Effect of low-intensity pulsed ultrasound on l929 fibroblasts

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Abstract

Introduction: Ultrasound has proven to be an important therapeutic resource regarding musculoskeletal disease and is routinely used in physical therapy and medicine both therapeutically and diagnostically. The aim of the present study was to analyse the effects with different ultrasound intensities in order to establish the ideal radiation level in cell cultures.

Material and methods: Fibroblast cell cultures were divided into five groups: group I – control (did not receive irradiation); group II – 0.2 W/cm^2 in pulsed mode at 10% (1 : 9 duty cycle); group III – 0.6 W/cm^2 in pulsed mode at 10% (1 : 9 duty cycle); group IV – 0.2 W/cm^2 in pulsed mode at 20% (2 : 8 duty cycle); and group V – 0.6 W/cm^2 in pulsed mode at 20% (2 : 8 duty cycle). Each group was irradiated with 24-h intervals, observing the following post-irradiation incubation times: 24, 48, 72 and 96 h; after 24 h of each irradiation, cultures were analysed using the MTT method.

Results: Analysis of the results following ultrasound irradiation demonstrated that the effect of ultrasound with 0.6 W/cm^2 in pulsed mode at 10% (1 : 9 duty cycle) was statistically significant in relation to ultrasonic irradiation in pulsed mode at 20% (2 : 8 duty cycle) ($p < 0.05$).

Conclusions: According to parameters used in the irradiation of cultivated fibroblasts, the pulse mode regime and the control of intensity are of fundamental importance for the optimal use of therapeutic ultrasound. Furthermore, low and medium intensities decreased cell damage, which establishes that acoustic pulsed energy induces the proliferation of fibroblast cells.

Key words: low-intensity pulsed ultrasound, inflammation, healing, repair, fibroblasts.

Introduction

Ultrasound has proven to be an important therapeutic resource regarding musculoskeletal disease and is used both therapeutically as well as diagnostically in medicine. Ultrasound delivers energy through a pressure field generated by the transducer that causes the molecules in the transmission medium to oscillate or vibrate. Mechanical stimulation of cell membranes occurs during the energy delivery process [1].

Ultrasound was introduced into the biological field about 70 years ago and is now widely used. However, research continues to be carried out to explore its field of action and get a better understanding of its effects and interaction with the biological medium. Despite the various advances, the

actual mechanisms of interaction between ultrasound/cavitation and cells remain far from being understood. Moreover, the manner in which ultrasound and cavitation augment cell membrane permeability is not yet clear. This is likely the result of a lack of methods for real-time monitoring of cavitation at the cell level [2].

Nonetheless, ultrasound is an indispensable tool for physical therapists and is commonly used to help in the treatment of soft tissue dysfunctions, including contractures, joint injuries, tendonitis, bursitis, musculoskeletal spasms and pain. It has good ability to penetrate tissue and can be focused into a small volume to give very high energy locally. This is an attractive characteristic from a clinical viewpoint, and has prompted extensive applications of low-intensity pulsed ultrasound (LIPUS) for therapeutic purposes in clinical practice. With regard to the physical effects produced by ultrasound, a significant response is obtained from biological tissues (thermal and mechanical) [3-5].

According to Milowska [6] and O'Brien Jr [7], mechanisms of action of ultrasound with biological means can be divided into thermal effects and non-thermal. Thermal acoustical effects occur when the energy is absorbed and transformed into heat; it depends on the absorption and dissipation of energy through the ultrasound. However, non-thermal effects cannot be classified as cavitational due to training and concentration of bubbles.

Ultrasonic irradiation leads to angiogenesis stimulation, increased blood circulation, and accelerated repair of fractures with the retardation of consolidation and pseudo-arthrosis [8, 9]. It also inhibits inflammatory activity, resulting in the production of chemical mediators that activate the proliferation of fibroblasts [10]. This activation leads to the early accumulation of endothelial cells in the tissue as well as the promotion of collagen synthesis through the stimulation of calcium influx and a change in membrane permeability, thereby favouring the synthesis and maturation of collagen as well as the formation of scar tissue. Moreover, collagen deposited following the application of therapeutic ultrasound is more resistant and better organized [11, 12].

Fibroblasts are the major cell type in connective tissue and are critical for tissue restoration and remodelling after injury [1, 13-15]. Thus, the aim of the present study was to analyse the effects with different ultrasound intensities in order to establish the ideal radiation level in cell cultures.

