



UNIVERSITÀ DEGLI STUDI DI TORINO

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Phototherapy and Nerve Injury: Focus on Muscle Response

Abstract

Preservation of biochemical processes in muscles is a major challenge in patients with severe peripheral nerve injury. In this chapter, we address the effects of laser irradiation and biochemical transformation in muscle, using in vitro and in vivo experimental models. The authors attempt to explain the possible mechanism of laser phototherapy applied on skeletal muscle on the basis of literature review and new results. A detailed knowledge of the evolution of endplates acetylcholine receptors and creatine kinase activity following laser irradiation can help to understand the therapeutic effect of laser phototherapy on muscle. This study showed that the laser phototherapy increases bio-chemical activity in intact muscle and thus could have direct therapeutic applications on muscle, especially during progressive atrophy resulting from peripheral nerve injury.

1. INTRODUCTION

Posttraumatic muscle denervation is a common and disabling out-come of various types of accidents which often involve young people ([Campbell, 2008](#)). Time to reinnervation is one of the most important determinants of functional outcome because muscle fibers progressively undergo an irreversible degeneration process, if reinnervation does not occur ([Battiston, Geuna, Ferrero, & Tos, 2005](#); [Wada, Katsuta, & Soya, 2008](#)).

The study of muscle changes following denervation is attracting more and more attention among clinicians, because of the relevance, number of traumas, and diseases which affect the neuromotor system. However the basic mechanisms that regulate the adaptation of muscle fibers to the absence of innervations are not fully understood. Muscle contraction and relaxation require the action of creatine kinase (CK), which is an important enzyme for supplying a source of energy for the muscle. Phospho-creatine, formed by the reaction of this enzyme, constitutes a reservoir of high-energy phosphate which is available for quick resynthesis of adenosine triphosphate (ATP). This high concentration of ATP is then accessible for muscle contraction. Following muscle denervation, the

level of CK and muscle weight decreases (Goldspink, 1976) and induces significant changes in acetylcholine receptor (AChR) density and distribution (Guzzini et al., 2008). Preservation of the muscle endplate structures and CK content is an important parameter for posttraumatic neuromuscular recovery.

Posttraumatic neuromuscular recovery continues to be a major challenge in restorative medicine. Among the various proposed methods, phototherapy has received increasing attention for enhancing nerve repair. The term phototherapy refers to the use of light for producing a therapeutic effect on living tissues. An extensive review of the literature (Gigo-Benato, Geuna, & Rochkind, 2005) showed that more than 80% of the experimental studies carried out so far on the use of laser phototherapy for promoting peripheral nerve repair led to a positive outcome on posttraumatic/postoperative nerve recovery. Our previous studies focused on the effectiveness of laser phototherapy in treating severely injured peripheral nerve and potential application on denervated muscle (Rochkind, Geuna, & Shainberg, 2009).

We chose to investigate the influence of low-power laser irradiation on intact muscle (in vivo) and muscle cell culture because it is accessible to study the cellular mechanism of laser–muscle interaction. Cell growth and differentiation are usually exclusive (Ontell, 1975). The myogenic cell culture provides a good in vitro model for studying the metabolic processes of the muscle tissue. During myogenesis, mononucleated cells derived from fetal skeletal muscle, when maintained in culture, withdraw from the cell cycle alignment and subsequently fuse to form multinucleated myotubes (Shainberg, Yagil, & Yaffe, 1971; Yaffe, 1969). These myogenic processes are associated with cessation of cell replication and elevation of a number of muscle-specific proteins (Kloosterboer, Faassen, Stroker-de Vries, & Hommes, 1979), including CK and AChR (Shainberg and Burstein, 1976).

2. PHOTOTHERAPY INCREASES SKELETAL MUSCLE BIOCHEMICAL ACTIVITY

In in vivo study, the influence of low-power laser irradiation on CK activity and the level of AChR in intact muscle to estimate biochemical transformation on cellular and biochemical levels during long-term period of time were investigated.

