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Contents

Lasers in medicine past, present and future.

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Laser in medicine represents the "poor cousin" of lasers in surgery but, in spite of its low profile, is potentially the medicine of the future. Einstein formulated the hypothesis of the generation of laser in 1916 [1] but it was not until 1960 that Maiman, in the Bell Laboratories, generated laser from ruby crystal (λ = 694nm) [2]. A number of early studies initiated research on low-level laser therapy (LLLT) using this wavelength, both clinically and experimentally [3-5].

One of the seminal studies from this period was that of Endre Mester, who treated non-healing chronic ulcers with ruby laser and found a remarkable rate of healing, especially of venous ulcers [6, 7]. Mester's study was the springboard for the continuing use of lasers for wound healing and research into mechanisms for the biological effects underpinning tissue repair commenced. Strong evidence for numerous effects on the cascade of cellular involvement in wound healing continue to be demonstrated [8-17]. Somewhat disappointingly however, there is still variability in the outcomes of clinical studies and standard meta-analyses cannot provide evidence of a strong effect [18-20].

Nevertheless the trend is strong and Enwemeka has used a novel statistical technique to demonstrate a clinically useful benefit [21, 22]. Effects of laser irradiation (LI) in wound healing are especially dose sensitive and in this clinical domain, low doses of laser stimulate and high doses inhibit [23]. Understanding and applying the biphasic biological effects of laser on cells in tissue is critical to optimising the "dose" [24]. This is even more complex in an individual patient where variation in sensitivity to LI is a confounding factor in clinical studies.

Alongside the use of lasers in wound healing, treatment of painful conditions was one of the other early clinical applications of LLLT [25-28]. Many different painful musculoskeletal conditions have been the subject of investigation and, in contrast to wound healing, the evidence base is now strong [29-32]. For example, a meta-analysis of LLLT in neck pain [33], established it as one of the most strongly evidence based of all treatments for neck pain.

This has now been supported in the report assessing neck pain treatments, by the World Health Organisation Committee of the Decade of the Bone and Joint [34]. Other painful conditions have accumulating evidence, such as tendinopathy [32], and lateral epicondylitis [32]. Analysis of laser parameters to identify optimal doses in addition to methodological assessment, have been critical to these meta-analyses, differentiating them from standard meta-analyses where no technological assessment is made [32, 35, 36]. It appears that the balance between stimulatory and inhibitory effects of LLLT is not as critical in pain modulation as in tissue repair. Importantly, the critical concept of "dose" is again emphasised in these studies.

Various mechanisms for the pain relieving effects of LLLT have been proposed since the first clinical studies were performed. These have included the gate control theory [37], endorphin release [38-40], serotonin increase [41, 42], neural inhibition [43-48] and anti-inflammatory effects [49-52], with these latter two effects the focus of intense investigation. The anti-inflammatory effects of laser have now been well documented in many experimental studies as well as clinical studies [50, 53-57], demonstrating that these effects are of the same order of magnitude as anti-inflammatory drugs, and indeed more effective in some instances. In one of the most difficult areas of management, LLLT for the treatment of chemotherapy-induced oral mucositis is one of the most important recent applications, which should be introduced without delay into mainstream practice, as this condition carries significant morbidity [58].

The capacity of LLLT to reduce inflammation is one of the most promising areas in medicine as the ease of application in primary care practice and safety, compared with NSAIDs, would make it an extremely cost-effective option for introduction to mainstream medicine. Other mechanisms for pain relieving effects which focus on neural inhibition, also underlie the benefit where drug therapies are limited by side effects and lack of efficacy. This is particularly so in chronic pain states, which is reaching epidemic proportions [59]. Specific anti-nociceptor effects are strongly supported

in the literature as is the capacity to reduce acute pain following injury and to prevent the progression from acute to chronic pain in the short term. Such "preventative" effects of "pre-emptive" treatment significantly reduced pain scores and drug intake when laser therapy was applied immediately post-operatively [60] and prevented recurrence of neck pain six months after treatment of an acute episode [61]. Neural inhibition may also have the capacity to reduce "wind up" as well as peripheral and central sensitisation by a cascade of effects from the peripheral nerve to the spinal cord and pain matrix [62]. These mechanisms are implicated in the progress of acute to chronic pain and induce long-term depression of pain in chronic pain states, such as fibromyalgia, which are very difficult to treat using conventional therapies.

Wound healing and treatment of painful conditions have been studied for many years, however, the first decade of the twenty-first century has seen research into a range of applications that offer novel treatment options for a range of neurological conditions.

Laser therapy as an adjunct to peripheral nerve and spinal cord repair presents an option for management for the near future [63-67]; laser therapy to the scalp within 24 hours of stroke reduces disability by about 25%[68]; laser (and LED) therapy for traumatic brain injury [69, 70] and depression [71] are also in the early stages of clinical development and offer novel approaches for difficult to treat conditions. Animal studies using models of myocardial infarction suggest another option for adjunctive treatment for minimising ischaemic damage [72]. Other areas at the frontier of research involves enhancement of stem cells viability [73-75] and enhancement of sperm motility [76, 77].

With all the potential of this therapy as well as the current evidence base for applications in pain and inflammation, one might ask why the notion that light might have a therapeutic potential in this new form is greeted with responses ranging from indifference to outright hostility, which was certainly the situation with the early literature [78]. There are many examples of currently applied light therapy such as the treatment of neonatal hyperbilirubinaemia, light therapy for psoriasis and seasonal affective disorder. The Nobel prize was won in 1903 by Neils Finsen for the treatment of tuberculosis with light. One reason for the resistance may have come about because the adoption of LLLT in clinical practice initially outstripped the evidence base, in addition to a lack of understanding of any mechanisms for the effects. This is very different from the introduction of drugs which undergo a series of experiments over many years from the Phase I laboratory based investigations to Phase III human clinical trials before finally, if at all, the drug becomes available to the public.

With LLLT, the safety and ease of application, meant that manufacturers could make and sell the devices to a wide variety of health practitioners, particularly alternative medicine practitioners, who rapidly took to the devices. The response of the medical profession was to attribute any benefits to placebo effects and therefore not attribute any credibility to LLLT. Manufacturers continued to make what appeared to be outrageous claims, further confirming in the medical profession's conservative and drug-oriented view that LLLT, which did not even have a heating effect, could "do anything".

One of the problems in critiquing laser therapy studies comes from both inside and outside the field. Firstly, many authors evaluating studies do not have the expertise to assess whether the technical aspects of the study fulfil the minimum criteria of effective dose and application, and conversely, those conducting studies either use inappropriate parameters or do not report them in sufficient detail to permit replication of the study. Added to this is the difficulty in measuring the precise "dose", and, even then, there is debate about what is the correct "dose".

In spite of these difficulties, there continues to be research into mechanisms of LLLT as well as clinical studies, which confirm significant benefit. While problems exist, mostly relating to understanding dosage, there is no doubt that laser medicine offers the potential for benefit across a range of difficult-to-treat clinical conditions. Patients will be the beneficiaries of the acceptance of LLLT into mainstream medicine. Laser Medicine is the energy medicine of the future.

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High intensity laser-therapy in hand osteoarthritis: a mixed protocol's proposal.

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ABSTRACT

Hand osteoarthritis (HOA) is a common chronic condition involving one or more joints of the thumb and fingers. Therapeutic approach in hand osteoarthritis must consider local interventions which are useful along the course of the pathology. Laser-therapy (Low Level Laser Therapy-LLLT-) is a possible useful instrumental therapy. High Intensity Laser Therapy (HILT) seems to be more effective than LLLT in pain and disability management of some forms of osteoarthritis, due to its higher intensity and to the depth reached by the laser ray. HILT may be used also in laser-acupuncture.

The aim of this study was to analyze the efficacy of HILT in patients with symptomatic HOA, using a mixed protocol, analgesic anti-inflammatory protocol plus laser-acupuncture.

18 out-patients with symptomatic HOA (II-III Kellgren-Lawrence Grading Index) were enrolled and evaluated by Australian Canadian Osteoarthritis Hand Index (AUSCAN) and Visual-Analogue Scale (VAS), before treatment (t0), after treatment (t1) and after 3 months (t2). The patients were treated with a mixed HILT protocol, analgesic plus laser-acupuncture treatment (4 sessions, once a week).

The patients showed a mean statistically significant improvement between t0 and t1 in AUSCAN Index and VAS, and improvement was found in 83% of the subjects (15/18). The improvement was mostly maintained at follow-up.

The mixed HILT protocol showed good results in a great percentage of HOA patients, with only 4 treatment sessions. We conclude that this kind of HILT protocol could be a good proposal for pain control and for improvement of patient's quality of life.

INTRODUCTION

Although underestimated, hand osteoarthritis (HOA) is an important pathology, with both epidemiological and clinical implications [1,2,3]. Estimates of the prevalence of symptomatic hand OA range from 13% to 26% and are greater in women [1]. Symptomatic hand osteoarthritis affects about 20 % of people over 55 years of age. HOA can be both painful and disabling: common symptoms of HOA include pain, swelling, limited range of motion, stiffness, aching at the base of the thumb. Physical function is compromised too: manual dexterity, fine motor control, and usual daily tasks may be difficult to perform. Osteoarthritis pathogenesis includes the contribution of biomechanical and metabolic factors and genetic [4,5] which gradually lead to articular joint tissues destruction. As the disease progresses, clinical features include joint pain, limitation of movement, tenderness, and episodic inflammation. Especially among the elderly, the impact of HOA on disability is important [5]. Pain control, together with the control of the disability progression are the two main targets of the therapeutic approach. European League Against Rheumatism (EULAR) recommendations for the management of HOA include a combination of pharmacological and non-pharmacological treatments [6]. Treatment options for hand osteoarthritis include oral medications (i.e., NSAIDs, analgesics), steroid injections, splinting, physical or occupational therapy and surgery. Rehabilitation treatments can offer significant benefits to patients with HOA, but research in this field is still poor [7].

Recent reviews analysed the effects of rehabilitative and non-surgical interventions in people with HOA [8,9]. Among physical therapy treatment, Low Level Laser Therapy (LLLT) has been often proposed for pain and flogosis control in osteoarthritis and in HOA too [10]. Nevertheless following the Cochrane Library reviews in LLLT no conclusions could be drawn on the optimal dose, the wavelength and the duration of treatment in osteoarthritis [11]. High Intensity Laser Therapy (HILT), a more recent laser application modality, can be more effective than LLLT in pain and flogosis control, due to its more intense and deeper effects [12,13,14]. In other forms of osteoarticular disease HILT treatment showed good results [15,16,17].

