

The effect of therapeutic ultrasound on fibroblast cells *in vitro*: the systematic review

Priscila Daniele de Oliveira, Deise Aparecida de Almeida Pires-Oliveira, Larissa Dragonetti Bertin, Stheace Kelly Fernandes Szezerbaty, Rodrigo Franco de Oliveira

Centro Universitário de Anápolis (UNIEVANGÉLICA) - Programa de Pós graduação, Anápolis, Goiás, Brasil.

Received: 17.05.2017

Accepted: 18.09.2017

Summary

Introduction: Therapeutic ultrasound is one of the most used physical resources in the area of physiotherapy for the treatment of injuries. However, the multiplicity of dosimetry used in clinical practice points to its indiscriminate use for pathologies that surround skeletal muscle and expresses the limitation of the available literature on the ideal dosimetric standardization to the tissue restoration, mechanism of action and its real effects on the treatment in question.

Objective: The objective of this study was to promote a systematic review about the different effects and the dosimetric parameters of therapeutic ultrasonic irradiation on the process of restoration of fibroblast cells *in vitro*.

Methods: To select the articles, three electronic data banks were consulted, with publication from January 2000 to September 2016. The studies were tracked by three freestanding reviewers, according to inclusion and exclusion criteria.

Results: 669 articles were selected and after the application of inclusion and exclusion criteria, 647 were excluded. Among the exclusions reasons there are: the utilization of another physical method, exclusive focus on another type of cell line, other experimental models or the use of another language, reaching at the end 22 studies directed to qualitative analysis.

Conclusion: The results of this study showed that the scientific basis is not enough to establish real effects and dosimetric parameters of therapeutic ultrasonic on the process of restoration of fibroblast cells *in vitro*, due to the lack of generalization and conflict of found results.

Key words:

Cell culture techniques. Fibroblasts. Ultrasonic therapy.

Efecto del ultrasonido terapéutico en células fibroblásticas *in vitro*: revisión sistemática

Resumen

Introducción: El ultrasonido terapéutico es uno de los recursos físicos más utilizados en el área de fisioterapia para el tratamiento de lesiones. Sin embargo, la gran cantidad de dosimetrías utilizadas en la práctica clínica muestra su uso indiscriminado para patologías que circundan el músculo esquelético y además expresa la limitación de la literatura sobre la estandarización dosimétrica ideal para la restauración del tejido, mecanismo de acción y sus efectos reales sobre el tratamiento en cuestión.

Objetivos: El objetivo de este estudio fue realizar una revisión sistemática sobre los diferentes efectos y parámetros dosimétricos de la irradiación ultrasónica terapéutica en el proceso de reparación de células fibroblásticas *in vitro*.

Material y método: Para la selección de los artículos fueron consultadas tres bases de datos para buscar publicaciones entre enero de 2000 y septiembre de 2016. La búsqueda de trabajos se realizó por tres revisores independientes, conforme a los criterios de inclusión y exclusión.

Resultados: Se seleccionaron 669 artículos y tras la aplicación de los criterios de inclusión, se excluyeron 647 estudios. Entre los motivos de exclusión están la utilización de otro medio físico, enfoque exclusivo de otro tipo de línea celular, otros modelos experimentales o el uso de otro idioma, quedando 22 estudios para el análisis cualitativo.

Conclusión: Los hallazgos de este estudio mostraron que la base científica todavía es insuficiente para el establecimiento de los efectos reales y parámetros dosimétricos de la irradiación ultrasónica terapéutica en el proceso de reparación de células fibroblásticas *in vitro*, por la falta de generalización y conflicto de los resultados encontrados.

Palabras clave:

Técnicas de cultivo de célula. Fibroblastos. Terapia por ultrasonido.

Correspondence: Priscila Daniele de Oliveira. E-mail: prisciladanielefsio@hotmail.com

Rodrigo Franco de Oliveira. E-mail: rfranco@yahoo.com.br

Introduction

The therapeutic ultrasound (TUS) is one of the most used physical resources in the physiotherapy area¹. However, the multiplicity of dosimetries used in clinical practice points the indiscriminate use of it to pathologies which surround the musculoskeletal², and express the limitation of available literature on the ideal dosimetric standardization to tissue restoration, mechanism of action and its real effects on the concerned treatment³.

