMFs

Cells were exposed to an ELF-PEMF produced by a pair of coils in the configuration of Helmholtz coils. In the central region of the system, corresponding to the area where the multiwell plate is located, the intensity was constant within about 3% of its maximum value. In order to perform the experiments described in this paper, the power parameters of the coils were set in order to have a field with 2 mT intensity of and 50Hz frequency. The experiment was carried out in a heated box, specially designed to contain the coils, to ensure temperature stability (37°C) in the

volume around the samples. The control samples were placed on the bench outside the coils, in an area where the field intensity produced by the coils was negligible. In order to study the dependence of the ELF-PEMF effects on the exposure time, cells were treated for 15min or 3h and the biological response was assessed immediately, 24h, 72h and 6d after the treatments. The control samples were maintained and processed in the same conditions except for the exposure to the ELF-PEMF.

Quantitative real-time RT-PCR: expression of differentiation markers

Expression of specific markers of muscle cell maturation (MyoD, myogenin and MHC) was evaluated by quantitative real-time qRT-PCR. Total RNA was isolated from cell cultures using High Pure RNA Isolation kit (Roche) according to the manufacturer's protocol.

Extracted RNA was diluted 1:10 in MilliQ water and RNA concentration was measured at 260 nm with a spectrophotometer (Lambda 45, Perkin Elmer). RNA retrotranscription into cDNA was performed with 200 ng of RNA in a total volume of 20 µl, including 4 µl of iScriptTM Reverse Transcription Supermix (5X, Bio-Rad), containing appropriate quantities of RNaseH+, dNTPs, oligo (dT), random primers, buffer, MgCl2, and reverse transcriptase.

The synthesis program included an initial incubation at 25°C for 5 min, followed by incubation at 42°C for 45 min and 48°C for 15 min. The reaction was inactivated by heating at 85°C for 5 min, and the reaction volume was finally increased up to 200 µl with MilliQ water.

Quantitative RT-PCR was performed with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) to determine the expression of the genes encoding for the differentiation markers considered. Results were normalized to the expression levels of a selected housekeeping gene, that is β-actin. Obtained cDNA (5µl) was mixed with 1µl of specific forward and reverse primers (8µM), 4µl of MilliQ and 10µl of SsoAdvancedTMSYBR®GreenSupermix (Bio-rad), containing appropriate quantities of hot-start Sso7f-fusion polymerase, SYBR®Green dye, dNTPs, MgCl2, and enhancers.

The thermal protocol was applied with one cycle of 30 s at 98°C for enzyme activation, followed by 40 cycles at 98°C for 3 s and 60°C for 7 s. After the last reaction cycle, the protocol provided a temperature ramp from 65°C to 95°C, at 0.5°C/s increments, to exclude unspecific products with melting curve results. All tests were carried out in triplicate. The cycle threshold (Ct) value relative of control sample was adopted as reference for the calculation of ΔΔCt (difference between ΔCt values deriving from difference between Ct of target and housekeeping genes) for the subsequent samples. The primer sequences (forward and reverse) of the investigated genes are reported below. For more details see [14].

MyoD
F: 5′-GCTCTGATGGCATGATGG-3′
MyoD
R: 5′-CACTCTTCCCTGGTCTGG-3′

Myogenin
F: 5′-TGAATGCAACTCCCACAG-3′
Myogenin
R: 5′-GCGAGCAAATGATCTCCT-3′

MHC
F: 5′- CTGGCTTCTGCTGATATTGA-3′
MHC
R:5′- CTTCTTGTTAGACATGATCTGGTA-3′

β-actin
F: 5′- CCACACCCGCCACCAGTTC- 3′

β-actin
R: 5′-GACCCATACCCACCATCACACC-3′

STATISTICS

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 ± standard deviation. Statistical significance was determined using a Student's t test. A p value lower than 0.05 was considered statistically significant. For Quantitative real-time RT-PCR, analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's post-test to test for significance, which was set at 5%. Results are presented as mean value ± standard deviation.

RESULTS

Viability and proliferation

In order to verify the effect of the exposure to ELF-PEMFs on cell viability and proliferation, Trypan Blue and MTT assays were carried out immediately, 24 h, 72 h and 6 days after treatments of 15 min or 3 h.

