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The influence of ultrasonic radiation of low intensity in cultured fibroblast cells

A influência da irradiação ultrassônica de baixa intensidade em cultura de células fibroblásticas

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ABSTRACT

Introduction: In vitro and in vivo put in evidence that the Low Intensity Pulsed Ultrasound therapy exerts a significant influence on cell function (cytoskeleton organization, stimulation of mitochondrial activity, ATP levels and plasma membrane). **Objective:** This study will analyze the radiation of low intensity pulsed ultrasound in fibroblast cells L 929. **Method:** In this study are presented the data from each exposure group average and standard deviation in each moment of evaluation (24 hours, 48 hours and 72 hours). The control group (received no radiation), 0.2 W/cm2 with 10% pulse regime (1: 9 duty cycle), 0.2 W / cm2 with 20% pulse regime (2: 8 cycle work), 0.4 W/cm2 with pulse scheme 10% (1: 9 duty cycle), 0.4 W/cm2 with pulse scheme 20% (2: 8 duty cycle). The analyzes will be performed through optical microscopy, MTT method 3 - (4,5-dimethylthiazol-2-yl) -2,5 diphenyl tetrazolium bromide, within the incubation times of 24, 48 and 72 hours. **Results:** Given the above study, the results presented in this project will be directed to increase the stimulation process and proliferation of fibroblast cells from the pulsed ultrasonic irradiation of low intensity, correlating with the healing process, neovascularization and repair. **Conclusion:** Therefore, the study of the effect of ultrasound from cell culture provides us with a simple and informative model on the significant aspects of the use of physical therapy in vivo system.

Keywords: Therapy ultrasonic; Fibroblasts; Cell culture technique.

RESUMO

Introdução: Estudos *in vitro e in vivo* colocam em evidencia que a Terapia de Ultrassom Pulsado de Baixa Intensidade exerce uma influência significativa sobre função celular (organização do citoesqueleto, a estimulação da atividade mitocondrial, níveis de ATP e membrana plasmática). **Objetivo:** A proposta deste estudo será analisar a irradiação do ultrassom pulsado de baixa intensidade em células fibroblásticas L 929. **Métodos:** Nesse estudo são apresentados os dados de cada grupo de exposição em média e desvio-padrão em cada momento de avaliação (24 horas, 48 horas e 72 horas). O grupo Controle (não recebeu radiação), 0,2 W/cm^2 com regime de pulso de 10% (1:9 duty cycle), 0,2 W/cm^2 com regime de pulso de 20% (2:8 duty cycle), 0,4 W/cm^2 com regime de pulso de 10% (1:9 uty cycle), 0,4 W/cm^2 com regime de pulso de 20% (2:8 duty cycle). As análises serão realizadas através de microscopia óptica, MTT método 3 - (4,5-dimethylthiazol-2-il) -2,5 diphenyl tetrazolium bromide, respeitando os tempos de incubação de 24, 48 e 72 horas. **Resultados:** Diante do estudo exposto, os resultados apresentados nesse projeto serão direcionados para o incremento do processo de estimulação e proliferação de células fibroblásticas a partir da irradiação ultrassônica pulsada de baixa intensidade, correlacionando com o processo de cicatrização, neovascularização e reparo. **Conclusão:** Portanto, o estudo do efeito do ultrassom a partir de cultura de células nos fornece um modelo simples e informativo sob os aspectos significativos do uso da terapia física no sistema *in vivo.*

Palavra-chave: Terapia ultrassônica; Fibroblastos; Técnica de cultura celular.

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INTRODUCTION

The therapeutic ultrasound (TUS) low intensity pulsed is constantly used as a treatment for acute or chronic disorders of the musculoskeletal system.[1] After all, this feature provides the stimulation of collagen synthesis in fibroblast and osteoblast cells, resulting thus in the optimization of tissue formation.[2,3]

Through the thermal and non-thermal mechanism, US act on cells causing disruption of various biological barriers such as the cellular membranes, altering the rate of diffusion and permeability of the membrane, triggering the cascade of anti-inflammatory reactions, with increased synthesis proteins, including collagen.[4,5]

Among the effects of ultrasonic applicability, it can be noted a significant increase in local metabolism of multiple tissues, leading to cell regeneration, through the efficacy in modulating microcirculation and vascular permeability, allowing the improvement of angiogenesis, increase in granulation tissue, increasing healing.[6,7,8]

According to Johns (2002), determination of cell culture is described as a device that provides review and qualify several changes occurring in vitro and in vivo studies, and among the in vitro studies the effect of ultrasound results from the cell type and the parameter used.[5,9]

