Volume [07] / 2011

Energy FOR Health

International journal
of information and scientific culture

volume [07]

OFFICIAL REVIEW OF **ASACAMPUS**

Energy for Health

International journal of information and scientific culture

ENERGY FOR HEALTH - n.07/11
Six-monthly scientific journal - Authorized by Court of Vicenza Italy, authorization number 1145/07 - Managing Editor: **Dott.Luigi Corti**
Editor: **ASA srl** Arcugnano (VI) Italy - Design Consultant: **DYN'ART** communication & marketing - Print: **CENTROSTAMPASCHIO** Litografia Schio (VI) Italy

Contents

Key words: measurement bias, decision making processes, measurement uncertainty

Physical grounds for understanding the interaction between electromagnetic fields and biological objects: results, bias and decision making.

A. Conti
ASAcampus Joint Laboratory,
Department of Clinical Physiopathology, University of Florence, Florence, Italy

I) INTRODUCTION

Our first contribution [1] was aimed at giving the basis to understand and discuss the interactions of electromagnetic fields with biological materials.

We focused on disturbance or perturbation, on error and inaccuracy of measurements. We concluded that some inaccuracies cannot be eliminated. We recalled both the deterministic and probabilistic physics concepts, stressing the fact that if we want to understand well the interactions between electromagnetic fields and live matter, we have to use at least molecular biology means, and therefore remember that we enter the quantum physics realm.
We stressed the concept of field in physics. We tried to make clear that the best way is to think about a property given to each point in space. But furthermore it remains to be understood that the electromagnetic field is not a static entity in space or time, but is something that propagates in space.

The final sentence was that "we are now ready to face all the issues to better understand the several possible mechanisms that explain the magnetic field interaction with biological matter, and its positive effects mainly on the human body".

When I approached the second part of my endeavour, I had a second thought. I pondered that I would not be able, as I promised, to face all the mechanisms that explain the magnetic field interactions with the living matter, if I did not take into account that all the results of our measurements, already affected by perturbation and inaccuracy, have to undergo a second step, namely the step of "reading" and "decision making".
My first contribution was mainly concerned with the physical, mainly unavoidable measurement limitations. This second contribution will be concerned with 'bias' and decision making.

II) BIAS AND DECISION MAKING

a) BIAS

The idea of 'measurement bias' evokes the idea of 'malpractice'. We tend to identify the measurement bias with a human error, where the scientist, or simply the person who does the measurement, introduces into the measurement a 'subjective contribution', which in some sense alters the 'objective result' of the measurement itself.
It is quite interesting to notice that the Working Group 1 of the Joint Committee for Guides in Metrology (JCGM/WG 1) in its 'Guide to the expression of uncertainty in measurement' [2], a very technical manual, among the 'many possible sources of uncertainty in a measurement' cites ten possible sources (see appendix), including ' d) inadequate knowledge of the effects of environmental conditions on the measurement or imperfect measurement of environmental conditions;'.
And furthermore the tenth source is ' j) variations in repeated observations of the measurand under apparently identical conditions', which applies very well to the measurement of an effect on biological materials.
And finally the statement that all the ten listed 'sources are not necessarily independent, and some of the first nine sources may contribute to the tenth source'.

Which can be the first conclusion after all our focus in the first contribution [1] on perturbation and inaccuracy of measurements, on deterministic and probabilistic physics concepts ? The first conclusion is the 'human reader factor' plays a very important role. And this fact should not be underestimated: when we think that we have automatized and computerized all the process, using all possible interfaces to avoid human intervention, the human touch is still there, in the design and realization of the interfaces.

b) DECISION MAKING

We are interested in the measurement of the interaction between
electromagnetic fields and biological objects. Therefore our decision is in fact a 'biological' decision, or even better a 'medical' decision.

When people think of medical decisions, especially in the field of diagnosis, our main idea is that medical decision making is a combination of virtuososhiip, statistics, experimentalism and evidence based practice [3]. In a sense all these elements, and not only the element 'experimentalism', are relevant for the determination of the 'goodness' of the experimental results.

The combination of these subjective and objective elements, recently proposed and discussed in a paper of our group, may certainly vary in quantity and quality, but the basic ingredients of this recipe may be considered constant and consistent.

Virtuosoship, or the artistic ingredient of medical decision making, is the oldest one. In the remote past scientific evidence was not available and the creative-technical dimension of the medical profession was the dominant one. We refer to the term "technical" since the western medical tradition beginning with Hippocrates (V-IV centuries B.C.) started, through intuition and observation, to mix theoretical cultural information with practical medical activity, and this combination of knowledge and art ("téchne" in Greek) was appreciated by patients. At that time physicians were highly considered and reputed even if a really scientific background for their daily practice was absent and their therapeutic armamentarium was extremely limited.

Between the eighteenth and the nineteenth centuries the statistical component started to be relevant in the practice of medicine. The study of probability, the Bayesian approach (Thomas Bayes, 1702-1761) and the development of statistical methods (Francis Galton, 1822-1911, and Karl Pearson, 1857-1936, may be mentioned for their work in this field) made the ever relevant observational abilities more structured and systematic. The quantitative definition of biological and human phenomena provided a numerical basis for the epidemiological consideration of facts and events in the nineteenth century; yet, the authority of ancient physicians and theories remained highly significant.

In the eighteen hundreds another great component of the current recipe of medical decision making, the experimental approach, emerged. In particular, it should be mentioned in this methodological context the contribution, in the second half of the nineteenth century, of scientists such as Claude Bernard (1813-1878) to the so-called "experimental reasoning". This French physiologist aimed at understanding and explaining the functions of human bodies and the laws regulating them. The subsequent development of physiology, and pathophysiology too, between the nineteenth and the twentieth centuries, led to the full identification of a "deterministic experimental approach" [3] still constituting a major component in the current combination of decision making in health care.

Last but not least, the "evidence based" approach is here discussed. This is the youngest element proposed, if and when the definition of "Evidence Based Medicine" elaborated by David Sackett is followed. However, Sackett himself acknowledges the role of Pierre Louis (1787-1872), a nineteenth century physician, in trying to integrate the semeiotic approach and the numerical method in the investigation of human biology and clinics. The harmonization of the statistical-epidemiological approach, of the clinical method and of the values and preferences of patients is considered today the characteristic profile of Evidence Based Medicine, a major element in modern medical decision making processes.

Currently the so-called "Narrative Medicine" approach further integrates the here proposed ingredients of the recipe of medical decision making, interestingly and correctly retrieving artistic-relational components in the scientific framework typical of the medicine of the third millennium.

Even more enlightening is the comparison between a so called subjective choice and an objective choice. We summarize here the results and considerations obtained in a second paper of our group, namely the one on decision making processes in sports and in medicine [4]. Intriguingly decision making processes in sports and in medicine may be recognized as containing common elements. As underlined in another section of this paper, in medicine both objective and subjective factors always operate in decision making. In sports, in turn, objective and subjective elements within rules determine referee verdicts. In sports, objective features concern those situations in which external factors allow no margin of doubt regarding the decision itself on the part of the referee, while subjective features involve personal evaluations resulting from subjective interpretations. With regard to the sports arena, modifications in refereeing processes have occurred over the years; in clinical medicine too there appears to be a continually changing situation due to the need for continuous adaptation to the varying principles, attitudes and expectations of human beings. Physicians are, like referees, called upon to make decisions, in their case regarding patients in conditions of different degrees of certainty and in various operational contexts. In the light of these methodological considerations, it appears really important to foresee and elaborate mechanisms of improvement in the two fields of sports and medicine; this appears even more relevant if the

extremely complex nature of "refereeing" itself is evaluated.

Why are these examples relevant in the context of the interaction between electromagnetic fields and biological objects ?
As already explained, both medical decision making as well as game refereeing imply a subjective and an objective part.
And the same is true when a scientist (the person who does a measurement) has to take a decision concerning a single measurement. Is this measurement relevant for the problem I would like to solve? Does it represent an objective measurement, or does it imply an amount of subjectivity, that is unacceptable in this kind of measurement? In a certain way one goes back through the ten possible sources evoked, among the ' many possible sources of uncertainty in a measurement', by the Joint Committee for Guides in Metrology (2, appendix).

III) CONCLUSION

We do not want to leave the reader in a state of complete indetermination. Scope of the paper is not to follow the Greek pre Socratic philosopher Parmenides, for whom in the end nothing changed, and so it was very difficult to make any statement.
On the contrary, we would like to stress the fact that measurements have to be done in order to assess the interaction between electromagnetic fields and biological objects. But the care in the judging of any measurement is never too important. The scientist should not remain paralyzed, because he would never be able to present any result.
He must always assess his results with the greatest possible care, beginning with the design of his experiment up to the final results and to the deductions drawn from the experiment itself.

BIBLIOGRAPHY

1. Conti A: Physical grounds for understanding the interaction between electromagnetic fields and biological objects: inaccuracy and fields. Energy for Health 2010; 6:4-7.
2. JCGM 100:2008 GUM 1995 with minor corrections
Evaluation of measurement data — Guide to the expression of uncertainty in measurement // Évaluation des données de mesure — Guide pour l'expression de l'incertitude de mesure. First edition September 2008. Accessed on February 10th, 2011 at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf
3. Conti AA, Conti A, Gensini GF: Medical decision making as a "historical" combination of art, statistics, experimentalism and evidence based practice. Vesalius. 2010;16:19–23.
4. Conti AA, Gensini GF, Galanti G, Conti A: Decision Making Processes in Sports and in Medicine: Refereeing the Game. Clin J Sport Med 2010; 20 (6):402-404.

APPENDIX

The ten possible sources evoked, among the many possible sources of uncertainty in a measurement, include:
a) incomplete definition of the measurand;
b) imperfect realization of the definition of the measurand;
c) nonrepresentative sampling — the sample measured may not represent the defined measurand;
d) inadequate knowledge of the effects of environmental conditions on the measurement or imperfect measurement of environmental conditions;
e) personal bias in reading analogue instruments;
f) finite instrument resolution or discrimination threshold;
g) inexact values of measurement standards and reference materials;
h) inexact values of constants and other parameters obtained from external sources and used in the data-reduction algorithm;
i) approximations and assumptions incorporated in the measurement method and procedure;
j) variations in repeated observations of the measurand under apparently identical conditions.
These sources are not necessarily independent, and some of sources a) to i) may contribute to source j). Of course, an unrecognized systematic effect cannot be taken into account in the evaluation of the uncertainty of the result of a measurement but contributes to its error. [2]

Key words: low level laser therapy, bone, fractures, osteoblasts.

Low level laser therapy for bone regeneration.

A.C. Rennó and P.S. Bossini
Department of Bioscience, Federal University of São Paulo (UNIFESP), Santos, SP, Brazil.

ABSTRACT
Since 1960, low level laser therapy (LLLT) has been used to stimulate a series of biologic tissues. Some authors have showed, trough experimental and clinics studies, the biostimulatory effects of LLLT on bone both in vivo and in vitro. Although the effects of LLLT have been demonstrated in many studies, the regulatory mechanisms of laser on tissues are poorly understood. Also, it has been postulated that there is an existence of a curve dose-response which means that the use of the appropriate parameters is effective on promoting an acceleration of bone healing. Then, the aim of this study was to show the state of the art with about the osteogenic effects of LLLT on bone cells and fracture consolidation. It was made a review in the databases including MEDLINE, EMBASE, Pubmed and Cochrane and the articles that met the inclusion criteria stated below were selected: papers published until December 2010 including the words "LLLT and bone and fracture", in the title, abstract, or keywords. In all studies, a fracture in tibiae or femur was induced and this injury was irradiated with LLLT. It was observed a wide variety of the parameters of LLLT used in the studies analyzed. Authors used different kinds of laser and different wave lengths, power, doses and time of application. Results obtained showed that LLLT can stimulate osteoblastic proliferation and can accelerate the consolidation of bone fracture. Although, a lot of studies state that LLLT contributes to accelerate the consolidation of bone fracture, further studies are necessary to investigate the effects of LLLT on bone tissue and to determine the best parameters to use.

INTRODUCTION
Laser is an acronym for "Light Amplification by stimulated Emission of Radiation" [1]. The first laser was demonstrated in 1960 and since then, it has been used for surgery, diagnostics and therapeutic medical applications. It is an electromagnetic energy and its physiological effects occur at the cellular level, stimulating or inhibiting biochemical and physiological proliferation activities by altering intercellular communication [2]. The action of LLLT is based on the absorption of the light by tissues, which will generate a series of modifications in cell metabolism. When the LLLT is applied to tissue, the light is absorbed by photoreceptors located in the cells, called chomophores. Once absorbed, the light can modulate cell chemical reactions and stimulate mitochondrial respiration, the production of molecular oxygen and ATP synthesis [3]. These effects can increase the synthesis of DNA, RNA and cell-cycle regulatory proteins, stimulating cell proliferation [4-6].
A significant body of evidence has now accumulated demonstrating that low level laser therapy (LLLT) is effective in reducing post-injury inflammatory processes, accelerating soft tissue healing and stimulating the formation of new blood vessels [4,5].
Since the decade of 70, some authors investigated the osteogenic potential of low level laser therapy (LLLT) and its use on healing of different connective tissues, including bone [7]. In 1971, a short report by Chekurov stated that laser was an effective modality in bone healing acceleration. Subsequently, other researchers studied the effects of LLLT osteoblast cell proliferation and bone healing after laser irradiation using histological, histochemical and radiographic measures [6, 8-10].
Many in vitro studies using osteoblastic cells showed that LLLT is capable of increasing mitochondrial activity [11,12], osteoblast DNA and RNA synthesis, bone nodule formation, osteocalcin and osteopontin gene expression and ALP activity, increasing osteoblast proliferation [13]. Ozawa et al (1998) [14] found a significant increase in the DNA synthesis in osteoblast cells after 830 nm laser irradiation. In a study investigating the effects of different dosages and wavelengths on osteoblast cells, our group observed that there was an increase in osteoblast proliferation and phosphatase activity after the irradiation of 830nm laser [6]. Kiyosaki et al (2010) [15] examined the effects of LLLT on osteoblasts via insulin-like growth factor I (IGF-I) signal transduction. The authors observed that laser therapy significantly increased the expression of IGF-I, Runx2 and calcium content in the mineralized nodules. Also, Aleksic et al (2010) [16] observed that low-level Er:YAG laser irradiation produced a significantly higher proliferation in laser-irradiated MC3T3-E1 cells at a fluence of 1 J/cm2, through the phosphorylation of extracellular signal-regulated protein kinase (MAPK/ERK).
Pires-Oliveira et al (2008) [17] observed that 830nm gallium-aluminium-arsenide diode laser (50 mW, 3 J/cm2) produced an intense grouping of mitochondria in the perinuclear region in osteoblasts cells, which culminated on the increase of cell proliferation. Xu et al (2009) [18] demonstrated that laser irradiation of 1.14 J/cm2, produced an increase of osteoblasts, stimulated alkaline phosphatase activity and indirectly inhibit osteoclast differentiation, by downregulating the RANKL:OPG mRNA ratio in osteoblasts. These authors suggest

that lasertherapy may play an important role in bone remodeling and should be valuable for the treatment of bone diseases such as osteoporosis. Stein et al (2008) [13] investigated the initial effect of low-level laser therapy on growth and differentiation of human osteoblast-like cells. SaOS-2 cells were irradiated with 670 nm laser. Over the observation period, cell viability, alkaline phosphatase activity and the expression of osteopontin and collagen type I mRNA were slightly enhanced in the irradiated cells compared with untreated control cells. Simizu et al (2007) [19] observed that osteoblast-like cells irradiated with a low-intensity Ga-Al-As laser (830 nm) presented a higher concentration of rIGF-I and area of bone nodules.