Acupuncture is a treatment with ancient and Eastern roots, but it plays a relevant role in some painful conditions and various kinds of arthritic and rheumatic pain, as reported in WHO documents and in a recent Cochrane Review [18,19]. Laser-acupuncture is a modern way to stimulate acupuncture points and its efficacy has been investigated in Western

countries [20,21]. High intensity laser spot may be used as a needle-like stimulus [22]. It probably works as a heath source to stimulate acupuncture points, in producing anti-nociceptive effects.
The present study was a "before-after" study. The aim was to evaluate the clinical and functional efficacy of the HILT treatment in patients affected by symptomatic hand osteoarthritis.

MATERIALS AND METHODS

Patients. Patients suffering for symptomatic HOA were recruited for this trial from outpatients of the Recovery and Rehabilitation Agency (AOU Careggi, Firenze). Informed consensus was obtained. Inclusion criteria required the presence of symptomatic HOA (following ACR criteria [23]), II-III grade of Kellgren-Lawrence Scale [24] on the radiological evaluation. Exclusion criteria were: therapy with oral anticoagulants, non compliant patients (cognitive impairment or psychiatric disorder), skin diseases.

The patients' evaluation included history and clinical examination, VAS (ref. val. 0-10) [25] and Australian/Canadian Hand Osteoarthritis Index (AUSCAN) [26]. AUSCAN Index (ref. val. 0-60) is a valid, disease specific self-administered questionnaire to assess the importance of pain, joint stiffness and physical disability in patients with osteoarthritis (OA) of the hand. Initial assessment (t0), before treatment, included AUSCAN Index and VAS Scale. The same assessment was repeated after the treatment (t1) and after three months (t2).

Treatment. After the initial assessment all the patients underwent the following treatment protocol: they were treated with High Intensity Laser Therapy, 4 sessions, 1 session a week, for four consecutive weeks. In the same session the patients were treated with a laser-acupuncture protocol, followed by the analgesic anti-inflammatory protocol (see Table I).

Data analysis. Data of patients were compared by Student' t-test.

RESULTS

Eighteen patients with symptomatic HOA, aged 52-73 years, were included in the study. Mean age was 68,4 years. The proportion of male (M) and female(F) patient was 3 M, 15 F . AUSCAN Index values at t0 were 39.6 ± 4.7; VAS scale values at t0 were 6.9 ± 2.2 (see Table II) At t1 the patients showed improvement in the scales points: AUSCAN values changed from 39.6 ± 4.7 to 16.4 ± 3.1 ($p< 0.05$). t1 VAS values were 2.9 ± 1.6, and this difference was statistically significant versus t0: $p< 0.001$ (see Table III). Two patients were lost at follow-up. t2 VAS values were 3.4±1.9 (t2 vs. t1: p = n.s.) . AUSCAN scale also showed little, non statistically significant, variations at t2 vs. t1: 16.4± 3.1 vs. 18.9± 4.3.(p: n.s.) (see Table IV). Improvement was found in 15 patients (83% of the subjects) at t1. At follow-up (3 months) 15/18 (83%) of the patients maintained the improvement. No side effects were found.

TABLE I: TREATMENT MIXED PROTOCOL

HILT treatment protocol: pulsed high power laser, Nd:YAG, λ1064 nm, 4 sessions, 1 session a week, laser-acupuncture treatment followed by analgesic anti-inflammatory program.

I) Laser-acupuncture was performed on the points 9LU, 10 LU, 11 LU, 3 LI, 4 LI, 3 SI, 8 PC, 3 SJ, 4 SJ, 8SJ, 36 ST, 10 sec/point, 10 Hz frequency, max 30 J /point.

II) The analgesic anti-inflammatory program was articulated in three phases (initial, intermediate and final phase), in manual scansion. Every phase is articulated in sub-phases in which increasing fluency (810-1170 J/cm²) and decreasing frequency (30-20 Hz) are administered, total energy 1000 J.

TABLE II: GROUP BASELINE CHARACTERISTICS (T0)

PATS. NUMBER	MEAN AGE	SEX	AUSCAN Scale	VAS	KELLGREN GRADE
18	68,4 (52-73)	3M, 15F,	39.6 ± 4.7	6.9 ± 2.2	II 6 pts. III 12 pts

TABLE III: PAIN AND FUNCTIONAL DATA BEFORE AND AFTER HILT TREATMENT

	TO (before treatment)	T1 (after treatment)	T-TEST p-value
VAS	6.9 ± 2.2	2.9±1.6	p<0.001
AUSCAN Index	39.6 ± 4.7	16.4 ± 3.1	p< 0.05

VAS= Visual Analogue Scale (0-10) // AUSCAN = Australian Canadian Osteoarthritis Hand Index (0-60)

TABLE IV: PAIN AND FUNCTIONAL DATA AT THE END OF HILT TREATMENT AND AT FOLLOW-UP

	TO (after treatment)	T1 (after 3 months)	T-TEST p-value
VAS	2.9±1.6	3.4±1.9	p = n.s
AUSCAN Index	16.4 ± 3.1	18.9 ± 4.3	p = n.s

VAS= Visual Analogue Scale (0-10) // AUSCAN = Australian Canadian Osteoarthritis Hand Index (0-60)

DISCUSSION

Recent years investigations agree on functional impact and disability of osteoarthritis of the hand, specially in the elderly, where it is central to daily living impairment. Pain control represents one of the principal tasks in HOA, especially in order to get over acute phases. To date we must register an unsatisfactory response to the various treatments proposed. In the present study we tried to investigate a mixed treatment which joints modern and ancient techniques.

Among instrumental physical therapy the effectiveness of laser-therapy has been often investigated with variable results. Despite a widespread use of this technique, a recent Cochrane review [11] didn't succeed in demonstrating a sure effect of low-level laser-therapy, mainly due to methodological causes of the studies (differences in number of cases, doses and wavelength of laser, etc.). Traditional laser-therapy, which is a low level laser therapy, has got some limits, especially related both to a poor penetration and to a little intensity of the light radiation. Experimental data seem to enhance the hypothesis that high intensity laser therapy may overcome these difficulties, and clinical studies in patients with osteoarthritis confirm its efficacy [12,13,14].

Together with high intensity laser-therapy treatment we combined laser-acupuncture. Acupuncture has nowadays reached EBM for several painful conditions, and its efficacy is accepted by scientific community [19]. Laser-acupuncture is a modern way to stimulate specific points, the same used for needle acupuncture [20]. HILT administered with HIRO.3, with a specific protocol, can work as acupuncture-like stimulation [22] The patients treated with our mixed protocol improved significantly, at the end of the treatment and at follow-up. This fact indicates a long-acting effect, as the results were maintained at distance. In our study, the treatment showed a great efficacy, achieving a rapid pain control and its maintenance till 3 months. The interesting data is that the results were obtained with a very short treatment, reached in only 4 sessions. May be that the immediate antalgic affect of HILT is maintained by the more slow effect of acupuncture, which is believed to re-equilibrate the whole energetic system of the organism. The hand points treated in fact are considered very important in Traditional Chinese Medicine for hand and fingers pain and stiffness.

A recent systematic review [8] establishes that certain rehabilitation interventions provide benefits in individuals with hand OA, but LLLT and acupuncture alone did not reach evidence for reducing both pain and improving function in hand OA. Laser-therapy resulted effective in improving ROM (Range of Motion) and acupuncture may be effective in pain control.

Nevertheless we tried a new way: our study is a preliminary work, which joints together the modern technology, which is High Intensity Laser Therapy, with a very ancient medical culture, acupuncture. The treatment resulted effective, safe and painless. We don't still know which is the best treatment in HOA patients. Certainly the optimal approach includes a combination of pharmacological and non-pharmacological strategies, tailored to the patient and to the phase of the disease.

CONCLUSIONS

From our data HILT appears to be a good medical instrument for pain control in HOA, with consequent improvement in patient's quality of life. It has a rapid and long lasting effect, it is a non invasive technique and no side effects were reported. The treatment protocol which perform analgesic anti-inflammatory treatment together with laser-acupuncture showed very good results in a few sessions. Our preliminary results suggest that this mixed HILT protocol may be a useful resource in the management of hand osteoarthritis patients

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Efficiency of MLS therapy in abarticular rheumatism revealed by digital thermography and visual analog scale.

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ABSTRACT

A lot of 22 patients with acute pathology soft-tissue injuries (shoulder periarthritis, tendinitis, epicondylitis, bursitis), which was divided into two groups. The first group (G1) received treatment with conventional electrotherapy (interferential or diadynamics current, ultrasound) and 100 mW laser. The second group (G2), received conventional therapy electrotherapy and MLS therapy. The aim of this study was to compare the anthalgic and anti-inflammatory effect of the MLS laser therapy and the mono-channel laser treatment in abarticular rheumatism, by digital thermography (outlining the local anti-inflammatory effect by the decrease in the cutaneous temperature), soft tissue echography (visualization of tissular modifications) and by clinical methods (the visual analogue pain scale). The decrease of VAS values to 5 or 10 days of treatment is more important for MLS therapy. It is also apparent decrease in the temperature difference between the affected and the healthy area.

INTRODUCTION

Abarticular rheumatism represents the inflammatory suffering of periarticular soft tissues (muscles, tendons, ligaments, burses). The clinical expression is the stiffness and pain of the affected areas. The causes include physical overstraining, wear, tissular degeneration. The clinical forms are: periarthritis, bursitis, tendinitis, myositis, and epicondylitis.

The most frequent form of periarthritis is the scapula-humeral periarthritis due to the anatomic characteristics of this joint, which provides great mobility. The most frequent causes are the degenerative lesions of tendons (especially those of supraspinatus and biceps), characterized by necrosis, which lead to partial fractures and calcifications. The wearing processes are frequent in people more than 40 years of age, but they are generally clinically masked; in the presence of certain factors (traumatisms, exposures to cold and wet conditions), an inflammatory process also occurs, followed by fibrosis. Clinical signs: localized or diffuse pain, suddenly or insidiously installed, emphasized in movement (abduction is the most painful movement), limitation of movements, muscular contraction, which may generate the blockage of the shoulder.

Burses are located among tendons, muscles, ligaments and bones, in the areas where tendons and muscles pass by several bone eminences. Their role is to reduce friction between these anatomic structures in movement, smoothing the slide and facilitating the moves. There are 80 burses on each side of the human body. The burse inflammation causes bursitis. There are multiple causes that may lead to their occurrence: overstraining (exercising excessive friction forces), vicious positions ("cleaning woman's knee", "policeman's heel"), direct traumatisms; some bursites may represent the manifestation of a systemic disease, such as rheumatoid polyarthritis or gout (in gout, the olecranian and prepatelar burses are the most frequently affected ones). The clinical symptomatology of bursitis is dominated by pain, which has the following characteristics: it is spontaneous or caused by pain or by the pressure upon the bursa, it is more intense at night, and it may irradiate to the related limb; in the case of superficial bursites (olecranian bursa, prepatelar bursa), the inflammation generates their tumefaction; the inflammation of the burses located nearby joints generated a certain degree of joint stiffness. The topographic perspective reveals numerous forms of bursitis. The most frequent and most important locations are at limbs ("goosefoot" bursitis, trochanteric bursitis, prepatellar bursitis – "cleaning woman's bursitis", achilian bursitis, calcaneal bursitis, hallux bursitis, subdeltoid bursitis, olecranian bursitis).

