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Contents

Laser therapy in the management of neuropathic pain: preliminary experience on 43 patients

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ABSTRACT

The aim of this case series is to report on the effect of MiS, a new laser therapy device which uses two synchronized emissions with 905 nm and 808 nm wavelengths, pulsed and continuous, respectively, with high peak power, in the management of neuropathic pain. A total of 43 patients (mean age: 53 years, from 23 to 85 years) presenting neuropathic pain associated to different anatomical areas, such as cervical zone, spine, foot/ankle, hand/wrist, shoulder, elbow, hip and knee were treated with laser therapy by MiS. Pain (VAS score) and functionality (therapist evaluation) were evaluated at the end of treatment. The severity of pain decreased over time and was lower at the end of treatment. MiS laser therapy demonstrated to be safe and effective in patients affected by neuropathic pain and represents a valuable tool for the management of these patients.

INTRODUCTION

Pain is described as a complex, subjective experience, involving the transduction of harmful environmental stimuli together with the cognitive and emotional processing by the brain [1,2]. Neuropathic pain is a form of chronic pain resulting from any kind of damage to the central or peripheral nervous system without nociception [3,5]. Neuropathic pain is a painful condition that may comprise different types of pathologies: such as postherpetic neuralgia, painful diabetic polyneuropathy, post-surgery neuropathic pain, multiple sclerosis, spinal cord injury, stroke and cancer. Patients with neuropathic pain often have spontaneous pain, allodynia, and hyperalgesia.

The estimation of the incidence and prevalence of neuropathic pain is difficult because of the lack of simple diagnostic criteria for large epidemiological surveys in the general population [6]. A portion of these patients is specifically affected by peripheral neuropathy and seek medical treatment to alleviate the pain and improve the function associated to conditions that are localised at several body levels: spine, being lumbar-sciatic pain a very common problem, cervical area, elbow, wrist and hand, knee, ankle and foot, hip. For instance, sciatica is a form of radicular pain, and is described as a disease of the peripheral nervous system. It is a very common condition and the main cause of absences from work, with great economic impact on society [7]. The trend in terms of life expectations getting longer suggests that more and more people will be experiencing this type of pain in their life. Chronic neuropathic pain is characterised by complexity of neuropathic symptoms, poor outcomes and difficult treatment decisions.

On the biological side, nerve inflammation plays an important role in the development and progression of neuropathic pain. For instance, recent studies have indicated that hypoxia-inducible factor 1a (HIF-1a) is crucial in inflammation [8], while other previous studies have identified the relationship between proinflammatory cytokines, and neuropathic pain development [9-12].

Therapeutic options are in many cases related to conservative treatment, consisting of modifying the pain-precipitating activity, biomechanical correction with physiotherapy or the use of antidepressants, analgesics and/or steroids [13,14]. Specifically, painkillers are the main drugs to treat pain, although these have shown only 30% effectiveness in patients with neuropathic pain [15-17]. Unfortunately, these drugs have undesirable side effects and, currently, there is a worldwide trend in opioid reduction for acute and chronic pain management [18-20]. Physical methods are an interesting alternative to the pharmacological treatment because of the absence of side effects.

Recent studies have reported the use of laser therapy in patients with peripheral somatosensory neuropathy and neuropathic pain [21,22]. Specifically, clinical studies on the effects of laser therapy on injured nerves reported an increase in nerve function [21]. Moreover, laser therapy demonstrated to be effective for promoting axonal growth in injured nerves in animal models [23-26].

Positive effects of MLS® therapy in promoting repair processes of peripheral nerves, acting on the recovery of the lesioned function and the muscle mass and inducing faster myelinization of the regenerated nerve fibers, have been reported by Gigo-Benato et al. [27].

In vitro studies were carried out to characterize the effect of MLS® pulse and have shown that MLS® treatment induces an increase of NLRP 10, a protein with anti-inflammatory action.

NLPR 10 inhibits the activity of caspase 1 and PYCARD protein complex, which promote the maturation of the inflammatory cytokines interleukin-1 β (IL-1β) and interleukin 18 (IL-18). Therefore, ultimately, NLPR 10 inhibits the production of pro-inflammatory interleukins IL-1β and IL-18, reducing inflammation [28]. The decrease in inflammation leads to a normalization of vascular function and thus to a decrease of the edema. Obviously, the decrease in inflammation and edema results in a decrease of pain symptoms, that are frequently present in patients.

MiS is a medical device for laser therapy which combines the synchronized pulse of traditional MLS® therapy with the high peak power typical of high intensity laser therapy. These specific characteristics allow MiS to act on pain and its causes, leading to significant and persistent improvement of pain symptomatology and concomitant recovery of functionality.

This case series collects the case reports of two physiotherapy centers that have treated 43 patients for peripheral neuropathy using MiS, a new laser therapy, reporting changes in pain and in function, and safety associated to the use of the device.

MATERIALS AND METHODS

This is a case series collecting patients from routine practice in two Italian physiotherapy centers: Fisiolab (Vicenza- Italy) and Rehability Center (Padova- Italy). Forty-three patients of both sexes affected by several conditions related to peripheral neuropathies have been included in the series.

During the treatment, patients and therapists wore safety glasses to prevent eye damage.

Diagnosis and instrumental evaluation (i.e. X-Ray, Ultrasounds, CT, MRI), when available, were recorded.

Additionally, patients were evaluated by the specialist performing the treatment before therapy start.

The patients that were included in this series received MiS (ASA Srl, Italy) treatment focused on the peripheral neuropathy as stand-alone treatment or as a part of their treatment programme.

MiS is a class IV NIR laser with two synchronised sources, one consists in 6 pulsed 905 nm laser diodes, the second is a continuous/frequency-modulate 808 nm laser diode. Maximum average power is 6W± 20%, while maximum peak power is 1kW. MiS has 2 interchangeable handpieces (with diameters of 2 and 5 cm).

The total number of sessions and the time of each session were adjusted based on each patient response to the treatment and ranged from 2 to 13 sessions, with a duration ranging from 6 to 20 minutes (according to body location). The used frequency was 30 Hz for all the body areas, while intensity was adapted to the anatomical site as follows: 80% for shoulder and hip, 70% for spine, 60% for elbow, wrist/hand, knee, ankle/foot and 50% for head and cervical area. Dosage was adjusted based on size of the area to be treated, patient and pathology characteristics and condition stage.

Trigger points, when present, were treated in all patients with the following parameters: Frequency: 10 Hz, time: 23 s, Intensity: 25%. In the trigger point phase, the hand piece was perpendicular to the treated points.

Pain evaluation was performed before and after each laser session using a Visual Analogue Scale (VAS) scale. It is a scale comprising 10 grades, with 10 representing 'unbearable pain' and 0 representing 'no pain'. It is a pain scale commonly used in the medical field, and it was shown to be a reliable and valid measure of pain [29,30]. Safety has been specifically assessed and the therapists recorded any side effect and/or rebound effect happened during the treatment. Functional evaluation and global assessment were reported by the specialist for each patient.

RESULTS

Demographic and clinical characteristics of all patients at baseline were recorded. Patients demographic characteristics are reported in Table I.

For 34 patients, peripheral neuropathy treatment was the focus of the overall therapy cycle, while 9 patients received other MiS treatments beside the peripheral neuropathy protocol (i.e. specific for edema, muscle pain and contracture) in their therapeutic path.

VAS pre and post treatment, along with change in VAS expressed as a percentage of the initial value are reported in Table II, divided by treated anatomic areas. As expected when dealing with neuropathic pain, average pain at baseline was moderate to severe (mean was >7 for all groups). Overall, VAS pre-treatment mean was 7,8 and VAS post-treatment mean was 1,6, corresponding to a decrease in pain of 79,5%. Pain completely disappeared in patients treated for elbow, hip and shoulder problems. Considering all the groups, improvement was at least 60% respect to baseline, meaning that initial pain score was reduced of above 60% at the end of the treatment cycle.