In all the samples, both treated and controls, and for all the different times of analysis, cell viability resulted higher than 95%. Therefore we did not observe significant effects of ELF-PEMFs on cell viability and, obviously, no difference between the two treatment times.

As shown in Fig.1, immediately after 15 min exposure, a moderate but significant decrease in cell proliferation was found. At the subsequent evaluation times (24h, 72h and 6 days), no significant differences were observed between treated samples and controls. As regard the longer treatment (3h), data obtained at all the times considered did not show any significant difference between treated and control samples and controls (Fig.2)

Cytoskeleton

Cytosketelon is an important cell structure since it allows both movement and shape

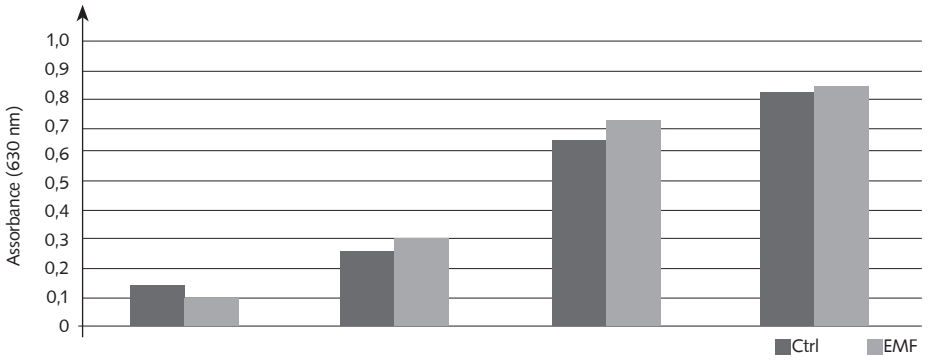

Figure 1: Cell proliferation assessed at 0h, 24h, 72h and 6days after 15 min of exposure to EMFs. Data were obtained by MTT assay.

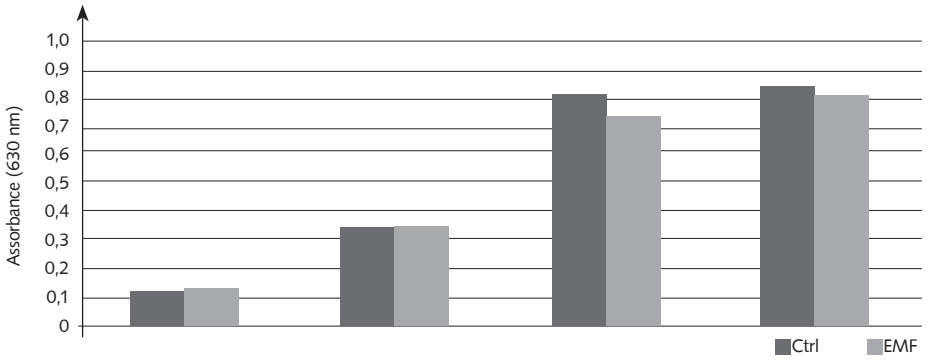

Figure 2: Cell proliferation assessed at 0h, 24h, 72h and 6days after 3h of exposure to EMFs. Data were obtained by MTT assay.

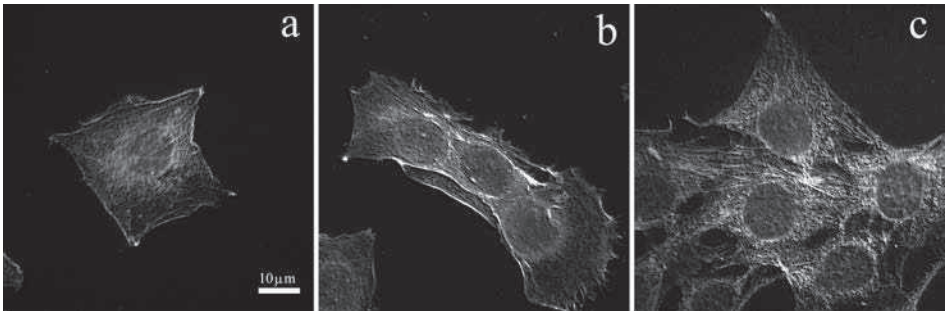

Figure 3: Actin expression assessed by immunofluorescence microscopy. Control (a) at 0h (b) and 72h (c) after 15 min of exposure to EMFs.