Tendinitis and tenosynovitis represent an inflammation of tendons, of synovial theca, respectively, covering the muscle tendons. De Quervain tenosynovitis and epicondylitis are the most frequent ones. Tenosynovitis of the short extensor and long abductor muscles of the thumb

is also named De Quervain disease. Patients find it difficult to hold objects in their hand, and they cannot lift weights. Lateral epicondylitis frequently occurs at tennis players, due to the degenerative modifications of the tendon from the common extensor of the fingers and of the short radial extensor muscle of the carpus. Medial epicondylitis occurs in golf players and it affects the radial flexor muscle of the carpus, which makes the hand flexion against some resistance become painful.

As all these affections of the periarticular soft tissues are associated to pain and, sometimes, to the limitation of the joint mobility, they may lead to an incapacity for work; moreover, as there are numerous cases which are caused by professional overstraining, their treatment must be prompt and intense. The therapeutic behaviour includes the administration of NSAD, general and local corticotherapy or physiotherapy in many forms (electrotherapy, ultrasounds, short waves, laser therapy), but the symptomatology is often protracted and rebellious to treatment.

Ever since it was discovered, the LASER has permanently extended its application field. Since 1970, laser has been successfully used, first of all in surgery, and then in medical specializations as well.

Controlled clinical studies of LLLT effectiveness in abarticular rheumatism (especially in shoulder periarthritis) showed different and sometime contradictory results. Some studies have demonstrated that low-level laser therapy is either totally ineffective in treating the scapular-humeral periarthritis [1-5] and the epicondylitis [4,6-7], but other studies proved higher efficiency compared with other therapeutic means when recommended dosages are used.[8-12]

The lasers used in physiotherapy have several common biological effects, but also particular effects, depending on the power and the type of the source.

The anti-inflammatory and anti-edematous effect. Lasers may influence the inflammation mechanisms at different levels. First of all, an active hyperemia is produced by the increased diameter and the decreased permeability of lymphatic vessels and capillaries, which generates a washing effect on the inflammatory substances (histamine, bradykinin, cytokine and lymphokine). Vasodilation increases the intake of oxygen and nutritive substances, an essential process in the repairing of the injured tissues. The laser stabilizes the membrane of the mastocytes (histamine producers) and stimulates phagocytes, which will eventually remove the harmful substances.

The analgesic effect. This effect is generated by several mechanisms. First of all, the laser induces the blockage of the action potential at the nociceptor level by the modification of the axonal membrane permeability. Then, the active hyperemia caused by heat and by the photochemical reactions encourages the drainage of the algogenic substances, eliminating the cause of the pain sensation. Pulsed emission lasers, especially the low-frequency ones, act on pain modulation by means of the big, myelinized fibers, according to the "gate" theory. [13] The laser eventually generates the production of morphinomimetic substances (endorphins and enkephalins), which have an analgesic action (a mechanism that is also proven by the emphasized increase in the urinary excretion of a serotonin degradation product - 5-Hydroxyindoleacetic acid, which precedes the amelioration of pain by several days). [14]

The biostimulation effect of Laser increases the ATP production, a phenomenon that encourages the cellular energetic processes. Mitochondria are the cellular organelle where these processes are developed, but the mediator between photons and the activation of biological processes has not been identified yet. Laser light may encourage cellular replication and RNA and proteins synthesis (collagen, for instance), facilitating the repairing processes. [13]

In comparison to the classical laser therapy, the MLS therapy has several special characteristics: it combines laser emissions with two wavelengths (808 and 905 nm), one in the continuous system and the other one in a pulsed system, with a maximum power of 1.1W. The advantage of this combination consists in better penetrability and in the possibility of increasing the emitted energy. Therefore, the pulsing system combines the stimulating effect on microcirculation with the advantage of an increased top power, but they have a low average energy, and the combination to a continuous laser wave secures an appropriate energetic intake. The synchronizing of the two wavelengths may transfer the energy towards the cellular sublayer in a more efficient manner than the emission of a single component. Thus, the MLS impulse has bigger antiphlogistic, bio-stimulating and analgesic effects than a continuous emission or a pulsed one, used separately or in combination, but unsynchronized. Enjoying the advantage of a bigger divergence of the diodes irradiation cones, the multidiode laser may have a spot of big dimensions – 50 mm. its wavelength and the energetic transfer method in relation to time.

MLS therapy creates the conditions for the achievement of numerous therapeutic effects, as it has an anti-inflammatory, anti-edematous, and analgetic action, which eventually leads to rapid ameliorations.

Starting from these theoretical premises, we have monitored the evolution under a complex physiotherapy treatment of a lot of patients with abarticular rheumatism, in order to compare the analgetic and anti-inflammatory effects of MLS laser therapy with traditional laser therapy. In order to monitor the analgetic effects, we have used the visual analogue scale of pain (VAS). As laser therapy generates an analgetic effect both by direct action at the nociceptor level, and by the production of morphinomimetic

substances, not merely as a consequence of the local anti-inflammatory effect, VAS is not appropriate to monitor the anti-inflammatory effects, and have therefore used a parameter which directly reflects local inflammation, that is the cutaneous temperature measured by digital thermography.

Digital thermography is a non-invasive physiological test. It is a valuable investigation that may provide medical alerts in relation to the modifications indicating a precocious stage of breast cancer, or it may be used in the exploration of unknown origin pain. It is also useful in the monitoring of treatment evolution for numerous diseases.

In 1965, Gershon-Cohen, a radiologist and researcher from Albert Einstein Medical Center, introduced thermography in USA. Ever since the last period of the 70s, numerous medical centres and independent clinics have used this exploration method on thousands of patients. In 1982, FDA (Food and Drugs Administration) approved thermography as a screening method for breast cancer, and, since 1990, it has been acknowledged as a diagnostic instrument by the American Academy of Physical Medicine and Rehabilitation. [15]

In the case of periarticular soft tissues affections, thermography may reveal the local vasomotor and inflammatory reaction by increased local temperatures and/or by the modification of the cutaneous heat map, as it is well-known that the heat symmetry principle is normally observed. [16]

Since it is a non-invasive method, the investigation may be repeated at any time throughout the treatment in order to monitor local modifications of temperature, which follow the evolution of the local inflammatory process.

MATERIAL AND METHODS

We have studied a lot of 22 patients presenting an acute abarticular pathology (scapulohumeral peri-arthritis, tendinitis, epicondylitis, and bursitis) (table I) which was divided in two parts. The first group (G1) made of 12 patients (8 women, 4 men) with an average age of 50,7 years received a conventional electrotherapy treatment (interferential and diadynamic currents, ultrasound) and 100 mW LASER. The second group (G2) made of 10 patients (6 women, 4 men) with an average age of 45,8 benefited from conventional electrotherapy and MLS therapy (table II).

TABLE I. DISTRIBUTION TYPES OF THE DISEASES STUDIED WITHIN BOTH GROUPS.

	G1	G2
Shoulder periarthritis	6	6
Epicondylitis	3	2
Quervaine's tenosynovitis	1	1
Prepatellar bursitis/housemaid's knee	2	1

TABLE II. DEMOGRAPHICAL DATA

	G1	G2
Mean age	50,7	45,8
Sex (F/M)	8/4	6/4

Both groups included all types of studied pathology, with a balanced distribution (fig 1. and fig. 2).

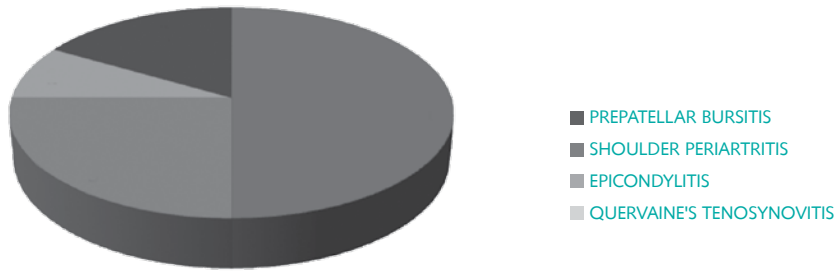

Fig. 1. Distribution types of the diseases studied within the group treated with the classic laser therapy

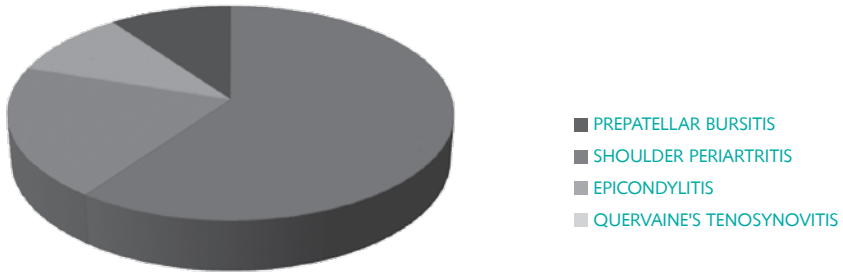

Fig. 2. Distribution types of the diseases studied within the group treated with the MLS laser therapy

METHODOLOGY

The patients received a physiotherapy session per day for 10 days. The first group was applied a laser therapy (830 nm wavelength) by non-contact technique with a 100 mW probe, in dosages of 6 J/cm2, by punctiform irradiation in 6-10 points. In order to treat the second group, we have used all the preset programs of the MLS M6 device, by scanning the affected area (with the 3300 mW probe) and after that the irradiation of the painful points (with the 1500 mW probe). The evolution of pain through the visual analogue scale (VAS) was monitored in both groups; the instant pain, the pain upon palpation and the pain upon mobilization initially, after 5, 10 days of treatment and 30 days after the termination of treatment was assessed; digital thermography and soft parts ultrasound were performed before starting the treatment and after the 10 session cure.

The thermo graphical determinations have been performed with a Flir B60 termocamera under controlled measurement conditions (22- 23 C room temperature, the patient needs 15 minutes for room temperature accommodation) and aimed to determine the modification of the temperature difference between the treated area and the contra lateral unaffected area.

Inclusion criteria: suggestive symtomatology for abarticular rheumatism with less than 3 weeks debut and the existence of ultrasound modifications.

Exclusion criteria: AINS previous or concomitant treatment, local/general corticosteroid therapy or the presence of some lesions in which the laser therapy is contraindicated: neoplasia, infectious cutaneous lesions, bleeding tendency body surfaces.