Also, many authors demonstrated that LLLT stimulates neoangiogenes at the site of fracture, increase collagen fiber deposition and promote higher bone cell proliferation, accelerating callus formation and fracture healing. Tajali et al (2010) [2] in a meta-analysis, stated that several studies in the literature indicates that LLLT can enhance biomechanical properties of bone during fracture healing in animal models.

However, LLLT needs better parameterization of variables to obtain the most appropriate stimulus, because many of the actual effects and limitations are not yet entirely clear and there is much controversy about its mechanism of action on tissues [6]. Moreover, many studies have produced no scientific validity for the low reliability of data because of methodological problems [20, 21]. However, excellent studies open this field, which in future will lead the individual with bone injuries to a faster return to their normal functions [22], avoiding the consequences of a prolonged immobilization, such as muscle mass loss and decrease in bone mineral density.

METHODS

A systematic search of four electronic databases including MEDLINE, EMBASE, Pubmed and Cochrane was performed and all the articles that met the inclusion criteria stated below were selected: papers published until December 2010 including the words "LLLT and bone and fracture", in the title, abstract, or keywords. Thus, ten studies were obtained, ranging between the years 2003 to 2010. In all studies, the authors produced bone defects in rats or rabbits and this injury was irradiated with LLLT.

LLLT IN OSTEOGENESIS

Several authors have demonstrated in experimental studies an acceleration of bone repair by the use of LLLT [23-25]. Mester et al. (1985) [26], suggest that the metabolic pathways are responsible for this healing effect, mainly due to the increased bioavailability of chemical energy (ATP) in cells.

Garavello-Freitas et al (2003) [23] examined the influence of daily energy doses of 0.03, 0.3 and 0.9 J of He-Ne laser irradiation on the repair of surgically produced tibia damage in Wistar rats. Laser treatment was initiated 24 h after the trauma and continued daily for 7 or 14 days. After 7 days, there was a significant increase in the area of neoformed trabeculae in tibiae irradiated with 0.3 and 0.9 J compared to the controls. At a daily dose of 0.9 J (15 min of irradiation per day) the 7-day group showed a significant increase in trabecular bone growth compared to the 14-day group. The Picrosirius-polarization method revealed bands of parallel collagen fibers (parallel-fibered bone) at the repair site of 14-day-irradiated tibiae, regardless of the dose. This organization improved when compared to 7-day-irradiated tibiae and control tibiae. These results show that low-level laser therapy stimulated the growth of the trabecular area and the concomitant invasion of osteoclasts during the first week, and hastened the organization of matrix collagen (parallel alignment of the fibers) in a second phase not seen in control, non-irradiated tibiae at the same period.

Pretel et al (2007) [8] evaluated bone repair in defects created in rat lower jaws after stimulation with infrared LLLT directly on the injured tissue. Bone defects were prepared on the mandibles of 30 rats allocated in two groups which were divided in three evaluation periods (15, 45, and 60 days), with five animals each: control group-no treatment of the defect; laser group-single laser irradiation with a GaAlAs (780nm, 35 mW; 178 J/cm²) directly on the defect area. The histological results showed bone formation in both groups. However, the laser group exhibited an advanced tissue response compared to the control group, abbreviating the initial inflammatory reaction and promoting rapid new bone matrix formation at 15 and 45 days. On the other hand, there were no significant differences between the groups at 60 days. The use of infrared LLLT directly to the injured tissue showed a biostimulating effect on bone remodeling by stimulating the modulation of the initial inflammatory response and anticipating the resolution to normal conditions at the earlier periods.

Liu et al (2007) [27] investigated the biological effects of LLLT on tibial fractures using radiographic, histological, and bone density examinations. Fourteen rabbits with surgically induced mid-tibial osteotomies were included in the study. Seven were assigned to a group receiving LLLT (LLLT-A) and the remaining seven served as a sham-treated control group (LLLT-C). A low-energy laser apparatus with a wavelength of 830 nm, and a sham laser (a similar design without laser diodes) were used for the study. Continuous outflow irradiation with a total energy density of 40 J/cm² and a power level of 200 mW/cm² was directly delivered to the skin for 50 seconds at four points along the tibial fracture site. Treatment commenced immediately post-surgery and continued once daily for 4 weeks. The results demonstrated that LLLT may accelerate the process of fracture repair or cause increases in callus volume and bone mineral density, especially in the early stages of absorbing the hematoma and bone remodeling.

Lirani-Galvão et al (2006) [28] compared the effects of LLLT and low-intensity pulsed ultrasound (LIPUS) on bone repair in rats. One group had the osteotomized limb treated with LLLT (GaAlAs laser, 780 nm, 30 mW, 112.5 J/cm²) and the second group with LIPUS (1.5 MHz, 30 mW/cm2, both for 12 sessions (five times

per week); a third group was the control. In the bending test, maximum load at failure of LLLT group was significantly higher. Bone histomorphometry revealed a significant increase in osteoblast number and surface, and osteoid volume in the LLLT group, and a significant increase in eroded and osteoclast surfaces in the LIPUS group. LIPUS enhanced bone repair by promoting bone resorption in the osteotomy area, while LLLT accelerated this process through bone formation.

Ribeiro and Matsumoto (2008) [29] studied the action of anti-COX-2 selective drug (celecoxib) on bone repair associated with laser therapy (735nm, 16J/cm²). A total of 64 rats underwent surgical bone defects in their tibias, being randomly distributed into four groups: negative control, animals treated with celecoxib, animals treated with LLLT and animals treated with celecoxib and LLLT. The animals were killed after 48 h, 7, 14 and 21 days. Statistical significant differences were observed in the quality of bone repair and quantity of formed bone between groups at 14 days after surgery for irradiated animals. COX-2 immunoreactivity was more intense in bone cells for intermediate periods evaluated in the laser-exposed groups. Taken together, such results suggest that low-level laser therapy is able to improve bone repair in the tibia of rats as a result of an up-regulation for cyclooxygenase-2 expression in bone cells.

Blaya et al. (2008) [24] evaluated the laser biomodulation of bone repair in cavities made in the femurs of rats. In Group I the complete surgical protocol to produce a bone defect was followed but without laser radiation (control). In Group II a continuous wave 830 nm infrared laser was used at 10 J/cm2 and 50 mW at each point of the surgical site. In Group III a continuous wave 685 nm infrared laser at 10J/cm2 and 35 mW was used at each point of surgical site. The animals were irradiated at intervals of 48 hours beginning immediately after the preparation of the defect and were sacrificed on the 15th, 21st, and 30th days. Greater degrees of new bone formation and vertical regeneration were found in the irradiated groups than in the control group. The authors concluded that laser therapy in this study protocol was efficient in promoting bone repair.

Javadieh et al (2009) [30] examined the effects of LLLT on a bone defect model in streptozotocin-induced diabetic rats. Twenty-eight rats were divided into five groups: 1 (diabetes, no LLLT), 2 (diabetes, LLLT high dose), 3 (diabetes, LLLT low dose), 4 (no diabetes, no LLLT), and 5 (no diabetes, LLLT low dose). A bone defect was made in the right tibia of rats in all groups. The defect in groups 2, 3, and 5 was treated with LLLT (890 nm, 70 W, 3000 Hz). Doses of 23.3 J/cm² for group 2 and 11.6 J/cm² for groups 3 and 5 were applied three times a week. The authors showed that LLLT with 11.6 J/cm² significantly increased bending stiffness and maximum force in diabetic rats compared with group 1.

Our group, in a study investigating the effects of LLLT during the process of bone healing, demonstrated that lasertherapy had a positive effect on bone consolidation. We used a 830nm laser, at 50J/cm2, during 7, 13 and 25 days to treat tibial bone defects in rats. The results pointed out intense new bone formation surrounded by highly vascularized connective tissue presenting a slight osteogenic activity, primary bone deposition was observed in the group exposed to laser in the intermediary (13 days) and late stages of repair (25 days). This was confirmed by morphometric analysis, in which statistically significant differences ($p<0.05$) were noticed when compared to control. Taken together, our results indicate that laser therapy improves bone repair in rats as depicted by histopathological and morphometric analysis, mainly at the late stages of recovery [31a].

Also, Favaro-Pipi et al (2010) [32] showed that 830nm laser (50 W/cm2, 50 J/cm², 30 mW) produced an increase in the expression of genes related to bone differentiation. The authors showed that laser irradiation produced an upregulation of BMP4, ALP and Runx 2 on day 25 after surgery, stating that laser therapy improves bone repair in rats as depicted by differential histopathological and osteogenic genes expression.

Pires-Oliveira et al (2010) [25] investigated the action of AsGA laser irradiation (904nm, 50mW, 50mJ/cm²) on bone repair in the tibia of osteopenic rats. The animals were randomly divided into eight experimental groups according to the presence of ovarian hormone (sham group) or the absence of the hormone (ovariectomy group), as well as being irradiated or non-irradiated. Low-level 904-nm laser accelerated the repair process of osteopenic fractures, especially in the initial phase of bone regeneration.

The same results were found by Kazem Shakouri et al (2010) [22] in a study showing an increased rate of bone mineral density and higher biomechanical properties in rabbits after laser irradiation (780nm, 4J/cm²). These authors stated that the use of laser could enhance callus development in the early stage of healing process and it should be recommended as an additional treatment in non-union fractures in humans.

It can be concluded that low level laser therapy acts as a proliferative stimulus on osteoblast cells and may accelerate bone metabolism and fracture healing. However, the mechanism by which LLLT acts on bone tissue is not fully understood [6]. Thus, there is a clear clinical need to understand the molecular details of the pathways that control bone formation after laser irradiation, which might allow to accelerate the healing of fractures and to treat the 5%–10% of fractures that fail to heal satisfactorily.

CONCLUSION

Many studies have demonstrated the positive effects of LLLT on bone metabolism, mainly in producing an increase in bone formation and accelerating fracture repair. Although the osteogenic effects of LLLT, there is no established protocol and there is a wide range of doses used by different authors, which are difficult to compare published results. Therefore, before LLLT can be used with confidence as a treatment within the clinical, it is necessary to investigate the mechanisms of action of this therapy to determine its safety and efficacy.