Thus, Pires-Oliveira et al. (2009) to irradiate cultured fibroblasts, they found that the dosimetry, the system clock and control the intensity are crucial in improving the use of therapeutic ultrasound. In addition, low and medium intensity decrease cell damage, stating that the acoustic energy pulse induces the proliferation of fibroblast cells. This fact provides a better knowledge of the behavior of cells and a higher molecular basis for the clinical observation that treatment with ultrasound in wounds promotes the repair of tissues.[10]

Thus, compared to the existence of a parameter window well below the ultrasound 1MHz frequency due to cavitation without significant cytotoxicity, shows the importance of the association of research between the action of therapeutic ultrasound and fibroblast cells. (10) Thus, the objective of this study was to analyze the cellular response after pulsed ultrasonic irradiation of low intensity L929 fibroblast cell culture.

METHOD

To carry out this experimental study were used fibroblast cells of connective tissue of mice - L929 lineage (ATCC CCL-1 NCTC) provided by the Instituto Adolfo Lutz-SP, Brazil. The study was approved by the Ethics Committee of Universidade Norte do Paraná (UNOPAR) under the Protocol 462478/2013.

Cell Cultivation

For cultivation, cells were routinely maintained in 25cm^2 dish (TPP, Switzerland, Europe) associated with MEM (Minimum Essential Medium, Gibco® - Invitrogen Corporation Grad Island, USA) supplemented with 10% Fetal Bovine Serum (CULTILAB, Brazil) and 1% antibiotic-Antimycotic (Gibco®, by Life Technologies), so that CO2 remained in greenhouses, in an atmosphere of 5% at 37 ° C (Thermo Forma Scientific, Waltham, MA). The cells used in this experiment followed the recommendations for use for in vitro toxicity test set out in ISO 10993-5.

Ultrasound

KLD® brand of equipment was used - (Biosistemas Electronic Equipment Ltda - Amparo SP-Brazil), model Avatar III, with 1 MHz head and Effective Radiation Area (ERA) of 1 cm2, calibrated by the manufacturer to perform of ultrasonic irradiation.

Irradiation

For the ultrasonic irradiation was used TPP 12-well plates with 24 mm diameter and 18 mm deep, containing 1x106 cells / ml. In order to evaluate the ultrasound stimulatory band The following groups were formed:

Control (not receive radiation)
0.2 W / cm2 with pulse scheme 10% (1: 9 duty cycle)
0.2 W / cm2 with pulse scheme 20% (2: 8 duty cycle)
0.4 W / cm2 with pulse scheme 10% (1: 9 duty cycle)
0.4 W / cm2 with pulse scheme 20% (2: 8 duty cycle).

For there to be a good coupling of the transducer interface (distance from the transducer to the layer of cells: 18 mm) and propagation of the mechanical wave, each pit has had its volume with MEM medium to the edge and the face of the ultrasound transducer, kept in the same position in relation to the irradiated pit.

During the course of the experiment, the culture medium was changed every 48 hours and on the application time, each well was irradiated for two minutes at room temperature. All experiments were performed in triplicate and after each period, cultures were evaluated by MTT cytotoxicity test.

Cell Cytotoxicity test by MTT

The cytotoxicity tests were performed by the method of MTT [3- (4,5-dimethylthiazol) -2,5-diphenyltetrazolium bromide]. The L929 cell cultures received ultrasonic irradiation at intervals of 24, 48 and 72 hours, and after 24 hours of each irradiation was performed MTT assay according to the following test: after removing the MEM medium, each well received 80 uL of MTT, a final concentration of 0.5mg / ml and incubated for 1 hour at 37 ° C in an atmosphere of 5% CO2; then was added to each well 400 uL of dimethyl sulfoxide (DMSO). The plates were subjected to stirring for 30 minutes in order to solubilize the formazan crystals. Thus, its concentration was measured spectroscopically by a microplate reader (ELISA Reader - SpectraCount - Packards istrument, Offeburg - Germany) at a wavelength of 570 nm.

Statistical Analysis

IBM SPSS version 20.0 statistical package was used. Initially held the Shapiro-Wilk test to verify the normality, like all values were normally distributed, the data are presented as mean and standard deviation, in addition, there was the ANOVA of

one factor test in three time periods (24 hours, 48 hours and 72 hours) to compare the different types based display in two comparison groups (Ultrasound low intensity pulsed mode at 10% and ultrasound of low intensity pulsed mode at 20%) and checking intergroup with equal dosages and different pulses we used the t test unpaired, 95% and 5% significance level.

RESULTS

Ultrasound low intensity pulsed mode at 10%

In Table 1 shows the data from each exposure group average and standard deviation in each moment of evaluation (24 hours, 48 hours and 72 hours). Comparisons between groups were performed at three predetermined periods and observed statistically significant differences between groups within 72 hours (F = 10.500, P = 0.011), however, in periods of 24 to 48 hours differences were found statistically significant (24 Hours, F = 0591, P = 0.583; 48 Hours, F = 3.086, P = 0.120).