The rats underwent laser treatment (He–Ne laser, 35 mW, 30 min) every day, for 14 days. Low-power laser irradiation was

delivered transcutaneously to the intact gastrocnemius muscle. Under general anesthesia, the rats were sacrificed and the gastrocnemius muscle (none-treated and laser-treated) was homogenized on day 7, 14, 21, 30, 60, 120, and 210 in both groups.

CK activity was measured by the specific spectrophotometrical method (Oliver, 1955; Rosaki, 1967) using a spectrophotometer at 340 nm and a Sigma kit at 7, 14, 21, 30, 60, 120, and 210 days in both intact (control) and laser-irradiated intact muscles.

Internal and membrane inserted AChR was quantitated by the ^{125}I - α -bungarotoxin on the same homogenates (Almon, Andrew, & Appel, 1974; Chin and Almon, 1980) at different time points 7, 14, 21, 30, 60, 120, and 210 days in both control and laser-irradiated muscles. The data obtained were evaluated as cpm of bound ^{125}I - α -bungarotoxin/mg protein. Radioactivity was assessed using Gamma Counter in both muscles.

In an in vitro study, the thigh muscles were dissected from 2- to 3-day-old rats. Single cells were dissociated by trypsin. The cells were preplated into a plastic culture dish and then plated 1.5×10^6 cells into collagen-coated dishes (35 mm, Nunc) containing 1.5 ml of growth medium. The growth medium contained Dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 2% chick embryo extract. The cultures were maintained at 37 °C in an atmosphere of 5% CO₂, 95% air, in a water saturable incubator. The cultures were then processed for protein and CK activity. DNA synthesis was investigated on two cell culture models: myo-blast cultures (young cells) and muscle fibers (mature cells) grown in culture for 7 days.

DNA synthesis was determined in the cells by measuring the incorporation of ^3H -thymidine for 2–3 h in the growth medium (0.5 mCi/ml).

Laser irradiation was applied on the cells directly using a 632.8-nm, 5-mW He–Ne laser, and diameter of laser beam 2.7 mm \times 2.7 mm. Level measurements of DNA and CK in young and mature skeletal muscle cells were performed at seven time points: control (for young and mature cells, respectively) and 1, 3, 7, 10, 14, and 21 min of laser irradiation. Total CK activity was measured in the cell homogenate.

Statistical analysis and calculations were done using MatLab software (Ver. 2008b, The MathWorks, Inc.). We used nonparametric statistics since the number of rats in each sample time point was too

small to evaluate normal deviation. The figure presentation is aligned with our statistics, thus all figures are presented with Median \pm Mad. All significance levels were calculated using a Mann–Whitney U test when samples are independent observations (e.g., when comparing the results between rats that were treated with laser and ones that had no treatment) and Wilcoxon signed-rank test when comparing two related samples (e.g., when comparing the results between laser-irradiated and nonirradiated leg of the same rat). Correlations between muscles in the same rat (laser-irradiated and nonirradiated) were calculated to evaluate the dynamic change of AChR and CK in time.

In vivo muscle response. Figures 4.1 and 4.2 present qualitative changes in amount of AChR and CK activity in irradiated (first 14 days) and non-irradiated intact gastrocnemius muscles. Laser irradiation significantly increased CK activity ($p < 0.05$) and AChR level ($p < 0.01$) in time periods of 30–60 days in comparison with the nonirradiated gastrocnemius muscles.

In vitro muscle cells response. Figure 4.3 shows the effect of 632-nm laser irradiation on the synthesis of DNA in young and mature skeletal muscle cells at seven time points (control, 1, 3, 7, 10, 14, 21 min of irradiation). Similar increase in DNA synthesis in both groups was found with tendency to decrease during the radiated time. Figure 4.4 shows the effect of 632-nm laser irradiation on the CK activity in young and mature skeletal muscle cells at seven time points (control [4;4 for young and mature cells, respectively]; min 1 [n ¼0;4]; min 3 [n ¼4;4]; min 7 [n ¼3;4]; min 10 [n ¼0;4]; min 14 [n ¼3;2]; min 21 [n ¼3;3]). In the laser-irradiated mature muscle fibers, the activity of CK was more expressed than in young cells and control level. Figure 4.5 shows in comparison the effect of 632-nm laser irradiation on CK activity and DNA synthesis in young and mature skeletal muscle cells. To evaluate the laser treatment effect, we calculated all cell results between 1 and 10 min as there were no trend change in these time points. The laser effect on CK activity increased in the mature cells in comparison with young cells ($p < 0.05$).

