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ORIGINAL RESEARCH

Effect of low-intensity pulsed ultrasound therapy on a fibroblasts cell culture

Efeito da terapia ultrassônica de baixa intensidade em cultura celular de fibroblastos

Efecto del ultrasonido de baja intensidad en cultivo celular de fibroblastos

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ABSTRACT | Within the physiotherapy practice there is a wide use of therapeutic ultrasound for the treatment of various musculoskeletal disorders. The aim of this study was to evaluate the effect of Low Intensity Ultrasonic irradiation with differents forms of pulse and intensity in cell culture of L929 fibroblasts (ATCC CCL-1 NCTC), in order to check cell viability and define parameters of dosimetry. For this purpose, it was used the application of pulsed ultrasound with a frequency of 1MHz in cultured fibroblast cells divided into five groups (control and instantaneous intensity of 0.3W/cm^2 - 10%, 0.3W/cm^2 - 20%, 0.5W/cm^2 - 10% and US 0.5W/cm^2 - 20% - 100Hz). Irradiation occurred at intervals of 24, 48 and 72 hours for two minutes and 24 hours after each irradiation test MTT [3 (4,5-dimethylthiazol) -2,5-diphenyltetrazolium bromide] was performed. Results showed that when comparing the values of viable cells by MTT method in the five groups, we could not find statistically significant difference in any of them, in these three conditions (24, 48 and 72 hours); while. Whereas, when performing the analysis of repeated measures in the different groups, it was found a statistically significant difference only in the group irradiated with ultrasound at 0.5W/cm^2 with pulse regime of 10% (p = 0.003). Based on these results, it is concluded that the Low Intensity Ultrasonic irradiation in L929 fibroblast cell culture, only in the group with an intensity of 0.5W/cm^2 -10% obtained numerical growth, with statistical significance in all periods evaluation.

Keywords | Dosimetry; Connective Tissue; Cell Culture Techniques; Ultrasonic Therapy

RESUMO | Dentro da prática fisioterápica verifica-se a ampla utilização do ultrassom terapêutico para tratamento das diversas afecções musculoesqueléticas. O objetivo deste estudo foi avaliar o efeito da irradiação Ultrassônica de Baixa Intensidade, com diferentes regimes de pulsos e intensidade, em cultura celular de fibroblastos L929 (ATCC CCL-1 NCTC), de modo a verificar a viabilidade celular e definir parâmetros de dosimetria. Para isso, utilizou-se a aplicação de ultrassom pulsado, com frequência de 1Mhz, em cultura de células fibroblásticas, divididas em cinco grupos (controle e com intensidade instantâneas de 0,3W/cm^2-10%; 0,3W/cm^2 -20 %; 0,5W/cm^2 -10% e US 0,5W/cm^2 -20 % - 100Hz). A irradiação ocorreu com intervalos de 24, 48 e 72 horas, por dois minutos, e após 24 horas de cada irradiação foi realizado teste de MTT Brometo de [3-(4,5-dimetiltiazol)-2,5-difeniltetrazólio]. Os resultados revelaram que ao compararem-se os valores de células viáveis pelo método MTT nos cinco grupos, não foi possível encontrar diferença estatisticamente significativa em nenhum deles, nos três momentos avaliados (24, 48 e 72 horas); enquanto que, ao se realizar a análise de medida repetida nos diferentes grupos, encontrou-se diferença estatisticamente significativa apenas no grupo irradiado com ultrassom a 0,5W/ cm^2 com regime de pulso

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de 10% (p=0,003). Com base nesses resultados, conclui-se que a irradiação Ultrassônica de Baixa Intensidade em cultura celular de fibroblastos L929, somente no grupo com intensidade de 0,5W/cm²-10% obteve o crescimento numérico, com significância estatística em todos os períodos de avaliação.

Descritores | Dosimetria; Tecido Conjuntivo; Técnicas de Cultivo de Célula; Terapia por Ultrassom.