This diversity of biological answers come from uncountable interactions of ultrasonic therapy with the cells and tissues, which have been studied for more than 50 years⁴. Among the biological answers, there is the stimulus to neuro-vascularization and leukocyte activity, to adenosine triphosphate production and collagen, to the speed of biochemical reactions, and yet, the significant influence TUS in the cell function in fibroblasts *in vitro* observed by Pires-Oliveira *et al*⁵.

In this context, notably, the fibroblast cells play an important role in the production of extracellular matrix (in the connective tissue) and collagen (in the fibrous tissue), being directly involved in the mechanisms of tissue repair and in phase of remodeling tissues^{6,7}.

Thus, when the ultrasound treatment is correlated to the fibroblast cells culture, it is observed a relevant complementation of studies *in vivo*, especially when the TUS potential is evaluated, since it is admitted the minimization of thermal effects of TUS, as well as the realization of analysis⁸⁻¹⁰. In this regard, it is important to emphasize that, commonly the biophysical effects of TUS were proved in experimental studies *in vitro*, while the same could not be described or analyzed *in vivo*¹¹.

Besides, with this technique of laboratorial manipulation *in vitro*, it is possible to achieve a strict control of uncountable variables involved, answer the questions in a more systematic manner and, finally, reach a further clarification about the use of TUS^{9,10}.

Therefore, the objective of this study was to accomplish a systematic literature review about the different effects of therapeutic ultrasonic irradiation and its dosimetric parameters on the process of fibroblast cells restoration.

Material and method

To select the articles of this systematic review three electronic data bank were consulted (PubMed, Bireme, Ebsco Host (Sport Discus), Scopus and Web of Science), being the research done on September 14, 2016.

In the search strategy, the keywords were selected by the terms "MeSH" and its matchings: "Ultrasonic Therapy", "Ultrasonics", "Cell Culture Techniques", "In vitro Techniques", "Fibroblasts", "Connective Tissue" and "Connective Tissue Cells", which were associated to the Boolean terms AND, OR and NOT, shown in Table 1. Some necessary adaptations have been done to meet the specificities of the search engine of each electronic data bank.

To be included in this review, the article should have the following criteria: publication between January 2000 and September 2016, present the text structure in English, French, Italian, Portuguese, Spanish or German, use the therapeutic ultrasound treatment as physical method, fibroblast cells *in vitro* or the biomodulating effect of ultrasound in the fibroblast repair process.

Table 1. Keywords and keyword combinations used to screen the systematic review.

("Ultrasonic Therapy"[Mesh] OR "Therapy, Ultrasonic" OR "Therapies, Ultrasonic" OR "Ultrasonic Therapies" OR "Ultrasonics"[Mesh] OR "Ultrasonic") AND ("Cell Culture Techniques"[Mesh] OR "Cell Culture Technique" OR "Culture Technique, Cell" OR "Culture Techniques, Cell" OR "Cell Culture" OR "Cell Cultures" OR "In Vitro Techniques"[Mesh] OR "In Vitro Technique" OR "Technique, In Vitro" OR "Techniques, In Vitro" OR "In Vitro as Topic" OR "In Vitro") AND ("Fibroblasts"[Mesh] OR "Fibroblast" OR "Connective Tissue"[Mesh] OR "Connective Tissues" OR "Tissue, Connective" OR "Tissues, Connective" OR "Connective Tissue Cells"[Mesh] OR "Cell, Connective Tissue" OR "Cells, Connective Tissue" OR "Connective Tissue Cell")

The papers which did not filled the inclusion criteria were excluded, and among them, there were the duplicated ones, with focus on another cell line, with another experimental model, with lack of essential information that affected the quality of the methods, the internal and external validity of the study and finally, the review of the articles. In addition to the exclusion criteria above mentioned, it was also included the summaries of events, editorials, consensus of physical means, validation of laboratory methods and manuals for clinical practice. At first, two freestanding reviewers (PDO and SKFZ) tracked the study searching for the title, abstract and key-words, and, in case of disagreement, a third reviewer was called (LDB). This way, after the phases of identification, screening and eligibility, all the studies potentially eligible (n=65) had their completed versions analyzed by these reviewers, according to the flowchart (Figure 1).