Material and methods

Cell culture

L929 fibroblast cells (Mouse conjunctive tissue – ATCC CCL-1 NCTC) (Instituto Adolfo Lutz – SP, Brazil) were routinely cultured in 25 cm^2 flasks (TPP, Switzerland, Europe) with MEM (Minimum Essential Medium) (Gibco^{TM} - Invitrogen Corporation, Grand Island, USA), supplemented with 10% FBS (fetal bovine serum) (Cultilab, Brazil) and kept in a humidified 5% CO_2 atmosphere at 37°C. The protocol was approved by the Research Ethics Committee of Univap, Protocol n° A061/CEP/2006. Conjunctive tissue of mouse cells was used in this experiment, since ISO 10993-5 recommends the use of this cell line for *in vitro* toxicity tests [16].

Ultrasound

Ultrasound KLD® – Biosistemas Equipamentos Eletrônicos Ltda, Brazil, model Avatar III, with one 1 MHz transducer and an ERA (effective radiation area) of 1 cm^2, duly calibrated by the manufacturer.

Irradiation

Before receiving ultrasonic irradiation, L929 cell cultures were sub-cultivated in four 12-well 100 mm^2 TPP® plates at a density of 1×10^6 cell/ml and separated into five groups: group I – control (did not receive irradiation); group II – 0.2 W/cm^2 in pulsed mode at 10% (1 : 9 duty cycle – 1 ms and 9 ms work interval); group III – 0.6 W/cm^2 in pulsed mode at 10% (1 : 9 duty cycle – 1 ms and 9 ms work interval); group IV – 0.2 W/cm^2 in pulsed mode at 20% (2 : 8 duty cycle – 2 ms and 8 ms work interval); and group V – 0.6 W/cm^2 in pulsed mode at 20% (2 : 8 duty cycle – 2 ms and 8 ms work interval). Each group was irradiated with 24-h intervals, observing the following times of incubation after irradiation: 24 h, 48 h, 72 h and 96 h; 24 h after each irradiation the cultures were analysed by the MTT method. For a good coupling of the ultrasound interface (transducer distance – 18 mm from the cell culture, according to the manufacturer's specifications) and propagation of mechanical waves, well volumes were completed to the brim with MEM medium; the irradiated well was always kept in the same position in relation to the transducer face of the ultrasound; each well received a 2-min irradiation at room temperature and plate heating was disregarded. The experiment was carried out in triplicate.

MTT cellular cytotoxicity test

The cytotoxicity experiments evaluated cultures that received ultrasonic irradiation at intervals of 24 h, 48 h, 72 h and 96 h through the MTT method [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] [17]; the MTT test was carried out 24 h after each irradiation according to the following assay: after the MEM medium was removed, each well received 80 µl of MTT to obtain a final concentration of 0.5 mg/ml and was incubated for 1 h at 37°C in a 5% CO_2 atmosphere. After this

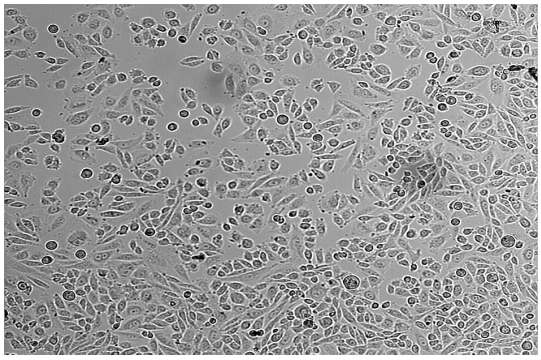

Figure 1. Cell cultures submitted to low-intensity ultrasound irradiation maintained shape and cell integrity

400 µl of DMSO (dimethyl sulfoxide) were added to each well. The plate was kept in agitation for 30 min for solubilization of formazan crystals. The crystal concentration was spectroscopically quantified by means of a microplate reader (Reader ELISA – SpectraCount – Packard Instrument, USA) at a wavelength of 570 nm.

Statistical analysis

Results are expressed as mean values ± SEM. Comparisons between groups were made using one-way analysis of variance (ANOVA) and *post hoc* Tukey-HSD test. Analysis was used to determine significant differences between groups. Values of $p < 0.05$ were considered statistically significant. Values were analysed using the GraphPad Prism 4.0 Demo statistical package.

Results

Analysis of results after LIPUS irradiation revealed that irradiated cells maintained their shape and cell integrity (Figure 1). The data demonstrate that L929 cell cultures submitted to low-intensity ultrasonic irradiation (0.2 W/cm^2 to 0.6 W/cm^2) maintained cellular morphology, thereby corroborating the results described by Hsieh [18].

The results reveal that irradiation with MTT reflected the number of living cells in the culture. There were significant increases after analysing the effect of LIPUS at 10% (1 : 9 duty cycle) and 20% (2 : 8 duty cycle) on fibroblast culture (Figure 2).