REFERENCES

1. Nissan J, Assif D, Gross MD, Yaffe A, Binderman I. Effect of low intensity laser irradiation on surgically created bony defects in rats. J Oral Rehabil., 2006, 33: 619-924.
2. Tajali SB, MacDermid JC, Houghton P, Grewal R. Effects of low power laser irradiation on bone healing in animals: a meta-analysis. J. Orthop. Surg. Res., 2010, 5: 1-10.
3. Stein A, Benayahu D, Maltz L, Oron U. Low-level laser irradiation promotes prolieration and differentiation of human osteoblasts in vitro. Photomed. Laser Surg., 2005, 23: 161-166.
4. Karu TI, Lubart R. Effects of low-power light on biological systems V. Amsterdam, Netherlands, 2000, Proceedings of SPIE, 01-17.
5. Dortbudak O. Biostimulation of bone marrow cells with a diode soft laser. Clin. Oral Implants Res., 2000, 16: 540-545.
6. Renno AC, McDonnell PA, Parizotto NA, Laakso El. The effects of laser irradiation on osteoblast and osteosarcoma cell proliferation and differentiation in vitro. Photomed. Laser Surg., 2007, 25: 275-280.
7. Yamada K. Biological effects of low power laser irradiation on clonal osteoblastic cells (MC3T3-E1). J. Japan Orthop. Assoc., 1991, 65: 101-114.
8. Pretel H, Lizarelli RF, Ramalho LT. Effect of low-level laser therapy on bone repair: histological study in rats. Lasers Surg. Med., 2007; 39: 788-796.
9. Coombe AR, Ho C, Philips JR, Chapple C. The effects of low level laser irradiation on osteoblastic cells. Clin. Orthod. Res., 2001, 4: 3-14.
10. Tamura K, Hosoya S, Hiratsuka K, Abiko, Y. Enhancement of mouse CDC46 gene expression in the osteoblast by laser irradiation. Laser Ther., 1998, 10: 25-32.
11. Trelles MA, Mayayo E. Bone fracture consolidate faster with low power laser. Lasers Surg. Med., 1987, 7: 36-45.
12. Hamajima S, Hiratsuka K, Tagawa T. Effect of low laser irradiation on osteoglycin gene expression in osteoblasts. Lasers Med. Sci., 2003, 18: 78-82.
13. Stein E, Koehn J, Sutter W, Wendtlandt G, Wanschitz F, Thurnher D, Baghestanian M, Turhani D. Initial effects of low-level laser therapy on growth an differentiation of human osteoblast-like cells. Wien Klin Wochenschr. 2008, 120: 112-117.
14. Ozawa Y, Shimizo N, Kariya G. Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells. Bone, 1998, 22: 347-354.
15. Kiyosaki T, Mitsui N, Suzuki N, Shimizu N. Low-level laser therapy stimulates mineralization via increased Runx2 expression and ERK phosphorylation in osteoblasts. Photomed. Laser Surg., 2010, Suppl 1: S167-72.
16. Aleksic V, Aoki A, Iwasaki K, Takasaki AA, Wang CY, Abiko Y, Ishikawa I, Izumi Y. Low-level Er:YAG laser irradiation enhances osteoblast proliferation through activation of MAPK/ERK. Lasers Med. Sci., 2010, 25: 559-69.
17. Pires Oliveira DA, de Oliveira RF, Zangaro RA, Soares CP. Evaluation of low-level laser therapy of osteoblastic cells. Photomed. Laser Surg., 2008, 26: 401-404.
18. Xu M, Deng T, Mo F, Deng B, Lam W, Deng P, Zhang X, Liu S. Low-intensity pulsed laser irradiation affects RANKL and OPG mRNA expression in rat calvarial cells. Photomed. Laser Surg., 2009, 2: 309-315.
19. Shimizu N, Mayahara K, Kiyosaki T, Yamaguchi A, Ozawa Y, Abiko Y. Low-intensity laser irradiation stimulates bone nodule formation via insulin-like growth factor-I expression in rat calvarial cells. Lasers Surg. Med., 2007, 39: 551-559.
20. Baxter GD. Therapeutic lasers: theory and practice. United States of America: Ed. Churchill Livingstone, 1-19, 1997.
21. Bossini PS, Fangel R, Habenschus RM, Renno ACM, Benze B, Zuanon JA, Neto CB, Parizotto NA. Low-level laser therapy (670nm) on viability of random skin flap in rats. Lasers Med. Sci., 2009, 24: 209-213.
22. Kazem Shakouri S, Soleimanpour J, Salekzamani Y, Oskuie MR. Effect of low-level laser therapy on the fracture healing process. Lasers Med. Sci., 2010, 25: 73-77.
23. Garavello-Freitas I, Baranauskas V, Joazeiro PP, Padovani CR, Dal Pai-Silva M, Cruz-Höfling MA. Low-power laser irradiation improves histomorphometrical parameters and bone matrix organization during tibia wound healing in rats. J. Photochem. Photobiol. B, 2003, 70: 81-89.
24. Blaya DS, Guimarães MB, Pozza DH, Weber JB, de Oliveira MG. Histologic study of the effect of laser therapy on bone repair. J. Contemp. Dent. Pract., 2008, 9: 1-8.
25. Pires-Oliveira DA, Oliveira RF, Amadei SU, Pacheco-Soares C, Rocha RF. Laser 904 nm action on bone repair in rats with osteoporosis. Osteoporosis Int., 2010, 21: 2109-2114.
26. Mester E, Mester AF, Mester A. The biomedical effects of laser application. Lasers Surg. Med, 1985, 5: 31-39.
27. Liu X, Lyon R, Meier HT, Thometz J, Haworth ST. Effect of lower-level laser therapy on rabbit tibial fracture. Photomed. Laser Surg., 2007, 25: 487-494.
28. Lirani-Galvão AP, Jorgetti V, da Silva OL. Comparative study of how low-level laser therapy and low-intensity pulsed ultrasound affect bone repair in rats. Photomed. Laser Surg., 2006, 24: 735-740.
29. Ribeiro DA, Matsumoto MA. Low-level laser therapy improves bone repair in rats treated with anti-inflammatory drugs. J. Oral Rehabil., 2008, 35: 925-933.
30. Javadieh F, Bayat M, Abdi S, Mohsenifar Z, Razi S. The effects of infrared low-level laser therapy on healing of partial osteotomy of tibia in streptozotocin-induced diabetic rats. Photomed. Laser Surg., 2009, 27: 641-646.
31. Fávaro-Pípi E, Feitosa SM, Ribeiro DA, Bossini P, Oliveira P, Parizotto NA, Renno AC. Comparative study of the effects of low-intensity pulsed ultrasound and low-level laser therapy on bone defects in tibias of rats. Lasers Med. Sci., 2010, 25: 727-732.
32. Fávaro-Pípi E, Feitosa SM, Ribeiro DA, Bossini P, Oliveira P, Parizotto NA, Renno AC Low-Level Laser Therapy Induces Differential Expression of Osteogenic Genes During Bone Repair in Rats. Photomed Laser Surg. 2011 [Epub ahead of print].

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

Effects of MLS laser on myoblast cell line C2C12.

L. Vignali, F. Cialdai and M. Monici.
ASAcampus, ASA Res. Div., Dept. Clinical Physiopathology, University of Florence, Florence, Italy.

ABSTRACT

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

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

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

MATERIAL AND METHODS

Cell Cultures

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

MLS Treatment

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

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

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

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

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

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

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

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

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

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

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

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

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

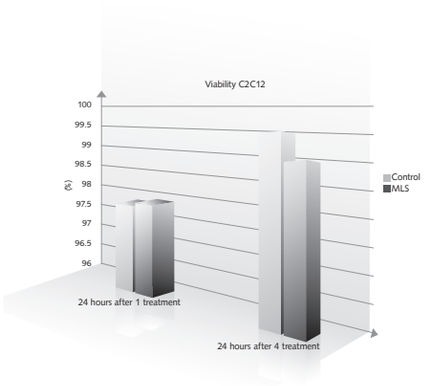


Fig.1. C2C12 Cell viability assessed 24 h after MLS treatment and 24 h after the fourth MLS treatments. (Control vs. MLS). Data were obtained by Trypan Blue assay.

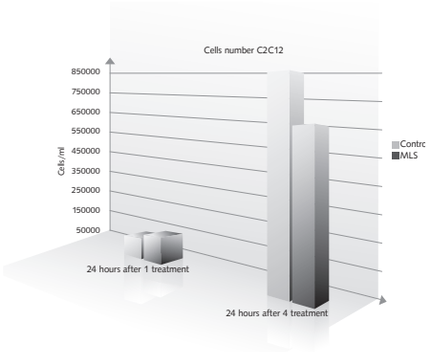


Fig. 2. C2C12 Cell proliferation assessed 24 h after MLS treatment and 24 h after the fourth MLS treatment. (Control vs. MLS). Data were obtained by Trypan Blue assay.

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

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

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

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

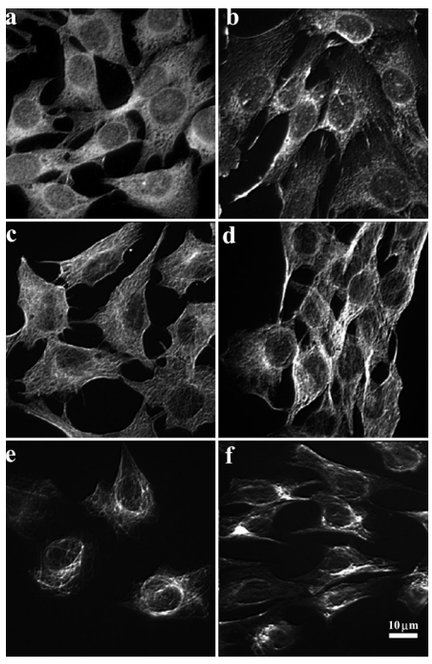


Fig. 3. Expression of cytoskeleton components assessed by immunofluorescence microscopy. Actin expression in control (a) and cells exposed to MLS treatment (b). Tubulin expression in control (c) and cells exposed to MLS treatment (d). Vimentin expression in control (e) and cells exposed to MLS treatment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

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

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

Extracellular matrix

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

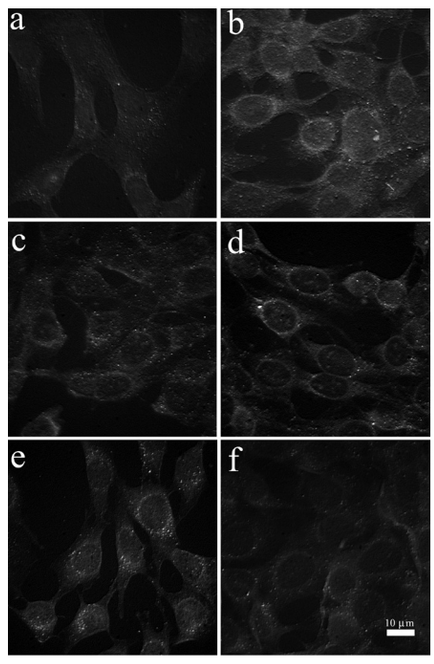


Fig. 4. Expression of extracellular matrix components assessed by immunofluorescence microscopy. Collagen I expression in control (a) and cells exposed to MLS treatment (b). MMP-2 expression in control (c) and cells exposed to MLS treatment (d). MMP-9 expression in control (e) and cells exposed to MLS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

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

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

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

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

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

Differentiation markers

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

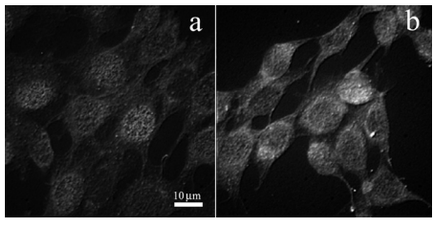


Fig. 5. MyoD expression assessed by immunofluorescence microscopy. Control (a) and cells exposed to MLS treatments (b).

DISCUSSION

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

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

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

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

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

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

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

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

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

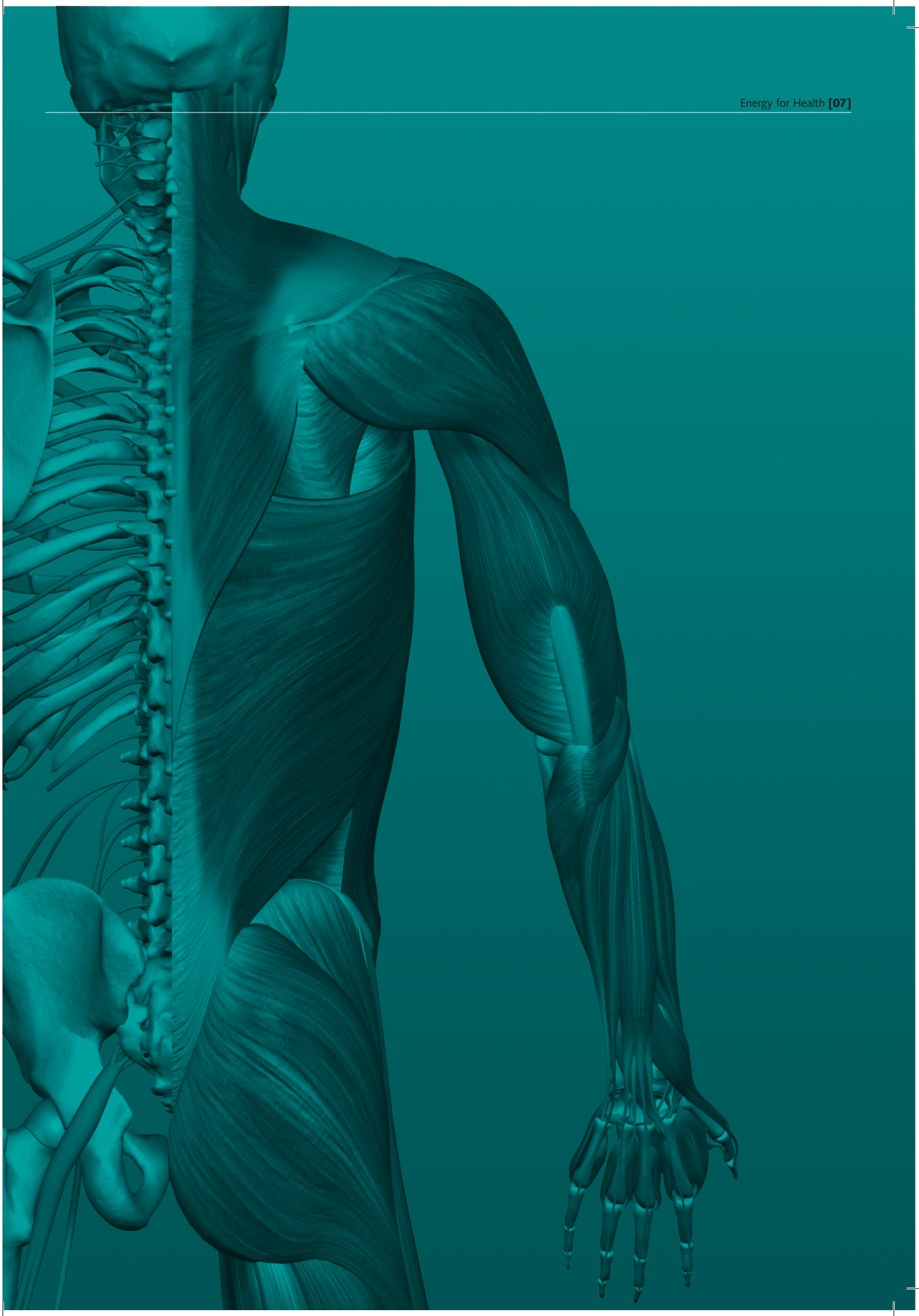
In conclusion, the results we obtained on cell viability and proliferation, structural changes of the cytoskeleton, MyoD, collagen I, MMP-2 and MMP-9 expression demonstrate that MLS treatment does not affect myoblast viability but can affect migration, differentiation and production of ECM molecules.