It has to be noted that some patients were not seeking medical treatment for pain, but for symptoms related to nerve irritation, as for example paraesthesia, dysesthesia, hyporeflexia, etc. In these cases, the treatment with MiS

Table I - Demographics

Sex	M=55,8% F=44,2%
Active (sport activity)	YES=46,5% % NO = 44,2% NA=9,3%
Age	Mean= 53 yrs (23 to 85)

Table II - VAS pre and post treatment divided by anatomical distribution of the treated areas

Area	Patient #	VAS Pre (mean)	VAS post (mean)	δVAS%
Spine	17	8,8	2,2	75%
Cervical area	3	8,3	3	63,9%
Elbow	4	9	0	100%
Knee	4	8	1,5	81,3%
Ankle/foot	3	7	2	71,4%
Hip (mainly pudendal nerve)	9	7	0	100%
Shoulder	1	9	0	100%
Wrist/Hand	2	7,5	2,5	66,7%
TOTAL	**43**	**7,8**	**1,6**	**79,5%**

gave excellent results and the therapists have reported strong improvements in sensitivity and dysesthesia reduction.

In general, looking at VAS value trend, it was possible to appreciate pain decrease during time, rather than intra-session. Some patients, reported fluctuation in VAS score between the sessions during the treatment cycle. This could be related to a prompt increase of physically demanding activities by the patients after perceiving benefit from the initial laser therapy sessions.

In general, laser treatment provided a positive impact on pain and function on the majority of the patients, only for 2 of them no significant improvement after the laser therapy cycle was reported.

DISCUSSION

Neuropathic pain can substantially impair quality of life as it often associates with other problems, such as loss of function, anxiety, depression, disturbed sleep and impaired cognition and physical therapies have been suggested as potential alternative for treatment [6]. The results of this case series show that patients treated with MiS for peripheral neuropathy had an improvement in terms of pain symptoms measured with VAS, even when starting from high VAS values, typical of neuropathic pain. The improvement was gradual and was normally seen after some sessions rather than at the end of each laser treatment, suggesting that MiS is able to induce biological responses whose effects depend on the evolution of the underlying biological processes over time, which could be interesting to address in future basic and clinical studies. MiS inherits the wavelengths (808 nm and 905 nm), the characteristic synchronized modulation of continuous and pulsed emissions, and the scientific evidence of the action mechanisms from MLS® laser therapy. Experimental and clinical research demonstrated that MLS® pulse exerts a positive effect in the treatment of many musculoskeletal diseases [31-34]. This effect is related to anti-inflammatory, anti-edema and tissue healing actions [28,35]. Besides relying on MLS® pulse features, MiS is characterised by a very high peak power in the order of kW. The modulation in short pulses allows to control the peak power avoiding damaging thermal effects.

In the literature, Kobiela Ketz et al [36] suggested that the reduction of hypersensitivity mediated by laser treatment in a model of neuropathic pain induced by spinal nerve injury could be exerted by modulating macrophages and microglia components. Preliminary *in vivo* investigation related to laser therapy use in neuropathic pain relief highlighted therapeutic effects that might be used for clinical application in neuropathic cases [37].

In the specific field of neuropathic pain, preclinical experiments carried out on animal models demonstrated that the treatment with MiS promotes the recovery of the myelin sheat in nerve fibres that have been damaged in the lesion area, as confirmed by histological and immunohistochemical evaluations [38]. These data support the concept that laser therapy by MiS could be a suitable tool in the management of neuropathic pain.

No rebound effect has been observed, thus confirming the safety of the device in this cases series, which included individuals with different characteristics, pathologies and stage of conditions.

Patients gave a positive feedback on the treatment feeling, especially when the 5 cm handpiece was used on large areas, as its shape allowed a sort of massage over the patient's skin, making the treatment well accepted and contributing to build compliance to session attendance.

CONCLUSION

This case series reports on the use of MiS in the management of 43 cases of neuropathic pain localised in different anatomical areas. Based on the results reported, the new MiS laser therapy demonstrated to be safe and effective in patients affected by neuropathic pain. Therefore, laser therapy by Mis may represent a valuable and well-accepted tool for the management of peripheral neuropathies.

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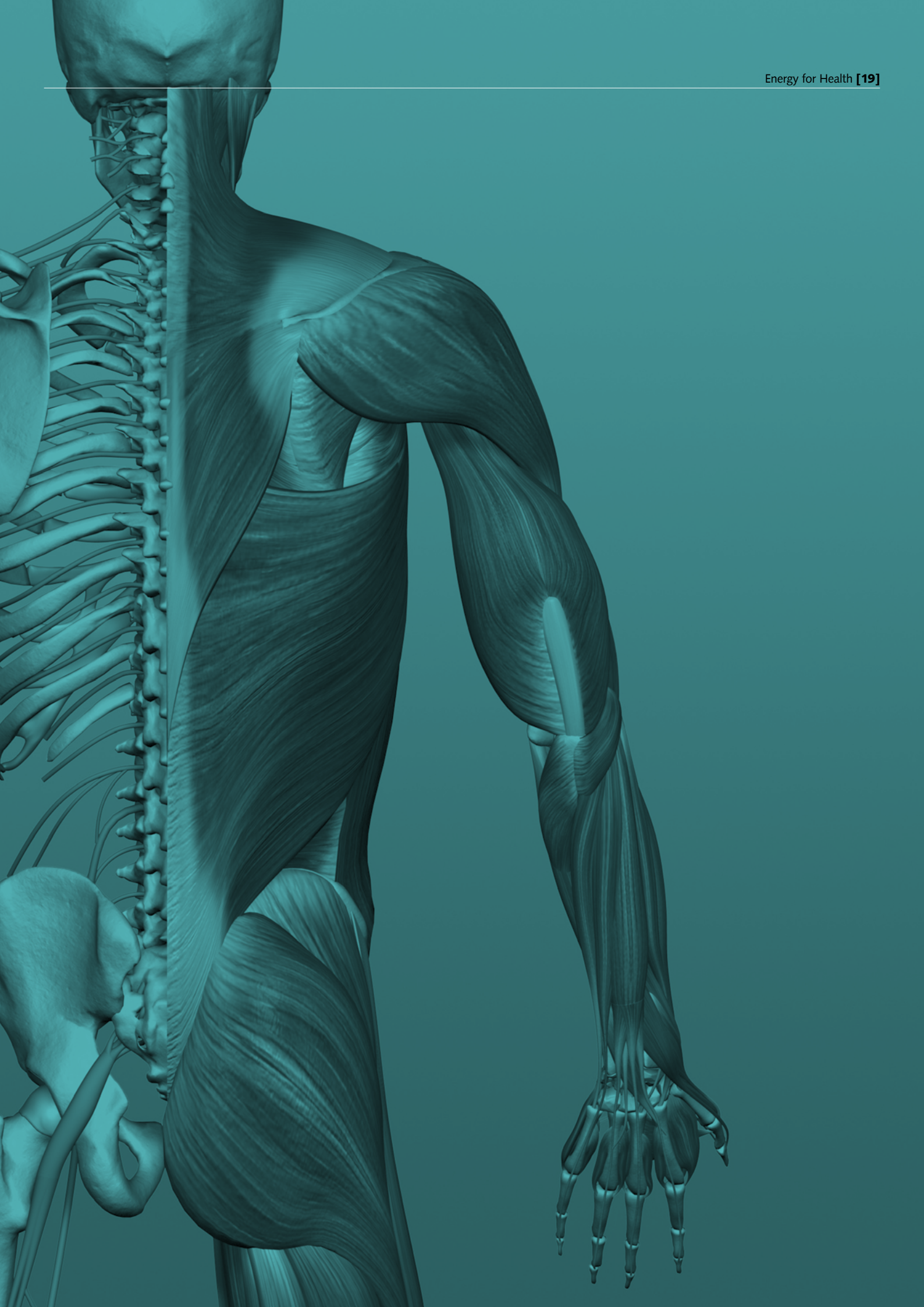