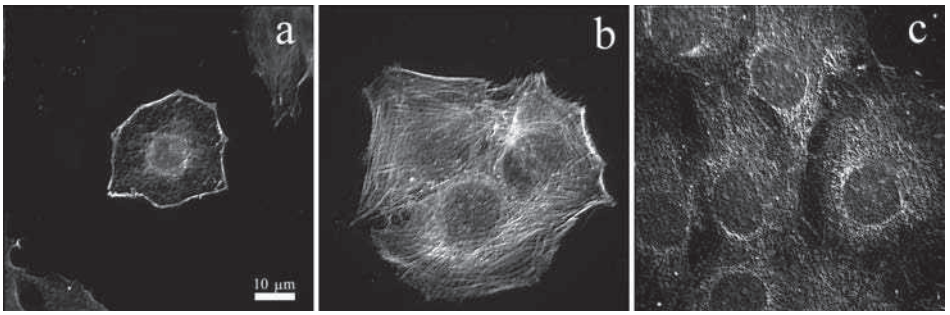

Figure 4: Actin expression assessed by immunofluorescence microscopy. Control (a), at 0h (b) and 6days (c) after 3h of exposure to EMFs..

modifications and has an important role in intracellular transport and signalling.

The morphological analysis of major cytoskeleton components, actin microfilaments and microtubules, which was performed by immunofluorescence microscopy, showed evident architectural alterations of both the cytoskeleton components considered in the samples exposed to ELF-PEMFs.

As expected, in control samples the actin microfilaments were distributed mostly in the perinuclear area and under the plasmamembrane, where they formed a thick layer (actin ring) (Fig.3a)

In cells exposed to ELF-PEMFs for 15 min, analyzed immediately after the treatment, the actin expression increased, in particular in the perinuclear area, and cells showed a higher number of filopodia (Fig.3b). In samples analyzed 72h after the treatment (15 min), cells showed some stress fibers arranged in bundles that allowed connections with neighboring elements (Fig.3c).

For longer exposure (3 h), an increase in actin stress fibers was observed immediately, 24 and 72 h after the treatment; cells merged to form syncytia-like structures (Fig.4b). In cells analyzed 6days after a 3 h exposure to ELF-PEMFs, actin resulted homogeneously distributed, the stress fibers and actin ring disappeared (Fig.4c).

As regards the microtubule network, in control samples the cells showed the expected organization pattern: microtubules radially distributed from the microtubule organizing centre, near the nucleus, towards the periphery of the cell (Fig.5a). In contrast, in treated cells analyzed immediately and 24h after the exposure, the microtubules were not radially distributed but formed a dense network (Fig.5b). 72h after 15min exposure to ELF-PEMFs, tubulin expression further increased (Fig.5c). In samples that had undergone the longer treatment (3 h) the tubulin expression strongly increased

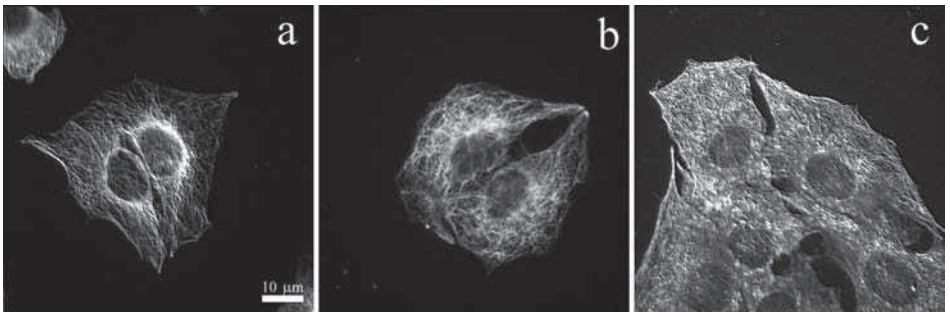

Figure 5: Tubulin expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 15 min of exposure to EMFs.