RESULTS

In both groups studied, was observed reduction of spontaneous pain (fig. 3), of pain on palpation (fig. 4) and of pain during mobilization (fig. 5), lower VAS values being more important in the MLS therapy, the differences between the two groups being statistically significant in all moments ($p<0.005$).

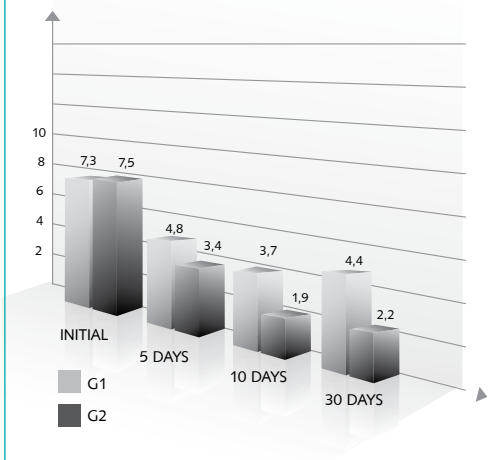

Fig. 3. Evolution of spontaneous pain – VAS score

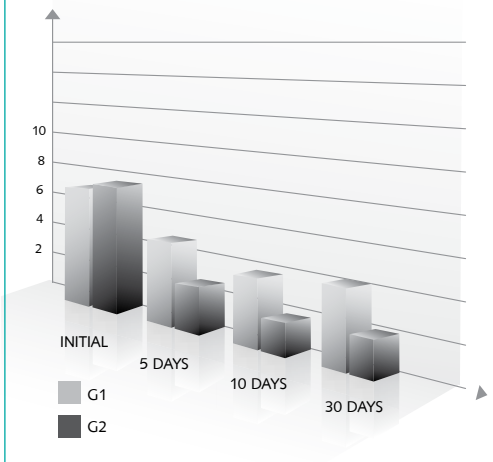

Fig. 4. Evolution of pain at palpation – VAS score

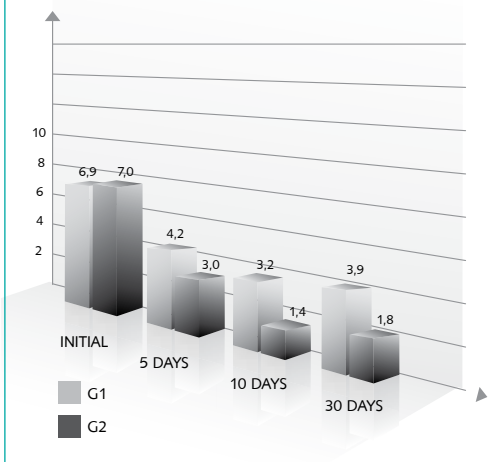

Fig. 5. Evolution of Pain during mobilization – VAS score

Also within both groups, the difference between the local temperature of the affected area and the contra lateral stimulated area has dropped, the reduction being more important in the group treated with the MLS laser therapy ($p=0,034$).

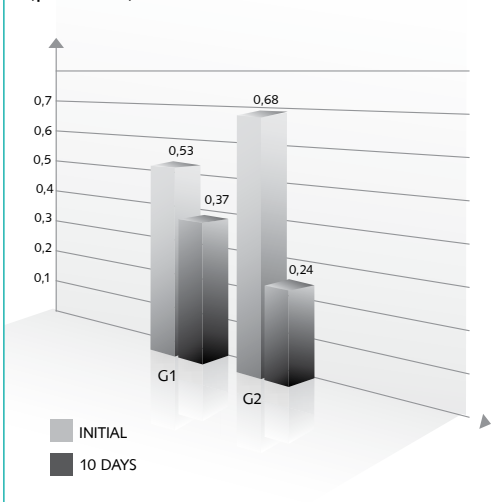

Fig. 6. Evolution of skin temperature difference ($p=0.034$)

DISCUSSIONS

The results from our study may support the importance of MLS therapy for analgesic and anti-inflammatory effects in patients with abarticular rheumatism over classical laser therapy. In this kind of pathology, pain control represents one of the principal tasks in order to get over acute phases. In this study, patients enrolled in the MLS therapy group reported very early (in some cases even after 2-3 treatments) a significant pain reduction and increased mobility of affected joints.

The results of this study also demonstrate anti-inflammatory effect of MLS therapy by lowering the local temperature measured by digital thermography.

Of the studied cases, an impressive evolution of the issued pursued, both pain and local inflammation shown through determining temperature by digital infrared thermography, was found in a prepatellar bursitis in which clinical

symptoms improved after the first treatment session and the temperature difference between the affected knee and the healthy one reduced from 3.8 °C to 0.3°C at the end of 10 treatment sessions (fig. 7). Could also be ascertained by ultrasonography of soft tissue the reduction of the amount of fluid from the

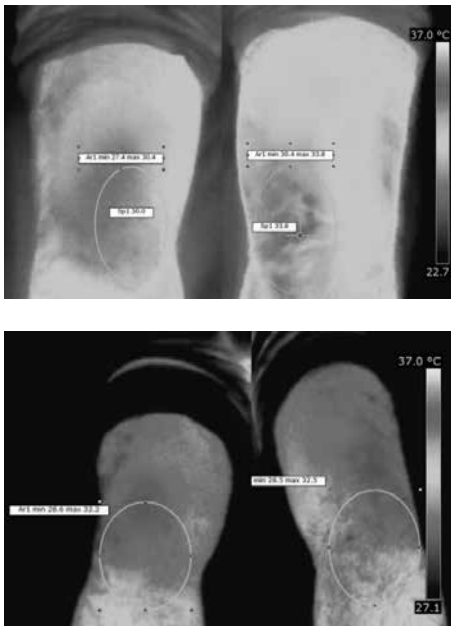

Fig. 7. Thermographic aspect of prepatellar bursitis before and after treatment

Unlike other forms of physiotherapy (including classical laser therapy), which according to literature data are not very effective in treating abarticular rheumatism, MLS therapy works quickly and efficiently in this type of pathology, reducing pain and functional impotence after a few treatment sessions. Because of the analgesic and anti-inflammatory effect MLS therapy may represent an alternative at administration of anti-inflammatory medications (corticosteroids or NSAIDs) and it has the advantage of fewer adverse effects and a much greater persistence for analgesic and anti-inflammatory effect over time.

CONCLUSIONS

- MLS therapy leads to a faster reduction of pain symptoms.
- In case of periarticular soft tissue diseases, the anti-inflammatory effect of MLS laser therapy may be confirmed through the reduction of temperature differences compared with the healthy contra lateral area.
- The analgetic effect obtained with MLS therapy is long lasting.

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Effects of low frequency electromagnetic fields on SHSY5Y cells - a neuroblast model.

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ABSTRACT

Electromagnetic fields (EMFs) are receiving increasing attention in basic research due to their ability to influence the behavior of cells and to evoke biological responses that may be important from a clinical point of view. In our study we analyzed the effects of exposure to low frequency EFM (2mT, 50Hz, 3 h) on a neuroblast model (SH-SY5Y cells).

These cells are a well-characterized model for studying in vitro differentiation towards a neuronal-like phenotype inducible by various exogenous agents. Viability and proliferation was assessed immediately after the treatment as well as after 24 h. No significant changes were observed in viability (>96%), while proliferation decreased (23%) after 24 h. Changes in cytoskeleton organization (analyzed by immufluorescence technique), in particular in actin microfilament, were also observed. These changes were accompanied by morphological modifications and the formation of cones and cytoplasmic extensions, or neurites and dendrites processes.

We finally monitored an increase in expression of markers typically expressed in neuronal differentiation: neurofilaments and NRF-1. In conclusion, our findings demonstrate that EMF exposure induces in SHSY5Y cells a biological response consisting in the remodelling and reorganization of the cytoskeleton and in the beginning of neuronal cell differentiation. A deeper knowledge of the mechanisms underlying the effects described above and a greater understanding of relationship between biological response and parameters variation could lead to concrete improvements in treatment protocols.

INTRODUCTION

Since many years, electromagnetic fields are widely used in medicine. Today, magnetic therapy is considered one of the most important physical therapies. It is experienced in many application and accepted by patients as a non invasive and easily to manage treatment. The main application in clinics are mostly addressed to the treatment of rheumatologic disease [1] and disorders characterized by bone loss, such as osteoporosis, and also to accelerate the healing of fractures [2,3]. In these fields, low frequency pulsed EMFs have shown to be the most effective

The molecular and cellular mechanisms underlying the systemic effects of EMsf are not completely understood, but recently many advancements have been made: it has been demonstrated that EMFs affect the permeability of the plasma membrane [4] by ion-channel or receptor redistribution, changes in activation kinetics of ion channels, reorientation of membrane phospholipids. Moreover, EMFs may modulate binding kinetics and ion bound or release from proteins [5].

In living tissues endogenous EMFs are generated by physiological activities such as the movements of the musculoskeletal system structures. Vibrations and contractions of human muscles induce in the underlying bone tissue low frequency EMFs which have an important role in maintaining bone mass. Bone cells are selectively sensitive to low frequencies: in the range 15-30 Hz, fields as low as 0.01mV/cm may affect the remodeling activity [6].

Different authors have shown that pulsed EMFs affect proliferation, differentiation and activity of osteoblasts, with stimulating or inhibitory effects depending on field type and exposure [7-9] expression of bone morphogenetic proteins [10,11], production of extracellular matrix [12].

Clinically, magnetic therapy has been also used to treat skin diseases [13]; chronic wounds in cancer patients undergoing radiation therapy [14], inflammation and edema [15]; the osteoarthritis of the knee [16,17]. Moreover, it has analgesic effect in fibromyalgia [18] and localized musculoskeletal pain [19].

The new medical frontiers are increasingly oriented toward complex and customized therapeutic protocols , in which physical medicine and biotechnology have a relevant role, whether used alone or combined with "traditional drug therapies".

Recently, the effectiveness of EMFs in the treatment of incontinence and rehabilitation of bladder and sexual function in radical prostatectomy [20] has been proved and new applications of EMFs to treat diseases that involve muscle and nervous tissue have been proposed.

The results of preliminary studies carried out in our laboratory suggested that pulsed low frequency EMFs, without altering cell viability, can influence the proliferation, differentiation and morphological characteristics of myoblasts and neuroblasts [21].
The present study was designed with the aim to confirm our preliminary findings on the effects of pulsed, low frequency EMFs on neuroblasts and to analyze how EMFs can interfere with the ability of neuroblasts to differentiate.