RESUMEN | En la práctica fisioterápica se utiliza bastante el ultrasonido como terapia para el tratamiento de diversos trastornos muscoloesqueléticos. Este artículo tiene por objetivo evaluar el efecto de la irradiación ultrasónica de baja intensidad, con diferentes regímenes de pulsos y de intensidades, en cultivo celular de fibroblastos L929 (ATCC CCL-1 NCTC), para verificar la viabilidad celular y establecer los parámetros de dosimetría. Se utilizó el ultrasonido pulsado, con frecuencia de 1Mhz, en un cultivo de células fibroblásticas, divididas en cinco grupos (con control y con la intensidad instantánea del 0,3W/cm²-10%; 0,3W/cm² -20%; 0,5W/cm² -10% y US 0,5W/cm² -20 % - 100Hz). La irradiación se llevó a cabo en intervalos de 24, 48 y 72 horas, durante dos minutos y después de las 24 horas de cada irradiación se realizó la prueba de MTT {Bromuro de [3-(4,5-dimetiltiazol)-2,5-difeniltetrazólio]}. Los resultados mostraron que en la comparación entre los valores de células viables por el método MTT en los cinco grupos evaluados no ha sido posible encontrar ninguna diferencia estadísticamente significativa en los tres momentos evaluados (24, 48 y 72 horas). En cambio, al llevar a cabo el análisis de medida repetida en los diferentes grupos, se obtuvo una diferencia estadísticamente significativa solamente en el grupo irradiado con ultrasonido a 0,5W/cm² con el régimen de pulso del 10% (p=0,003). Basándose en estos resultados se concluyó que la irradiación ultrasónica de baja intensidad en cultivo celular de fibroblastos L929 obtuvo el aumento sólo en el grupo con intensidad de 0,5W/cm²-10%, con significancia en todos los periodos de evaluación.

Palabras clave | Dosimetría; Tejido Conectivo; Técnicas de Cultivo de Células; Terapia por Ultrasonido.

INTRODUCTION

Therapeutic ultrasound (TUS) is verified to be widely used for the treatment of several acute and chronic musculoskeletal illnesses[1,2] in the physical therapy practice, when one oversees the resources which are used in clinical rehabilitation. That is mainly due to the fact it is a safe method which prevents patients from being exposed to risks from invasive procedures[3].

That method has been present in the clinical practice for more than six decades[4]. However, Wardejn and McMeeken[5] highlight the lack of scientific evidence in regards to its therapeutic effects and to the normalization of its application, and the available articles confirm the contradiction above, concerning standardization and the very tissue healing, as they are short of evidence on the actual biologic effects, action mechanisms, or concrete determination of criteria that are indicated for that kind of treatment[4,6,7].

That shows the importance of constantly investigating its clinical results and studying the relationship between dosages and biological responses, in order to achieve dosimetric consensus for the related physical agent[1,8]. After all, as Ishikawa et al.[9], point out, the correct application of [10] TUS is essential, not only to support the exposure levels which induce significant biological repercussion, but also to protect patients. From that perspective, an array of thermal and non-thermal physiological effects which TUS induces in biological tissues through vibration is found on several studies[10].

Considering those effects, one can mention fibroblast activation, increased collagen extensibility and scarring, diminished inflammatory cells (leukocytes and macrophages) through cell metabolism acceleration, protein synthesis, osteogenesis, calcium cycle activation, angiogenesis (with a consequent increase in blood circulation and perfusion), growth factor production, reduced muscular spasms, articular rigidity, and, finally analgesia[11-18].

In this context, based on the studies from Zhou et al.[19], TUS effects are highlighted to have a close connection with cell types when applied *in vitro*, which favors DNA synthesis in periosteum, osteoblasts, and fibroblasts.

Finally, based on the considerations by Johns[20], the relevance of culture cell studies is highlighted when they are correlated with ultrasound action, once they allow complementing *in vivo* investigations, strictly controlling the countless variables, and also potentializing knowledge in regards to that therapeutic tool.

Thus, this study aimed at evaluating the effect of low-intensity pulsed ultrasound irradiation with different pulse and intensity regimes in an L929 fibroblast cell culture, in order to check for cell viability and to define dosimetry parameters.

METHODOLOGY

This study is characterized as experimental, and, in order that it be conducted, fibroblast cells from the connective tissue of mice of L929 lineage (ATCC CCL-1 NCTC) were used. They were provided by Instituto Adolfo Lutz (SP), Brazil. The study was approved by the Ethics Committee of Universidade Norte do Paraná (UNOPAR), under protocol no. 462,478/2013.