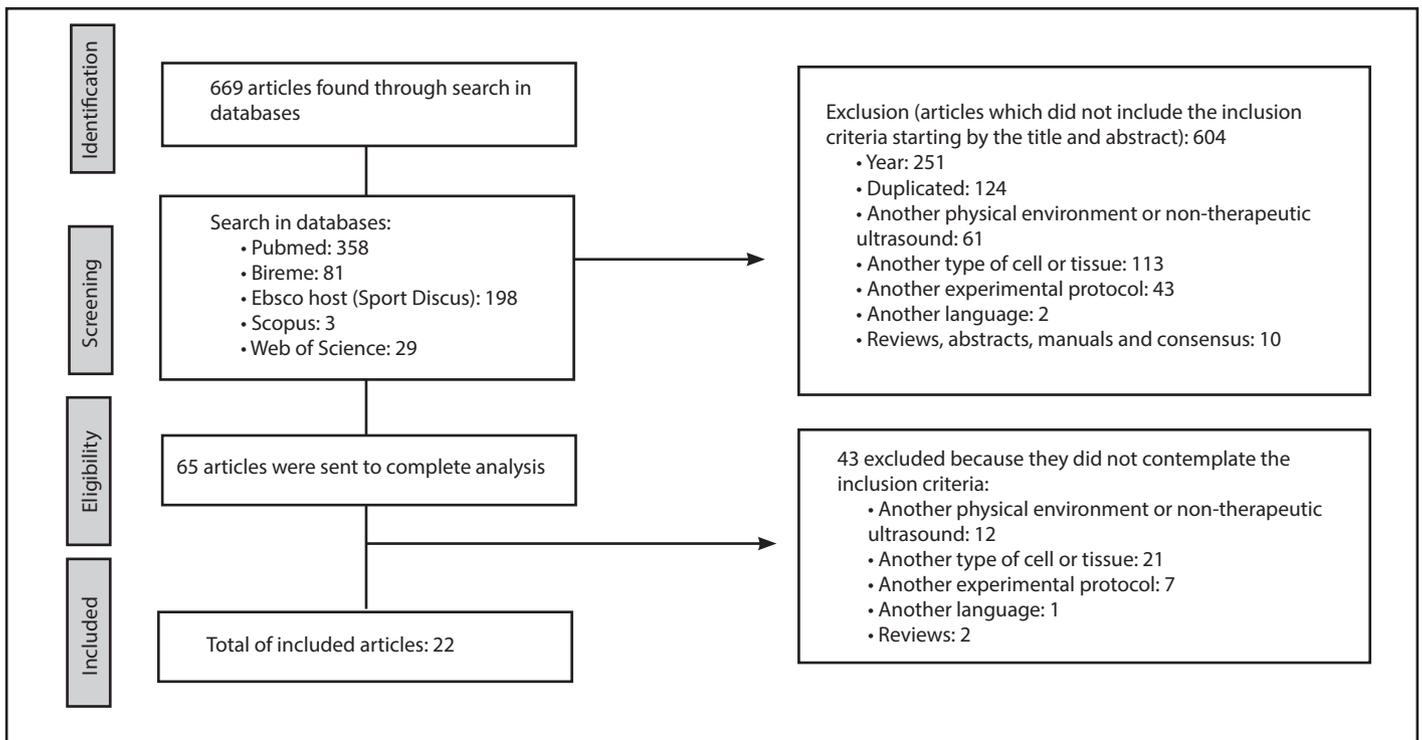
The critical evaluation of the studies was observed by the methodological quality in the results obtained through gold standard tests, by the integrity of the evaluations, and the mode adopted for laboratory manipulation in cell culture.