MATERIALS AND METHODS

Cell Culture

The human neuroblastoma cell line SH-SY5Y (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI medium supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and maintained at 37 °C in a humidified atmosphere (5% CO2/95% air). All the reagents for cell cultures were from Sigma Chemical Co. (St. Louis, MO, USA). Tissue plastic-ware was from Bibby Sterilin (Staffordshire, UK). For the exposure to EMFs, cells were seeded in 24-well plates.

Cell viability and count

Cell viability after exposure to EMFs 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 cells were centrifuged and resuspended in a solution of phosphate buffered saline (PBS) and Trypan Blue (dilution factor: 2) and counted, after 5 min of incubation, using Neubauer hemocytometer.

Immunofluorescence microscopy

At the end of the experiments, cells were fixed for 5 min in cold acetone, then washed in PBS. After blocking unspecific binding with PBS containing 3% bovine serum albumin, cells were incubated overnight at 4°C with the specific antibodies: anti-actin, anti-tubulin, anti-vimentin, anti-$\alpha 5\beta 1$ integrin, anti-Nrf-1 and anti-neurofilament antibodies. Cells were then incubated with the FITC (fluorescein isothiocyanate) conjugated specific secondary antibodies (specifically: anti-mouse IgG for anti-tubulin, anti-$\alpha 5\beta 1$, anti-neurofilament; anti-rabbit IgG for anti-NRF-1; anti-mouse IgM for anti-vimentin). 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. 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). Image analysis was performed by extracting, for each cell image, the region of interest (ROI) by appropriate software (Image Pro Plus, Mediacybernetics, Inc., Silver Springs,Maryland). Then, the mean pixel value (16 bit, gray level) related to the mean fluorescence intensity and therefore to the specific epitope detection was calculated.

Statistics

Experiments were carried out in triplicate. For immunofluorescence analysis, at least 30 cells per slide were scored in 10 random fields/slide, and the data were expressed as mean ± SD. Statistical significance was determined using a Student's t test. A p value lower than 0.05 was considered statistically significant.

Elecromagnetic fields exposure

Cells were exposed to an EMFs produced by a pair of coils in the configuration of Helmholtz coils. The intensity in the central region of the system 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 such as to expose the cells to intensity of 2 mT and frequency of 50Hz. The experiment was carried out with a heating / hot air circulation to ensure that the temperature around the multiwell containing cells was stable at about 37 °C. The control cells are placed on the bench outside the coils, in an area where the field intensity produced by the coils is negligible.

Quantitative real-time RT-PCR for neuronal marker transcripts

Total RNA to be subjected to RT was extracted from SHSY5Y cells, exposed and not exposed to EMF. Non-exposed cells were taken as control. Total RNA isolation and cDNA synthesis were obtained as reported previously [22]. Primers and probes were TaqMan gene expression Assays ID Tau: Hs00902193_m1; SYP: Hs00300531_m1; MAP2: Hs00159041_m1 (Applied Biosystems, Foster City, CA, USA): The PCR mixture (25µL final volume) consisted of a 1 X final concentration of Assay-On-Demand mix, 1 X final concentration of Universal PCR master mix (Applied Biosystems) and 20 ng cDNA. Each measurement was carried out in triplicate. The mRNA quantification was based on a comparative Ct method according to the manufacturer's instructions (Applied Biosystems) and data were normalized to ribosomal 18S RNA expression (assay ID Hs99999901_s1; Applied Biosystems). Results were expressed as the fold increase in mRNA untreated cells.

RESULTS

Viability and proliferation

In order to verify the effect of the EMF exposure on cell viability and proliferation, Trypan blue assays were carried out at 0 h and 24 h after 3h exposure to the EMF.
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 96% in all the samples.
Immediatly after EMF exposure, cell proliferation did not change significantly, but it showed a decrease of the ~ 22% after 24h (Fig. 2).

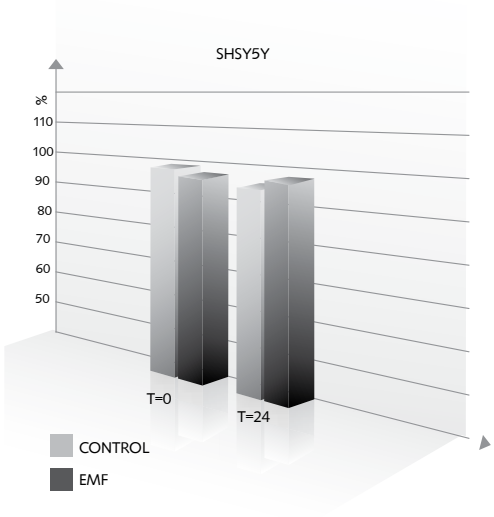

Fig. 1 Cell viability assessed at 0h and 24h after exposure to EMFs. Data were obtained by Trypan Blue assay.

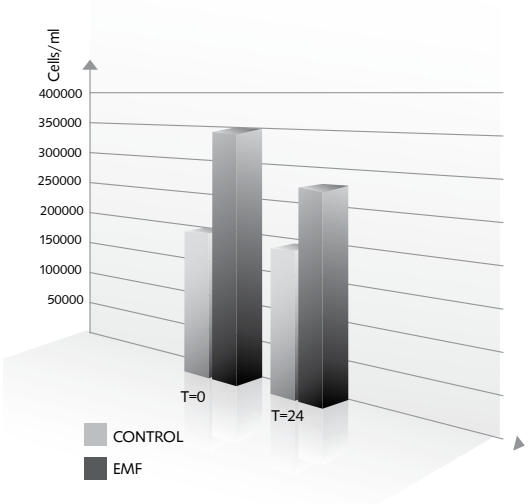

Fig. 2 Cell proliferation assessed at 0h and 24h after exposure to EMFs. Data were obtained by Trypan Blue assay.

Cytoskeleton

Cytoskeleton is an important structure for the cell since it allows both movement and shape modifications and has an important role in intracellular transport and signalling. 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 SHSY5Y exposed to EMF was studied by immunofluorescence microscopy and image processing.

The results of the analysis showed a reorganization of all three components of the cytoskeleton following exposure to EMF. In controls, actin microfilaments were distributed as expected: more thickened just below the plasma membrane and in the perinuclear area; stress fibers were arranged in parallel (Fig.3A). In cells exposed to EMF the concentration of actin increased, in particular in the peripheral region.

Stress fibers were organized to form cones, structures that precede the formation of neuritis and dendrites (Fig.3B).

In samples analyzed 24h after EMF exposure, cells showed branched extensions that allowed connections with neighboring cells, producing a network

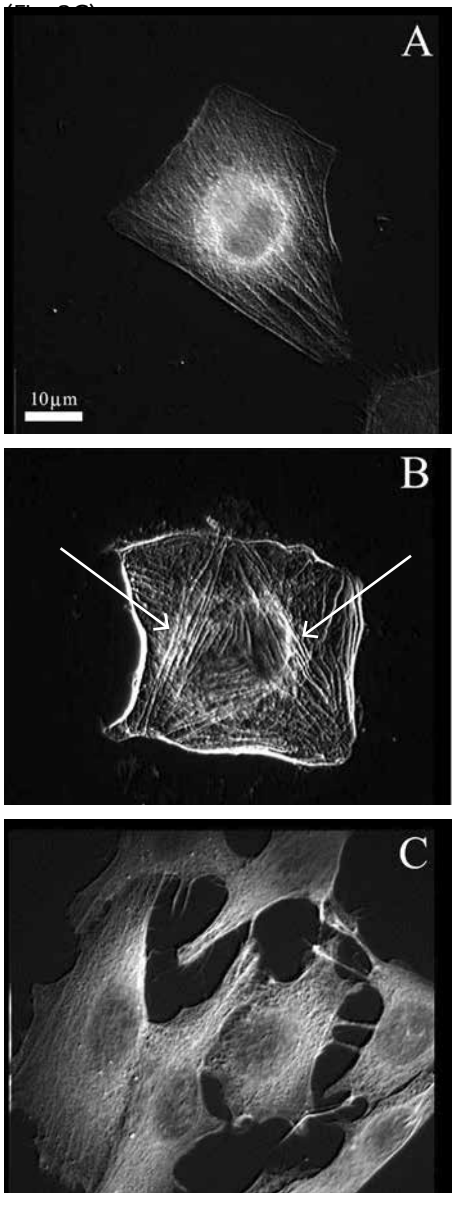

Fig. 3 Actin expression assessed by immunofluorescence microscopy. Control (A) 6h after exposure to EMFs (B) and 24 hours after exposure to EMF (C).

Specific staining for tubulin revealed that the exposure to EMF did not change significantly the expression of the protein, rather the distribution of the microtubules, which are formed by self-assembling of tubulin monomers. In controls, as expected, microtubules branched radially from the "organizing center" near the nucleus, while in treated samples the microtubules were organized into bundles that would go in the dendrites/neurites that were forming (Fig.4A, Fig.4B).

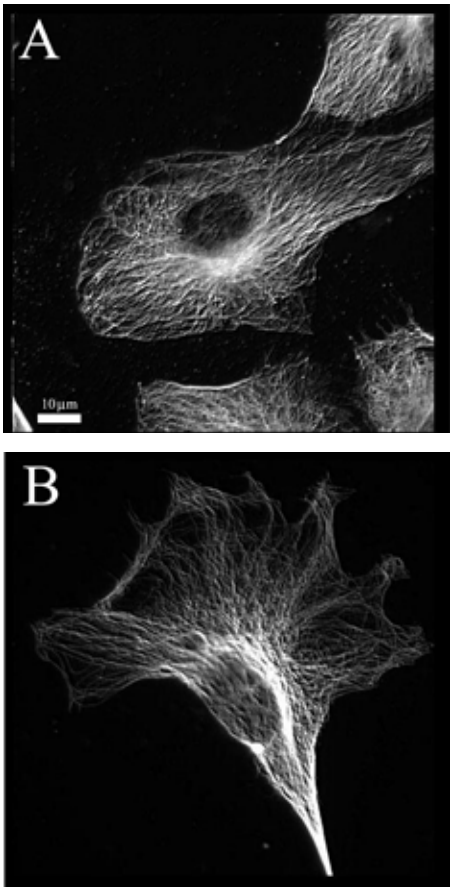

Fig. 4 Tubulin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

Vimentin is the major constituent of the intermediate filaments. In controls, they formed bundles mainly distributed in the perinuclear area (Fig. 5A). In treated samples the expression of the protein increased and we observed the formation of a dense ring around the nucleus, as shown in Fig. 5B.

