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● *Original Contribution*

OPTIMAL ULTRASOUND EXPOSURE CONDITIONS FOR MAXIMIZING C2C12 MUSCLE CELL PROLIFERATION AND DIFFERENTIATION

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Abstract—Described here is an *in vitro* systematic investigation of the effects on C2C12 myoblasts of exposure to finely controlled and repeatable low-intensity pulsed ultrasound of different frequencies (500 kHz, 1 MHz, 3 MHz and 5 MHz) and different intensities (250, 500 and 1000 mW/cm^2). An in-house stimulation system and an ultrasound-transparent cell culture well minimized reflections and attenuations, allowing precise control of ultrasound delivery. Results indicated that a 3 MHz stimulation at 1 W/cm^2 intensity maximized cell proliferation in comparison with the other exposure conditions and untreated controls. In contrast, cell differentiation and the consequent formation of multinucleated myotubes were maximized by 1 MHz stimulation at 500 mW/cm^2 intensity. The highly controlled exposure conditions employed allowed precise correlation of the ultrasound delivery to the bio-effects produced, thus overcoming the inconsistency of some results available in the literature and contributing to the potential of ultrasound treatment for muscle therapy and regeneration. (E-mail: a.salgarella@santannapisa.it) © **2017 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Low-intensity pulsed ultrasound, Ultrasound stimulation, Frequency, Intensity, C2C12, Myoblasts, Muscle regeneration, Proliferation, Differentiation, Bio-effects.**

INTRODUCTION

Soft tissue regeneration is one of the main goals of tissue engineering and regenerative medicine. Such disciplines combine engineering and life sciences to restore, maintain, replace and improve the functions of tissues and organs that are lost because of injuries or diseases (Langer and Vacanti 1993; Wobma and Vunjak-Novakovic 2016). In particular, skeletal muscle regeneration represents an interesting research domain. Thanks to resident stem cells and precursor cells, skeletal muscle is constantly renewed in response to injury, damage or aging (Zouraq et al. 2013). According to Musarò (2014), muscle injury and regeneration processes can undergo five interrelated and time-dependent phases, the first of which is degeneration (necrosis), which is followed by inflammation, regeneration, remodeling and maturation/functional repair. After the intervention by macrophages for removal of necrotic cellular debris, satellite cells (stem cells localized between the basal lamina and the muscle fiber membrane) and other myogenic progenitors are activated and undergo proliferation, differentiation and fusion to one another or to undamaged portions of the fibers, thus forming new myofibers or myofiber segments (Ciciliot and Schiaffino 2010). Some of these processes reiterate the sequence of events observed during embryonic myogenesis (Musarò 2014). Severe damage from diseases such as muscular dystrophies or traumatic lesions such as contusions and strains (which are common in sports medicine) causes necrosis of myofibers, stimulates the inflammatory response and triggers the consequent regeneration process (Ciciliot and Schiaffino 2010). Several approaches have been proposed to improve and accelerate muscle regeneration. In the most critical cases, the self-repair capability of skeletal muscle can be seriously impaired by subject aging, severe muscle diseases such as congenital myopathies characterized by progressive muscle wasting and weakness or large loss of mass after trauma, aggressive tumor removal and prolonged denervation. In these cases, invasive regenerative medicine approaches can be pursued, and new genetic and cell therapy strategies have been proposed. For example, myogenic cells from embryonic stem cells and

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induced pluripotent stem cells have been considered for the reconstruction of whole muscle tissue parts, by engineering functional 3-D skeletal muscle tissues using biocompatible smart materials (Bach et al. 2004; Juhas et al. 2016; Tedesco et al. 2010). Along this research line, several efforts have focused on the role of surface topography (Altomare et al. 2010; Bajaj et al. 2011; Ricotti et al. 2012), material stiffness (Engler et al. 2004), surface electrical properties (Ricotti et al. 2011) and electromechanical inputs (van der Schaft et al. 2013) on myogenesis.