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

REFERENCES

1. Nakano J, Kataoka H, Sakamoto J, Origuchi T, Okita M, Yoshimura T. Low-level laser irradiation promoters the recovery of atrophied gastrocnemius skeletal muscle in rats. Exp Physiol, 2009, 94(9):1005-1015.
2. Hudlicka O, Brown M, Egginton S. Angiogenesis in skeletal and cardiac muscle. Physiol Rev.,1992, 72(2):369-417.
3. Deveci D, Marshall JM, Egginton S. Muscle ischaemia in rats may be relieved by overload-induced angiogenesis. Exp Physiol, 2002, 87:479-488.
4. Almeida-Lopes L, Rigau J, Zangaro RA, Guidugli-Neto J, Jaeger MM. Comparison of the low lever laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg Med, 2001, 29(2):179-84.
5. Liu H, Dang Y, Wang Z, Chai X, Ren Q. Laser induced collagen remodeling: a comparative study in vivo on mouse model. Laser Surg Med, 2008, 40(1):13-9.
6. Kovács IB, Mester E, Görög P. Stimulation of wound healing with laser beam in the rat. Experentia, 1974, 30:1275-1276.
7. Mochizuki-Oda N, Kataoka Y, Cui Y, Yamada H, Heya M, Awazu K. Effects of near-infrared laser on adenosine triphosphate and adenosine diphosphate contents of rat brain tissue. Neurosci Lett, 2002, 323(3):207-10.
8. Kujawa J, Zavodnik L, Zavodnik I, Buko V, Lapshyna A, Bryszewska M. Effect of low-intensity (3.75-25 J/cm2) near-infrared (810 nm) laser radiation on red blood cell ATPase activites and membrane structure. J Clin Laser Med Surg, 2004, 22(2):111-7.
9. Oron U, Ilic S, De Taboada L, Streeter J. Ga-As (808 nm) laser irradiation enhances ATP production in human neuronal cells in culture. Photomed Laser Surg, 2007, 25(3):180-2.
10. Passerella S, Casamassima E, Molinari S, Pastore D, Quagliarello E, Catalano IM, Cingolani A. Increase of proton electochimical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by helium-neon laser. FEBS Lett, 1984, 175:95-99.
11. Karu TI. Low intensity laser light action upon fibroblasts and lymphocytes. In: Ohshiro T, Calderhead RG, eds. Progress in laser therapy. Selected papers from the October 1990 ILTA congress. Wiley, West Sussex, England, 1991, 175-179.
12. Moore P, Ridgway TD, Higbee RG, Howard EW, Lucroy MD. Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation in vitro. Laser Surg Med, 2005, 36(1):8-12.
13. Chen CH, Hung HS, Hsu SH. Low-energy laser irradiation increases endothelial cell proliferation, migration, and eNOS gene expression possibly via P13K pathway. Lasers Sung Med, 2008, 40(1):46-54.
14. Rochkind S, Nissan M, Alon M, Shamir M, Salame K. Effects of laser irradiation on the spina cord for the regeneration of crusched peripheral nerve in rats. Laser Surg Med, 2001, 28(3):216-219.
15. Rochkind S, Ouaknine GE. New trend in neuroscience: low-power laser effect on peripheral and central nervous system (basic science, preclinical studies). Neurol Res, 1992, 14:2-11.
16. Gigo-Bennato D, Geuna S, Castro Rodrigues A, Fornaro M, Boux E, Battiston B, Tos P. Low power laser biostimulation enhances nerve repair after end-to-side neurorrhaphy: a double-blind randomized study in the rat median nerve model. Lasers in Medical Science, 2004, 19(1):57-65
17. Rochkind S, Leider-trejo L, Nissan M, Shamir MH, Kharenko O, Alon M. Efficacy of 780 nm Laser Phototherapy on Peripheral Nerve Regeneration after Neurotube Reconstruction Procedure (Double-Blind Randomized Study). Photomed Laser Surg, 2007, 25(3): 137-143. Doi 10. 1089/pho.2007.2076.
18. Conlan MJ, Rapley JW, Cobb CM. Biostimulation of Wound Healing by Low-energy laser irradiation. A review. J Clin Periodontol, 1996, 23:492-496.
19. Yaakobi T, Maltz L, Oron U. Promotion of bone repair in the cortical bone of the tibia in rats by low energy laser (He-Ne) irradiation. Calcif Tissue Int., 1996, 59(4):297-300.
20. Oron U, Yaakobu T, Oron A, Mordechovitz D, Shofti R, Hayam G, Dror U, Gepstein L, Wolf T, Haudenschild C, Ben Haim S. Low energy laser irradiation reduces formation of scar tissue following myocardial infraction in dogs. Circulation, 2001, 103
21. Baptista J, Martins MD, Pavesi VC, Bussadori SK, Fernandes KP, Júnior DD, Ferrari RA. Influence of Laser Photobiomodulation on Collagen IV During Skeletal Muscle Tissue Remodeling After Injury in Rats. Photomed Laser Surg., 2011, 29(1):11-17.
22. Liu XG, Zhou YJ, Liu TCY, Yuan JQ. Effects of Low-Level Laser Irradiation on Rat Skeletal Muscle Injury after Eccentric Exercise. Photomed Laser Surg, 2009, 27(6): 863–869 DOI: 10.1089=pho.2008.2443.
23. Weiss N, Oron U. Enhancement of muscle regeneration in the rat gastrocnemius muscle by low energy laser irradiation. Anat Embryol (Berl), 1992, 186:497-503.
24. Bibikova A, Belkin V, Oron U. Enhancement of angiogenesis in re gene rating gastrocnemius muscle of the toad (Bufo viridis) by low-energy laser irradiation. Anat Embryol (Berl), 1994, q90:597-602.
25. Amaral AC, Parizotto NA, Salvini TF. Dose-dependecy of low-energy HeNe laser effectin regeneration of skeletal muscle in mice. Laser Med Sci, 2001, 16:44-451.
26. Bischoff R. A satellite cell mitogen from crushed adult muscle. Dev Biol, 1986, 115:140-147.
27. Bischoff R, Heintz C. Enhancement of skeletal muscle regeneration. Dev Dyn, 1994, 201:41-54.
28. Kawiak J, Brzóska E, Grabowska I, Hoser G, Stremi□ska W, Wasilewska D, Machaj EK, Pojda Z, Moraczewski J. Contribution of stem cells to skeletal muscle regeneration. Folia Histochem Cytobiol., 2006, 44(2):75-9.
29. Huang YY, Chen AC, Carroll JD, Hamblin MR. Biphasic dose response in low level

light therapy. Dose Response, 2009, 7(4):358-83.
30. Karu TI, Kolyakov SF. Exact action spectra for cellular responses relevant to phototherapy. Photomed. Laser Surg, 2005, 23:355-361.
31. Wong-Riley MT, Liang HL, Eells JT, Chance B, Henry MM, Buchmann E, Kane M,Whelan HT. Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: role of cytochrome c oxidase. J Biol Chem.,2005, 280(6):4761-71. Epub 2004 Nov 22.
32. Beauvoit B, Evans SM, Jenkins TW, Miller EE, Chance B. Correlation between the light scattering and the mitochondrial content of normal tissues and transplantable rodent tumors. Anal Biochem., 1995, 226(1):167-74.
33. Silveira PC, Silva LA, Fraga DB, Freitas TP, Streck EL, Pinho R. Evaluation of mitochondrial respiratory chain activity in muscle healing by low-level laser therapy. J Photochem Photobiol B, 2009, 95(2):89-92. Epub 2009 Jan 21.
34. Small J, Rottner K, Hahne P, Anderson KI. Visualising the actin cytoskeleton. Microsc Res Tech, 1999, 47(1):3-17.
35. Skall O, et al. Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericyte. The Histochemical Society, 1989, 37(3):315-321.
36. Carson DD. Extracellular matrix: forum introduction. Reproductive Biology and Endocrinology, 2004, Jan 7;2:1.
37. Chiquet M, Sarasa-Renedo A, Huber F, Flück M. How do fibroblast translate mechanical signals into changes in extracellular matrix production ? Matrix Biology, 2003, 22:73-80
38. Fraser RD, MacRae TP, Suzuki E. Chain conformation in the collagen molecule. J Mol Biol.,1979, Apr 15;129(3):463-81.
39. Rossert J, Terraz C, Dupont S. Regulation of type I collagen genes expression. Nephrol Dial Transplant.,2000, 15 Suppl 6:66-8.
40. Rutges JP, Nikkels PG, Oner FC, Ottink KD, Verbout AJ, Castelein RJ, Creemers LB, Dhert WJ. The presence of extracellular matrix degrading metalloproteinases during fetal development of the intervertebral disc. Eur Spine J., 2010, Aug;19(8):1340-6. Epub 2010 Apr 10.
41. Löffek S, Schilling O, Franzke CW. Biological role of matrix metalloproteinases: a critical balance. Eur Respir J., 2010, 22.
42. Abeles AM, Pillinger MH.The role of the synovial fibroblast in rheumatoid arthritis: cartilage destruction and the regulation of matrix metalloproteinases. Bull NYU Hosp Jt Dis. 2006, 64(1-2):20-4.
43. Puri PL, Iezzi S, Stiegler P, Chen TT, Schiltz RL, Muscat GE, Giordano A, Kedes L, Wang JY, Sartorelli V. Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. Mol Cell., 2001, Oct;8(4):885-97.
44. Pessina P, Conti V, Pacelli F, Rosa F, Doglietto GB, Brunelli S, Bossola M. Skeletal muscle of gastric cancer patients expresses genes involved in muscle regeneration. Oncol Rep., 2010, Sep;24(3):741-5.
45. Ferreira MP, Ferrari RA, Gravalos ED, Martins MD, Bussadori SK, Gonzalez DA, Fernandes KP. Effect of Low-Energy Gallium-Aluminum-Arsenide and Aluminium Gallium Indium Phosphide Laser Irradiation on the Viability of C2C12 Myoblasts in a Muscle Injury Model. Photomed Laser Surg, 2009, 27(6):901–906. DOI: 10.1089=pho.2008.2427.
46. Peplow PV, Chung TY, Baxter GD. Laser Photobiomodulation of Proliferation of Cells in Culture: A Review of Human and Animal Studies. Photomedicine and Laser Surgery, 2010, 28(1):S3–S40 DOI: 10.1089/pho.2010.2771.
47. Penn BH, Bergstrom DA, Dilworth FJ et al. A MyoD generated feed-forward Cicuit temporally patterns gene expression during skeletal muscle differentiation. Genes Dev., 2004, 18:2348-2353.
48. Monici M, Basile V, Cialdai F, Romano G, Fusi F, Conti A. Irradiation by pulsed Nd:YAG laser induces the production of extracellular matrix molecules by cells of the connective tissues. A tool for tissue repair. In: "Biophotonics: Photonic solutions for Better Health Care", J. Popp, W. Drexler, V.V. Turchin and D.L. Matthews Eds., Proc. of SPIE vol. 6991, 69912K1-10, 2008 doi: 10.1117/12.782865.
49. Ricci R, Pazos MC, Eller Borges R, Pacheco-Soares C. Biomodulation with low-level laser radiation induces changes in endothelial cell actin filaments and cytoskeletal organization. Journal of Photochemistry and Photobiology B: Biology, 2009, 95:6–8.
50. Khaitlina SY. Functional specificity of actin isoforms. Int Rev Cytol, 2001, 202:35-98.
51. Komati H, Naro F, Mebarek S, De Arcangelis V, Adamo S, Lagarde M, Prigent AF, Némoz G. Phospholipase D Is Involved in Myogenic Differentiation through Remodeling of Actin Cytoskeleton. Mol Biol Cell, 2005,16(3): 1232–1244.
52. Yong-Deok K, Seong-Sik K, Seok-Jun K., Dae-Woo K, Eun-Suk J, Woo-Sung S. Low-level laser irradiation facilitates fibronectin and collagen type I turnover during tooth movement in rats. Lasers Med Sci, 2010, 25:25–31. DOI 10.1007/s10103-008-0585-8.
53. Lewis MP, Tippett HL, Sinanan ACM, Morgan MJ, Hunt NP. Gelatinase-B (Matrix Metalloproteinase-9; MMP-9) secretion is involved in the migratory phase of human and murine muscle cell cultures. Journal of Muscle Research and Cell Motility, 2000, (21)3: 223-233.



Key words: wound healing, pressure ulcers, decubitus ulcers, MLS laser therapy, open wound healing, dogs

MLS laser Therapy in dogs with pressure ulcers and open wound: case reports.

L. Dragone[a], S. Palladini[a], and M. Glazar[b],
a) Ambulatorio Veterinario "Dog Fitness", Reggio Emilia, Italy
b) ASA, Vicenza, Italy

ABSTRACT

The aim of this report is to describe the clinical application of MLS Laser Therapy as adjuvant therapeutic technique in combination with topical wound management for treatment of pressure ulcers and open wound in four dogs.
The dogs have sustained trauma, were hospitalized in the physical therapy veterinary clinic and had wounds that had to be treated concurrently with neurologic conditions. Pressure ulcers and open wound were managed by topical wound medications and contemporaneously were irradiated twice daily with MLS pulse. The wounds were measured at the beginning of the treatment and at the end of the therapy and showed a reduction in size during the course of treatments.
MLS laser therapy may be useful as adjuvant therapeutic tecnique for treatment of decubitus ulcers and open wounds in dogs.

INTRODUCTION

Management of wounds is an important part of physical therapy and rehabilitation in humans and animals. Patients that have sustained trauma often have wounds that must be treated concurrently with other conditions. Proper wound care, along with some of the newer modalities, as laser therapy, should be applied for successful treatment of open wounds. Many veterinary patients have orthopedic or neurologic conditions that result in prolonged recumbency, placing them at risk for decubital ulcers. When these wounds occur, appropriate treatment is critical to limit morbidity [1].
Some wounds fail to progress in an orderly and timely manner through the biologic sequences comprising the phases of healing, resulting in a non healing or poorly healing wound. The location of a wound over a bony surface or joint may result in delayed healing owing to difficulty in maintaining approximation of found edges [1]. A pressure sore or decubital ulcer is localized injury to the skin and/or underlying tissue usually over a bony prominence, as a result of pressure, or pressure in combination with shear, resulting in local or regional tissue ischemia [2]. The progression of pressure sores is influenced by several other factors like direct pressure, including shear forces, friction and moisture [3,4,5]. Underlying conditions, such as neurologic injuries (paralysis), vascular diseases causing impaired circulation, metabolic diseases (diabetes mellitus or hyperadrenocorticism), and malnutrition, can place animals at much greater risk for the development of pressure sores. Most pressure sores observed in veterinary medicine occur in nonambulatory patients or in patients that cannot or are unwilling to change their body position [1]. Common anatomic locations for pressure sores include the greater trochanter, tuber ischium, calcaneus, lateral malleoulus of the tibia, and the lateral aspect of the fifth digit of the paw in the pelvic limbs and the acromion, olecranon, and lateral epiconyle of the humerus, and the lateral aspect of the fifth digit of the paw in the thoracic limbs [1].
Prevention of pressure sores is certainly more cost effective than treating them; however, this is often easier said than done. The prevalence of pressure sores in people in the United states is reported to be between 1.3 to 3 million, and pressure sores are estimated to affect 5% to 10% of hospitalized patients [6,7]. Pressure sores are a source of numerous complications contributing to high rates of morbidity and mortality in humans. Treatment of pressure sores can result in huge costs to the health care system [3,7,8]. Although similar statistics are not available in veterinary medicine, pressure sores are similarly known to be a cause of increased patient morbidity and expense to the owner.
National Pressure Ulcer Advisory Panel (NPUAP) and European Pressure Ulcer Advisory Panel (EPUAP) developed a common international definition and classification system for pressure ulcers in humans [2]. These classification schemes are used to determine treatment protocols and can similarly be applied to animals. According to International Pressure Ulcer Classification System four levels of injury are described (Tab. I).