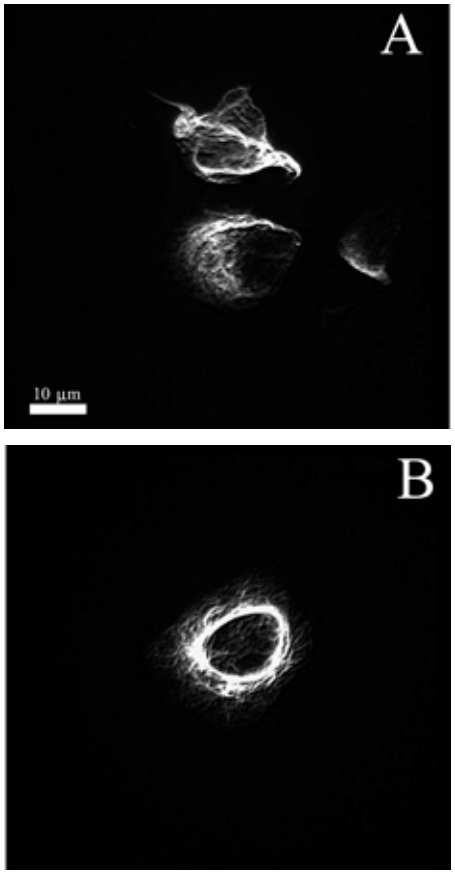

Fig. 5 Vimentin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

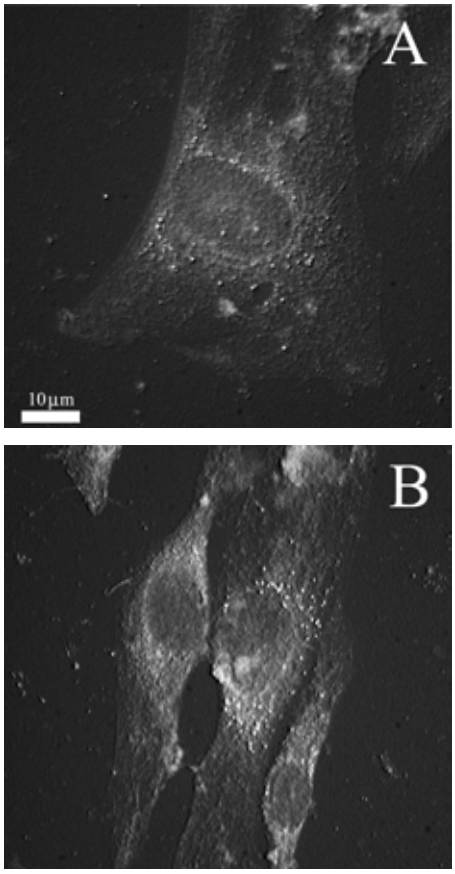

Fig. 6 Integrin expression assessed by immunofluorescence microscopy. Control (A) and 24h after exposure to EMFs (B).

Integrins are transmembrane proteins composed of two subunits α and β (there are 20 different integrins with different combinations of α and β). They bind to short sequences of aminoacids found in many extracellular matrix components, including collagen, fibronectin and laminin and also serve to anchor some components of the cytoskeleton. In all the samples the $\alpha 5\beta 1$ integrin was expressed at very low levels, in agreement with literature [20], which reports a down-regulation of this protein during the differentiation of early neuronal precursors. There were no significant differences between control (Fig. 6A) and treated (Fig. 6B).

Gene expression analysis by Real Time PCR

Evaluation of the expression of some genes involved in neurogenesis and development of synapses in nerve cells was assessed by the presence of transcribed mRNA of MAP2, TAU and synaptophysin.

The values shown in the graphs were calculated from the ratio between the amount of mRNA present in treated and control samples. Figure 7 shows MAP2 expression at 0h and 24h after EMF exposure. MAP2 gene encodes for a microtubule-associated protein (MAP2). The proteins of this family are involved in the construction of microtubules, important process in neurogenesis. In samples analyzed immediately after treatment, there was a marked decrease in the MAP2 transcript (approximately 90%) compared to the control. After 24h, the amount of mRNA in treated sample increased, returning to values comparable to the control. In controls the amount of transcript did not change at 24h from the treatment.

In controls the amount of transcript does not vary, both immediately after treatment and after 24 hours. This confirms that the cells did not undergo any stimuli that induce changes in the production of the transcript.

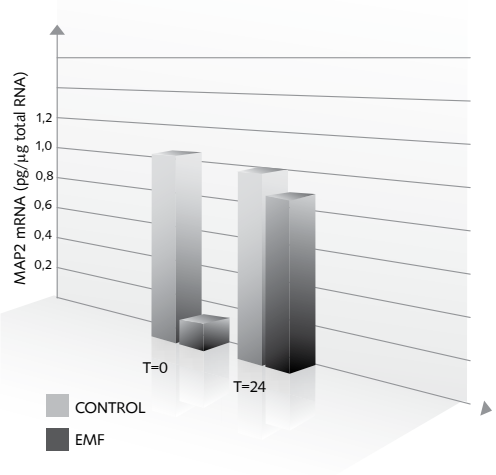

Fig. 7 Amount of MAP2 gene expression in SHSY5Y cells. * p<0.05

Figure 8 shows the amount of TAU transcript immediately after the treatment and measured after 24 h. The product of TAU is a protein (TAU) which promotes the elongation of microtubules by binding to the surface of neurites of protofilaments [23].

In samples analyzed at 0h after EMF exposure there is a 12-fold greater amount of transcript compared with control. After 24 h the amount of TAU transcript return at control values.

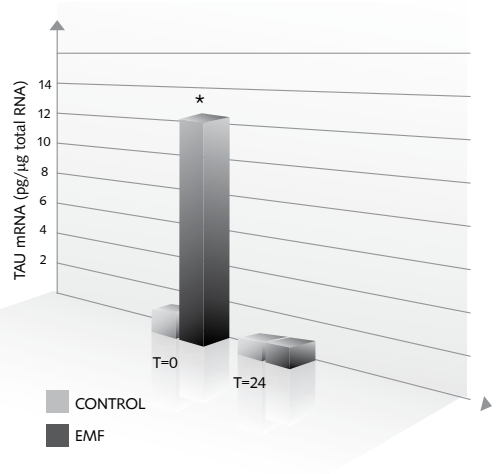

Fig. 8 Amount of TAU gene expression in SHSY5Y cells. * p<0.05

In Fig. 9 is reported the expression of SYP analyzed at 0h and 24h after EMFs exposure. Synaptophysin, the product of the gene SYP, is a membrane glycoprotein present in synaptic vesicles in neurons. The protein participates in the formation of a channel between the synaptic vesicle and presynaptic membrane through which flow the neurotransmitters [24].
Immediately after the treatment, SYP increased of about 6-7 times compared to control samples. After 24h from the treatment, the expression in treated samples returned similar to the control, as happened for TAU. Therefore we observed an increase in transcription of TAU and SYP immediately after EMF exposure, while transcription of MAP2 was inhibited. After 24h from EMF exposure the transcription of the genes returned at basal values.

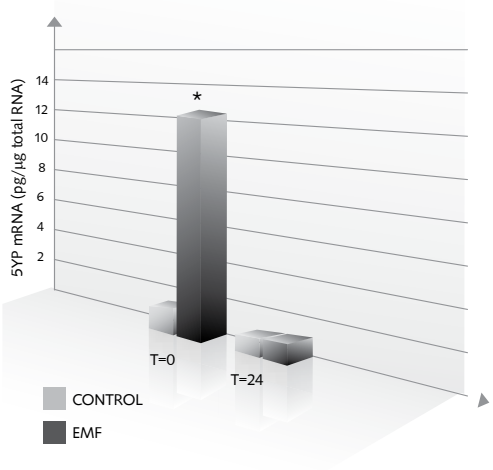

Fig. 9 Amount of SYP gene expression in SHSY5Y cells. * p<0.05

Neuronal marker expression assessed by immunofluorescence analysis

Since in our experiments we observed that the exposure to EMF induced in SHSY5Y cells morphological changes and increase in expression of genes involved in neurite growth, we decided to deeper investigate on possible role of EMF exposure in differentiation process. Therefore we assayed, by immunofluorescence microscopy and image analysis, the expression of two markers of neurogenesis: Nrf-1 and neurofilaments.
NRF-1 is a transcription factor associated with the regulation of neurite outgrowth, thus it is considered a marker of neuronal differentiation.
We found that Nrf-1 increased significantly in neuroblasts exposed to EMF compared with controls and the protein distribution appeared more punctiform (Fig. 10A and 10B).

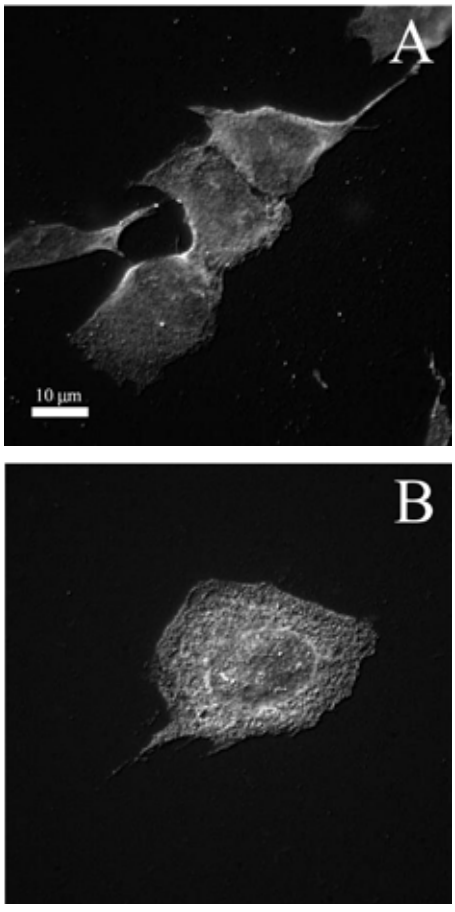

Fig. 10 NRF-1 expression assessed by immunofluorescence microscopy. Control (A) and after exposure to EMFs (B).

Also the expression of neurofilaments, in particular medium neurofilament (NF-M), significantly increased in cells exposed to EMF. Figs. 11B show that the protein accumulated in the perinuclear area. NF-M is a member of the intermediate filament family and an important component of neuronal cytoskeleton. Therefore it is considered a major marker of neuronal differentiation.

DISCUSSION

The long-term goal of our studies is to propose new clinical applications of magnetic therapy for the treatment of disorders involving muscle and nerve tissue. The aim of the experiments reported in this paper was, in particular, to investigate the effect of EMF on nerve cells and neuronal differentiation. Our experimental model was the cell line SH-SY5Y, a human neuronal model widely used for studies on neurogenesis. In fact SH-SY5Y cells can differentiate into neuronal direction in response to appropriate stimulation. The differentiation process is characterized by morphological changes and increase in expression of specific markers of neuronal differentiation. Cells form growth cones, and then long neuritic processes, through cytoskeleton rearrangement.