Thereafter, when analyzing the established criteria to determine the validity and reliability of the selected studies, the information were collected, in which were discarded the possibility of a meta-analysis due to the heterogeneity of data of the included studies, classifying this study as a qualitative systematic review.

The extraction and tabulation of the data of the documents obtained at the end of the scan was delimited in predefined fields (cell line, ultrasound parameters used, presence of biological effects after the ultrasonic treatment with statistically significant differences between the groups, or absence of biomechanical effects, or non-significant differences between the study control and the treated groups), and then, for the accuracy of the variables collection, the final database was again compared to the original sources by the evaluators and, lastly, interpreted.

Results

The results were presented in a PRISMA flow diagram¹² (Figure 1), describing the main phases of the systematic review, which are: identification, screening, eligibility and included.

Figure 1. Flow chart of triage of studies. This flowchart is according PRISMA Statement 2009¹².

The searches in the databases resulted in 669 articles and after the application of the criteria inclusion, 647 studies were excluded. Among the reasons of exclusion, there was the use of another physical method, such as laser, nano-particle emitters, scaller, softwares or use of non-therapeutic ultrasound for diagnosis. A total of 65 studies passed in the first three phases of filtering, and after reading the full texts, another 43 works were eliminated.

Regarding the exclusive presence of another cell line (such as chondrocytes, macrophages, mesenchymal cells, osteoblasts, cardio myocytes, mammary adipocytes, stromal cells, myofibroblasts, liposomes, odontoblasts, osteocytes, macrophages, phagocytes, fibronectin, osteoclasts), of tissue (vascular, tumor, muscle, cartilage, bacteria, tooth, venous ulcer, gum, vertebral disc, dentine, collagen, skin) and of experimental models (*in vivo*, with titanium association, biomaterials, nanoparticles, tissue engineering, measurement of attenuation, of temperature variation and wave propagation), in both stages of sorting, 156 and 28 works were excluded, respectively.

Furthermore, with regard to revisions, manuals, event summaries and consensus, 10 manuscripts were retained and finally, it has been also withdrawn three articles that appeared in another language (Chinese).

It is worth mentioning that the inclusion of the studies was conditioned by the absence incomplete outcomes due to missing data, any other loss involving the laboratory routine of the cell culture with its relevant weights or other easily detectable possible bias problems, to rule out the possibility of low methodological rigor and thus achieve more credible results.

Regarding the critical evaluation of the studies, since the methodology in question does not meet the criteria of available classifications (experimental studies in animals or clinical trials) it was not possible to

calculate the final bias, since these are cell studies *in vitro*. In this sense, as the available information was not sufficient to classify the methodological aspect as having a high or low risk of bias, the domain receives the uncertain risk classification.

Then, after a wide search, a total of 22 articles were selected for this review, summarized and displayed in a chart (Table 2) to qualitative analysis. The selected studies observed the ultrasound action on rats and mice^{15-18,19,21-27,29-31,33}, hamsters²⁸, rabbits³² and humans^{13,14,19,20,28}.

In relation to TUS parameters, it was observed the most diverse dosimetries, in such a way that the doses varied from 0.002 to 2 W/cm², and in frequencies of transducer there was the prevalence of 0.02 MHz (20 kHz)²⁹ over the 3 MHz^{30,31}, and another study did not mention this last parameter³².

Concerning the emissions, the pulsed stood out, which appeared in 18 out of 22 analyzed papers, having the other ones approached together the continuous and pulsed emissions, or not reporting the type of emission used^{14,18,26,31,32}. Regarding the treatment time, it was analyzed short-term duration applications from 10 seconds to 60 minutes.