During the past 30 years there have been numerous reports indicating the potential of laser biostimulation in the facilitation of the wound healing process. In vitro data suggest that laser therapy facilitates collagen synthesis [9], keratinocyte cell motility [10], and growth factor release [11], transforms fibroblasts to myofibroblasts [12] and accelerates angiogenesis [13,31]. Many authors of clinical studies have reported the benefits of low level laser

Category/Stage	Description
Category/Stage I	Non-blanchable erythema Intact skin with non-blanchable redness of a localized area usually over a bony prominence. The area may be painful, firm, soft, warmer or cooler as compared to adjacent tissue
Category/Stage II	Partial thickness Partial thickness loss of dermis presenting as a shallow open ulcer with a red pink wound bed, without slough. May also present as an intact or open/ruptured serum-filled or sero-sanginous filled blister. Presents as a shiny or dry shallow ulcer without slugh or bruising
Category/Stage III	Full thickness skin loss Full thickness tissue loss. Subcutaneous fat may be visible, but bone, tendon or muscle are not exposed. Slough may be present, but does not obscure the depth of tissue loss. May include undermining and tunneling. The depth of a Category/Stage III pressure ulcer varies by anatomical location. Bone/tendon is not visible or directly palpable.
Category/Stage IV	Full thickness tissue loss Full thickness tissue loss with exposed bone, tendon or muscle. Slough or eschar may be present. Often includes undermining and tunneling. The depth of a Category/Stage IV pressure ulcer varies by anatomical location. Category/Stage IV ucers can extend into muscle and/or supporting structures (e.g. fascia, tendon or joint capsule) making osteomyelitis or osteitis likely to occure. Exposed bone/muscle is visible or directly palpable.

Tab. I. International Pressure Ulcer Classification System

therapy (LLLT) on tissue healing, but others have shown no effect [14-16]. The data from appropriately designed studies indicated that LLLT should be considered as an adjuvant therapy for refractory wound-healing disorders, including in diabetic patients, although many of the in vivo studies lacked specific information on dosimetric data and appropriate controls [17]. LLLT also resulted beneficial in treating difficult wounds in metabolically compromised patients, as it was demonstrated in a study on wound healing in diabetic rats [18], while another study reviewed the literature regarding the overall treatment effects of laser phototherapy on tissue repair and concluded that LLLT represented an effective treatment [19]. To our knowledge, untill today only one previous report has been published on the clinical application of laser therapy to promote closure of chronic skin wound in a dog [20].

This case report describes the clinical use of MLS laser therapy as adjuvant therapeutic technique in three dogs with pressure ulcers developed as a consequence of neurologic disorders and in a dog with an open skin wound followed by secondary closure. The dogs were hospitalized in a veterinary physiotherapy and rehabilitation center in Reggio Emilia and the rehabilitation program and wound management were performed one/twice day by veterinarians.

MLS Laser Therapy was carried out using a MLS M1 vet laser device (ASA, Arcugnano (Vi), Italy), equipped with synchronized combination of continuous and pulsed emissions. Continuous emission was produced by an InGa(Al)As laser with the following parameters: wavelenght 808 nm; maximum power 1000 mW; continuous wave; spot area 3,14 cm^2. Pulsed emission was produced by an InGaAs/GaAs laser with the following parameters: wavelenght 905 nm; maximum power 25 W; pulse frequency 2000 Hz; pulse duration 200 ns; spot area 3,14 cm^2. MLS laser treatment was performed on the wounds every day, after cleaning procedure and before applying topical wound management products.

The wounds were cleaned with chlorhexidine gel and the medication was done by applying products for topical wound management.

Case 1

An eight-year-old male mixed breed dog, weighing 13 kg, was presented in the clinic after the right hemilaminectomy at T12 to T13 performed for intervertebral disk disease. The examination revealed a non-ambulatory paraparesis and bilateral tuber ischium stage III pressure ulcers developed because the dog was allowed to drag itself only on his front paws. The wounds were full thickness, 2,1 x 2,1 cm on the left tuber ischium, 1,9 x1,9 cm on the right tuber ischium, but bone was not exposed (Fig. 1). The dog was hospitalized and put on the rehabilitation programme consisting in hydrotherapy and passive and active exercises repeated twice daily. The pressure sores were treated with MLS Laser Therapy and topical wound medications two times a day.

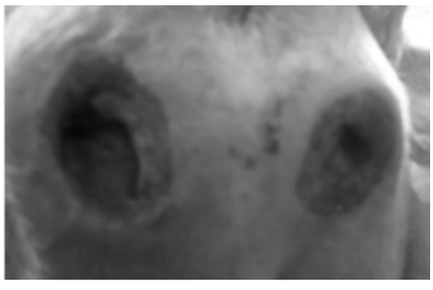


Fig. 1. decubitus ulcers at the first examination in a mixed breed dog

The ulcers were thoroughly cleansed with Clorexyderm Spot Gel (ICF, Cremona, Italy), treated with MLS laser therapy (Contaminated Wound Healing programme, continuous wave, scanning mode, time 2 minutes, energy delivered 126,9 J) and then dressed topically with Repy Gel (Innovet, Saccolongo, Italy). The irradiation field included the entire

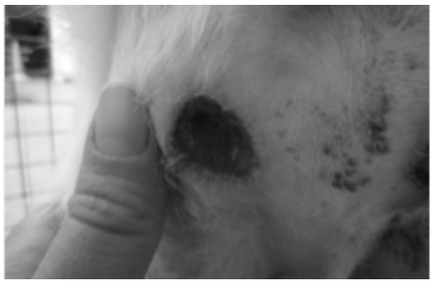


Fig. 2. decubitus ulcers 12 days after treatment onset

wound plus a 2 cm margin around the wound. Treatments were given for 12 consecutive days and pressure sores were measured twice, once at the beggining of the treatment and at the 12th day of the treatment. At the second measurement the dimensions of the ulcers were 1,2 x 1,2 cm for the left ulcer and 1,3 x 1,3 cm for the right ulcer and the depth of the wounds was diminished (Fig. 2, 3). The dimensions of the wounds were reduced by 42,8% and 31,6% respectively in 12 days.

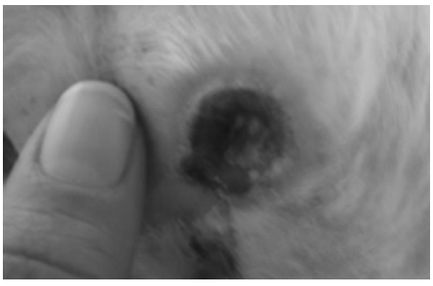


Fig. 3. decubitus ulcers 12 days after treatment onset

Case 2

A thirteen-year-old female mixed breed dog, weight 32 kg, was presented in the clinic after the right hemilaminectomy performed at T3 to T7 for discospondylitis. The patient was severely paraparetic and non-ambulatory. It presented category IV pressure ulcer on the right great trochanter. Initially, the pressure ulcer was 1,5 cm wide (Fig. 4). The patient was hospitalized, put on the rehabilitation programme that included hydrotherapy and passive and active exercises performed two times a day and started the antibiotic therapy with clindamycin 11 mg/kg BID.

The pressure sore was treated with

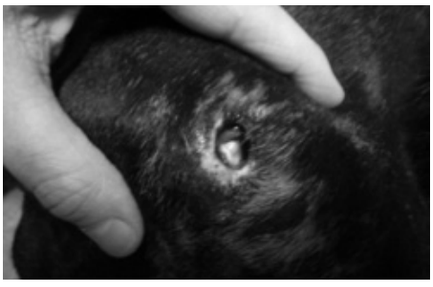


Fig. 4. great trochanter pressure sore at the first examination

MLS Laser Therapy and topical wound medications twice daily. The ulcer was thoroughly cleansed with Clorexyderm Spot Gel (ICF, Cremona, Italy), treated with MLS laser therapy (Contaminated Wound Healing programme, continuous wave, scanning mode, time 2 minutes, energy delivered 126,9 J) all over the wound and on the margin of the wound and then dressed topically with a solution of Betadine (Viatris, Milano, Italy) and sugar. The second measurement of the pressure ulcer, 4 days after the first measurement, revealed a reduction of the wound dimensions from 1,5 cm to 1 cm (33,3%) (Fig. 5).

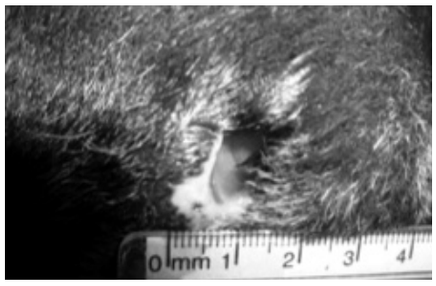


Fig. 5. pressure sore 4 days after onset of therapy

Case 3

A ten years old male Dobermann, weight 30 kg, was examined in the clinic after the ventral slot performed at C5 to C6 and at C6 to C7 for Wobbler syndrome. At the reception the dog was severely tetraparetic, non-ambulatory, not able to stand and presented noticeable muscle atrophy of the shoulders and hind legs and maintained constantly the lateral recumbency, although the recumbency was changed every 4 hours. The patient started the rehabilitation programme including hydrotherapy and passive and active excercizes twice daily. The pressure ulcers at both great trochanters were developed 1 month after the admission to the clinic. Initially, the diameter of the right decubitus ulcer was 5 cm and of the left decubitus 4,6 cm (Fig. 6). The ulcers were thoroughly cleansed with Clorexyderm Spot Gel (ICF, Cremona, Italy), treated with MLS laser therapy (Contaminated Wound Healing

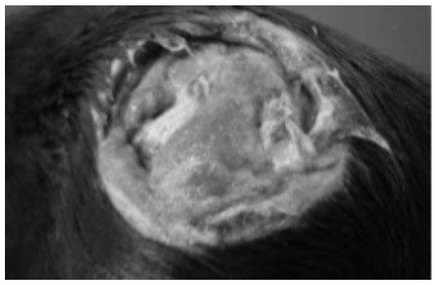


Fig. 6. great trochanter decubitus ulcer at the first examination

programme, continuous wave, scanning mode, time 2 minutes, energy delivered 126,9 J) and then dressed topically with a solution of Betadine (Viatris, Milano, Italy) and sugar. The irradiation field included the entire wound plus a 2 cm margin around the wound. After 5 days of MLS laser therapy and topical wound management the diameter decreased to 4,5 cm on the right and to 4,1 on the left trochanter. After 10 days of treatment the diameter of the right decubital ulcer was 4,1 cm and 3,9 cm of the left one. After 18 days of therapy the dimensions of the wound decreased further to 3,6 cm on the right side and to 2,9 cm on the left side (Fig. 7). In 48 days of therapy the ulcer on the right trochanter was reduced by 28% and on the left trochanter by 36,9%.

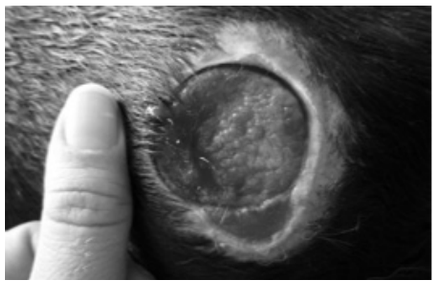


Fig. 7. the ulcer after 48 days of treatment

Case 4

A three years old male mixed breed dog, weight 26 kg, was examined in the clinic after the stabilisation of the vertebral fracture and luxation at T2 to T3 and the amputation of the second digit of the right hind limb. At the reception the dog was tetraparetic and non-ambulatory. The patient was hospitalized, put on the

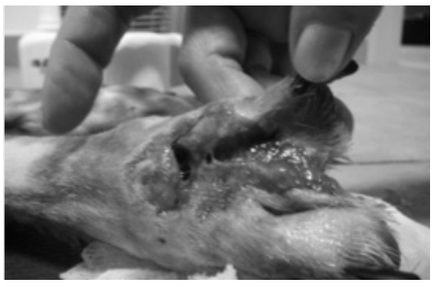


Fig. 8. chronic wound on the right hind limb, after the amputation of one digit

rehabilitation programme that included hydrotherapy and passive and active excercizes performed two times a day and started the antibiotic therapy with clindamycin 11 mg/kg BID. The skin lesion that developed in situ of the amputation had to close by second intention healing and was managed as open wound (Fig. 8). The wound was treated with MLS Laser Therapy and topical wound medications twice daily. It was thoroughly cleansed with Clorexyderm Spot Gel (ICF, Cremona, Italy), treated with MLS laser therapy (Contaminated Wound Healing programme, continuous wave, scanning mode, time 2 minutes, energy delivered 126,9 J) all over the wound and on the margin of the wound and then dressed topically with Hypermix (RI. MOS., Modena, Italy). The wound healed completely after 32 days of therapy (Fig. 9, 10).