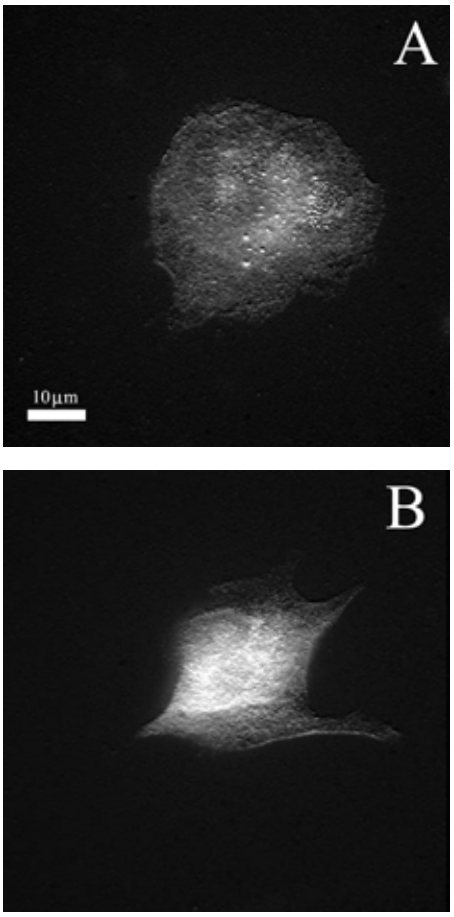

Fig.11 Neurofilament protein (NF-M) expression assessed by immunofluorescence microscopy Control (A) and after exposure to EMFs (B).

The results of our experiments showed that the EMF treatment did not produce changes in cell viability, evaluated immediately after exposure and after 24h. Cell proliferation did not change immediately after the treatment, while we observed a moderate but significant decrease after 24h. A decrease in cell proliferation associated with unchanged viability suggested the hypothesis that the exposure to EMF induced neuroblast differentiation.

Since it was shown that extremely low frequency EMF induces changes in cell morphology [19] and normally morphological changes occur also during differentiation, we studied the effect of EMF on cytoskeleton organization. Data, obtained by immunofluorescence microscopy, revealed that EMF exposure induced a significant rearrangement both in microtubles and in the network of actin microfilaments. In control samples, as expected, actin microfilaments were concentrated under the plasma membrane and in the perinuclear area, while after 6 h and even more after 24h from exposure to EMF we observed a reorganization of actin microfilaments with stress fibers which formed cones and then cytoplasmic extensions. These results agreed with the reports presented by other authors [16]. Also the microtubule network changed after exposure to EMF. In treated samples there was a significant increase in tubulin expression, especially in the cytoplasmic extensions. This findings are even more significant because they are associated with the increase in Tau expression. It is well known that, during early phases of neuronal differentiation, Tau is concentrated in the nascent axon and an intracellular redistribution of tubulin occurs [17].

The hypothesis that the exposure to EMF could promote, in our cell model, a differentiation process was further confirmed by the increase in expression of Nrf1 and NF-M, assessed by immunofluorescence analysis. As explained above, these proteins are considered major markers of neuroblasts differentiation and have a key role in the process of neuronal maturation.

Also the results of real time PCR supported the hypothesis that cells exposed to EMF began a differentiation process toward the neuronal line. In fact, after the treatment, we found downregulation of MAP2, overexpression of synaptophysin and TAU. MAP-2 function is not required when cell disassembles microtubules in the cell body to give rise to the formation of neurites, while TAU is required to add new subunits to microtubules which are forming in the neuritis [23]. Synaptophysin, as mentioned above, is involved in synapse formation and release of neurotrasmitter. [24]

In summary, rearrangement of microtubules and actin microfilaments, with formation of cones and cytoplasmatic extension, joined with increase in expression of neuronal markers and changes in expression of genes involved in cytoskeleton organization and neurite formation strongly agree with the hypothesis that exposure to EMF may induce neurogenic differentiation.

24h after exposure, the expression of synaptophysin, TAU and MAP2 returned to control value but the formation of cellular processes continued to progress, with the appearance of branched cytoplasmic extensions. This means that the modulation of protein expression and the morphological changes are part of a complex biological response that, once triggered by exposure to EMF, proceeds even after the cessation of the stimulus.

In conclusion, the data presented here indicate that the exposure to a 50-Hz and 2 mT EMF may significantly affect neuronal differentiation of SHSY5Y by upregulating the expression of genes involved in neurite formation and inducing cytoskeleton rearrangement.

The progress of the differentiation process is unequivocally proven by the increase in expression of specific markers of neurogenesis, such as NF-M and Nrf1, and the gradual growth of neurites.

These findings open perspectives for future application of EMFs in tissue engineering, tissue regeneration and repair. From the clinical point of view, the reported results indicate that there are bases to study EMFs application for recovering nerve tissue function. Additional studies are needed to further understand the cellular and molecular mechanism underlying the biological responses induced by exposure to EMF, since a better knowledge of these mechanisms can lead to significant improvements in the therapeutic protocols.

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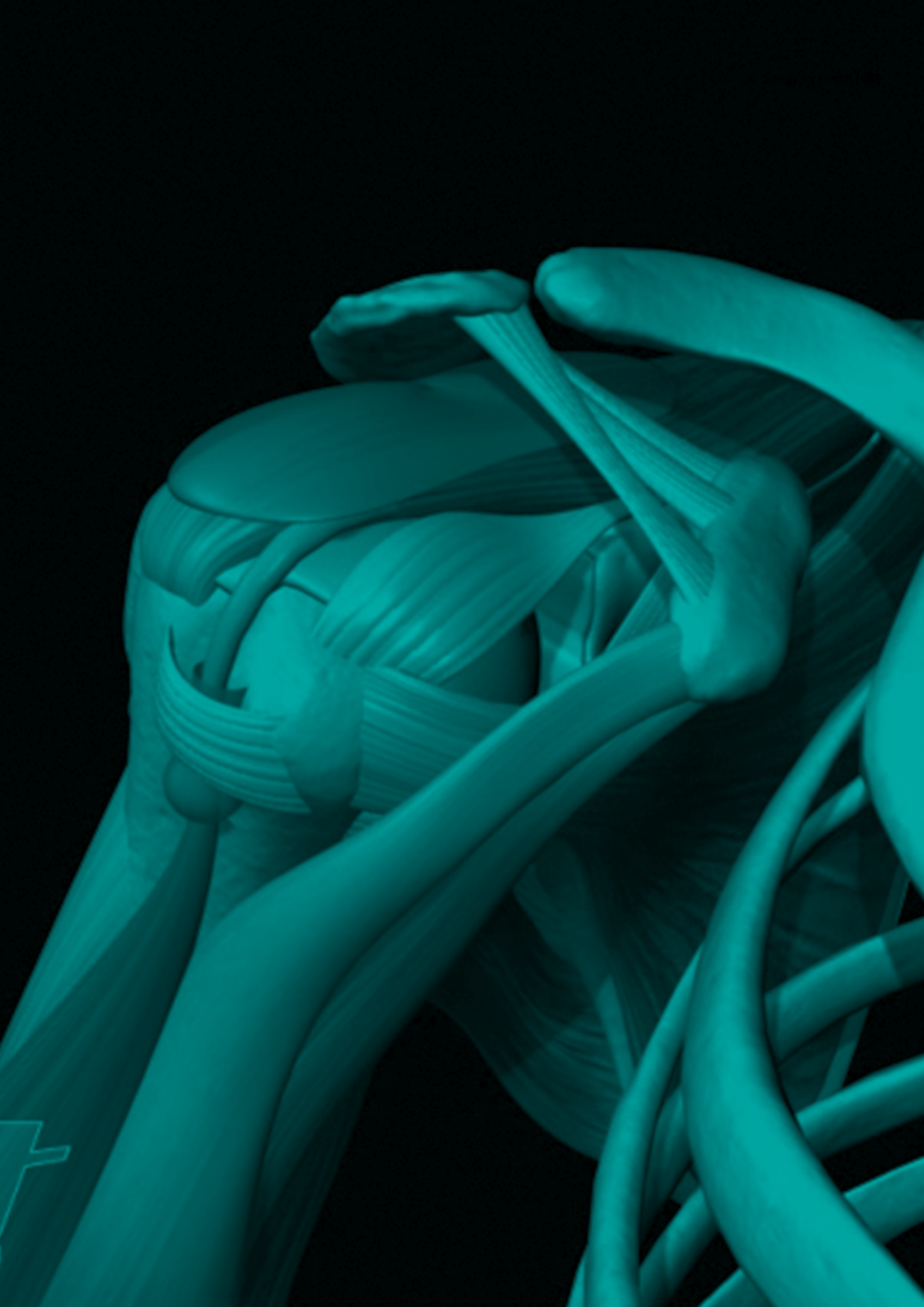

The effects of low level laser therapy on irradiated cells: a systematic review.

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ABSTRACT

The aim of this systematic review was to elucidate the effects of low level laser on irradiated cells concerning cell structure, viability and DNA damage. A search of health science databases (Cochrane Library and PubMed) was performed. The main key words used were "Low level laser therapies" [Mesh] OR "low intensity laser therapy" OR "low energy laser therapy" OR phototherapy AND "ultra structural changes" OR "ultra structural analysis" OR mitochondria [Mesh] OR "DNA damage" [Mesh]. The inclusion criteria comprised laboratory studies that used LLL with its wavelength ranging from 632 to 1064 nm and a delivery of 1 to16 J/cm^2, evaluating the cell structure by means of DNA or ultra-structural analysis. The articles selected were carefully read and data of interest were tabulated. Eight studies were included in this review. Three articles have performed cell viability tests showing an increase in viability when low fluences (5 J/cm^2) were applied and a decrease with higher fluences (higher than 10 J/cm^2). Five articles used transmission electron microscopy to identify any type of ultra structural morphology alteration in irradiated cells and a few differences were found such as the presence of giant mitochondria, and several cytoplasmic collagen-containing phagosomes. Two articles performed a DNA test (Comet Assay) and verified that damage appears when using higher fluences (higher than 10 J/cm^2). The effects of LLL irradiation on cell structure and on its DNA vary depending on the laser parameters of irradiation. Adequate dosages can accelerate wound healing, stimulate cell proliferation and decrease the DNA damage. Lower dosages do not seem to stimulate cells and higher dosages seem harmful to cell viability and increase DNA damage.

INTRODUCTION

The use of low intensity laser as a therapeutic modality was originally introduced in Europe by Professor Endre Mester who reported its earliest clinical application in Medicine in 1968. He described that the healing process of chronic ulcer was faster when it was irradiated with argon laser [1]. Since this study was published, the number of studies on the medical application of low-level laser therapy has grown steadily and there has been an increasing clinical use of laser, in vivo and in vitro, for a variety of medical conditions [1].