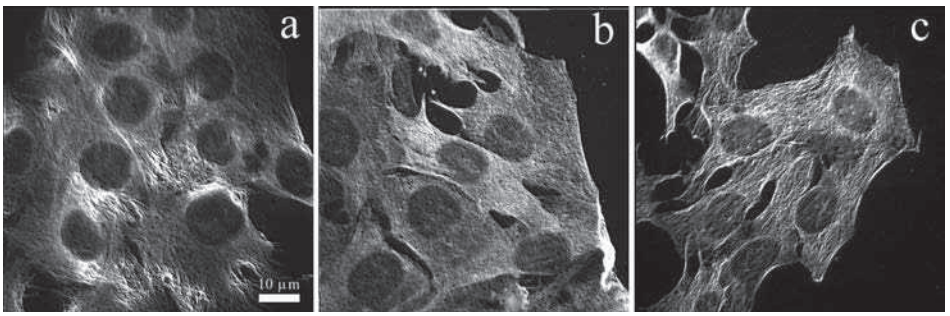

Figure 6: Tubulin expression assessed by immunofluorescence microscopy. Control (a) at 72h (b) and 6days (c) after 3h of exposure to EMFs.

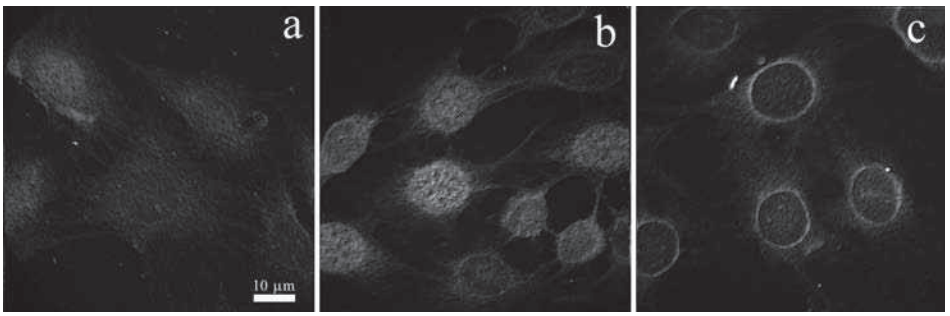

Figure 7: MyoD expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 15 min of exposure to EMFs.

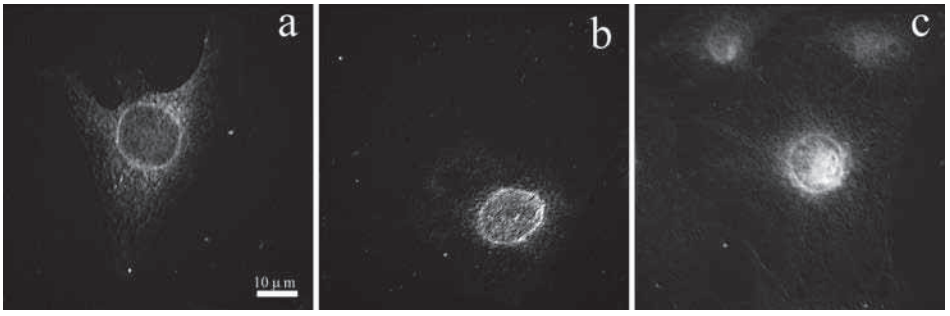

Figure 8: MyoD expression assessed by immunofluorescence microscopy. Control (a) at 24h (b) and 72h (c) after 3h of exposure to EMFs.

after 24 h and the protein remained highly expressed also after 72 h and 6 days, while the microtubule organizing centre disappeared.(Fig.6).

Expression and subcellular localization of MyoD

MyoD is a member of the Muscle Regulatory Factors family. It is a transcription factor and plays a pivotal role in the complex mechanism of skeletal muscle cell differentiation. The effects of ELF-PEMFs on its expression and intracellular distribution were analyzed by immunofluorescence microscopy.

In control samples (Fig.7a) MyoD showed a very low expression and cytoplasmic localization. While, 24 h after a 15 min treatment, MyoD expression increased in myoblasts and showed a nuclear localization (Fig.7b). The effect was reversed 72 h after the treatment (Fig.7c): MyoD did not show nuclear localization but again appeared distributed in the cytoplasm surrounding the nucleus.

In cells exposed to the ELF-PEMFs longer treatment (3 h), MyoD increased and presented a nuclear localization, the expression was particularly significant after 24 h and 3 days after treatment. After 6 days the expression decreased (Fig.8)..