The Table 2 also shows that the biological effects promoted were bigger DNA¹³ and protein⁵ synthesis, a significant increase of cell proliferation^{18,22,27,29,32,33} and incidence of micronucleus³⁰, reorganization of actin cytoskeleton¹³, endocytic cell activity²⁰, improvement in the efficiency of membrane permeability^{27,31}, increasing in gene transfer rate¹⁵, of potential of osteogenic differentiation¹⁹ and of the reticulum activity⁵, as well as the amplification of vacuoles in cytoplasm¹⁴, of Ca⁺² available²¹, of collagen contents and glycosaminoglycan²⁵, of the transfection of microbubbles rate²⁶, the size of the molecules of entry³¹, and finally the induction of focal adhesion²⁴ and efficiency in absorption³¹.

Table 2. Relation of scientific articles found with their respective cell lines, ultrasonic parameters and biological effects.

Study	Cell Line	Ultrasound Parameters	Biological effects
Zhou <i>et al.</i> , 2004 ¹³	Human foreskin fibroblasts	F: 1.5 MHz; I: 30 mW/cm ² ; DC: 20%; TE: 6 or 11 min	+/-
Lai and Pittelkow, 2007 ¹⁴	Fibroblasts neonatal foreskin	F: 40 kHz (mist) 1.0 cm from the cell culture; I: 0.002 W/cm ² ; TE: 7, 15 and 30s	+/-
Chen <i>et al.</i> , 2007 ¹⁵	3T3-MDEI (Embryonic mouse fibroblast), C2C12 e CHO	F: 1 MHz; DC: 20%; I: from 0.5 to 2 W/cm ² ; TE: 5 to 80 s.	+/-
Oliveira <i>et al.</i> , 2008 ¹⁶	Mouse fibroblast (L929)	F: 1 MHz; I: 0.2 e 0.6 W/cm ² ; DC: 10 and 20%; TE: 2 min;	+
Oliveira <i>et al.</i> , 2008 ¹⁷	Mouse fibroblast (L929)	F: 1 MHz; I: 0.1, 0.2, 0.6, 0.8, 1.0 and 2.0 W/cm ² ; DC: 10 and 20%; TE: 2 min	+/-
Tomankova <i>et al.</i> , 2009 ¹⁸	NIH3T3 (mouse fibroblast cells) and B16FO (mouse melanoma cells)	F: 1 MHz; I: 2 W/cm ² ; TE: 10 min; control group; DC: did not report	+/-
Mostafa <i>et al.</i> , 2009 ¹⁹	Human gingival fibroblasts (HGF)	F: 1.5 MHz; I: 30 mW/cm ² ; DC: pulsed; TE: 5 or 10 min	+/-
Pires-Oliveira <i>et al.</i> , 2009 ⁵	Mouse fibroblast (L929)	F: 1 MHz; I: 0.2 e 0.6 W/cm ² ; DC: 10 and 20%; TE: 2 min;	+
Hauser <i>et al.</i> , 2009 ²	Human foreskin fibroblasts	F: 1.5 MHz; I: 30 mW/cm ² ; DC: 20%; TE: 6 min	+/-
Tsakamoto <i>et al.</i> , 2011 ²¹	Mouse fibroblast (L929)	F: 1 MHz; pressure amplitude 0.4 MPa (peak to peak); DC: 20%; TE: 60s	+/-
Oliveira <i>et al.</i> , 2011 ²²	Mouse fibroblast (L929)	F: 1 MHz; I: 0.2 e 0.6 W/cm ² ; DC: 10 and 20%; TE: 2 min	+/-
Grimaldi <i>et al.</i> , 2011 ²³	Murine fibroblasts (NIH-3T3)	F: 1 MHz; I: 307 and 46 mW/cm ² ; DC: 75%; TE: 5, 15, 30, 45 and 60 min;	+/-
Roper <i>et al.</i> , 2012 ²⁴	Mouse embryonic fibroblasts (MEFs)	F: 1.5 MHz; I: 30 mW/cm ² ; DC: 20%; TE: 0, 10, 30 and 60 min	+
Bohari <i>et al.</i> , 2012 ²⁵	3T3 mouse fibroblasts	F: 1 MHz; I: 0.2 W/cm ² ; DC: 20%; TE: 5 min;	+/-
Zhang <i>et al.</i> , 2012 ²⁶	Mouse embryonic fibroblast cells (NIH3T3)	I: 0–11 W/cm ² , pulse repetition frequency (PRF, 50–50.000 Hz), duty ratio (10 to 50%), TE: 0–120s, and microbubble volume concentration (0 to 10%)	+/-
Domenici <i>et al.</i> , 2013 ²⁷	Murine fibroblasts (NIH-3T3)	F: 1 MHz; I: 11.8, 15.2 and 19.3 mW/cm ² ; DC: 75%; TE: 5, 15, 30, 45 and 60 min;	+
Duvshani-Eshet <i>et al.</i> , 2013 ²⁸	Human foreskins fibroblasts and baby hamster kidney	F: 1 MHz; I: 2 W/cm ² ; DC: 30%; TE: 30 min;	+/-
Samuels <i>et al.</i> , 2013 ²⁹	3T3 mouse fibroblasts	F: 20 kHz; I: 50 and 200 mW/cm ² ; DC: 10% and 20%; TE: 15 min	+/-
Udroiu <i>et al.</i> , 2014 ³⁰	Murine fibroblasts (NIH-3T3)	F: 1 and 3 MHz; I: 7.1, 11.8, 15.2 and 19.3 mW/cm ² (for the 1 MHz exposure); 1.0, 4.9 and 7.0 mW/cm ² (for the 3 MHz exposure); DC: 75%; TE: 5, 15, 30, 45 and 60 min;	+/-
Domenici <i>et al.</i> , 2014 ³¹	Murine fibroblasts (NIH-3T3)	F: 1 and 3 MHz; TE: 5, 15, 30, 45 and 60 min; DC: 75% for 1 MHz and 100% for 3 MHz; I: 0.11, 0.12 and 0.09 W/cm ² (for 1 MHz); 0.01, 0.04 and 0.06 W/cm ² (for 3 MHz)	+/-
Li <i>et al.</i> , 2015 ³²	Fibroblasts of rabbit ears scar	F: did not report; I: 0.5 W/cm ² TE: 10, 30, 60, 90s;	+/-
Oliveira <i>et al.</i> , 2015 ³³	Mouse fibroblast (L929)	F: 1 MHz; I: 0.3 e 0.5 W/cm ² ; TE: 2 min; DC: 10 and 20%	+/-