After completion of the post-test found that the groups were statistically significant differences only within 72 hours, in which cells exposed to ultrasound of 0.2 W/Cm² had higher cell growth compared to control and the same was observed with cells exposed to ultrasound of 0.4 W/cm², with no statistically significant difference between groups 0.2 and 0.4 W/cm² - 10%. All comparisons made in these three time periods are presented in Table 2 and Figure 1.

Ultrasound low intensity pulsed mode at 20%.

In Table 3 shows the data from each exposure group average and standard deviation in each moment of evaluation (24 hours, 48 hours and 72 hours). Comparisons between groups were performed at three predetermined periods and there was a statistically significant difference between groups (48 hours, F = 5.823, P = 0.039; and 72 Hours, F = 25,922; P <0.001), but was not observed difference in 24-hour period (F = 2.044, P = 0.210). The mean values for each group at each time period are shown in Table 3, and the values of the comparisons are shown in Table 4 and Figure 2. After the completion of the post-test pulse length with groups behaved 20% similarly to groups with a pulse length of 10%.

So that, within 72 hours, cell growth in groups 0.2 and 0.4 W / Cm² was higher when compared to the control group (P <0.05), however, there was no statistically significant difference between 0.2 and 0.4 W/Cm² dosages - 20%. All comparisons made in these three time periods are presented in Table 3 and Figure 2.

The analysis between groups with equal doses and different pulse was no statistically significant difference between the means only between the groups 72 hours at dose 0.4 W/cm² (P = 0.02), where the pulse 20% showed a greater increase in cell viability when compared with the pulse by 10% (154.3 ± 6.3, 129.6 ± 0.5).

Table 1. Cell growth values in percent according to the different types of display in three time periods.

Groups	*24 Hours*		*48 Hours*		*72 Hours*	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Control	90.0%	0.00	94.00%	0.0	98%	0.0
0.2 W/Cm²	98.0%	15.6	106.6%	17.0	125.3%	15.8
0.4 W/Cm²	93.6%	0.5	114.6%	5.1	129.6%	0.5

Table 2. Comparisons made using one-way ANOVA with Bonferroni post-test and ultrasound of low intensity pulsed mode at 10%.

Comparisons	*24 Hours*	*48 Hours*	*72 Hours*
Control vs. 0.2 W/Cm²	0.958	0.545	0.032*
Control vs. 0.4 W/Cm²	1.000	0.147	0.017*
0.2 W/Cm² vs. 0.4 W/Cm²	1.000	1.000	1.000

* Statistically significant difference, P < 0.05.

Table 3. Cell growth Values in percent according to the different types of display in three time periods.

Groups	*24 Hours*		*48 Hours*		*72 Hours*	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Control	113.0%	0.00	114.0%	0.00	118.0%	0.00
0.2 W/Cm²	101.6%	13.5	137.0%	14.7	148.6%	9.6
0.4 W/Cm²	100.6%	4.7	120.0%	1.7	154.3%	6.3

Table 4. Comparisons made using one-way ANOVA with Bonferroni post-test and ultrasound of low intensity pulsed mode at 20%.

Comparisons	*24 Hours*	*48 Hours*	*72 Hours*
Control vs. 0.2 W/Cm²	0.436	0.050	<0.001*
Control vs. 0.4 W/Cm²	0.356	1.000	<0.001*
0.2 W/Cm² vs. 0.4 W/Cm²	1.000	0.153	1.000

* Statistically significant difference, P < 0.05.

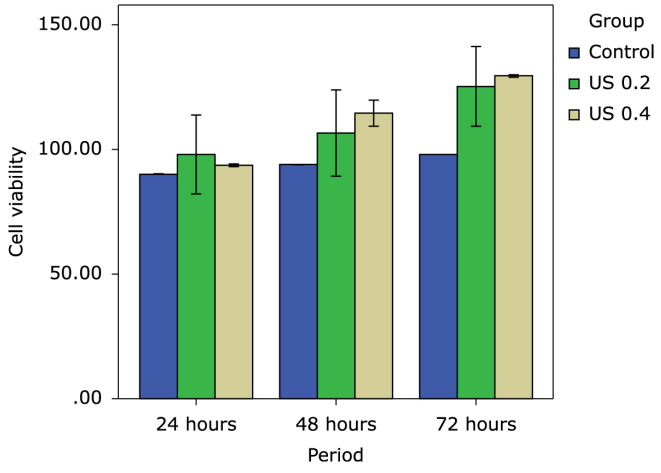

Figure 1. Cell growth in percent according to the different types of exposure.