Gene expression analysis by Real Time PCR

Myogenesis consists of numerous ordered steps that require a wide variety of transcription factors which control proliferation and differentiation. The expression of genes involved in myogenesis was assessed by RT-PCR analysis. MyoD is an early-differentiation marker involved in the commitment of precursor cells to a myogenic fate, whereas myogenin and MHC (Myosin Heavy Chain) expression is associated with terminal differentiation.

In samples analyzed 24h after treatments (Fig.9), there were no differences between controls and treated samples, except for

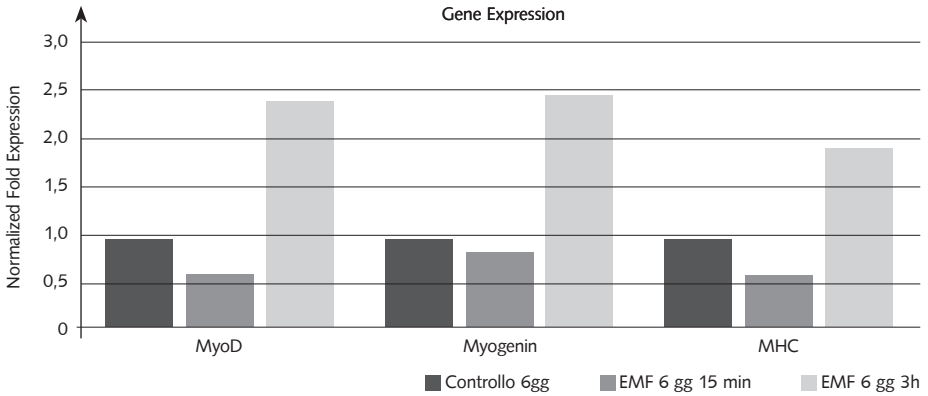

Figure 9: MyoD, Myogenin, and MHC expression 24h after 15 min of exposure to EMFs

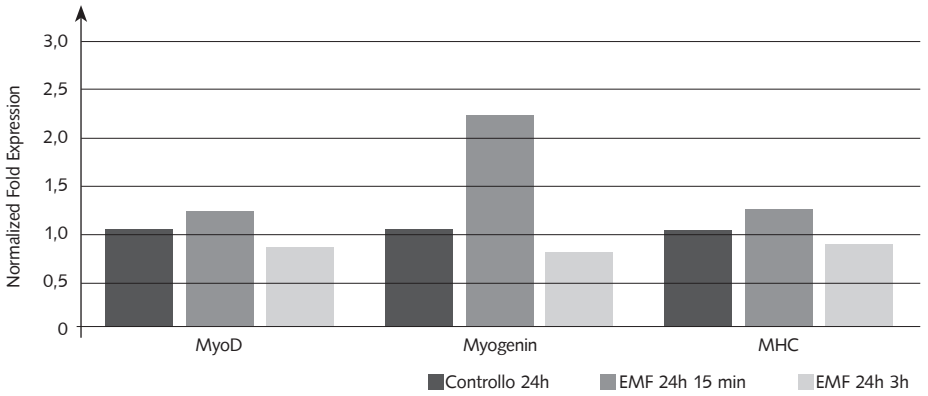

Figure 10: MyoD, Myogenin, and MHC expression 24h after 3h of exposure to EMFs

myogenin mRNA transcript. We observed a marked increase in myogenin mRNA transcript (more than 2 fold) in C2C12 cells after 15min of exposure to ELF-PEMFs, compared to the control.

In Fig.10 is reported the expression of mRNA transcripts 6d after the ELF-PEMFs treatments. In cells exposed for 3 h MyoD, myogenin and MHC expression increased at least 2 fold, compared to the control. Otherwise, in cells treated for 15 min, MyoD and MHC transcripts decreased 0.5 fold.

DISCUSSION

The goal of this study was to analyze the behavior of myoblasts exposed to ELF-PEMFs in order to open possible future perspectives of clinical application for treating muscle disorders after accurate evaluation of suitable parameters for effective stimulation. Our experimental model was the C2C12 cell line, a mouse myoblast model widely used for studies on myogenesis and repair mechanisms in muscle tissue.