Exposure time (TE), Minutes (min), Seconds (s), Duty Cycle (DC), Intensity (I), Frequency (F), (+) present or statistically significant, (-) absent or not statistically significant.

On the other hand, in these papers, the inhibitory effects or the effects which did not have significant statically results comprehended the non-significant increase of cellular viability^{29,33} or a significant decrease on the number of cells^{15,17,25,26,30-32}, absence of significant alterations on the Collagen-I expression¹⁹, on the observed morphology¹⁴ or on the actin fibers²⁸, and even, the rupture of cell membrane²¹. Specifically, on high intensities, there was fast collapse of cell membranes²⁶, besides the loss of adhesion, plasmatic membrane retractions and verification of cellular fragmentation¹⁷.

Due to the heterogeneity of the obtained database, with different cell types, treatment doses and several methods of analysis, it was not possible to carry out the statistical analysis for a meta-analysis.

Discussion

The main results of this systematic review show that still exists a gap in standardization of dosimetries of ultrasonic therapy and its respective correlations with biological effects on fibroblast cells *in vitro*,

due to the scarce available literature found with scientific evidences referring to the subject.

Such divergences about the biological responses appear on the studies frequently, due to the lack of agreement in relation to the intensities, time of application and type of pulse^{5,13-33}. As can be seen on Zhou *et al.*¹³ experiments, made on human fibroblasts, in which the low intensities of TUS, spite of inducing the DNA synthesis, did not activate EGFR (epidermal growth factor receptor), with periods of 6 or 11 minutes and 30 mW/cm² intensity or in the study of Hauser *et al.*²⁰, which triggered a peak in metabolism by means of endocytotic vesicles.