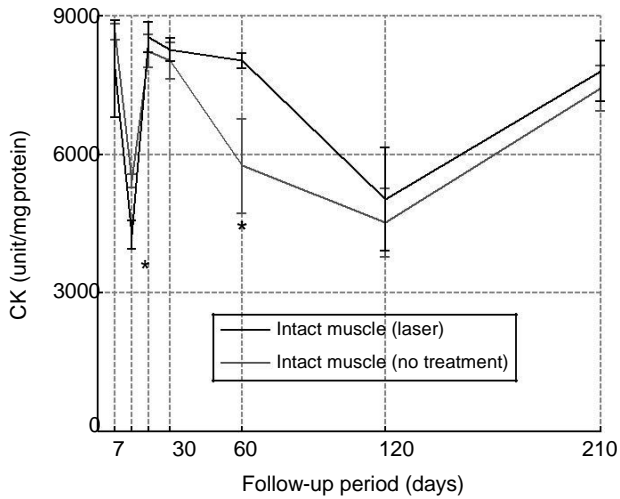


Figure 4.1 Longitudinal level measurements of CK levels in intact gastrocnemius muscle in rats that underwent laser irradiation (black lines) and rats that had no additional treatment (gray lines). Statistically significant change in CK levels between laser-irradiated intact muscles and nonirradiated intact muscles is presented with black asterisks. Asterisks denote significant p-values ($p < 0.05$; $p < 0.01$).

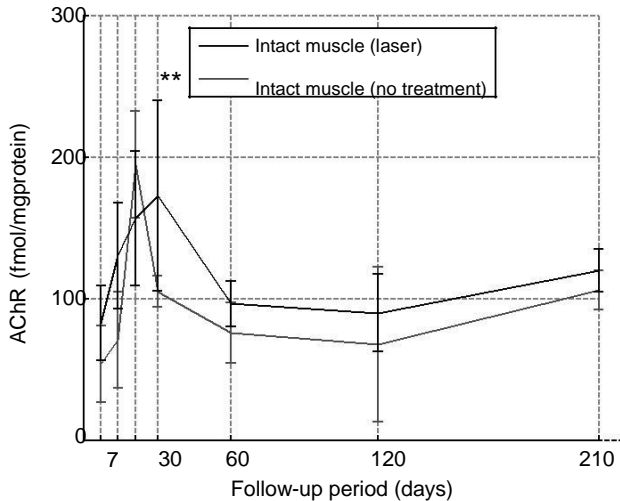


Figure 4.2 Longitudinal level measurements of AChR levels in intact gastrocnemius muscle in rats that underwent laser irradiation (black lines) and rats that had no additional treatment (gray lines). Statistically significant change in AChR levels between laser-irradiated intact muscles and nonirradiated intact muscles is presented with black asterisks. Asterisks denote significant p-values ($p < 0.05$; $p < 0.01$).

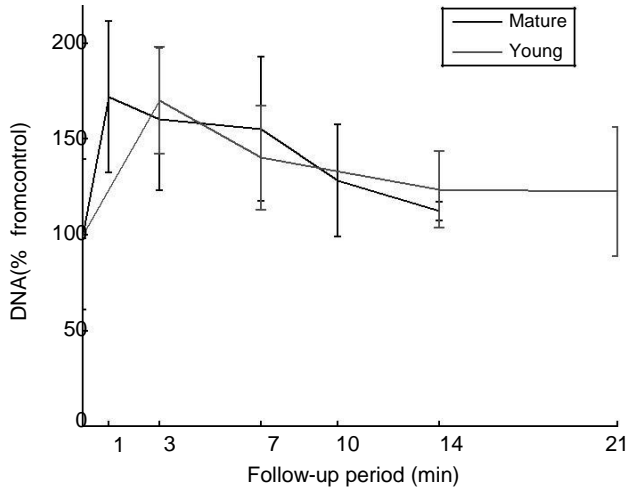


Figure 4.3 Effect of 632-nm laser irradiation on the synthesis of DNA in young and mature skeletal muscle cells. Longitudinal level measurements of DNA synthesis that underwent laser treatment at seven time points (control for young and mature cells, respectively), 1, 3, 7, 10, 14, and 21 min.