The following results were obtained after analysing the effect of LIPUS at 10% (1 : 9 duty cycle) on fibroblast cultures. After 24 h, the group of cells irradiated at an intensity of 0.2 W/cm^2 to 0.6 W/cm^2 exhibited increased cellular viability when compared with data for the group of non-irradiated cells (p = 0.001). However, when comparing the effect of irradiated cells at 0.2 W/cm^2 and 0.6 W/cm^2, the results did not show statistical significance (p = 0.06). After 48 h, these groups of cells exhibited the same behaviour (non-irradiated and irradiated at 0.2 W/cm^2 and 0.6 W/cm^2) as during the first 24 h (p = 0.001). Seventy-two h after irradiation, group II and group III irradiated cells exhibited a significant increase in cell viability when compared with viability values from the control group (non-irradiated) (p = 0.001). An interesting fact about this period of time was the significant increase in cell viability exhibited by cells irradiated at intensity of 0.6 W/cm^2 when compared with values for cells irradiated at 0.2 W/cm^2 (p = 0.01) 96 h after the first irradiation. The groups of cells irradiated at 0.2 W/cm^2 and 0.6 W/cm^2 tended to maintain their growth or pattern the same way as presented in the analysis 72 h after irradiation (Table I).

The following results were obtained after analysing LIPUS mode at 20% (2 : 8 duty cycle). After 24 h, irradiated group IV and group V cells exhibited an increase in cell viability when compared with data for the group of non-irradiated cells (p = 0.01). Cells irradiated at 0.2 W/cm^2 exhibited a significant increase in cell viability when compared with values for cells irradiated at 0.6 W/cm^2 (p = 0.01). Forty-eight h after irradiation, cells maintained the same growth

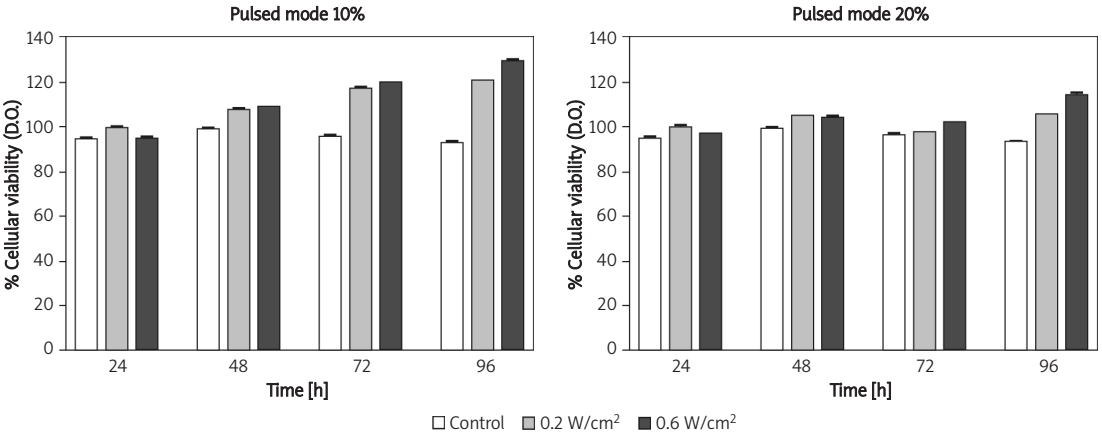

Figure 2. Cellular growth due to ultrasonic irradiation and cultivation time; cellular viability of control populations

Table I. Significance values obtained from variance analysis of means of experiments by one-way ANOVA and Tukey's HSD *post hoc* test, for cells irradiated by ultrasound in pulsed mode at 10% (1 : 9 duty cycle)

Time [h]	Control vs. 0.2 W/cm^2	Control vs. 0.6 W/cm^2	0.2 W/cm^2 vs. 0.6 W/cm^2
24	0.001*	0.001*	0.06
48	0.001*	0.001*	0.065
72	0.001*	0.001*	0.01*
96	0.001*	0.001*	0.01*

**For values with statistical significance*

pattern. However, when comparing the effect between cells irradiated at 0.2 W/cm^2 and 0.6 W/cm^2, the results revealed no statistical significance (p = 0.08). In the 72-h period, behaviour was constant, similar to the 48-h period after the first irradiation. However, it should be pointed out that there was a discrete tendency towards a decrease in cell growth values after 48 h. After 96 h, growth values for cells irradiated at 0.2 W/cm^2 and 0.6 W/cm^2 were significantly higher than those for non-irradiated cells (control) (p = 0.001). There was significantly higher cell growth among cells irradiated at 0.6 W/cm^2 than cells irradiated at 0.2 W/cm^2 (Table II).