In contrast, Lai and Pittelkow¹⁴ with similar line cell and dose of 0.002 W/cm², reported the absence of difference in morphology and mitosis activities between the treated cells and controls, except on the activation of keratinocyte growth factor (KGF), of c-Jun N-terminal Kinase (JNK) and of extracellular regulated Kinase (ERK), however, with expositions of 7, 15 and 30 seconds. Yet, with higher intensities (2 W/cm²) and longer irradiation time (30 minutes), other authors did not observe relevant impacts on actin fibers in human fibroblasts²⁸.

On the other hand, in fibroblast culture of rabbit ears, the control group, only with ultrasound (I: 0.5 W/cm²; TE: 10s), presented a high survival rate, but, in 30, 60 or 90 seconds there was a decrease of survival levels, pointing a "dose-effect" relation between the treatment durability established and apoptosis³².

At the same time, several authors studied the TUS influence using fibroblasts from rats, and, not differently from others, presented many results^{15,18,23-31,33}. Like Grimaldi *et al.*²³, who showed a sensibility of these treated cells (1MHz; 307 and 46 mW/cm²; 75%; 5, 15, 30, and 60 minutes) and the lack of association with nuclear division rate in these experimental conditions.

As well as Domenici *et al.*²⁷, differing the last one only in intensity (11.8, 15.2 and 19.3 mW/cm²), concluded that the lipid membrane alterations, with consequent high efficiency on molecular absorption by cellular permeability, are related to the sub cavitation manners applied. Still, in tests with 3 MHz frequency, other researchers pointed that the fibroblasts did not present improvements on absorption efficiency (0.01, 0.04 and 0.06 W/cm²)³¹ or positive cellular viability, with analog time to the former ones (1.0, 4.9 and 7.0 mW/cm²)³⁰.

Considering these facts, it is important to highlight the remarkable presence of the bioeffects with the use of equipment with a frequency of 1 MHz^{18,21,22,25,28,33} or even with 20 kHz and, even when compared to 3 MHz²⁹, some authors verified more expressive results with 1 MHz and, they argue, that it is due to the fact that the energy that reaches the cell monolayer is smaller with 3 MHz, as well as they indicate that the loss of energy imposed by the attenuation, absorption, reflection and refraction^{30,31}, since these transferred behaviors to the tissues are intrinsic to both kind of TUS, mechanic and thermic²⁵.

When the applied doses were higher (2W/cm²), having as examples researches done by Tomankova *et al.*¹⁸, specifically the negative control group of NIH-3T3 demonstrated a bigger percent of viable cells than its control, confronting data from Chen *et al.*¹⁵, in which this number decreased drastically.

In the meantime, on mice fibroblasts studies, the main results showed low and medium intensities (F:1 MHz; I: 0.2, 0.3, 0.5 and 0.6 W/cm²; DC: 10 and 20%, TE: 2 min), with reflection on the cell proliferation

extension^{16,17,33}, of the endoplasmic reticulum performance and protein synthesis, being these dosimetries considered beneficial for this biological tissue activity^{5,16,17,33}, but with no exact definition of which is the most indicated dose^{5,16,17,33}. However, with 0.08, 1.0 and 2.0 W/cm², there was a cell growth limitation, appearance of morphologic deformation (membrane retraction) or even, the complete loss of adhesion and cell destruction¹⁷.

Lastly, the findings of this study express that the biophysical properties of ultrasound may unleash biological effects on different biological tissues in different ways. But, due to the heterogeneity of results, there is the necessity of a broader investigation so that these mechanisms can be clearly elucidated.

Conclusion

The analysis of the selected articles for this systematic review shows that the scientific basis is not enough to stablish the real effects and dosimetric parameters of therapeutic ultrasonic irradiation on the process of restoration of fibroblast cells *in vitro*, due to the lack of generalization and conflict of found results. Hence, new studies should be done aiming at the protocol standardization and its interaction with the biological tissue concerned.

Limitations

The authors assume the risk of language bias due to the exclusion of articles published in Chinese.

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