Cell Culture

L929 fibroblast cells were routinely cultivated in 25cm^2 dishes (TPP, Switzerland, Europe) with MEM (Minimum Essential Medium, Gibco® – Invitrogen Corporation, GradIsland, USA) that was supplemented with 10% fetal bovine serum (Cultilab, Brazil) and 1% antibiotic (Gibco®, by Life Technologies). Cells were kept in a CO_2 incubator, in a 5% atmosphere at 37°C (Thermo Forma Scientific, Waltham, MA). The cells used in this experiment followed the usage recommendations for *in vitro* toxicity tests figuring in ISO 10993-5.

Ultrasound

Ultrasound irradiation was conducted with a device of brand KLD® – (Biossistemas Equipamentos Eletrônicos Ltda.), model Avatar III, with a 1MHz transducer, BNR (beam nonuniformity ratio) ≤6, and with a 1cm^2 effective radiation area (ERA). The equipment was properly calibrated by the manufacturer.

Irradiation

After the culture became confluent, trypsinization was conducted in the 12-well TPP dishes with 24 cm in diameter and 18 mm in depth, in a 1x10^6 cells/mL density. Following that, cells were left sedimenting for 24 hours (overnight). After that, the intervention in 24, 48, and 72-hour intervals was started, by observing the following separation among groups (Table 1):

In order to get good coupling of the ultrasound interface (cell layer away from the 18 mm transducer) and mechanical wave propagation, the well volumes were filled up with MEM medium up to their rims, and each well was irradiated and always kept in the same position in regards to the ultrasound transducer face.

Table 1. Description of experiment groups and respective dosimetries

Group	Instant Intensity (W/cm^2)	Pulse Regime (%)	Average Intensity (W/cm^2)	Work Cycle	Frequency (Hz)
G1	Control group (not irradiated)				
G2	0.3	10	0.03	1:9	100
G3	0.3	20	0.06	1:4	100
G4	0.5	10	0.05	1:9	100
G5	0.5	20	0.10	1:4	100

In regards to the application time, the irradiation for conducted at room temperature for 2 minutes in each well, in the 24, 48, and 72-hour intervals, without the dish being heated. All experiments were conducted in triplicate, and, after each period, cultures had their cell viability evaluated through MTT toxicity test.

Cytotoxicity test through MTT

The cytotoxicity was tested for through MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. L929 cell cultures received ultrasound irradiation in the 24, 48, and 72-hour intervals. MTT assays were conducted 24 hours after each irradiation, according to the following procedure: after the MEM medium was removed, each well received 80 µL MTT, in a final 0.5mg/mL concentration, and was incubated for 1 hour at 37°C, in a 5% CO_2 atmosphere; then, 400µL dimethyl sulfoxide (DMSO) was added to each well. Dishes were kept under agitation for 30 minutes, for formazan crystals to be solubilized. Their concentrations were spectroscopically quantified through a microplate reader (ELISA Reader – Spectra Count – Packards Instrument, Offeburg – Germany), in a 570nm wavelength.

STATISTICAL ANALYSIS

The results were expressed in average values and standard deviation, with their normalities being verified through Shapiro-Wilk test. In order to compare and check for expressive differences among groups, Analysis of Variance (ANOVA) and post-hoc Tukey HSD test were used. Repeated Measures ANOVA was used