Effect of MLS® Laser Therapy on Pain and Satisfaction for Musculoskeletal Conditions: A Retrospective Study

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ABSTRACT

What most often runs in parallel with injuries, chronic joint damage, and post-operative wounds is pain. Pain management is a duty that physicians must assist patients with on a daily basis. There is an abundance of pain-reducing techniques used in clinics today, including opiate pain medications and steroid injections, with new medications and technologies continuing to be developed. MLS® Laser Therapy is a growing pain-reducing technique that utilizes light to produce anti-inflammatory and analgesic effects. This retrospective chart analysis study was designed to evaluate patient pain and satisfaction for a variety of musculoskeletal conditions before and after treatments with MLS® Laser Therapy. The study included post-hoc charts available at a laser pain center in Sedalia, Missouri, United States. The average decrease in reported pain was 46% at (or after) three treatment sessions and 55% at (or after) six treatment sessions. The average patient satisfaction for all 11 conditions was 71% satisfied or better, while the average doctor-reported improvement for all conditions was 67%. Results indicate MLS® Therapy Laser as a pain free, noninvasive alternative to reducing pain and increasing satisfaction for a variety of musculoskeletal conditions.

INTRODUCTION

Pain caused by musculoskeletal conditions is a worldwide commonality that transverse age and demographics. These conditions do not only affect older populations but also impact individuals throughout the age spectrum. Musculoskeletal conditions are the second largest contributor to disability worldwide with persistent pain conditions accounted largely for by musculoskeletal conditions [1]. Commonly, patients with musculoskeletal pain will seek medical treatment, and one such noninvasive treatment option is laser therapy.

Light Amplification via Stimulated Emission of Radiation (LASER) is a device that, by design, amplifies photons to create and emit a beam of light that is classified by its wavelength within the electromagnetic spectrum. This wavelength, measured in nanometers (nm), dictates the nature and intended purpose of the laser. Therapy lasers, with wavelengths ranging from 600nm to 1000nm, penetrate skin and tissue as photons are not strongly absorbed by hemoglobin or water in the body [2].

Lasers for Low Level Light Therapy (LLLT) have been designed with low emission to ensure the treated tissue temperature does not rise more than a few degrees above normal body temperature [2]. LLLT treatments have demonstrated significant and effective results in decreasing muscle fatigue in elderly women [3], as well as improving circulation in treated areas and reducing pain in knee osteoarthritis [4].

The Multiwave Locked System (MLS®), a type of LLLT laser, has been shown to decrease inflammation and increase the biostimulation effect on tendons [5], increase functionality of ligaments by decreasing thickness and decreasing patient pain [6], and increase myoblast function thus increasing recovery of damaged muscle tissue [7]. MLS® Laser Therapy has also shown significant clinical improvement in vascular conditions such as Raynaud's Phenomenon [8].

The MLS® M6 Therapy Laser emission precisely synchronizes dual wavelengths consisting of 808nm and 905nm, as well as combining continuous and pulsed emissions, resulting in optimum clinical effectiveness [7]. The MLS® emission provides more efficient results while using less energy in considerably reduced times compared to traditional LLLT [9].

MLS® is being utilized by many medical practitioners in the United States and Europe to reduce pain and inflammation in a variety of patients. Many patient success stories have been a result of treatments with MLS®, including in orthopedic practices. This retrospective chart review analyzed 235 charts who received MLS® Laser Therapy at a single medical facility with the purpose to identify

the reported patient pain and satisfaction following treatment sessions.
Eleven pre-determined orthopedic conditions were identified to review.

METHODS

Chart Selection

Medical diagnoses were assigned to each patient by an orthopedic medical doctor prior to laser therapy. The 11conditions identified for this study were based on commonality and available resources: knee arthritis, lumbar pain, shoulder arthritis/pain, post-operative total knee replacement (TKR)/ post-operative total hip replacement (THR), neck pain, plantar fasciitis, wound, hip arthritis/pain, contusion/ sprain, tendonitis, and post fracture/ joint jam. Only charts representing one of these 11 conditions were selected for review and analysis.
Since MLS® Laser Therapy is cumulative in effectiveness, multiple treatment sessions were recommended to patients. Due to incongruencies in number of completed treatment sessions across conditions, the time points chosen to analyze during this chart review were after the 3rd treatment session and after the 6th treatment session.
Inclusion criteria included: diagnosis and treatment for knee arthritis, lumbar pain, shoulder arthritis/pain, TKR/THR, neck pain, plantar fasciitis, wound, hip arthritis/pain, contusion/sprain, tendonitis, or post fracture/ joint jam and at least 3 MLS® Laser Therapy treatment sessions. The energy density (fluence) was in the range at 3.6-7.0 J/cm^2. Charts were excluded if the condition was outside the 11 specified, did not complete at least 3 treatment sessions, or did not have Visual Analogue Scale (VAS) recorded.

Procedures

Each chart was analyzed to determine the decrease in pain (from Treatment Visit 1 to Treatment Visit 3 and again from Treatment Visit 1 to Treatment Visit 6), the overall patient- reported satisfaction (satisfied with MLS® outcome or not satisfied with MLS® outcome), and post-hoc doctor reported improvement (Poor, Fair, Good, or Excellent). Doctor-reported improvement was based upon the patients' reports on VAS for pain at the time of treatment sessions (see Table I). In addition, the various settings of the laser, including frequency, duration of administration, and intensity were recorded. For all post-hoc analysis, a single study team member reviewed all charts and recorded necessary information on case report forms.

Table I - Criteria for Pain Improvement

Average VAS Pain Improvement	Doctor Rating
<0%	Poor
0% to 30%	Fair
30% to 60%	Good
60% to 100%	Excellent

RESULTS

The percent decrease in pain were calculated for each selected condition. These results were averaged in Table II. The averages are represented across top with conditions along the side. In each, "n" represents number of charts available for that analysis.

Table II - Decrease in Pain, Reported Satisfaction, and Reported Improvement for All Conditions

Condition Reported	Average % decrease in pain in or after 3 treatments	Average % decrease in pain after 6 treatments[1]	Overall patient satisfaction (in average %)	Overall doctor reported improvement (based on individual assessment of pain improvement; measured in average %)
Knee Arthritis	51 n= 14	66 n=2	86 satisfied or better	86 good or better
Lumbar	49 n= 26	53 n=10	71 satisfied or better	64 good or better
Shoulder Arthritis/ Pain	39 n= 30	47 n=4	60 satisfied or better	53 good or better
Post Op TKR/THR	57 n=38	87 n=11	76 satisfied or better	76 good or better
Neck Pain	47 n=19	74 n=3	63 satisfied or better	63 good or better
Plantar Fasciitis	78 n=6	0[2] n=1	100 satisfied or better	100 good or better
Wound	13 n=7	N=0	25 satisfied or better	25 good or better
Hip Arthritis/ Pain	48 n=7	25 n=1	71 satisfied or better	57 good or better
Contusion/Sprain	51 n=18	77 n=3	80 satisfied or better	75 good or better
Tendonitis (Ankle, Wrist, Fingers, Elbow, Shoulder)	45 n= 46	48 n=4	77 satisfied or better	71 good or better
Post Fracture/ Jam	34 n= 27	75 n=2	70 satisfied or better	66 good or better

[1] Most patients did not receive 6 or more treatments

[2] Only one patient had more than 3 treatments for Plantar Fasciitis. For this patient: after 6 treatments there was no decrease in pain and at the conclusion of all treatments (22) pain was 75% improved.