The laser light emitted is polarized and coherent and may be absorbed by different tissues [1]. Tissue biostimulation is only possible if irradiated cells have molecular photoacceptors that absorb light and enter into a state of excitation triggering an intracellular cascade of signals leading to a measurable biological effect [2]. The transduction of the primary photosignal and its amplification in the cell leads to a photobiological macroeffect, such as an increased cell proliferation or DNA synthesis. Irradiation of cells at certain wavelengths can also activate some inner components and biochemical reactions, and the whole-cell metabolism can be altered [3]. Some studies revealed a new ultra structural conformation of irradiatied cell organelles [4,5] and cell morphology [6-8].

The possible damaging effects of laser irradiation are still highly contested. Light absorption induces the production of reactive oxygen and nitrogen species that are involved in subsequent free radical reactions and lead to a modification of biomolecules and changes in cell function [9].

The present systematic review was focused on this question: what are the effects of low level laser on irradiated cells concerning cell structure, viability and DNA damage?

METHODS

A literature search was performed on Cochrane Library and PubMed. The main key words used were "low level laser therapies" [Mesh] OR "low intensity laser therapy" OR "low energy laser therapy" OR phototherapy AND "ultra structural changes" OR "ultra structural analysis" OR mitochondria [Mesh] OR "DNA damage" [Mesh]. The inclusion criteria comprised laboratory studies that used LLL with wavelengths ranging from 632 to 1064 nm and a delivery of 1 to16 J/cm^2, evaluating the cell structure by means of DNA or ultra-structural analysis. The articles selected were carefully read and data on the following issues were extracted

Author and year	Experimental model	Laser type and wavelength	Fluence applied (J/cm²)	Viability in irradiated cells (pM ATP*)	Viability in NI cells and survival rate (pM ATP*)	Survival rate in irradiated cells	Survival rate in NI cells	Ultra structure and cell organization in irradiated cells in comparison with control (NI)	DNA damage in irradiated cells	DNA damage in NI cells	Evaluation method
Bayat, 2007	Rabbits	He-Ne (632,8 nm)	13	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Increased number and depth of filopodia and increased density of fibrillary fletwork of extracellular matrix	Not evaluated	Not evaluated	TEM
Delbari, 2007	Rats	He-Ne (632,8 nm)	0.01 1.2	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Larger collagen fibril diameter	Not evaluated	Not evaluated	TEM
De Araújo, 2007	Rats	He-Ne (632,8 nm)	1	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Faster organization of collagen fibrils and inflammatory response	Not evaluated	Not evaluated	TEM
Houreld, 2007	Human fibroblasts	He-Ne (632,8 nm) Diode (830 nm)	5 16	Not evaluated	Not evaluated	96% 90%	95%	Not evaluated	Not evaluated	Not evaluated	Trypan Blue exclusion and apoptosis
Hawkins, 2006	Human fibroblasts	He-Ne (632,8 nm)	0.5 2.5 5 10 16	9.38 10.68 10.46 8.16 8.72	10.31	Not evaluated	Not evaluated	Not evaluated	36.53% 35.82% 32.53% 34.15% 34.55%	33.63%	Comet Assay and CellTiter-Glo
Bortoletto, 2004	Culture cells Hep-2	GaAlAs (635 nm)	10	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Mitochondrial alteration produced increase in ATP synthesis and granular aspect within the first 24 hours.	Not evaluated	Not evaluated	TEM
Kujawa, 2004	Culture cells B14 (hamsters) and human erytrocites	CTL 1106MX (810 nm)	3.75 7.50 11.25 15.00	Not evaluated	Not evaluated	— 98% 96% 95%	100%	Not evaluated	11.5% 13.2% 17.9% 21.8%	11.1%	Method of Mosman and Comet Assay
Manteifel, 1997	Lymphocytes	He-Ne (632,8 nm)	56	Not evaluated	Not evaluated	Not evaluated	Not evaluated	Reduced number of mitochondria, but increased area (giant mitochondria)	Not evaluated	Not evaluated	TEM

*pM ATP indicates the amount of energy a cell can produce, more energy means that the cell is working well, in abnormal condition.
NI= non irradiated cells; TEM= Transmission Electron Microscopy
Cell survival % indicates how many cells were still alive and in normal condition after laser irradiation.

and tabulated: author and publication year; laser type and wavelength, fluence, cell viability, survival rate, ultra structure and DNA damage.

RESULTS

Search for articles retrieved 59 articles, but only eight met the inclusion criteria after title and abstract reading (Manteifel, 1997; Bortoletto, 2004; Kujawa, 2004; Hawkins, 2006; De Araújo, 2007; Bayat, 2007; Delbari, 2007; Houreld, 2007) [10-13,7,8,6,14]

Cell viability

In three of the eight articles included, cell viability tests were performed [12-14]. The results presented an increase in cell viability (%) when low fluences were applied and a decrease with higher fluences.

In the study performed by Hawkins [13], the cell viability assay showed that normal cells exposed to a single dose on 2 consecutive days responded with an increase in cell viability after 0.5 J/cm^2 (P=0.033) and 5 J/cm^2 (P=0.046), while at higher doses (10 and 16 J/cm^2) there was a decrease in cell viability. A dose of 2.5 J/cm^2 did not increase or decrease cell viability when compared with the normal non-irradiated control.

Kujawa [12] affirmed that light irradiation did not produce any substantial change in cell survival. Houreld [14] observed that diabetic-wounded fibroblast cells [human skin fibroblast cell WS1 maintained in a diabetic-induced condition obtained by adding glucose in culture medium (17mMol/l) and characterized by the presence of a central scratch in the monolayer that simulates the wound] irradiated at a wavelength of 632.8 nm with a fluence of 5 J/cm2 showed a significant increase in viability when compared to diabetic-wounded non-irradiated cells (P=0.001) and cells irradiated with 16 J/cm2. Cells irradiated at a wavelength of 830 nm with a fluence of either 5 or 16 J/cm^2 and incubated for one hour showed no significant change in the viability percent. Cell irradiated with 16 J/cm^2 to all three wavelengths showed a decrease in viability. On the other hand, diabetic-wounded cells irradiated with 16 J/cm^2 showed a significant increase in caspase-3 and 7 in comparison with normal and diabetic-wounded non-irradiated cells (P<0.000) and cells irradiated with 5 J/cm^2 (P=0.021). When irradiated at a wavelength of 1064 nm with either 5 or 16 J/cm^2, there was no significant change in viability, although cells irradiated with 16 J/cm^2 showed a decrease when compared with normal and diabetic wounded non-irradiated cells (P=0.066 and P=0.076, respectively) [14]. The results were summarized in Table 1.

Cell ultra structure and organization

Five articles used transmission electron microscopy to identify any type of ultra structural morphology alteration in irradiated cells [5-8,10,11]. The articles were divided into two subgroups: the first with two studies, which focused their analysis on mitochondria [5,11], and the second with the other three, which concentrated on cell morphology and characteristics [6-8].

Mitochondrial alteration after irradiation produced an increase in ATP synthesis and granular aspect during the first hours, but after 24 hours the aspect was similar to that of the control group [11]. Another aspect was the presence of giant mitochondria in the irradiated group; the number of mitochondria was reduced when compared with non-irradiated cells, but the total area of all mitochondria was similar for experimental and control groups [5].

Delbari [6] studied the difference between fibril diameter of transected medial collateral ligament (MCL) in rats irradiated and non irradiated with He-Ne laser and observed that fibril diameter in the irradiated group was larger than in control group but density was similar. Bayat [7] used transmission electron microscopy on rabbit articular cartilage to evaluate chondrocytes and demonstrated that the nucleus of control and experimental groups presented the same characteristics: euchromatin nucleus in all cells. He observed a significant difference in the quantity and depth of filopodia, which means that irradiated chondrocytes worked more than normal chondrocytes. Another observation was that the experimental group had a moderate density of fibrillary fletwork of extracellular matrix while the control group presented a low density. De Araújo [8] observed that laser radiation reduced the local inflammation and appears to influence the organization of collagen fibrils in the repairing areas. The results were summarized in Table 1.

DNA damage

Two articles performed the Comet Assay test to verify DNA damage [12,13]. Damage appeared when high fluences were applied: Kujawa [12] observed DNA damage using 15 J/cm^2 and Hawkins [13] using 10 J/cm^2 and 16 J/cm^2. On the other hand, dosages that are too low do not seem to stimulate wounded fibroblast cells (cells characterized by the presence of a central scratch in the monolayer that simulates the wound). Hawkins [13] found DNA damage on wounded fibroblast cells irradiated by 0.5 J/cm^2 .

The dose used was possibly too low to stimulate cell function restoration and to initiate the healing process. When different doses were used in this study (2.5, 5 and 10 J/cm^2), there was no significant increase in DNA damage. On the contrary, there was a decrease in DNA damage when a dose of 5 J/cm^2 was used, indicating a repair process [12]. These results indicates that, as the dose increases beyond the optimum dose, the amount of DNA damage also increases [12,13]. The results were summarized in Table 1.

DISCUSSION

As we approach the fiftieth year of laser therapy use, there are still many open

questions such as the exact mechanism of its action; the correct dosage for a certain medical condition; the effect in cellular viability; DNA damage; and influence on surrounding tissues. This review was focused on verifying the effects of LLL irradiation on cell structure and organization and possible DNA damage.
Five articles used transmission electron microscopy to identify ultra structural morphological alterations in irradiated cells [5-8,10,11]. No harmful effects were observed in these studies. Only desired effects, such as the presence of giant mitochondria [5], increased fibril diameter [6], increased number and depth of filopodia and increased density of fibrillary network of extracellular matrix [7], faster organization of collagen fibrils and inflammatory response [8] were observed.

The selected articles reported the observation of significant DNA damage when high fluences were applied (higher than 10 J/cm2) and an inability to heal wounded cells when applying low fluence [12,13]. An adequate dosage maintained cell viability, improved cell proliferation and slightly decreased DNA damage [13]. The secret of LLL therapy seems to be the choice of the adequate type of laser and its parameters of irradiation. Using the correct laser device, wavelength, fluence rate and radiant exposure, no degenerative or lethal alterations occur within the cells or the tissue surrounding the irradiated areas [5-8,11,12-14].

CONCLUSION

This review concluded that the effects of LLL irradiation on cell structure and on its DNA vary depending on the laser parameters of irradiation. Adequate dosages can accelerate wound healing, stimulate cell proliferation and decrease DNA damage. Lower dosages do not seem to stimulate cells and higher dosages seem to be harmful to cell viability and increase DNA damage.

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3. Gmünder FK, Cogoli A. Effect of space flight on lymphocyte function and immunity. In: Fregly MJ, Blatteis CM, eds. Handbook of Physiology. Oxford:University Press, 1996, vol. 2, pp 799-813.

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