The findings of this research show that the exposure to ELF-PEMFs did not affect cell viability but, depending on exposure time and elapsed time between exposure and implementation of the proliferation assay, was able to mildly inhibit cell proliferation. However, the magnitude of the effect did not result statistically significant, except for the proliferation assay performed immediately after the short treatment (15 min), where the decrease in proliferation was definitely significant.

The literature is rich of reports describing the effect of PEMFs on proliferation. The results are controversial: some authors describe an increase [15] and some others a decrease [16] in cell proliferation induced by PEMFs, thus suggesting that the effect strongly depends on the exposure parameters (frequency, intensity and exposure time) used. These are often different, therefore the various studies are difficult to compare.

The analysis of major cytoskeleton components, actin microfilaments and microtubules, by immunofluorescence microscopy demonstrated that the exposure to ELF-PEMFs strongly affected cell morphology and cytoskeleton organization.

Actin has a crucial role in cytoskeleton organization, cell motility and contraction and is also considered a marker of myogenic differentiation. After exposure to ELF-PEMFs myoblasts generally showed an increase in actin expression, disappearance of the actin ring under the plasmamembrane, increase in filopodia, appearance of stress fibers. Sometimes the cells were aligned and tended to merge to form tube-like or syncytia-like structures.

These data confirm results we obtained on previous studies both on neuroblasts [11] and agree with reports by other authors [17].

It is known that ELF-PEMFs, and in general physical stimuli, cause the redistribution of focal adhesion on the plasmamembrane to create new contacts between cell and extracellular matrix and stress fibers have a key role in the reorganization of focal adhesions [18].

Also the microtubule network changed after exposure to PEMFs-ELFs. In treated samples tubulin expression generally

increased, the classical radial distribution of the microtubules disappeared and sometimes also the microtubule organizing centre became indistinguishable, microtubules formed a dense network and sometimes they appeared even fragmented.
The outcomes of our observations indicate that ELF-PEMFs may strongly alter cytoskeleton organization and, consequently, cell morphology, interaction with the extracellular microenvironment and motility. It is known that microtubules, throughout their assembly and disassembly, and stress fibers represent the mold [19] and the scaffold Sanger et al. [20], respectively, to build new myofibrils.

These effects on cytoskeleton are even more significant since they are associated to changes in MyoD expression and distribution, as shown by immunofluorescence analysis. Some aspects of cytoskeletal rearrangement and morphological changes that occur during differentiation are mediated by transcriptional and translational induction of regulators of the process. MyoD, in myoblasts induces not only the expression of muscle-specific genes, in particular myogenin, but also elongation and fusion into multinucleated myotubes [21].
Taken together, the data on cytoskeleton components and MyoD expression suggest that ELF-PEMFs can trigger myoblasts differentiation in a time dependent manner.

The results of the RT-PCR assay further support this hypothesis because in myoblasts exposed to the shorter treatment (15 min) we observed, after 24 h, a significant upregulation of myogenin and weak upregulation of MyoD and MHC, while in cells exposed to the longer treatment (3 h), after 6 days, an upregolation of MyoD, myogenin and MHC was found. As mentioned above, these myogenic regulatory factors transactivate skeletal muscle specific differentiation genes that contain an E-box motif, a DNA binding site with general consensus sequence CANNTG. For example MyoD and myogenin can bind to E-boxes in the regulatory region of mouse desmin gene and transactivarte desmin gene in vitro [22].

In summary rearrangement of microfilaments and microtubules, over-expression and nuclear localization of MyoD, formation of stress-fibers and syncytia-like structures, increase in expression of genes involved in myogenesis regulation are consistent with the hypothesis that exposure to ELF-PEMFs can induce myogenic differentiation.

In conclusion the ELF-PEMFs are physical stimuli recognized and elaborated by cells. The outcomes of this study clearly show that ELF-PEMFs treatment induces morphological and functional changes that ELF-PEMF treatment induces morphological and functional changes that could underlie the early stages of a differentiation process toward myogenesis. The exposure time seems to play a fundamental role in establishing the effect: the genes encoding for factors which regulate myogenesis are differently expressed when comparing the two exposure times considered. Further studies are needed to better understand the relationship between duration of treatment and modulation of the involved pathways.
However, these findings open perspectives of future application of ELF-PEMFs in order to favour myogenesis and muscle tissue repair.