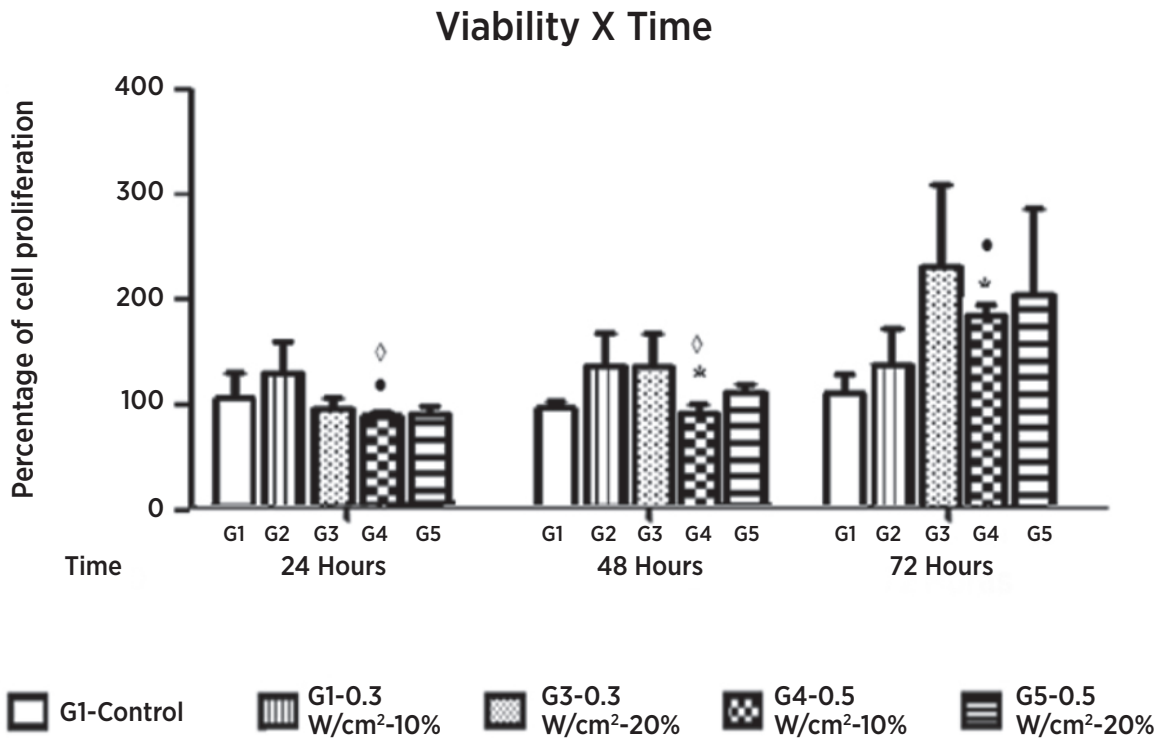
in between evaluations. SPSS, version 20.0 software was used in the statistical analysis, whose confidence interval was 95%, and values of $p<0.05$ were considered to be statistically significant.

RESULTS

No statistical significance was found among the viable cell values and the groups (G1=control, G2=0.3–10%, G3=0.3–20%, G4=0.5–10%, and G5=0.5–20%) through MTT method, in the three evaluated times (24, 48, and 72-hours) (Figure1).

When the repeated measure analysis was conducted in the different groups, statistic significance was only found in the group that was irradiated with 0,5W/cm² ultrasound with a 10% pulse regime (p=0.003). In that group, cell viability speed was increased from 24 to 48 hours, from 48 to 72 hours, and from 24 to 72 hours.

However, despite the low number of evaluated samples, it is important to mention certain differences in absolute values, concerning cell viability, especially in group G3, which was irradiated with 20% pulsed ultrasound in a 0.3W/cm² intensity, which may suggest that, even though no statistically significant differences had been found in all experimental groups, a considerable growth took place in the number of irradiated cells. Such fact allows understanding that ultrasound has not operated in a harmful way to cells, and the numbers in no groups were decreased, as the fibroblasts remained inert to the ultrasound action, because all groups experienced growth in their total values (Figure 1).



◊ statistically significant differences between the first and second evaluations (24/48 hours)

* statistically significant differences between the second and the third evaluations (48/72 hours)

• statistically significant differences between the first and the third evaluations (24/72 hours)

Figure 1. Cell viability comparison among groups at the different evaluated times.G1-not irradiated; G2- received US 0.3 W/cm² irradiation (10%); G3- irradiated with US 0.3 W/cm² (20%);
G4- group irradiated with US 0.5 W/cm² (10%), and G5- US 0.5 W/cm² (20%)

DISCUSSION

According to Silva et al.[16], the therapeutic effects of ultrasound do not depend exclusively on the intensity, mode of emission, and frequency, but they are also directly subject to the intervention duration. In this study, the therapeutic application time was standardized in two minutes, based on the conclusion from Oliveira et al.[21], who confirmed increase cell viability with this same application protocol.