Patients with plantar fasciitis, when treated with MLS® Laser Therapy, reported the largest average decrease in pain (78%) and overall were the most satisfied with their results (100%). Patients receiving treatment after total knee or hip replacements reported the second largest average decrease in pain by 57% at (or after) 3 treatments (e.g. pre-treatment VAS score of 8 to post treatments VAS score of 3.5). The top 4 conditions in order of improvement are listed in Table III. The top 4 conditions in order of satisfaction are listed in Table IV.

Average overall patient satisfaction for all 11 conditions was 71% satisfied or better (range 100% to 25%). Average overall doctor reported improvement for all conditions was 67% (range 100% to 25%).

Table III - Condition in Order of Improvement in or After 3 Treatments

Plantar Fasciitis
Post-Op TKR/THR
Contusion Sprain
Knee Arthritis

Table IV - Overall Satisfaction at Conclusion of Treatment

Plantar Fasciitis
Knee Arthritis
Contusion Sprain
Tendonitis

DISCUSSION

Of the 235 total patient charts analyzed, none experienced pain during or after laser therapy as a direct result of treatment. Few possible side effects were noted but all resolved quickly with no lasting effects.

Overall, among the 11 conditions analyzed, the average decrease in reported pain was 46% at (or after) 3 treatment sessions. This equates to a patient having a pain reported at 8 out of 10 prior to MLS® Laser Therapy and at (or after) 3 treatments having a pain level of 4.32 out of 10. At (or after) 6 treatment sessions, the average decrease in reported pain was 55%, thus equating in pretreatment pain of 8 out of 10 and resulting in pain of 3.6 out of 10.

When reviewing the data, it's noted that most patients did not receive 6 or more treatment sessions. It can be estimated that results may continue to trend in the same direction if more treatments occurred since MLS® is cumulative in effectiveness.

Since pain is universally consistent with injuries, chronic joint damage, and post- operative wounds, pain management options for patients are essential. Results of this study indicate MLS® Therapy Laser as a possible pain-free, noninvasive alternative to reducing pain for a variety of musculoskeletal conditions.

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In vitro biological responses to electromagnetic fields exposure of peripheral nervous system cells

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ABSTRACT

An important goal in neuroscience is the study of new therapeutic strategies to promote nerve regeneration in peripheral neuropathies. Evidences from the literature suggest that the application of electromagnetic field (EMF) might be a valid approach to promote nerve regeneration, even if the molecular mechanisms underlying these positive effects are not clearly defined. Aim of our work is to characterize EMF safety and effects on rat Schwann cells (SCs) cultures as an *in vitro* model of nerve cells involved in axonal integrity. SCs were exposed to EMF with different experimental settings and cell viability, proliferation, migration ability and specific myelin markers were analyzed.

Data suggest that EMF is not a toxic stimulus for SCs even when it is applied repeatedly, moreover a "chronic" EMF exposure induces an increased proliferation without affecting cell differentiation. In conclusion, a repeated EMF exposure might represent a tool to improve regenerative ability of myelin producing SCs on peripheral nerves.

INTRODUCTION

Peripheral neuropathies are a group of heterogeneous diseases, characterized by alterations of peripheral nerves structure and functions. Peripheral neuropathies are produced by different etiopathogenetic causes including genetic and metabolic diseases (diabetes, alcoholism, nutritional deficiencies), infective disorders (bacterial or viral), exposure to drugs (e.g. chemotherapy-induced) or environmental toxins and traumatic injuries. Neuropathies are often extremely debilitating and able to significantly compromise the life quality of affected individuals.

The identification of new therapeutic strategies and devices to avoid nerve degeneration and to promote nerve regeneration is therefore an important goal in the field of neuroscience. In literature there are several evidences suggesting the use of electromagnetic fields (EMF) to stimulate peripheral nerves regeneration. Indeed, the application of low frequency fields (20/50 Hz) seems to represent a promising tool to promote nerve regeneration in clinical practice [1, 4]. However, few papers include details regarding the possible cell target affected by the EMF exposure [5, 6]. Therefore, the identification of the molecular mechanisms and the target cells may be fundamental to develop more efficient and strong therapeutic strategies to promote nerve regeneration.

The general **aim** of our work was to characterize EMF effects and viability on cell potentially related to peripheral nerves regeneration. To this purpose, an *in vitro* model of rat Schwann cells (SCs), the main peripheral nerve cells responsible of nerve and axonal integrity, were exposed to EMF with different experimental settings.

MATERIAL AND METHODS

Cell cultures

Rat SCs cultures were prepared from sciatic nerve accordingly to the method commonly used in the laboratory of Prof. Magnaghi [7]. Briefly, sciatic nerves from 3-day-old rats were digested with collagenase and trypsin, the cell pellets suspended in Dulbecco's modified Eagle's medium (DMEM, Serotec, Oxford, UK), supplemented with 10% FCS (Gibco-Life Technologies, Italy) and plated onto Petri dishes. Cells were routinely maintained in DMEM, 10% FCS, 2 μM forskolin, 200 μg/ml bovine pituitary extract (BPE, Invitrogen, Italy); before being used for each different assay, cells were treated for 48h with 4 μM forskolin.

EMF exposure

According to the specific experimental assays, SCs were plated in Petri dishes and exposed to EMF using the commercial ASA PMT QS device equipped with the Flexa applicator from ASA S.r.l.

(Vicenza, Italy). The different protocols and exposure times used are listed in Table I. SCs used as control were plated in same culture conditions, without EMF exposure. For the "chronic" treatment, SCs were exposed 5 consecutive times, every 24 hours each, using the maximum power (3mT), with a frequency of 50Hz for 30 minutes each exposure (Table I).

Proliferation assay

SCs proliferation was performed by cell count: 60 000 cells were plated into 35 mm petri dishes and collected after 48 and 72 h, with Trypsin 0.05%-EDTA 0.02% in DMEM (PBS, Euroclone, Italy). The cell suspension was then counted under an optic microscope.

MTT assay

Cell vitality was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This is a rapid colorimetric assay measuring the cell mitochondrial activity. Adherent cells were stained with MTT solution (0.5 mg/ml) for 30 min at 37 °C and absorbance was measured at 570 nm.

Soft agar

Soft agar colony formation assay is a well-established method for characterizing the anchorage-independent growth of cells in vitro. Cells were grown on a layer of soft agar, thus preventing from adhering to the culture plate. Cells were then exposed to EMF according to the "chronic" protocol (see Table I). After 25 days, colonies were stained with crystal violet and counted.

Scratch assay

The scratch assay was used to evaluate the migratory ability of SCs. Scratch assay is a reliable and approved method to measure cell migration in response to tissue injury. An artificial gap (so called "scratch") is experimentally created on a confluent cell monolayer by a mechanical smear. The cells on the edge of the newly created gap move toward the opening, to close the "scratch" until new cell–cell contacts are established again. Images are captured at the beginning and regular intervals during cell migration, and the comparison of the images determines the rate of cell migration. In our experimental conditions, sub-confluent cells in monolayer were scratched with a plastic tip. Wounded monolayers were washed with fresh medium to remove dead cells; images of the scratched monolayers were captured immediately after the scratch (t0), 2h, 6h, 24h and 48h after with the Axiovert 200 microscope (Zeiss, Jena, Germany) and the MetaVue software (Molecular Devices, Sunnyvale, CA, USA). Distances between cell fronts were measured with Image-ProPlus 6.0 (MediaCybernetics, Bethesda, MA, USA), considering at least six measurements from the top to the bottom.