CONCLUSIONS

In conclusion, this study revealed that a short-period application of HILT biostimulating protocol is more effective in pain reduction and in functional ability improvement than no treatment in patients with symptomatic knee OA. Thus, HILT can be an important instrument in pain control contributing to the long-term management of chronic painful knee. The study confirms the safety of the technique.

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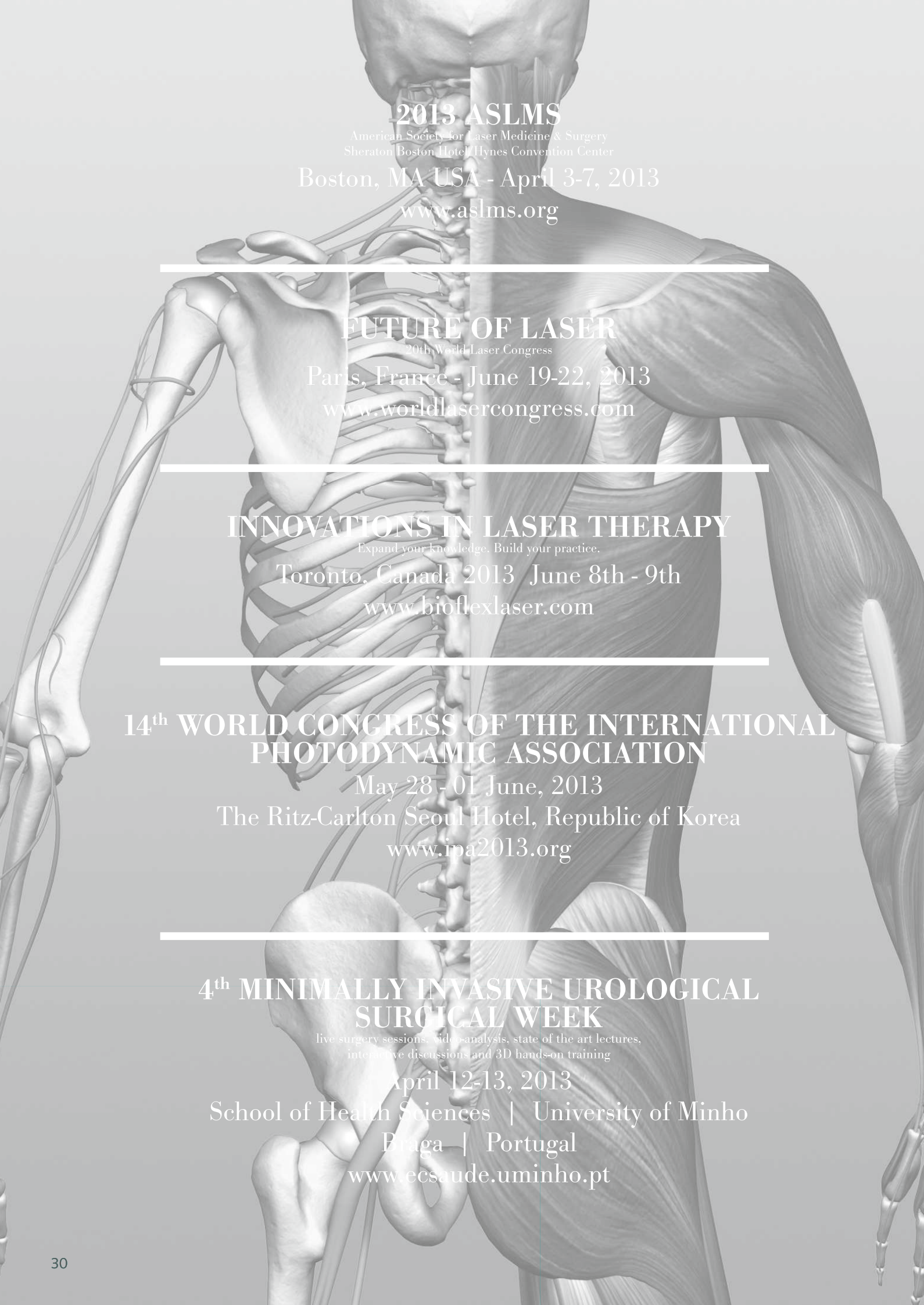

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