Discussion

Ter Haar [19] observed that pulsed ultrasound may alter the cell structure and functioning. This is due to its transient cavitation effect as well as a change in volume and pressure caused by bubbles formed in the liquid medium, which when hitting one another release energy that may break chemical bonds, thereby producing reactive free radicals and provoking chemical changes in the cells. A change in pressure exerted by bubbles may modify the permeability of the cellular membrane to calcium and sodium ions, increasing protein synthesis. Furthermore, organelles may be altered due to irradiation forces.

A number of research groups have shown that bubbles under LIPUS scanning conditions increase the permeability of the cell membrane to external substances and enhance their uptake in the cavitation process [2]. According to Kodama *et al.* [20] the viability of cells is different at each position. This would appear to indicate that fluid motion is involved in cell membrane damage and subsequent molecular delivery.

Zhou *et al.* [13] showed that a single low-intensity pulsed treatment is able to promote DNA synthesis in fibroblast cells. Daily repeated ultrasound stimulation, which resembles the clinical schedule, substantially increases cell numbers, demonstrating that a physical stimulus (acoustic pulsed energy) can promote cell proliferation, which is a prerequisite for injury healing.

The athermal mechanisms have a considerable effect on therapeutic results (tissue regeneration), increasing permeability, diffusion of the cell membrane and intracellular calcium as well as changing the electric activity of the tissue [18, 21].

However, several parameters must be carefully controlled in order to minimize cell damage, including the acoustic pressure level, flow properties and temperature [22]. Furthermore, a wide range of ultrasound parameters have been tested by varying acoustic frequency, pressure, energy exposure time and duty cycle. Experimental systems and acoustic conditions have generally been designed to produce cavitation, which is believed to be responsible for bioeffects [23]. The destructive effect of ultrasound is likely to occur under the influence of high intensities, which suggests that cavitation is the physical mechanism responsible for cell alterations. Membrane alterations and the consequent destruction of several kinds of chicken embryo cells submitted to ultrasound irradiation have been observed [24].

According to Demir *et al.* [25] and Lowe *et al.* [26], ultrasound has demonstrated efficiency in the stimulation of fibroblasts, establishing that intensities from 0.1 W/cm^2 to 0.5 W/cm^2 accelerate

Table II. Significance values obtained from variance analysis of means of experiments by one-way ANOVA and Tukey's HSD *post hoc* test, for cells irradiated by ultrasound in pulsed mode at 20% (2 : 8 duty cycle)

Time [h]	Control vs. 0.2 W/cm^2	Control vs. 0.6 W/cm^2	0.2 W/cm^2 vs. 0.6 W/cm^2
24	0.01*	0.01*	0.01*
48	0.01*	0.01*	0.08
72	0.01*	0.01*	0.068
96	0.001*	0.001*	0.001*

**For values with statistical significance*

the inflammatory phase of repair, suggesting that ultrasound intensities of about 0.5 W/cm^2 in pulsed mode and a frequency of 1 MHz or 3 MHz promote healing.

Marking with rhodamine-phalloidin allowed the analysis of alterations in the cell structures of a single cell. The data obtained from the groups that received ultrasonic irradiation indicate that intensities between 0.2 W/cm^2 and 0.6 W/cm^2 are beneficial to cells [27]. This corroborates the work of Oliveira *et al.* [28], which established that energy levels of 0.2-0.6 W/cm^2 of ultrasound were quite effective in stimulating fibroblasts and cell repair.

Dyson *et al.* [29] report that an increase in intensity tends to reduce the number of viable cells; cell death in the 1st h after irradiation suggests an alteration in the membrane of cultivated cells.

Thus, based on therapeutic advances resulting from technological progress in recent decades, ultrasound is a resource that has achieved good results as a healing therapy. In the inflammatory phase of tissue repair, an acceleration of the process is observed, increasing the release of growth factors through the degranulation of mastocytes, platelets and macrophages. In ultrasound therapy, the proliferation phase is begun early, reducing its duration, and is followed by the re-modelling phase. Hence, scar contraction is an important step that seems to accelerate the release of these growth factors stimulated by the ultrasound [11].

In conclusion, according to parameters used in the irradiation of cultivated fibroblasts, the pulse mode regime and the control of intensity are of fundamental importance for the optimal use of therapeutic ultrasound. Furthermore, low and medium intensities decreased cell damage, which establishes that acoustic pulsed energy induces the proliferation of fibroblast cells and provides a better knowledge of the cellular behaviour and a molecular basis for the clinical observation that ultrasound treatment of wounds promotes tissue repair.

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