Immunofluorescence

SCs morphology of control and "chronically" EMF exposed cells was evidenced by the cytoskeletal protein actin. Cells were seeded on slides and fixed in 4% paraformaldehyde, then stained with Phalloidin-FITC for f-actin (1: 250, Sigma) and Alexa 488 (green) as secondary antibody (1 : 800, Gibco-Life Technologies). Slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA) and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI). Controls for the specificity of antibodies included a lack of primary antibodies. Confocal microscopy was carried out using a Zeiss LSM 510 System (Gottingen, Germany) and images were processed with Image Pro-Plus 6.0.

Gene expression

The expression of SCs biomarkers, such as the main myelin-forming protein of the peripheral nerves, glycoprotein P0 and protein PMP22, was assessed by **qRT-PCR** on total RNA.

RNA extraction, purification and quantitation

Total RNA was extracted with trizol (Gibco-Life Technologies) according to the manufacturer's instructions and then quantified with Nano-Drop2000 (Thermo Scientific, Waltham, MA, USA).

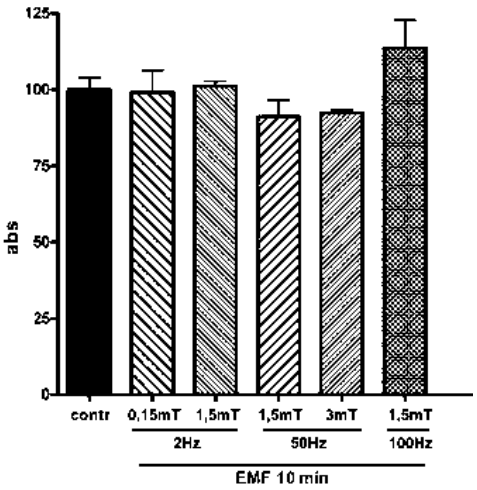
Real-Time PCR

Reverse transcription was performed on 1 µg of total RNA from each sample according to the manufacturer's protocol (iScript cDNA synthesis kit, BioRad, Segrate, Italy) using random primers. qPCR was done in singleplex in CFX96 Touch™ Real-Time PCR Detection System (BioRad, Segrate, Italy) by using SYBR Green dye (SsoAdvanced SYBR Green Supermix, Bio-Rad, Segrate, Italy) and specific set of primers as follows:
P0: 5'-CCTGCTCTTCTCTTCTTTG-3' and 5'-CACAGCACCATAGACTTC-3';
PMP22: 5'-TCCTGTTCCTTCACATCG-3' and 5'-TGCCAGAGATCAGTCCTG-3';
α-tubulin: 5'-TCGCGCTGTAAGAAGCAACACC-3' and 5'-GGAGATACACTCACGCATGGTTGC-3';
β2-microglobulin: 5'-TGCTTGCAGAGTTAAACACGTCAC-3' and 5'-TTACATGTCTCGGTCCCAGGTG-3'.
Data analysis was performed using the CFX Manager 2.0 software (Bio-Rad, Segrate, Italy). Each sample was analysed in triplicate.
Data was normalized for α-tubulin and β2-microglobulin Ct value. Relative mRNA levels were then calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) and data expressed as fold induction

Statistical analysis

Data were statistically evaluated by GraphPad Prism 4.00 (San Diego, CA, USA). Statistical significance between groups was determined by means of an unpaired Student's t-test. P-values < 0.05 were considered as significant.

Figure 1 - Cell viability, measured as cell mitochondrial activity by the colorimetric MTT assay, in control and EMF-exposed SCs. EMF was applied for 10 minutes with different frequency (2, 50 and 100 Hz) and intensity (0,15, 1,5 and 3 mT). Data are expressed as Absorbance ± SD.



RESULTS

Figure 1 shows cell viability, measured as cell mitochondrial activity by the colorimetric MTT assay. A single 10 minutes exposure to EMF, applied according to different protocols as described in Table 1 (frequency: 2, 50 and 100 Hz; intensity: 0,15, 1,5 and 3mT), does not induce any statistically significant modification of cell viability.

In **Figure 2**, cell proliferation was evaluated by cell count after EMF exposure (time, frequency and intensity combinations are listed in **Table I**): EMF was applied once but with different duration (10 and 30 minutes) and cells were counted 48 and 72 hours after EMF exposure. None of the exposure protocols applied produced any significant change in SCs proliferation, neither at low (2Hz) nor at high frequencies (100 Hz), for none of the used intensity (0,15 – 3mT), even when the duration of the exposure was longer (30 minutes).

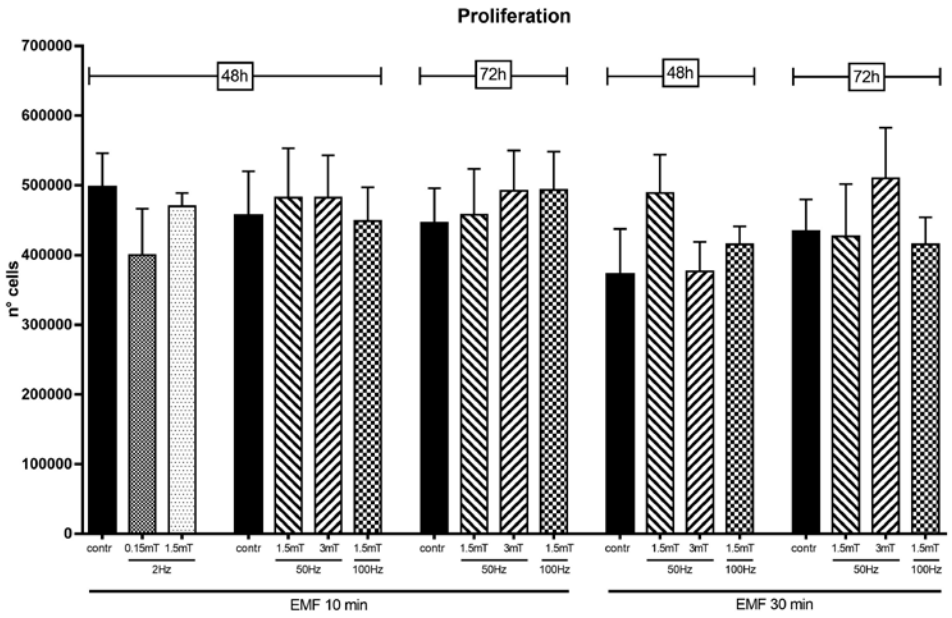
The scratch assay was used to evaluate the migratory ability of SCs. In a first set of experiments (**Figure 3**), SCs were exposed for 10 minutes to different combinations

Table I - Different combination of EMF frequency and intensity

Frequency	intensity		Duration
2 Hz	0,15 mT	(5%)	10′
2 Hz	1,5 mT	(50%)	10′
50 Hz	1,5 mT	(50%)	10′
50 Hz	3 mT	(100%)	10′
100 Hz	1,5 mT	(50%)	10′
50 Hz	1,5 mT	(50%)	30′
50 Hz	3 mT	(100%)	30′
100 Hz	1,5 mT	(50%)	30′
50 Hz	3 mT	(100%)	30′ x 5 time (every 24h)

Different protocols and exposure times are listed in the Table. Intensity is also indicated as % value relative to max potency of the device used.