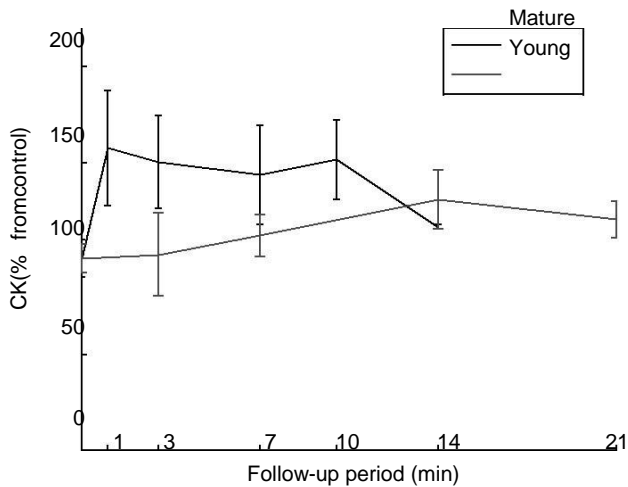


Figure 4.4 Effect of 632-nm laser irradiation on CK activity in young and mature skeletal muscle fibers. Longitudinal level measurements of CK level that underwent laser irradiation at seven time points: control (for young and mature cells, respectively), 1, 3, 7, 10, 14, and 21 min.

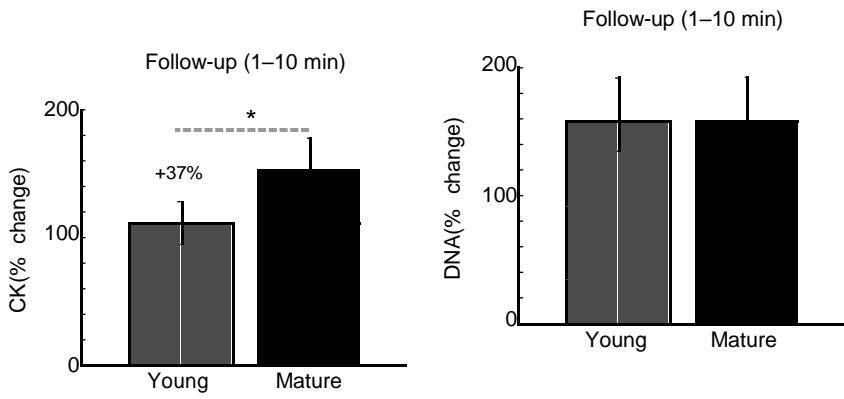


Figure 4.5 Comparative effect of 632-nm laser irradiation on CK activity and DNA synthesis in young and mature skeletal muscle cells. To evaluate the laser treatment effect, we calculated all cells results between 1 and 10 min as there is no trend of change in these time points. Asterisks denote significant p-values ($p < 0.05$).

3. DISCUSSION AND FUTURE PERSPECTIVES

In this study, we have investigated the influence of low-power laser irradiation on CK activity and the amount AChRs in intact gastrocnemius muscle (in vivo), and on the synthesis of DNA and CK in muscle cells (in vitro) in order to estimate biochemical transformation on cellular and biochemical levels.