DISCUSSION

Wound healing is a biologically complex sequence of overlapping events and is a natural restorative response to tissue injury [1]. The dynamic series of interrelated processes is divided into the inflammatory, proliferative and remodelling phases. The duration of each stage will vary with the wound type, management, microbiologic and other physiologic factors [21]. Each phase involves biochemical mediators such as cytokines, growth factors, and other cellular components that stimulate or inhibit the cellular responses that facilitate healing [1]. The biologic process for wound healing is the same for all wounds, although the specific mechanisms may vary. Superficial and partial-thickness wounds complete

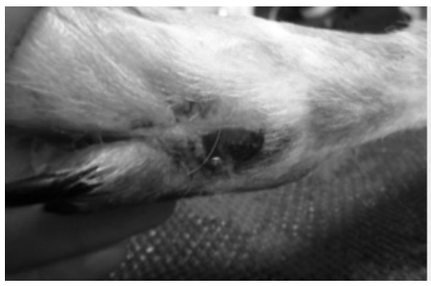


Fig. 9. the wound 15 days after treatment onset

Fig. 10. the wound after 32 days of therapy. Note that the wound has completely healed.

healing principally through epithelialization and progress through the repair process more quickly than full thickness wounds that rely primarily on contraction. Chronic wounds may lack an orderly progression through wound healing phases, allowing for prolonged inflammation, repeated injury, and infection [1]. Many of the regimens and therapeutic interventions designed to facilitate the wound healing process, as laser therapy, influence the various phases of the process. Methods to promote wound healing must not interrupt these biological processes.

Laser therapy is thought to stimulate wound healing by inducing vasodilatation that increases nutrients and growth factors supplies, activates fibroblasts and increases collagen synthesis, an essential protein for tissue repair and regeneration [22,23]. In damaged tissues, where recovery of nervous functions is normally slow, laser stimulation accelerates nervous cells regeneration, revitalising the insensitive areas [24,25,26]. It also induces lymphatic and vascular regeneration [27,28,29] and increases and accelerates angiogenetic processes [13,30]. Finally, laser therapy prevents the formation of hypercheratotic lesions formation because it reduces the formation of cicatricial tissue following a skin lesion [31].

To authors' knowledge, in veterinary medicine only one report on laser therapy in wound healing in a dog has been published [20] and our aim with this case reports was to present our experience with MLS laser therapy used as adjuvant therapy for decubitus ulcers and wound healing in unfavorable conditions for healing. In the cases presented in this report, the MLS laser therapy was used in combination with topical wound management to promote healing and to accelerate the formation of cicatricial tissue in decubitus ulcers and an open wound. The four dogs in this case series showed deep pressure ulcers and a chronic open wound and all cases followed the process of second intention healing. All dogs have sustained trauma and had wounds that had to be treated concurrently with other conditions. Hydrotherapy was included in the rehabilitation programme of all dogs and it probably represented a delying factor in the healing process, as hydrotherapy is usually contraindicated when there is a presence of infected skin lesions. However, considering the concurrent illness of the patients and the benefits of acquatic exercise in their conditions, it was decided that the delayed healing of the ulcers and wounds was a minor problem that could be handled with MLS laser therapy and topical wound medication. The patients did not show signs of discomfort during the treatments and the therapy was easy to handle and quick to perform.

In summary, we consider MLS laser therapy an effective and valid adjuvant therapy for refractory wound healing disorders as decubitus ulcers and open wounds that have to heal by second intention in dogs, even in conditions that dely healing as those experienced during the treatment of our patients. However, the advantage of the laser therapy to promote wound healing has still not been uneqivocally established by appropriate studies in veterinary medicine. For veterinary patients, carefully designed clinical trials using laser therapy for treatment of open wounds or decubitus ulcers may help define its effectiveness.

REFERENCES

1. Hanks J, Spodnick G. Wound healing in the veterinary rehabilitation patient. Vet Clin Small Anim, 2005, 35: 1453-1471
2. European Pressure Ulcer Advisory Panel and National Pressure Ulcer Advisory Panel. Prevention and treatment of pressure ulcers: quick reference guide. Washington DC: National Pressure Ulcer Advisory Panel. 2009.
3. Sørensen JL, Jørgensen B, Gottrup F. Surgical treatment of pressure ulcers. Am J Surg, 2004, 188 (1A Suppl): 42-51
4. Brem H, Lyder C. Protocol for the successful treatment of pressure ulcers. Am J Surg, 2004, 188 (1A Suppl): 9-17
5. Edlich RF, Winters KL, Woodward CR, et al. Pressure ulcer prevention. J Long Term Eff med Implants, 2004, 14(4): 285-304
6. Lyder CH. Pressure ulcer prevention and management. JAMA, 2003, 289:223-6
7. Allman RM, Laprade CA, Noel LB, et al. Pressure sores among hospitalized patients. Ann Intern Med, 1986, 105:337-42
8. O'Brien SP, Gahtan V, Wind S, et al. What is the paradigm: hospital or home health care for pressure ulcers? Am Surg, 1999, 65:303-6
9. Abergel RP, Meeker CA, Lam TS, Dwyer RM, Lesavoy MA, Uitto J. Control of connective tissue metabolism by lasers: recent developments and future prospects. J Am Acad Dermatol, 1984, 11: 1142-150
10. Haas AF, Isseroff RR, Wheeland RG, Rood RA, Graves PJ. Low energy helium-neon laser irradiation increases the motility of human keratinocytes. J Invest Dermatol, 1990, 94: 822-26
11. Yu W, Naim JO, Lanzafame RJ. The effects of photo-irradiation on the secretion of TGF and PDGF from fibroblasts in vitro. Lasers Surg Med Suppl, 1994, 6:8
12. Pourreau-Schneider N, Ahmed A, Soundry M, et al. Helium-neon laser treatment transforms fibroblasts into myofibroblasts. Am J Path, 1990, 137: 171-78
13. Mirsky N, Krispel Y, Shoshany Y, Maltz L, Oron U. Promotion of angiogenesis by low energy laser irradiation. Antioxid Redox Signal, 2002, 4(5): 785-90
14. Allendorf JD, Bessler M, Huang J et al. Helium-neon laser irradiation at fluences of 1, 2 and 4 J/cm2 failed to accelerate wound healing as assessed by wound contracture rate and tensile strenght. Lasers Surg Med, 1997, 20: 340-45
15. Hunter J, Leonard L, Wilson R, Snider G, Dickson J. Effects of low energy laser on wound healing in a porcine model. Lasers Surg Med, 1984, 3: 285-90
16. Saperia D, Glassberg E, Lyons RF. Demonstration of elevated type I and III procollagen mRNA level in cutaneous wounds treated with helium-neon laser: Proposed mechanism for enhanced wound healing. Biochem and Biophysical Res Comm, 1986, 138: 1123-28
17. Schindl A, Schindl M, Pernerstorfer-Schoen H, et al. Low intensity laser therapy in wound healing: a review with special respect to diabetic angiopathies. Acta Chir Aust, 2001, 33(3): 132-7
18. Reddy GK, Stehno-Bittel L, Enwemeka CS. Laser photostimulation accelerates wound healing in diabetic rats. Wound Repair Regen, 2001, 9(3): 248-55
19. Enwemeka CS, Parker JC, Dowdy DS, et al. The efficacy of low-power lasers in tissue repair and pain control: a meta-analysis study. Photomed Laser Surg, 2004, 22(4): 323-9
20. Lucroy MD, Edwards BF, Madewell BR. Low-intensity laser light-induced closure of a chronic wound in a dog. Vet Surg, 1999, 28:292-95
21. Wound management. In: Aiello S, Mays A, eds. The Merck veterinary manual. Eight edition. Whitehouse Station: Merck & Co. Inc, 1998, pp 1255
22. Almeida-Lopes L, Rigau J, Zângaro RA, Guidugli-Neto J, Jaeger MM. Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg Med, 2001, 29(2): 179-84
23. Liu H, Dang Y, Wang Z, Chai X, Ren Q. Laser induced collagen remodeling: a comparative study in vivo on mouse model. Lasers Surg Med, 2008, Jan;40(1): 13-9
24. Rochkind S, Ouaknine GE. New trend in neuroscience: low-power laser effect on peripheral and central nervous system (basic science, preclinical and clinical studies). Neurol Res, 1992, 14: 2–11
25. Gigo-Benato D, Geuna S, Castro Rodrigues A, Fornaro M, Boux E, Battiston B, Tos P. Low-power laser biostimulation enhances nerve repair after end-to-side neurorrhaphy: a double-blind randomized study in the rat median nerve model. Lasers in Med Science, 2004, 19: 57-65
26. Rochkind S, Leider-Trejo L, Nissan M, Shamir MH, Kharenko O, Alon M. Efficacy of 780-nm Laser Phototherapy on Peripheral Nerve Regeneration after Neurotube Reconstruction Procedure (Double-Blind Randomized Study). Photomedicine and Laser Surgery, 2007, 25(3): 137-143
27. Maier M, Haina D, Landthaler M. Effect of low energy laser on the growth and regeneration of capillaries. Laser in Medical Science, 1990, 5(4): 381-386
28. Rebeiz E, April MM, Bohigian RK, Shapshay SM. Nd-YAG laser treatment of venous malformations of the head and neck: an update. Otolaryngol Head Neck Surg, 1991, 105(5):655-61
29. Goldman MP, Fitzpatrick RE, Ruiz-Esparza J. Treatment of port-wine stains (capillary malformation) with the flashlamp-pumped pulsed dye laser. J Pediatr, 1993, 122(1):71-7
30. Garavello I, Baranauskas V, da Cruz-Höfling MA. The effects of low laser irradiation on angiogenesis in injured rat tibiae. Histol Histopathol, 2004, 19(1):43-8
31. Alster T, Zaulyanov L. Laser scar revision: a review. Dermatol Surg, 2007, 33(6):770

Key words: Pulsed High Intensity Laser, Wound Healing, Diabetic Foot Ulcer.

Effect of pulsed high intensity Nd:YAG laser in treatment of chronic diabetic foot ulcer.

A.A. Ebid[a], A.A. Thabet[b], O.F. Helal[c]

a) Lecturers Physical Therapy, Department for surgery, Faculty of Physical Therapy, Cairo University, Egypt.
b) Lecturers Physical Therapy, Department for Obstetrics and Gynecology, Faculty of Physical Therapy, Cairo University.
c) Lecturers Physical Therapy, Department of Internal Medicine, Faculty of Physical Therapy, October 6 University, Egypt

ABSTRACT

Delayed wound healing specially in diabetic ulcer is continuing challenge in rehabilitation medicine despite some recent advances in understanding of its basic principles and problems in wound healing that continue to cause significant morbidity and mortality. The aim of this study was to determine the effect of Pulsed High Intensity Nd:YAG Laser in the treatment of chronic diabetic foot ulcer (Deep Ulcer grade 2) and suggest laser protocol for wound healing. Forty patients suffering for chronic diabetic foot ulcer as a complication of diabetes mellitus, aged 40-70 years (mean age 58.17±9.83), were included. Patients were randomized for treatment in two groups. In the group A (HILT group), twenty patients received 24 session of pulsed high intensity Nd:YAG laser according to designed protocol, 3 times per week in addition to standard medical treatment which is given for diabetic foot patients. In the group B (Standard Medical Therapy Group), twenty patients received standard medical treatment for 24 sessions, three times per week. The result of this study revealed that there was statistical significant reduction in wound surface area for group (A) after 12 and 24 sessions. The results have demonstrated the objective effect of pulsed high intensity Nd:YAG laser in treatment of chronic diabetic foot ulcer. Therefore, pulsed high intensity Nd:YAG laser is effective, innovative, non invasive, non expensive and can be used as a new trend physical therapy modality in the treatment of chronic diabetic foot ulcer.

INTRODUCTION

Diabetes mellitus is the most common serious chronic disease which is characterized by hyperglycemia, metabolic abnormalities and long term complications involving eyes, skin, nerves and blood vessels. More than 220 million people had diabetes by the year 2010 and the majorities have type II diabetes [1].

Wound healing and tissue repair are complex processes that involve a dynamic series of events including clotting, inflammation, granulation tissue formation, epithelization, collagen synthesis and tissue remodeling [2].

Diabetic foot ulcer and subsequent foot amputation continue to cause considerable morbidity among persons with diabetes. Foot ulcer had been recognized as an important antecedent of lower extremity amputation in multiple studies. Progress has occurred in understanding the pathogenesis of these complications [3].

In recent years low intensity laser photostimulation has gained considerable recognition and importance among treatment modalities for various medical problems including wound repair processes, musculo-skeletal complications and pain control [4]. Also, laser is a new therapeutic tool used to reduce pain and to accelerate healing process of wounds [5].

Therapeutic lasers use monochromatic light in the 630 to 905 nm range, known as the "therapeutic window" [6]. High Intensity Laser Therapy (HILT), performed with a pulsed Nd:YAG laser, is characterized by a wavelength of 1064 nm that allows it to penetrate and spread more easily through the tissue due to not having endogenous chromophores able to efficiently absorb 1064 nm radiation. Moreover, with Nd:YAG pulses it is possible to deliver power peaks of up to 1000 Watt for times of 200µ seconds: extremely elevated peak intensity W/cm^2 in very brief times. Such a high intensity in such a short time prevents the heat accumulation by the tissues as happens with the use of Nd:YAG laser with continuous emission. These features result in a greater propagation of the radiation in the tissues with a very low histolesive risk, leading to the possibility of treating deep tissues and structures.

At the same time, the photothermal effect can be controlled in terms of patient safety and comfort by modulating pulse intensity and frequency [7].

Photostimulation promotes tissue repair by accelerating the production of collagen and promote overall connective tissue

stability in wound healing [2].
Powell et al [8] mentioned that fifteen percent or more of people with diabetes sustain one or more foot wounds during their life time and they are fifteen times more likely to suffer from non traumatic lower extremity amputation than people without diabetes. In addition, Margolis et al [9] revealed that diabetic patients with lower extremity ulcers were hospitalized longer on average than those who were hospitalized and did not have ulcers. Whereas half of all lower extremity amputations in hospitalized patients occurred in diabetic patients. They also assured that those with lower extremity amputation have a diminished quality of life, increased health costs, and more likely to have the contra lateral limb amputation, and are more likely to die within the next five years than those with no amputation.