With respect to less severe muscle injuries, typical of sports activities, less invasive strategies have been suggested to improve tissue healing and regeneration. First, immediate treatment of an injured skeletal muscle usually follows the RICE principle (rest, ice, compression and elevation) with the aim of minimizing bleeding into the injury site. Then, different therapies can be exploited, such as rehabilitation exercises, non-steroidal anti-inflammatory drugs or glucocorticoids and hyperbaric oxygen therapy (Cheung et al. 2003; Järvinen et al. 2007). It has been reported that microcurrent electrical neuromuscular stimulation can reduce signs and symptoms of muscle damage, strongly suggesting that it can facilitate the regeneration of injured skeletal muscles (Fujiya and Goto 2016). Moreover, it is known that skeletal muscle is sensitive to mechanical stimulation, which can be provided by means of massage and exercise, by using smart materials or by exploiting therapeutic ultrasound (Cezar et al. 2016; Cheung et al. 2003; Järvinen et al. 2007; Teixeira and Duarte 2016).

Ultrasound is commonly used for imaging and diagnostic applications and has the advantage over other imaging modalities of being non-ionizing. It has been found that ultrasound can also be used for therapeutic purposes, in both low- and high-intensity ranges. Several bio-effects of ultrasound exposure at the tissue, cellular and protein levels have been reported in the literature (Khanna et al. 2009; Marchioni et al. 2009; Martin 2009). Healing properties have been assigned to low-intensity pulsed ultrasound (LIPUS) stimulation (Khanna et al. 2009). At the cellular level, LIPUS has been observed to increase proliferation and migration of aortic endothelial cells and increase proliferation of fibroblasts, Schwann cells and other cell types (Martin 2009).

With respect to muscle regeneration, as reported by Abrunhosa et al. (2014), therapeutic applications of ultrasound have been used in physiotherapy and rehabilitative settings since the 1950s to induce both thermal and mechanical bio-effects. Despite the wide use of this instrument in clinical practice, *in vitro* and *in vivo* studies aimed at evaluating the bio-effects triggered by ultrasound have had rather divergent results.

Concerning *in vivo* tests, Wilkin et al. (2004) and Markert et al. (2005) found no evidence that LIPUS enhances and accelerates skeletal muscle regeneration after contusion injury. In these studies, the authors stimulated rats at different intensities and in pulse mode at a frequency of 3.3 MHz and continuously at a frequency of 3 MHz. In contrast, Fisher et al. (2003) proved that rat muscles receiving pulsed ultrasound at a frequency of 870 kHz responded in a positive way if compared with the response to continuous stimulation and no stimulation (controls), with significant production of contractile proteins. Chan et al. (2010) reported enhancement of regenerative myofiber formation after injury when mice muscles were stimulated with LIPUS at a frequency of 1.5 MHz, whereas Matsumoto et al. (2014) suggested that pulsed ultrasound irradiation can inhibit the development of disuse muscle atrophy in rats (partly via activation of satellite cells) by stimulating muscles in continuous mode at 1 MHz. Nagata et al. (2013) observed a reduction in the number of inflammatory infiltrate cells and an increase in the size of regenerating myofibers in the experimental group of C57BL/6 mice treated with pulsed ultrasound at 1 MHz, in comparison with the control group.

With respect to *in vitro* tests, Chan et al. (2010) performed differentiation experiments on C2C12 cells, a murine skeletal muscle cell line, which exhibited an increase in myogenin protein during differentiation because of LIPUS treatment at 1.5 MHz. Nagata et al. (2013) observed enhancement of myogenin mRNA expression in the same cell line as a result of LIPUS treatment at 3 MHz, simulating an inflammatory environment. Abrunhosa et al. (2014) compared the effects of continuous and pulsed ultrasound treatment at a frequency of 1 MHz on primary cultures of chick skeletal muscle cells, determining in both cases an increase in differentiation in stimulated cells compared with non-stimulated control cells, with better results in the case of continuous stimulation. Ikeda et al. (2006) reported that LIPUS pushes the differentiation pathway of C2C12 cells more toward the osteoblast and/or chondroblast lineage than the myoblast lineage by activating phosphorylation of ERK1/2 and p38 MAPK.

The wide range of exposure parameters and metrics used in the studies discussed here makes it difficult to observe clear correlations between ultrasound exposure conditions and muscle regeneration efficacy. This is due mainly to the lack of standardization of stimulation protocols and the lack of systematic investigations that compare different exposure conditions. In addition, the different biological models used and the different types of injuries analyzed contribute to the incoherence of the results. As reported by Abrunhosa et al. (2014), ultrasound is broadly used in the frequency range