Posttraumatic nerve repair and prevention of muscle atrophy represent a major challenge of restorative medicine. Among the possible explanations for the incomplete restoration of very long term denervated muscle are failures of regenerating nerves to reach all of the atrophic muscle fibers and establish mature muscle–nerve contacts (Fu and Gordon, 1995; Irintchev, Draguhn, & Wernig, 1990). It was shown earlier that the sites of former endplates could be detected in surviving skeletal muscle fibers even after 17 months (Sunderland and Ray, 1950) and 2 years of muscle denervation (Debkov, Kostrominova, Borisov, & Carlson, 2001). Nevertheless, it is very difficult to assess the functional condition of those long-term denervated sites of former neuromuscular junctions with respect to their capacity to accept growing axons if reinnervation was to occur. Considerable interest exists in the potential therapeutic value of laser phototherapy for restoring denervated muscle resulting from peripheral nerve injury. We have previously suggested that the function of denervated muscles can be partially preserved by temporary prevention of denervation-induced biochemical changes (Rochkind et al., 2009). The function of denervated muscles can be restored, not completely but to a very substantial degree, by laser treatment, initiated at the earliest possible stage postinjury. Iyomasa, Garavelo, Iyomasa, Watanabe, and Issa (2009) found that the treatment of lesioned muscles with low-level He–Ne laser therapy could increase mitochondrial activity in muscular fibers and activate fibroblasts and macrophages and stimulate angiogenesis. Schwartz, Brodie, Appel, Kazimirsky, and Shainberg (2002) found that, after irradiation of muscle cell cultures (632 nm, 3 J/cm², 20 mW), there was a rise in the levels of nerve growth factor, which is a neurotrophic factor secreted by skeletal muscles that influences the survival and regeneration of sympathetic and sensitive neurons in the

peripheral nervous system. Other neurotropic growth factors are also bio-stimulated by laser therapy, such as GAP-43 ([Shin et al., 2003](#)) and fibroblast growth factor ([Ihsan, 2005](#)).

The data collected from different experimental studies support our results and help to understand the mechanism of influence of low-power laser irradiation (visible and near-infrared wavelengths) and muscle tissue. It has been demonstrated that reactive oxygen species (ROS) formation and augmented collagen synthesis are elicited by traumatic muscular injury, effects that were significantly decreased by laser treatment ([Silveira et al., 2013](#)). Evaluation of mitochondrial respiratory chain complexes and succinate dehydrogenase activities after traumatic muscular injury shows that the laser treatment may induce an increase in ATP synthesis, and that this may accelerate the muscle healing process ([Silveira et al., 2009](#)) and delay fusion of cultured myoblasts ([Wollman and Rochkind, 1993](#)). The increase in muscle fibers area and mitochondrial density after laser treatment was reported after muscle toxic injury ([Amaral, Parizotto, & Salvini, 2001](#)). The process of regeneration in denervated muscles was markedly enhanced in muscle that was irradiated by laser prior to injury, probably by the activation (stimulation of proliferation and/or differentiation) of cells in the muscles that are “recruited” and participate in the process of regeneration ([Bibikova and Oron, 1995](#)). In model of prolonged muscle ischemia, laser treatment decreased posttraumatic changes in CK and lactate dehydrogenase ([Lakyová, Toporcer, Tomečková, Sabo, & Radoněk, 2010](#)). A positive effect on muscle metabolism was found after cryolesion injury, whereas Cyclo-oxygenase 2 (COX-2) immunoreexpression was lower in laser-treated group. COX-2 is a key enzyme in conversion of arachidonic acid to prostanoids ([Renno et al., 2011](#)). The expression of COX-2 is relevant to many pathological processes, including inflammation and tissue repair. In the present study, laser irradiation significantly increased CK activity and AChR level in time periods of 30–60 days in comparison with the nonirradiated gastrocnemius muscles. At the cell level, we also found increased DNA synthesis and CK activity in young and mature skeletal muscle. The induced biochemical changes may be attributed to trophic signal for increased activity of CK, thus preserving a reservoir of high-energy phosphate available for quick resynthesis of ATP. These findings are supported by early results by [Bolognani and Volpi \(1991\)](#) and [Passarella et al. \(1984\)](#) who showed that laser irradiation increased ATP production in the mitochondria. The present study and our previous publication ([Rochkind et al., 2009](#)) suggest that laser

photo-therapy may enhance biochemical activity of the muscle to overcome stress conditions.

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