The ulcer healing and its effects on the elongation of hospitalization period are major economical problems that face the physical therapists and other team members of ulcer rehabilitation. The importance of this study arises from the severity of diabetic foot ulcer which leads to serious complications such as: delayed wound healing, amputation and risk of infection. Diabetic foot ulcer problem may impair the primarily daily living activities due to the reduction of muscle power and the limitation of range of motion of peripheral extremities. Therefore, this study was designed to evaluate the effect of pulsed high intensity Nd:YAG laser in treatment of diabetic foot ulcer as a new physical therapy method for promoting healing process of chronic diabetic foot ulcer and to suggest a treatment protocol for wound healing by using pulsed high intensity laser.

MATERIALS AND METHODS:

Forty patients with chronic diabetic foot ulcer participated in this study. The patients suffering for unilateral or bilateral grade 2 deep ulcer diabetic foot ulcers (according to Wagner [10] Ulcer Classification System) lasting longer than two months were recruited in the study. The recruitment occurred via collaboration with general surgeons and wound care specialists serving outpatients clinics. All patients were selected from diabetic foot clinic in Alnour Hospital. All participants were informed about the nature and the purpose of the study; patients were examined by physician before the study to determine inclusive and exclusive criteria. Demographic information was obtained from standardized interview including age, sex, occupation, residence and special habits. Physical examination and history evaluation were conducted for all patients included weight, height, diabetes duration, ulcer duration and site, inspection of foot and palpation of peripheral pulses (anterior tibial artery and dorsalis pedis). Patient received an Arabic instruction manual that contains general advices about foot hygiene; nail care and foot wear to avoid future injuries. Patients were asked to do regular analysis of serum blood glucose level to maintain it within normal ranges during the program.
Subjects Criteria: Inclusive Criteria: patients with type II diabetes mellitus and grade 2 (Deep Ulcer) diabetic foot ulcers were referred from diabetic foot clinic in Alnour Hospital. Patient's age ranged from 40 to 70 years (mean age 58.17±9.83). All patients were male, and they did not receive any prior physical treatment for diabetic foot ulcer management. Exclusive Criteria: Patients had any pathological conditions or associated injuries which may affect the result of the study, patients had skin disease or any disease which leads to ulcer other than diabetes as venous or arterial ulcers, patients with malignancy, patients had any type of osteomyelitis associated with diabetic foot ulcer.
The patients randomly divided into two groups. Group A (HILT Group): twenty patients received application of pulsed high intensity Nd:YAG Laser (HIRO 3.0, ASA srl, Italy) for 24 sessions (about 8 weeks), three days per week in addition to standard medical treatment which is given for diabetic foot patients. Laser application was supplied immediately after standard medical treatment. HILT was given by 5 mm probe and energy densities 4 J/cm^2 with 1 cm distance from ulcer surface (non-contact), according to protocol designed for wound healing (Table 1). Group B (Standard Medical Therapy Group): twenty patients received standard medical treatment for 24 sessions. They were instructed to receive the treatment three times per week in the Physical Therapy Department; they received routine treatment of foot ulcer in form of hypoglycemic medications such as insulin injection to control blood glucose level, systemic antibiotics against microorganisms according to culture tests, debridement for removal of necrotic tissues and foreign bodies when needed, irrigation of the wound by normal saline solution twice daily, dressings after irrigation of the ulcer, and finally it was covered with sterile gauze.

Grade-0	High risk foot and no ulceration.
Grade -1	Superficial Ulcer.
Grade -2	Deep Ulcer (cellulitis)
Grade -3	Osteomyelitis with Ulceration or abscess.
Grade -4	Gangrenous Patches. Partial foot gangrene.
Grade -5	Gangrene of entire foot.

Tab. I. Wagner's classification for diabetic foot disease (Adopted from Levin and O'Neals).

Wound surface area (WSA) was measured by tracing the wound perimeter as reported by Kloth and Feedar [11] and by using a digital camera.
In the transparent method the patient was positioned in a comfortable position with exposure of the affected foot, double sterilized transparent plastic films were placed directly flat and attached to the skin around the wound area with avoiding any movement and distortion of the foot. Ulcer margins were traced by the same investigator to establish

reliability of measurements [12]. The ulcer perimeter was traced by using the film-tipped transparency marker. Each ulcer was traced three times to establish measurement reliability. After tracing, the side of the transparency film facing the ulcer was cleaned with a piece of cotton and alcohol. Carbon paper was placed over the 1-mm-squared metric graph paper. The traced transparency film was placed over the carbon paper with white paper in between and the tracing was transcribed onto the metric graph paper. WSA was calculated by counting the number of square millimeters on the metric graph within the wound tracing. The mean value of the three trials was calculated and taken to be the WSA. WSA measurements were taken at zero time ("pre"), after 12 sessions ("post -1"), and after 24 sessions ("post-2"), and after two weeks of follow up.

The digital camera was placed through a constant distance on a tripod from patient's foot to capture a colored picture of ulcer to detect the size changes of the ulcer before treatment and through 12 and 24 sessions of treatment. The environmental conditions such as patient position, camera distance and orientation and lighting level were controlled.

A 10 cm ruler was included in each photograph field to allow calibration during subsequent measurement procedures.

Designed protocol for laser applications (Table 1):

After calculating the area of the wound (Wound Surface Area (WSA) expressed in (cm^2), the dose applied for each wound was 4 J/cm^2 in each phase of laser treatment with a 10 minute total duration for all phases.

For example, for WSA=20 cm^2 the dose in each phase is 80 Joules.

RESULTS

Statistical analysis: Data were collected and statistically analyzed using repeated measures and ANOVA test to verify the hypothesis and control both within and between variabilities with significance of 0.05. The data collected for both groups before treatment (pre), after 4weeks (12 sessions, (post- 1) and after 8 weeks (24 sessions, (post -2) were compared with each other.

Phase	Frequency (Hz)	Mode of application	Time (Minutes)	Total Energy
Initial	25	Fast Scanning	3	According to the size of each wound (WSA) expressed in (cm^2)
Intermediate	15	Applied at the periphery of the wound *(Fixed points)*	4	
Final	25	Slow Scanning	3	

Tab. I. Treatment protocol of Pulsed High Intensity Nd:YAG laser for wound healing.

1-Results for group (A): The mean value and standard deviation of WSA (cm^2), in group (A) before application of laser (pre) was 8.10±2.35, post-1 was 4.05±1.46 and post-2 was 0.65 ±0.58, there was significant decrease in the WSA after 4 weeks and 8 weeks compared to initial measurement (before treatment), $p>0.05$ as shown in table (2)

2-Results for group (B): The mean value and standard deviation of WSA (cm2), in group (B) at the beginning of the study (pre) was 8.75±2.48, post-1 was 7.75± 2.20 and post-2 was 6.40 ± 2.22, there was decrease in the WSA after 4 weeks and 8 weeks compared to initial measurement (pre), $p>0.05$ as shown in table (2).

3-Comparing the mean values of WSA in group (A) and group (B) before treatment (pre), after 4 weeks (post-1) and after 8 weeks (post-2) we found that before treatment (pre), there were no significant differences between the two groups, $p > 0.05$. After 4 weeks (post-1), there was a significant reduction in WSA in group (A) compared to group (B) ($p < 0.05$). After 8 weeks (post-2), there was a significant reduction in WSA in group (A) compared to group (B), ($p < 0.05$), as shown in figure (1).

DISCUSSION

Delayed wound healing specially in diabetic ulcer is continuing challenge in rehabilitation medicine despite some recent advances in understanding of its basic principles and problems in wound healing that continue to cause significant morbidity and mortality. A great number of studies have been conducted on acceleration of wound healing, attainment of normal breaking strength and prevention of keloid and scar formation by using many physical methods such as therapeutic ultrasound, laser therapy and electrical stimulation [13].

This study was designed to investigate the effect of pulsed high intensity Nd:YAG laser on chronic diabetic foot ulcer. The result of this study showed that, there was significant decrease in WSA in group (A), after 4 and 8 weeks of laser treatment, compared to group (B).

Hawkins and Abrahamse [14] investigated the effect of multiple exposures to Low Intensity Laser Therapy (LLLT) on cell response, using as experimental model wounded skin fibroblasts. They demonstrated that correct energy density or fluency and the number of exposures can stimulate cell response in terms of cell migration and proliferation by stimulating mitochondrial activity and maintaining viability without causing additional stress or damage to the cells. Results indicate that the cumulative effect of lower doses determines the stimulatory effect.

Several indices of tissue repair are positively affected by laser treatment. In vivo studies and clinical reports indicated that laser therapy promotes wound healing by accelerating collagen synthesis [15,16], inflammation course, healing time and strength acquisition [17]. These results are consistent with previous reports that have demonstrated elevation of several metabolic indices of ATP synthesis [18], fibroblast proliferation, [16] and collagen synthesis, as well as increases in the biomechanical indices of tissue healing.

Laser stimulation leads to increased production of ATP from ADP molecules.

Wound Surface Area (WSA cm2)						
Groups	**HILT group (A)**			**control group (B)**		
Time of measurements	pre	Post-1	Post-2	pre	Post-1	Post-2
Mean	8.10	*4.05*	0.65	8.7500	7.7500	6.4000
SD ±	2.35	1.46	0.58	2.48945	2.20347	2.22781
F value (df)	86.46 (2)			242.67 (2)		
P value	P>0.05			P>0.05		

Tab. 2. The WSA for group (A) and group (B) at (pre), (post-1) and (post-2).

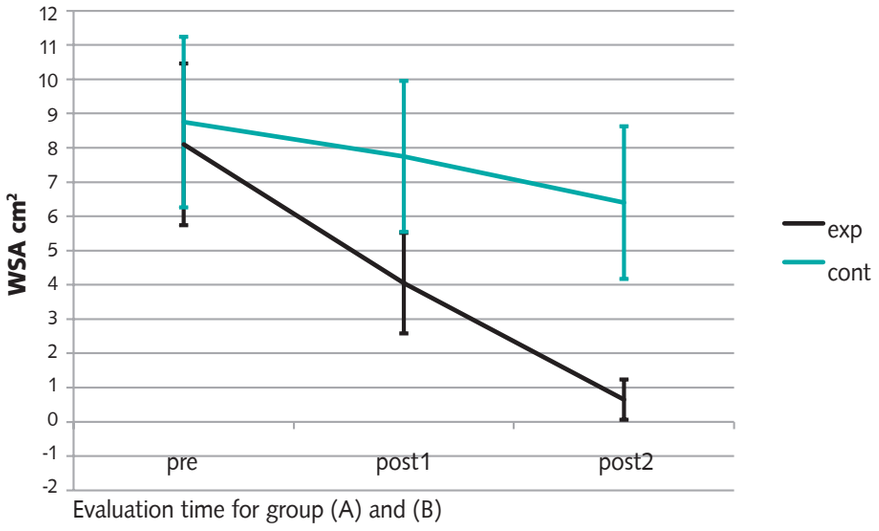


Figure (1): The mean values of WSA for group (A) and (B) at (pre), (post-1) and (post-2)

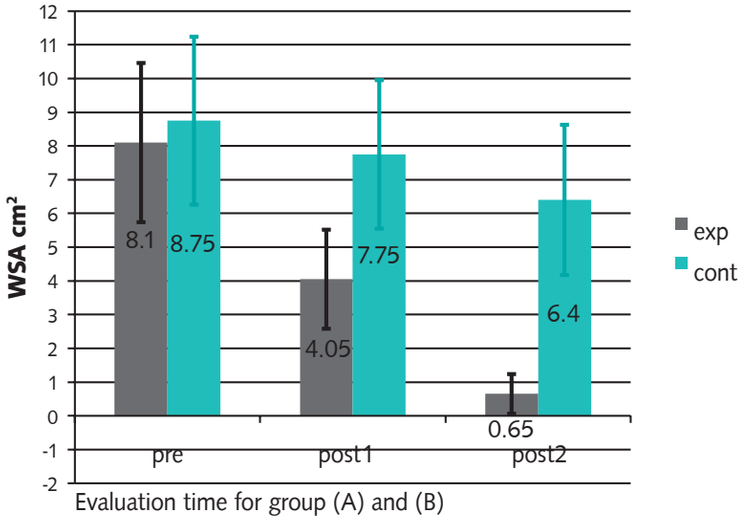


Figure (2): The mean values of WSA for group (A) and (B) at (pre), (post-1) and (post-2)

These processes occur in mitochondria, although the mediator between the action of photons and biochemical processes activation has not yet been identified. From numerous experiments and studies in vitro, it is evident that laser light is able to induce cell replication and synthesis of RNA and proteins (eg, collagen), promoting the healing process [19,20].

Our protocol has been designed on the basis of the study of Madrado et al [21], which evaluated the effects of laser therapy in experimental cutaneous wound healing and concluded that a dose of 4 J/cm^2 was more effective to that of 8 J/cm^2. In addition, Hawkins and Abrahams [22,23] reported that a dose of 5 J/cm^2 generated by a HeNe laser stimulates mitochondrial activity, leading to re-establishment of cellular functions, and induces proliferation and migration of fibroblasts, thus hastening wound closure. In contrast, a dose of 10 J/cm^2 was associated with a significant amount of cellular and molecular damage.

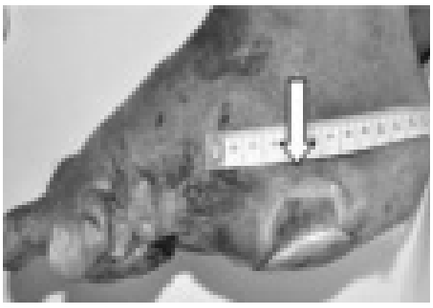
Pereira et al [24] studied the effect of a 120 mW GaAs diode laser on fibroblasts, and concluded that a dose of 3 J/cm^2 stimulated fibroblast proliferation without impairing procollagen synthesis. Pourzarandian et al [25] studied the effect of Er: YAG laser irradiation on cell growth of cultured human gingival fibroblasts and concluded that the optimal energy density for stimulation was 3.37 J/cm^2. The results indicate that Er: YAG laser irradiation may benefit wound healing.

It has been reported that in the case the tissues are affected by chronic phatological conditions, longer intervals between treatments are required, with a maximum of two or three sessions/week [26]. Clinical practice has shown that deeper is the wound or target tissue, more treatments are required and, as a general rule, it is better to use 3-4 treatments/week with moderate doses than using higher doses and fewer treatments [27].