Figure 2 - Cell proliferation measured by cell count 48 and 72 hours after EMF exposure, in control and EMF-exposed SCs. EMF was applied for 10 or 30 minutes with different frequency (2, 50 and 100 Hz) and potency (0,15, 1,5 and 3 mT). Data are expressed as number of cells ± SD.



of frequency and potency (**Table 1**), starting from 2 Hz to 100 Hz, the maximum frequency obtained by Flexa. Optical images were acquired at time 0, 2, 6 and 24 hours after EMF exposure; these images were used to measure the distance covered by the migrating front of the cells. Migration ability was calculated as the distance covered in the specific time. In a second set of experiments (**Figure 4**) we focused special attention to the higher frequency and potency, to longer exposure time (30 minutes) and images were acquired up to 48 hours after EMF exposure. As shown in **Figure 3 and 4**, none of the exposure protocols used is able to significantly modify the migratory capacity of SCs. Cells cover almost the same distance either when are exposed to low (2 Hz) or high EMF frequency (100 Hz). No differences were evidenced at none of the selected EMF intensity (0,15 – 3mT), even when cells were exposed for longer period (30 minutes).

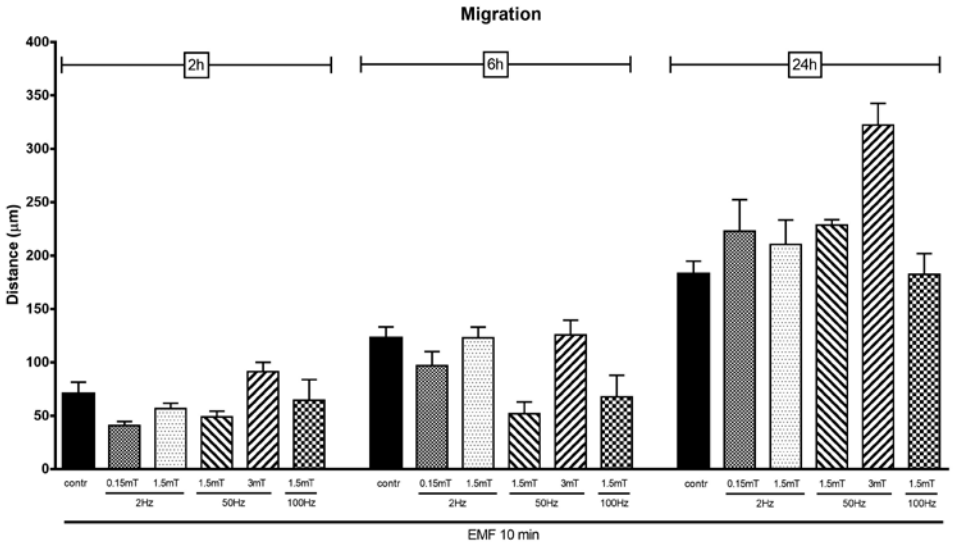


Figure 3 - Migration ability evaluated by scratch test in control and EMF-exposed SCs. EMF was applied for 10 minutes with different frequency (2, 50 and 100 Hz) and intensity (0,15, 1,5 and 3 mT). Optical images were acquired at time 0, 2, 6 and 24 hours after EMF exposure; data are expressed as the distance (µm) covered by the migrating front of the cells ± SD.

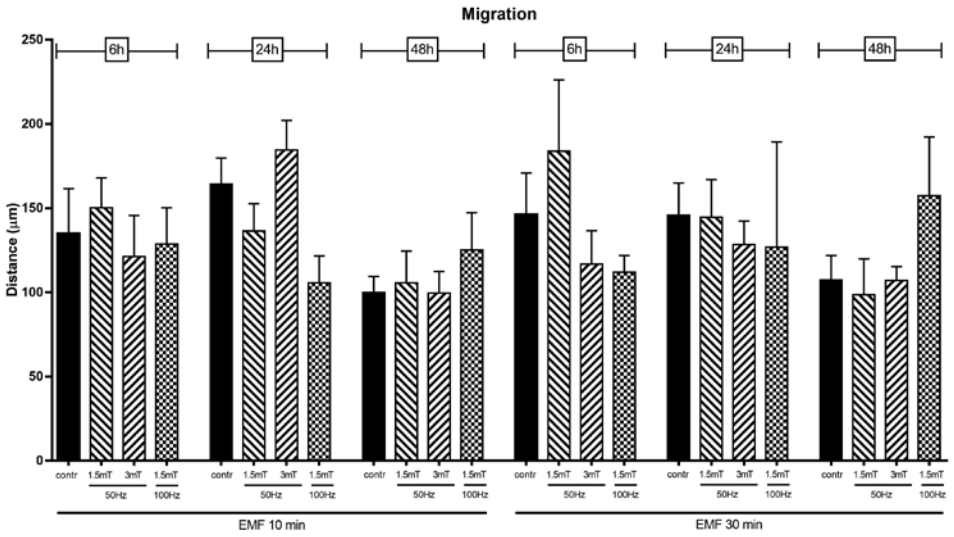
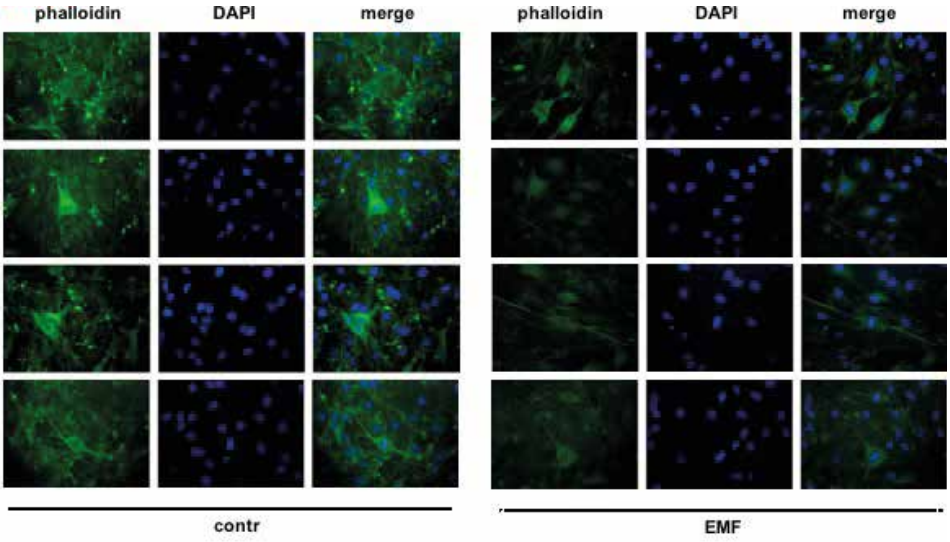


Figure 4 - Migration ability evaluated by scratch test in control and EMF-exposed SCs. EMF was applied for 10 or 30 minutes with different frequency (50 and 100 Hz) and intensity (1,5 and 3 mT). Images were acquired 6, 24 and 48 hours after EMF exposure. Data are expressed as the distance (µm) covered by the migrating front of the cells ± SD.

Figure 5 - Staining of the actin cytoskeleton by phalloidin immunofluorescence (green) in control and EMF-exposed SCs. Nuclei are stained with DAPI (blue). EMF was applied "chronically": 5 consecutive exposure every 24 hours each, with a frequency of 50Hz and a intensity of 3mT, 30 minutes each exposure four different fields are shown.



