Biological response \textit{in vitro} of skeletal muscle cells treated with different intensity continuous and pulsed ultrasound fields

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Abstract. Therapeutic ultrasound has been used in physiotherapy to accelerate tissue healing. Although the ultrasonic wave is widely used in clinical practice, not much is known about the biological effects of ultrasound on cells and tissues. This study aims to evaluate the biological response of ultrasound in primary cultures of chick myogenic cells. To ensure the metrological reliability of whole measurement process, the ultrasound equipment was calibrated in accordance with IEC 61689:2007. The skeletal muscle cells were divided in four samples. One sample was used as a control group and the others were submitted to different time and intensity and operation mode of ultrasound: 1) 0.5 W/cm\textsuperscript{2} continuous for 5 minutes, 2) 0.5 W/cm\textsuperscript{2} pulsed for 5 minutes, 3) 1.0 W/cm\textsuperscript{2} pulsed for 10 minutes. The samples were analyzed with phase contrast optical microscopy before and after the treatment. The results showed alignment of myogenic cells in the sample treated with 0.5 W/cm\textsuperscript{2} continuous during 5 minutes when compared with the control group and the other samples. This study is a first step towards a metrological and scientific based protocol to cells and tissues treatment under different ultrasound field exposures.

1. Introduction

Skeletal muscle’s function are locomotor activity, postural behavior and assist breathing. After direct trauma or resulting from indirect causes such as neurological dysfunction or innate genetic defects, the skeletal muscle is affected by injury (Charge & Rudnicki, 2004).

In physiotherapy, the ultrasound wave has been used over then 50 years, such as therapeutic appeal, for the treatment of injuries. Commonly, this has been used in rehabilitation for care skeletal muscle disordered. Depending on the treatment and degree of heating in tissue, this equipment can operate in two forms: athermal or thermal. The athermal ultrasound wave is characterized by increase the temperature in tissue less than 1\degree C, for that, the pulsed mode of application is used. The thermal effects produced in tissue are usually applied in continuous mode application (Locke & Nussbaum, 2001). However, the degree of heating depends not only in the intensity of the ultrasound wave but
also in the duration of application, the correct setting of these parameters is important to avoid irreversible changes in the biological tissue (Zeqiri, 2007).

The ultrasound wave produces an acoustic vibration and this effect induces changes in the cell membrane, such as an increase in cell permeability, and changes in ionic concentrations within the cell (Locke & Nussbaum, 2001; Ter Haar, 1998). Moreover, it has been shown that the ultrasound wave induces an increase in protein synthesis and in the levels of intracellular calcium, promotes fibroblasts activity and angiogenesis, accelerates the healing of bone fractures, among other functions. Despite these effects, there is no scientific evidence to explain how the ultrasound wave interacts with tissue (Nishikori et al., 2001). To avoid damage in the biological tissue, the absolute maximum effective intensity should be less than or equal to 3.0 W/cm$^2$ (IEC 61689, 2007). Laboratories with recognized expertise in the area have procedures and systems for reliable measurement evaluate this and other metrological parameters of physiotherapy equipment (Alvarenga & Costa-Felix, 2009). There is no agreement in the literature on the best way to treat each lesion. The present study was performed to examine the interference of different ultrasound treatment conditions on a model of in vitro growth of primary skeletal muscle cells.

2. Experimental Procedure

2.1. Primary skeletal muscle cell culture

Primary cultures of mononucleated cells were prepared from pectoral muscles of 11-day-old chick embryos (Mermelstein et al., 2005) from Tolumeci’s farm (Rio de Janeiro, Brazil). Fragments of breast muscle were incubated in a plate for 15 min under a humidified 5% CO2 atmosphere at 37°C with calcium-magnesium-free solution (CMF) containing 0.25% trypsin (Sigma, USA), to assist the dissociation of cells. The contents of the plate was placed in a tube with the culture medium 8-1-0.5 (80% minimum essential medium – MEM, 10% horse serum and 0.5% chick embryo extract), and the cells were centrifuged. Trypsin activity was stopped by addition of this culture medium. Cells collected in the pellet were dispersed by repeated pipetting with culture medium. The resulting suspension was filtered, and the cells were plated at an initial density of $5 \times 10^5$ cells/35-mm culture dishes (Corning, EUA) previously coated with rat-tail collagen. Cells were grown under a humidified 5% CO2 atmosphere at 37°C for 24 hours, following which ultrasound wave treatment was performed.

2.2. Treatment with ultrasound waves

Before treatment, ultrasonic transducer was evaluated according to IEC 61689:2007. The transducer diameter of this equipment was 35 mm and operating at frequency of approximately 1 MHz. The coupling between the transducer and culture dish was achieved by the use of vaseline. The cell cultures were divided in four different treatment groups, in which the duration and the intensity of the ultrasound waves were different. One group was used as a control (with no treatment) and the others received treatments according to table below. Cells that were grown for 24 hours were subjected to ultrasound wave treatment.

| Mode of Operation | Intensity (W/cm²) | Time of Treatment (Minutes) |
|-------------------|------------------|---------------------------|
| Pulsed            | 0.5              | 5                         |
| Pulsed            | 1.0              | 10                        |
| Continuous        | 0.5              | 5                         |
2.3 Phase contrast optical microscopy
To investigate the possible changes in cell morphology and differentiation, skeletal muscle cells (treated and untreated) were analyzed in an inverted optical microscope (Axiovert 100, Carl Zeiss, Germany). Cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, followed by membrane permeabilization with 0.5% Triton X-100 in PBS (3 times for 10 min each) at room temperature (Mermelstein et al., 2005). Phase contrast images were acquired immediately before, immediately after, and 24 and 48 hours after the ultrasound wave treatments.

Figure 2 – Fluxogram showing when the images were acquired with and without ultrasound treatment.

3. Preliminary results and discussion
Chick embryonic skeletal muscle cells grown in culture were analyzed after the different ultrasound wave treatments. It was observed that low intensity-pulsed ultrasound wave induces cell proliferation, whereas low intensity-continuous ultrasound wave induces muscle differentiation. Furthermore, high intensity-pulsed ultrasound wave induced cellular death. Control and treated cells are currently being analyzed by indirect immunofluorescence microscopy, SDS-PAGE electrophoresis and Western blotting, in order to study the molecular and cellular basis associated with the observed changes in the cell cultures.
Figure 3 – (a) Control cell culture grown for 24 hours. (b) The same culture as shown in (a) was observed after 72 hours.

Figure 4 – (a) Cell culture treated with continuous ultrasound treatment (0.5W/cm$^2$ intensity) during 5 minutes. (b) The same culture as shown in (a) was observed after 48 hours of ultrasound treatment.

Figure 5 – (a) Cell culture treated with pulsed ultrasound treatment (1.0 W/cm$^2$ intensity) during 10 minutes. (b) The same culture as shown in (a) was observed after 48 hours of ultrasound treatment.

Figure 6 – (a) Cell culture treated with pulsed ultrasound (0.5 W/cm$^2$ intensity) during 5 minutes. (b) The same culture as shown in (a) was observed after 48 hours of ultrasound treatment.
4. Conclusion
This work concludes that depends on operated mode of equipment, the intensity adjusted and the
duration of treatment the biological response of ultrasound in primary cultures of chick myogenic cells
will be different.

Acknowledgements
This work was supported by National Institute of Metrology, Standardization, and Industrial Quality
(Inmetro) and Fundação Carlos Chagas Filho de Apoio à Pesquisa do Estado do Rio de Janeiro
(FAPERJ)

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