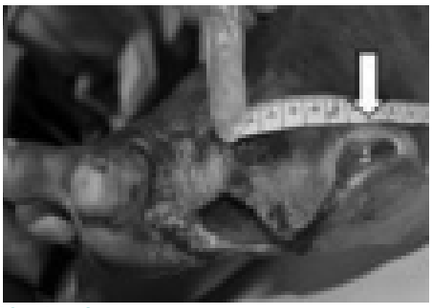
In agreement with outcomes of previous studies our results completely support

Figure (3): Differences in wound surface area by using digital camera

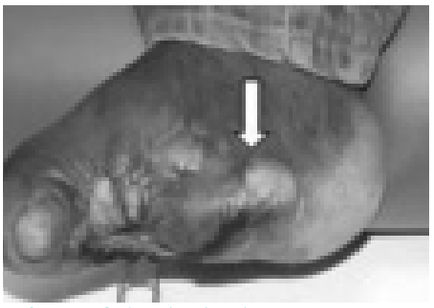
Case 1



Pre treatment



After 4 weeks

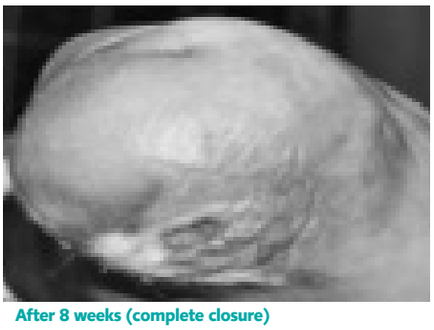


After 8 weeks (complete closure)

Case 2

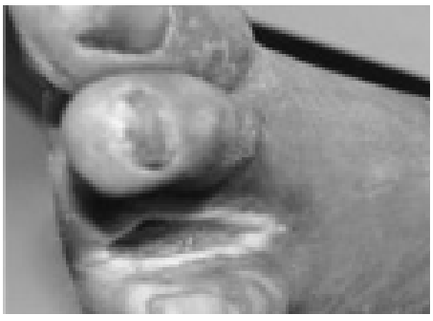
Pre treatment

After 4 weeks

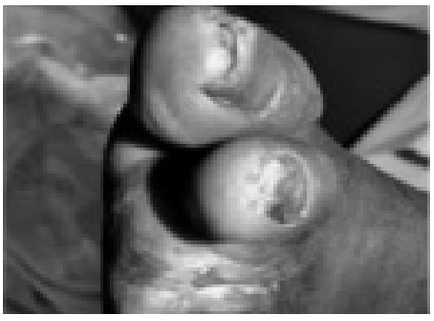


After 8 weeks (complete closure)

Case 3



Pre treatment



After 4 weeks

After 8 weeks (complete closure)

the hypothesis that healing process of diabetic foot ulcer is favoured by pulsed high intensity Nd:YAG laser. In fact the findings show that the application of pulsed high intensity Nd:YAG laser, according with the described protocol, is safe and effective for the treatment of grade 2 chronic diabetic foot ulcer, and induces a significant decrease in WSA as demonstrated by evaluation of the wounds at 4 and 8 weeks.

CONCLUSION

The common lower extremity problem associated with diabetes is the development of foot ulcers. The delayed ulcer healing and its effects on the rate of recovery and period of hospitalization are serious and functional problems. Therefore the selection of the appropriate treatment modalities is one of the big challenges to deal with those patients and the rehabilitation teams. The findings of this study are important to the specialists who work in the field of physical therapy and rehabilitation because suggest advanced physical therapy modalities in treating one of the most complicating problems with diabetic patients. The advantages to develop new strategies and standardize protocols for treating diabetic foot ulcer result in decrease of cost and faster time of wound healing.

REFERENCES

1. Anjuman GM, Atour R, Anila J. Changes In Gycosylated Proteins In Type 2 Diabetic Patients With And Without Complications. J Ayub Med Coll Abbottabad, 2005, 17 (3): 1-5.
2. Reddy GK, Stehno-Bittel L, Enwemeka CS. Laser photostimulation accelerates wound healing in diabetic rats. Wound Repair Regen. 2001, 9(3): 248-255.
3. Boyko EJ, Ahroni JH, Cohen V, Nelson KM, Heagerty PJ. Prediction of Diabetic Foot Ulcer Occurrence Using Commonly Available Clinical Information. Diabetes Care, 2006, 29 (6):1202-1207.
4. Fahey TJ, Sadaty A, Jones WG, Barber A, Smoller B, Shires GT. Diabetes impairs the late inflammatory response to wound healing. J. Surg. Res., 1991, 50: 308-313.
5. Sabbahi SA, Abdel-Hameed Z. Does laser prevent physiologic muscle atrophy? An histological study. Energy For Health, International journal of information and scientific culture, 2009, 3(3).
6. Stadler I, Lanzafame R, Oskoui P, Zhang R, Coleman J, Whittaker M. Alteration of skin temperature during low level laser irradiation at 830nm in a mouse model. Photomedicine and Laser Surgery, 2004, 22(3): 227-231.
7. Monici M, Cialdai F, Fusi F, Romano G, Pratesi R. Effects of pulsed Nd:YAG laser at molecular and cellular level. A study on the basis of Hilterapia. Energy FOR Health; International journal of information and scientific culture, 2009, Volume 3 Number [03].
8. Powell MW, Carnegie DH, Burke TJ. Reversal Of Diabetic Peripheral Neuropathy And New Wound Incidence: The Role Of MIRE. Adv. Skin Wound Care, 2004, 17: 295-300.
9. Margolis D Tylor J, Hoffstad O et al. Daibetic Neuropathic Foot Ulcers, The Association Of Wound Size, Wound Duration, And Wound Grade On Healing. Diabetes Care, 2002, 25(10):1835-39.
10. Andrew JM, Boulton V, Kyte L. Diabetic foot problems and their management around the world. In Levin and O'Neals "The diabetic Foot" 6th edition. Mosby, Inc. 2001; 266.
11. Kloth LC, Feedar JA. Acceleration of wound healing with high-voltage monophasic pulsed current. Phys Ther, 1988, 68: 503-8.
12. Haghpanah S, Bogie K, Wang X et al. Reliability Of Electronic Versus Manual Wound Measurement Techniques. J. Arch. Phys. Med. Rehabil, 2006,87: 1396-1402.
13. Demir H, Balay H, Kirnap M. A Comparative Study of the Effects of Electrical Stimulation and Laser Treatment on Experimental Wound Healing In Rats. Journal Of Rehabilitation Research & Development, 2004, 41(2): 147-154.
14. Hawkins D, Abrahamse H. Effect of multiple exposure of low- level laser therapy on the cellular responses of wounded human skin fibroblasts. Photomed Laser Surg, 2006, 24:705-14.
15. Reddy GK, Stehno-Bittel L, Enwemeka CS. Laser photostimulation of collagen production in healing rabbit Achilles tendons. Lasers Surg. Med, 1998, 22: 281–287.
16. Enwemeka CS. Ultrastructural morphometry of membrane bound intracytoplasmic collagen fibrils in tendon fibroblasts exposed to He:Ne laser beam. Tissue Cell, 1992, 24: 511–523.
17. Schindl A, Schindl M, Pernerstorfer-Schon H and et al. Low intensity laser therapy: a review. J. Invest. Dermatol, 2000, 48: 312–326.
18. Oren DA, Charney DS, Lavie R et al. Stimulation of reactive oxygen species production by an antidepressant visible light source. Biol. Psychiatry, 2001, 49: 464–467.
19. Smol'ianinova NK, Karu TI, Zelenin AV. Activation of the synthesis of RNA in lymphocytes following irradiation by a He-Ne-laser. Radiobiologia, 1990, May – Jun, 30(3): 424-426.
20. Manteĭfel' VM, Karu TI. Increase of number of contacts of endoplasmic reticulum with mitochondria and plasma membrane in yeast cells stimulated to division with He-Ne laser light. Tsitologia, 2004, 46(6): 498-505.
21. Medrado AR, Pugliese LS, Reis SR, Andrade ZA. Influence of low level laser therapy on wound healing and its biological action upon myofibroblasts. Lasers Surg Med, 2003, 32(3): 239-244.
22. Hawkins D, Abrahamse H. Biological effects of helium-neon (632.8 nm) laser irradiation on normal and wounded human skin fibroblasts. Journal of Photomedicine and Laser Surgery. 2005b, 23(3): 251-259.
23. Hawkins D, Abrahamse H. The role of fluence in cell viability, proliferation and membrane integrity of wounded human skin fibroblasts following helium-neon laser irradiation. Lasers in Surgery and Medicine, 2006a, 38(1): 74-83.
24. Pereira AN, Eduardo C de P, Matson E, Marques MM. Effect of low power laser irradiation on cell growth and procollagen synthesis of cultured fibroblasts. Lasers Surg Med, 2002, 31(4): 263-267.
25. Pourzarandian A, Watanabe H, Ruwanpura SM, Aoki A, Ishikawa I. Effect of low level Er:YAG laser irradiation on cultured human gingival fibroblasts. J. Periodontal, 2005, 76(2): 187-193.
26. Ohshiro T, Calderhead RG. Low Level Laser Therapy: A practical introduction. Published by Wiley and Sons, Inc. New York and Brisbane,1988, pp. 17, 28,29, 30, 33, 34, Olsen J.E., Schimmerling W. and Tobias C.A. 1980 Laser action spectrum of reduced excitability in nerve cells. Brain Res. 204, 436-440.
27. Tunér J, Hode L. Laser Therapy — Clinical Practice and Scientific Background. Prima Books AB, Grangesberg, Sweden. Chapter 1. Some basic laser physics. pp. 12, 21, 22, (ISBN: 91-631-1344-9), 2002.

Guide for Authors

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, LLLT, HILT, magnetotherapy. 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.

ARTICLE CATEGORIES

Articles are full-length papers presenting complete descriptions of original research, which have not been published and are not being considered for publication elsewhere.
Letters to the editor will be accepted and published if considered pertinent to the aim of the journal by the editorial board.
Reviews are topical overviews on emerging areas of research. They summarize key problems, concepts, experimental approaches, and research opportunities that characterize a subject area. Reviews should not include previously unpublished research results. The Editors generally invite them; authors who wish to submit a review should first consult with the Editors.

MANUSCRIPT SUBMISSION

To keep the review time as short as possible, the authors are requested to submit manuscripts (both text and art) in electronic form to the executive editor of "Energy for Health", Dr. Monica Monici, using the following e-mail address: monica.monici@asalaser.com. Manuscripts submitted via any other method will be returned. The manuscript must be accompanied by a cover letter outlining the significance of the paper. Authors are requested to read carefully the instructions (also available at the web site www.asalaser.com) and to follow them for the preparation of their manuscript.

PREPARATION OF MANUSCRIPTS

Manuscripts must be written in clear, concise, grammatical English. Authors unfamiliar with English usage are encouraged to seek the help of English-speaking persons in preparing their manuscripts. Manuscripts should be double-spaced.

TITLE PAGE

The title page (page 1) should include:
- A concise and informative title (capital bold font; not exceeding 120 characters)
- The name(s) of the author(s) (lower-case bold font, initials in capital letters)
- The affiliation(s) and address(es) of the author(s) (italics font)
- The name of the corresponding author, with complete address, e-mail address, telephone and fax numbers

ABSTRACT

Each paper must be preceded by an abstract (page 2) that summarizes in no more than 250 words a brief introduction, the aim of the study, materials and methods; main results and conclusions. It shouldn't contain any reference.

KEYWORDS

After the abstract, in the same page, a list of 4-6 keywords should be supplied for indexing purposes.

INTRODUCTION

The introduction should describe the state of the art, give a short review of pertinent literature, state the purpose of the investigation. It should be as concise as possible, without subheadings.

MATERIALS AND METHODS

The "materials and methods" section should follow the introduction and should provide enough information to enable the experiments to be reproduced.

Patients (clinical studies): typology of patients (age, sex....), criteria for enrolment in the study, etc.
Experimental model: cellular, animal, etc.
Instruments: laboratory instruments used for the research.
Methodology: protocols and evaluation mode.
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).
Data analysis: data-analysis method, statistical analysis.

RESULTS

This section should describe the outcome of the study without any comment. Data should be presented as concisely and clear as possible.

DISCUSSION

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.

REFERENCES

Reference should be made only to articles that are published or in press. The list of references should only include papers that are cited in the text. They must be progressively numbered (in square brachets) in the order in which they appear in the text and listed at the end of the paper in numerical order. Each reference should cite article title and the authors. Abbreviations of journal titles should follow those used in Index Medicus.
References with correct punctuation should be styled as follows:

Reference to a journal publication:
1. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature, 2003, 423: 337-342.

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.

FIGURES

All figures should be cited in the text and consecutively numbered with arabic numbers. Figures should be exclusively in TIFF or JPG format, with a minimum resolution of 300 dpi. Figure legends must be brief, self-sufficient explanations of the illustrations and double spaced. The legends should be prepared in a separate file in rtf format.

TABLES

All tables should be cited in the text and consecutively numbered with roman numbers.
Each table should have a title and a legend (double spaced) explaining the table content and any abbreviation used. Each table should be prepared in a separate page.

ABBREVIATIONS

Abbreviations should be defined at first mention preceded by the extended name.

COPYRIGHT

The author(s) guarantee(s) that the manuscript is their original work, submitted exclusively to the journal and will not be published elsewhere without the consent of the copyright holders.
Upon an article being accepted for publication, the right of publication, as well as rights of translation, of granting reproduction licences, of storage in electronic retrieval systems, of producing special impressions, photocopies, and microcopies are transferred to the publishers.

After manuscript acceptance the corresponding author is responsible for: 1) obtaining from coauthors permission to transfer copyright; 2) obtaining written permission to republish or reproduce all previously published material.
In any case, the journal will be not responsible for the lost of manuscript.

PEER REVIEW

The practice of peer review is to ensure the good quality of the published papers. It is an objective process carried out on all reputable scientific journals. When a manuscript is submitted to "Energy for Health" it is assigned by the Executive Editor to a member of the Editorial Board, based on expertise. If the manuscript is consistent with the aims of the journal, the Editor sends it to colleagues for review, then decides to accept or reject the manuscript on the basis of the referee comments.

WWW.ASACAMPUS.IT

ASA Research Division