In regards to the pulsed emission mode that was used, it corroborates findings from Cunha et al.,[22], who regard the related modality to be more efficient than the one of continuous pulse. That fact, within the process of tissue repair in rats, signaled optimization in the organization and aggregation of collagen fiber bundles, as the continuous work cycle was found to show disorganization in the material, as an example of a harmful effect in the healing.

Lirani-Galvão et al.[23] and Hsieh[24] report that pulsed ultrasound has a relevant behavior, which related to therapeutic effects such as: increased permeability, membrane and intracellular calcium diffusion.

In turn, the benefits of 0.5W/cm^2 dosage (pulsed at 10 and 20%, and instant intensity) that were also found in our results may also be linked to the association of athermal effects, which could be responsible for the related stimulation in the transportation of substances and modified cell membrane permeability[25].

The findings in this experiment also reinforce the studies by Demir et al.[26], in which the therapeutic ultrasound has been shown to be efficient in the stimulation of fibroblast cultures, with intensities between 0.1 and 0.5W/cm^2, making the inflammatory phase of healing quicker. Likewise, Lowe et al.[27] and Oliveira et al.[28] also confirmed their effectiveness in the healing phase, using ultrasound dosages with intensities of approximately 0.5W/cm^{2}in pulsed modes (10 and 20%–100Hz) and 1MHz frequency.

On the other hand, when using the 0.3 W/cm^2 intensity (pulsed at 10% and 20%, 100Hz frequency, and instant intensity) in order to highlight the non-thermal action TUS, the employed dosage may not have been enough to cause significant biophysical implications, as they are intensity-dependent[29].

Also, one may mention that Lim et al.[30], in an odontoblast culture, verified increased cell viability when it is associated to low-intensity pulsed ultrasound therapy (1MHz, 0.5W/cm^2, pulsed at 20% and 50%) in 10 minutes of intervention. Thus, the ultrasound application time (two minutes) in groups 0.3W/cm^2 (pulsed at 10% and 20$, with 1MHz frequency, and instant intensity) may not have been enough to promote biomodulatory responses.

This way, other studies on those subjects differ in some aspects from our results, and they discuss the increased bone metabolism in rats (with a 0.3W dosage and 1MHz frequency)[14,17], or the increased number of fibroblasts and collagen alignment through the use of pulsed ultrasound (20%) at a 0.5W/cm^2[13] intensity.

However, it is important to point out authors who have not obtained stimulatory results - such as Artifon et al.[25], with the 0.5W/cm^2 group in the soleus muscles of rats, and Frasson et al.[31], who have not verified increased numbers of fibroblast cells or blood capillaries in calcaneus tenectomies, when pulsed TUS is applied (20% with intensities of 0.3W/cm^2 and 1.5W/cm^2).

In that context, cell culture also contributes to their findings, by pointing out that ultrasound effects are correlated to the parameters used and to the cell type, and it is capable of ensuring the proliferation of fibroblasts[21], osteoblasts[32], and chondrocytes[33].

Corroborating such statements, other *in vitro* studies say that, after different incubation periods (24, 48, and 72 hours), cell viability, as analyzed by MTT method, was not found to have significant differences[15]. Likewise, Bohari et al.[34] found the same results in regards to a fibroblast culture.

This way, we point out that in our study it was possible to verify that, the same way as Artilheiro et al.[15], cell numbers were increased as expected. Even though that increase has not been significant in all groups, TUS has not caused cell viability to be inhibited.

Finally, through the therapeutic advances that are based on technological developments taking place in the last few years, ultrasound became a resource with promising results in scar healing; that is related to the fact that increased release of growth factors takes place through the granulation of platelets, mast cells, and macrophages during the initial inflammatory phase of healing.

Considering that, the proliferation phase is started earlier, reducing its time interval, and operating in its remodeling phase. With that, scarring, which is sped up through the release of those growth factors, may be stimulated by ultrasound[35].

CONCLUSION

Based on the results from this study, 0.3 W/cm² (10%–20%) and 0.5W/cm² (20%) low-intensity pulsed ultrasound irradiation can be concluded to percentually increase cell viability in an L929 fibroblast culture. In turn, only the group with 0.5W/cm² (10%) treatment obtained numerical growth with statistic significance in all evaluation periods (24, 48, and 72 hours).

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