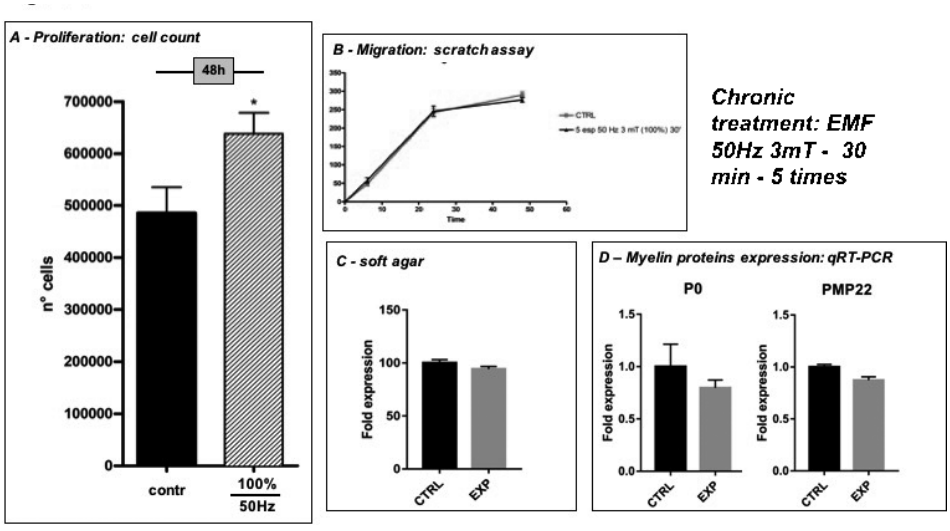


Figure 6 - Effects of a "chronic" EMF exposure (5 consecutive exposure -50Hz, 3mT, 30 min - every 24h each) on SCs proliferation, migration and myelin protein expression. **A)** Cell proliferation measured by cell count 48 hours after EMF exposure in control and EMF-exposed SCs: data are expressed as number of cells ± SD; *p<0,05 vs contr **B)** Migration ability evaluated by scratch test in control and EMF-exposed SCs: data are expressed as the distance (μm) covered by the migrating front of the cells ± SD. C) Soft agar growth in control and EMF-exposed SCs: the number of stained colonies in EMF-exposed SCs are expressed as a fold induction vs Contr. D) Myelin protein P0 and PMP22 expression assessed by qRT-PCR: data are normalized vs α-tubulin and expressed as fold induction vs control SCs.

Figure 5 shows a staining of the actin cytoskeleton by phalloidin immunofluorescence in control and EMF exposed SCs. EMF was applied "chronically", that is 5 consecutive times, every 24 hours each, using the maximum power (3mT), with a frequency of 50Hz for 30 minutes each exposure. Immunofluorescent images show SCs with their classic flat and spindle-shaped form, that does not change even after EMF exposure. **Figure 6** reports the results obtained after the "chronic" EMF exposure as to SCs proliferation, migration and functionality. A prolonged and repeated EMF does not influence either SCs migration rate (**Figure 6B**), or the expression of P0 and PMP22 (**Figure 6D**) however, "chronic" EMF exposure induces a statistically significant increase in proliferation (**Figure 6A**) assessed by cell count, but not in the ability to growth in soft agar (**Figure 6C**), a measure of the anchorage-independent growth.

DISCUSSION

Cell proliferation and migration are physiologic phenomena strictly related to the regenerative ability of a tissue. This is also true for peripheral nerves and for Schwann cells, the main cell type involved in the regenerative process of nerve tissue. In the data here presented we tested the possibility for EMF to promote nerve regeneration, by means of an "in vitro" approach, using rat SCs as the experimental tool for our studies. Our hypothesis was that if EMF exerts any effect on SCs proliferation and migration, this might be predictive of a regenerative effect of EMF on peripheral nerve. EMF positive effects on nerve regeneration is widely discussed in the literature [4] but there is no general agreement about the application protocol as to the intensity, time intervals and frequency [8]. Also, the molecular mechanisms through which EMF exerts its effects are not well defined, spanning from its influence on NGF levels, cytokines secretion, Ca++ channel modulation, intracellular ROS production etc.; only few of these mechanisms have

been also demonstrated in SCs [9]. The different experimental settings used in this paper were designed to clarify some possible mechanisms of EMF action on peripheral SCs. EMF does not represent a toxic stimulus for SCs in culture: the different experimental settings tested, as listed in Table I, never induced any variation in cell proliferation and vitality, even when cells were exposed to EMF with repeated applications. This clearly indicate that SCs maintain an healthy state even when EMF is applied repeatedly and for longer period, thus representing a good experimental model for the evaluation of the effects of EMF chronic exposure on cell proliferation and migration. To our knowledge, this is the first time in which repeated application of EMF is considered as an experimental tool to mimic what happens in clinical use. No positive effect of EMF on cell growth was seen with one short exposure (10 minutes), for none of the different potencies and frequencies applied. Only the EMF repeated exposure for 5 times of 30 minutes each, mimicking a "chronic" in vivo exposure, induces a modest, but statistically significant, increase in SCs proliferation. This is particularly relevant as EMF is applied repeatedly and for a long period of time, when used in therapy.

The cell growth in soft agar, an assay for the assessment of the anchorage-independent growth, was used to evaluate whether the increased proliferation was related to changes towards a less differentiated phenotype. No difference was evidenced between control and EMF-exposed cells, suggesting that SCs increased proliferation does not affect cell phenotype. This is also proved by the expression of the two myelin proteins, P0 and PMP22, considered specific markers of SCs differentiation, the expression of which is almost the same between control and exposed SCs.

In conclusion, our findings evidenced a positive and promising effect of the chronic EMF exposure, generated by the ASA PMT QS, on the SCs in vitro, that may be summarized as follow:

1. EMF exposure does not seem to cause toxicity or morphology/differentiation changes on exposed SCs, for 2Hz – 100 Hz frequencies and 0,15 – 3mT intensity.
2. SCs morphology, growth, vitality, migration and myelinating capacity are not influenced by low frequency intensity EMF exposure.
3. Conversely, a high intensity (3mT), long-time (30 minutes) and repeated (5 times) exposure, even if does not produce signs of cell toxicity, induces an increase in SCs proliferation.

All together, these data are in line with those recently published by our group [10] showing that SCs, exposed to high intensity EMF (50 Hz, 0,1T) for 10 minutes, are able to proliferate and to migrate significantly better than control cells. The effect appears after 24 hours, but it becomes statistically significant for longer exposure times (48 and 72 hours). Furthermore, a second exposure to EMF, 24 hours later, further increases cell proliferation, suggesting an additive effect. Thus, the EMF, when applied at 50Hz frequency and high intensity (0,1T), exerts a pro-proliferative and pro-migration activity on SCs in culture [10].

We assume that the chronic EMF exposure is promising and might be predictive of regenerative ability following therapeutic application of the device to the peripheral nerves.

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Key words: Necrotizing fascitis, Photobiomodulation, Laser therapy, Wound healing

Laser therapy in the treatment of necrotizing fasciitis – a case report.

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ABSTRACT

The aim of this report is to highlight the benefits of the MLS® laser system in the management of necrotizing fasciitis (NF).

A 6-year old, cross-bred bitch, presenting necrotizing fasciitis in the perineal region and hindquarters, is admitted with septic shock and remains in intensive care for three days. The necrotic tissue is cleaned out, wet dressings are applied together with the use of a MPHI Multiwave Locked System (MLS®) laser. After necrotic tissue has been completely removed, skin resurfacing and regrowth of hair were achieved in 45 days, and the resulting scars are small and supple.

The results establish that the application of MLS® therapy in the treatment of necrotizing fasciitis helps to reduce drug therapies and recovery times.

INTRODUCTION

Nectrotizing Fasciitis (NF) is often associated with systemic signs of sepsis and septic shock, which arise as a consequence of the release of bacterial toxins and a systemic inflammatory response.