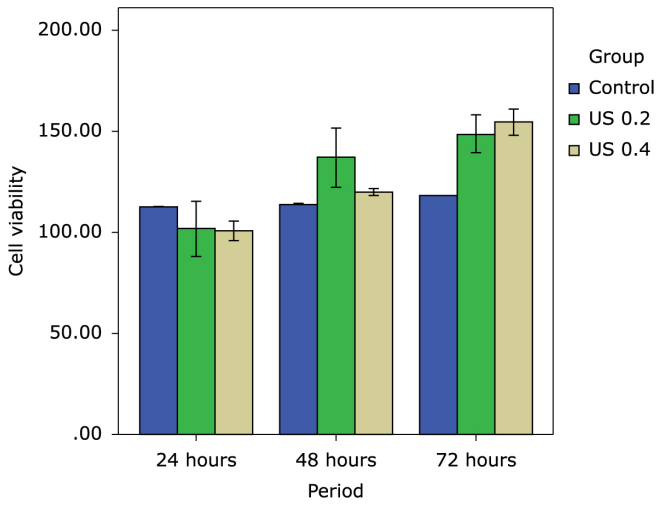

Figure 2. Cell Growth in percent according to the different types of exposure.

DISCUSSION

Based on our findings, according to the analysis of the cellular response after pulsed ultrasonic irradiation low intensity fibroblast cell culture L929, it was observed that in the pulse scheme Groups of 10%, only in the period of 72 hours there was a statistically significant difference compared to their respective controls. This, according to Silva et al. (2010), it is due to the fact that the biological responses of ultrasound are not only dependent on frequency, or pulse intensity, but also relate to the duration of the intervention.[11,12]

Thus, this same group submitted to 10% pulse regimes, dosages of 0.2 and 0.4 W/cm² showed no statistically significant differences between them in any of the three evaluation periods, on the other hand, Oliveira et al. (2008) fibroblasts to undergo the action of ultrasonic 0.2 W/cm² - 10% difference detected at all times (24, 48 and 72 hours).[13]

Accordingly, in a similar study, the authors investigate the doses of 0.2 and 0.6 W/cm² with pulse 10% and 20% in fibroblast cultures also found a significant increase in cell viability in periods of 24, 48, 72 and 96 hours. According to studies Pires-Oliveira et al (2008) ultrasonic irradiation has been quite effective on cell metabolism activation, confirming that the use of pulsed low intensity ultrasound ranging between 0.1 to 0.5 W/cm² which proves to be effective, accelerating the inflammatory phase of tissue repair, corroborating our findings.[13]

Studies Tascam et al (1997) analyzed cell culture fibroblast L 929, with intensity from 0.2 to 0.6 W/cm² was observed maintenance of cell shape and integrity, which is against our findings where ultrasound 20% 0.4 W/cm² was more efficient.[14]

According to Liang et al., (2004) they reported that by increasing the intensity of UST observed a decrease in cell viability and increase the transfection rate (transmission and expression of exogenous DNA by a cell, Greenleaf et al ., (1998) where the tolerable maximum intensity was 1 W/cm². Over 1 W/cm² there was a large decrease in cell viability in the order of 75% and a significant decrease in the transfection rate, ± 3, 7%. These findings indicate that cellular viability decreases with exposure time and intensity of the therapeutic ultrasound and high intensity levels would certainly cause cell death.[15,16] Demir works (2004) show the use of physical means, such as ultrasound and laser used in order to accelerate the repair phase as well as improvement of scar contraction, the results become significant in vivo.[17]

Given the above, cell culture involving experiments have several advantages compared to those directly in living organisms. In the case of cell culture, the experimental conditions can be controlled more rigorously, by manipulating the growth conditions and medium used in the culture of a specific cell type. However more studies are needed to understand the association of physical media in stimulating, repair, angiogenesis and inflammation processes.

CONCLUSION

We conclude that from the parameters used in pulsed ultrasound therapy of low intensity L fibroblast cell culture 929, we can infer that both the pulse regime, intensity and frequency are key to better application of ultrasound in relation to phase tissue repair , particularly as regards the early hours of the repair process, where we observe a significant effect on cell viability with respect to the low intensity of 0.4 w/cm².

AUTHORS CONTRIBUTION

RFO and DAAPO development of the research project, the study definition, contribution to the drafting of the text and approved the final version; LDB, ODP, JAS, SKFS performed the intervention of the study, contributed to the drafting of the text and approved the final version; JPMS participated in the definition of the study and statistical analysis and contributed to the drafting of the text. All authors read and approved the final version.

COMPETING INTERESTS

The authors declare no conflicts of interest.

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