In veterinary medicine, beta-hemolitic Streptococcus is the microorganism which is most frequently involved. When the skin barrier is broken through puncture wounds, micro-organisms reach the subcutaneous tissue and fasciae. Once the subcutaneous space is reached, the tissue is destroyed locally as a consequence of the production of exotoxins and bacterial proteases. The toxins cause necrosis, and necrotic tissue serves as a locus for bacterial proliferation and so forth. The progression can be very quick and septic shock and organ failure can occur within hours.

MATERIALS AND METHODS

The case concerns a 6-year-old, cross-bred, sterilised bitch of medium size, 12 kg in weight. It was admitted to the emergency room with septic shock. The owner reported that the animal had been missing for 24 hours. It presented two puncture wounds in the perianal region, which appeared extremely painful, hot and show local inflammation.

After 24 hours the perineal region, both cranial and distal, became greyish, with necrotic tissue and absence of bleeding, with exudative and odorous fluid in the thickened subcutaneous area Fig.1.

Figure 1 - Necrotic tissue affecting the skin, subcutaneous tissue and fasciae.

Figure 2 - Appearance of the lesions on day 20.

The infectious nature was determined by means of cytology of the exudates, and it was possible to observe degenerated neutrophils and abundant coccoid bacteria arranged in rows. In the analytical study it was possible to observe leucopenia, thrombocytopenia (due to consumption and activation of the coagulation), hypoalbuminemia, hypoglycemia, and elevated liver enzymes. Through the use of ultrasound, fluid and gas were visible between the subcutaneous region and fascial planes, in a fuzzy manner. There were no foreign materials and an abscess was ruled out. On the basis of the physical examination and the outcome of the tests, the clinical diagnosis of NF was made. The patient was admitted to the intensive care unit and provided with treatment for shock in order to hemodynamically stabilize it. Antibiotic coverage was provided with ampicillin 30-50 mg/kg/8h, IV (Ampicillin® 500 mg, Biosano) and amikacin 10 mg/kg/24h, IV (Amikacin® 250 mg, Normon). Pain was controlled with methadone 0.4 mg/kg/4h SC (Metasedin® 10 mg, Esteve) and an infusion of ketamine (Imalgene®, Merial) during the first 24 hours. In less than 24 hours, the subcutaneous tissue and deep fasciae were easily separated and an aggressive surgical debridement of the infected necrotic tissues was carried out. These debridement procedures were repeated 3 times, at 3-day intervals.

After the first surgery the general state of the patient evolved favourably.

In addition to the surgical management, wet dressings were applied with honey, and Balsam Peru and Castor oil (Linitul®, Alfasigma) every three to four days until complete re-epithelialization was achieved.

The treatment protocol included 10 sessions of laser therapy throughout the affected area. Mphi Vet Orange equipment was used with two sources of wavelength. A diode laser that emits at 905 nm wavelength, 1-2000 Hz and a peak power of 25W and another diode laser with a wavelength of 808 nm, which may be continuous (1.1W peak power) or frequenced (1-200 Hz, 550 mW). A duty cycle of 50% was selected. It was used in sweep mode over an area of 150 cm^2 (including 3 cm of the healthy edges) and modifying the total area depending on the surface to be treated. Before the first surgical cleaning, the protocol for acute inflammation (3 sessions: 0, 12 and 36 hours) was used. In the successive sessions, the infected wound protocol was used and subsequently the one for non-infected wound was used. Treatment schedule is reported in Table I. Lesion appearance after 20 days is shown in Fig.2.

Figure 3 - Scar tissue.

RESULTS

After 20 days, laser therapy has been suspended but the patient was still monitored to control lesion healing. Full recovery was attained after 45 days of treatment (Fig.3). The lesion showed gradual and progressive improvement from the first treatment session, thus speeding up the repair of damaged tissues. The infectious process is stopped and grayish and cornered necrotic tissues are removed in the first days. The extensive affected surface soon appears covered by reddish granulation tissue and a bright appearance. The margins of the lesion are contracting at a speed greater than expected and it is also noticeable the regrowth of the hair at the edges of the scar tissue.

DISCUSSION

When the patient was admitted, it was possible to observe two puncture wounds. In the beginning, the lesions observed did not seem to correspond with the gravity of the general state of the patient. NF is an underdiagnosed pathology and the definitive diagnosis is made by means of culture and histopathology. The rapid progression and threat to life of the patient justify the fact that therapeutic decisions are made on the basis of presumptive diagnosis, as was done in this case. The aggressiveness and timeliness of the surgical debridement are the only predictive variables of successful progress [1,2].

Treatment is based on the stabilisation of the patient, pain management, antibiotherapy and cleaning out of the affected tissue. The use of fluoroquinolones is not recommended because they can induce bacteriophage encoders of super-antigen genes that can lead to increased bacterial virulence in these patients. The use of NSAIDS is not recommended either because of the potential negative effect on the immune system, facilitating the spread of infection [3].

In this case report photobiomodulation was used, with excellent results. The speed of wound healing was higher than expected, and it was not necessary to perform reconstructive plastic surgery. Scar tissue was flexible and not painful, consistent with results described in the

literature [4].

The application of laser therapy significantly reduced the length of the first phase of debridement and infection control. The application of the described clinical protocol allowed the tissues to heal faster. Laser can help tissue stimulation, therefore decreasing the time needed for wound healing.

The final result is that due to the application of laser therapy, the necrosis stage is controlled much more quickly and tissue damage is reduced, healing is accelerated and a complete regeneration of tissues is achieved, without the need to resort to reconstructive surgery. In addition, this reduces the time and amount of medication that needs to be administered to the patient, with the consequent benefits for the patient, with a decrease in the emergence of bacterial resistance and savings for the owner.

The author finds that this technique is a useful tool as an adjunct to other treatments, improving the effectiveness of those treatments, with no observed adverse effects, and providing added quality.

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Table I - Treatment schedule.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10	Day 13	Day 16	Day 20	Day 20-40
Lasertherapy MLS®	√ √	√		√		√		√	√	√	√	√
Amikacin/24 h Ampicillin/24 h	√	√	√	√	√	√	√					
MLA Infusion	√											
Carprofen			√	√	√	√	√	√				
Buprenorphine Patch		√			√							
Wet dressings				√		√		√	√	√	√	√

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The aim of "Energy for Health" is to spread the results of research on the application of laser and magnetic field in biology and medicine. The journal will publish studies which involve basic research and clinical trials: laser-tissue interaction, effects of laser and electromagnetic field on cells.
Attention will be focused on studies devoted to explain the molecular and cellular mechanisms at the basis of the effects produced by laser and magnetotherapy.

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Experimental model: cellular, animal, etc.
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"In the case that laser sources are considered, authors are requested to specify all the necessary technical data pertinent to the experiment(s): laser type and wavelength, emission mode (continuous, pulsed), laser power (peak and average power in case of pulsed emission), laser beam dimensions, beam intensity (Watt/cm^2 spot area), total energy dose on the irradiated area in a single treatment (J/cm^2), duty cycle. In case of laser treatment of cultured cell models, as well as in vivo and ex vivo treatments, authors are requested to specify the dimensions of the treated region, treatment duration and timing modalities (e.g. one session, multiple sessions)."
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The discussion should be an interpretation of the results and their significance, also with reference to works by other authors. The relevance of the results in the research and clinical applications should be explained.

CONCLUSIONS

They should be concise and effective, with reference to possible involvements in the future.

ACKNOWLEDGEMENTS

Concise acknowledgements may be addressed to persons, public and private organizations, companies.

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Reference to a book:
2. Michaeli W. Extrusion Dies. Hanser Publishers, Munich, Vienna, New York, 1984.

Reference to a chapter in an edited book:
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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