Annals of Anatomy 195 (2013) 57–67

Contents lists available at SciVerse ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.de/aanat

Research article

Cell specific ultrasound effects are dose and frequency dependent

A. Schuster[a], T. Schwab[a], M. Bischof[a], M. Klotz[a], R. Lemor[b], C. Degel[b], K.-H. Schäfer[a,c,*]

[a] Department of Biotechnology, University of Applied Sciences Kaiserslautern, Germany
[b] Department of Ultrasound, Fraunhofer Institute for Biomedical Technology, Germany
[c] Clinic of Pediatric Surgery, University of Mannheim, Germany

ARTICLE INFO

Article history:
Received 1 February 2012
Received in revised form 19 March 2012
Accepted 20 March 2012

Keywords:
Ultrasound
Energy density
Frequency
Epithelial cells
Endothelial cells
Neuroblastoma
Adenocarcinoma

ABSTRACT

Ultrasound is widely used in clinical practice, mostly in diagnostic studies, but increasingly in therapeutic applications as well. This may be the case in acceleration of wound healing or treatment of cancer. Still, little is known about the direct effect of frequency or energy density of the ultrasound upon the cells themselves. We therefore investigated the impact of three different protocols using high, medium and low energy densities at three different frequencies on normal endothelial and epithelial as well as carcinoma cell lines (neuroblastoma and adenocarcinoma cell lines).

Proliferation of endothelial and epithelial cell lines was significantly increased depending on the frequency and energy density applied. No influence on actin cytoskeleton formation was seen in these cells after treatment, while a significant decrease in the density of microvilli and the length of filopodia in the epithelial cell line could be noted. The proliferation rate of the carcinoma cell lines was reduced and cells destroyed. Apoptosis was induced in the adenocarcinoma cells after ultrasound exposure. Additionally, the expression of neurofilament was increased in neuroblastoma cells as evidence of beginning differentiation.

So, different settings of frequency and energy density in an ultrasonic treatment protocol lead to different impacts on proliferation, morphology and differentiation and might be used to stimulate or inhibit the growth of individual cell types.

1. Introduction

Ultrasound is an important form of acoustic energy, which is widely used in therapeutic and diagnostic approaches. Ultrasound is defined as acoustic sound of a frequency above the audible range between 20 kHz and 1 GHz and shows a longitudinal wave in fluid and gas. The ultrasound waves are propagations of pressure and density fluctuations. While the major focus is on diagnostic applications, ultrasound is currently increasingly used in therapeutic applications. Medical ultrasound can be applied alone or in combination with drugs for the treatment of diabetes (Prausnitz et al., 2004), cancer (Kennedy, 2005), stroke (Sacco et al., 2007) as well as for the treatment of thrombosis (Atar and Rosenschein, 2004). Ultrasound acts as an acoustic pressure wave and applies mechanical stress indirectly into the tissues. Several studies have been performed to investigate tissue responses to ultrasound, and also the effect of ultrasound upon the cell itself. It could be demonstrated that ultrasound promotes osteogenesis (Chen et al., 2003), protein synthesis (Hasanova et al., 2011) and calcium uptake. Obviously, the stimulation of various cell functions through the second messenger calcium (Mortimer and Dyson, 1988) and DNA synthesis (Chen et al., 2003) can be influenced by ultrasound. The induction of DNA synthesis is cell type dependent. Ultrasound promotes DNA synthesis in human osteoblast (Chen et al., 2003), in gingival fibroblast (Reher et al., 1998) and periosteal cells (Leung et al., 2004), but not in chondrocytes (Zhang et al., 2003). The molecular mechanisms by which ultrasound induces DNA synthesis or cell proliferation are widely unknown. So far, studies dealing with the influence of frequency or defined energy densities are rare (Ramli et al., 2009).

Here, we investigated the effect of low, medium and high energy density ultrasound at three individual frequencies upon different cell types. Cells from different sources, such as epithelial, endothelial or cancer tissues were exposed to a series of ultrasound protocols. A human cardiac microvascular endothelial cell line (hcMEC) and a canine kidney epithelial cell line (MDCK) were used as epithelial representatives, while a neuroblastoma (Neuro2A) and an adenocarcinoma cell line (HT29) were investigated to study effects upon cells of carcinoma origin. The primary goal of this study was to investigate how frequency and energy density of ultrasound affects cell behaviour such as proliferation or cell morphology. To achieve this, screening for the general effect of

* Corresponding author at: University of Applied Sciences Kaiserslautern, Amerikastr. 1, D-66482 Zweibrücken, Germany. Tel.: +49 0631 3724 5418; fax: +49 0631 3724 5305.
E-mail address: karl-herbert.schaefer@fh-kl.de (K.-H. Schäfer).

0940-9602/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.aanat.2012.03.008

ultrasound on different cell lines was performed. In this screening, three different frequencies were used with the maximum intensity technically possible. This resulted in three different energy densities, thus making it impossible to compare the frequency effect alone. We therefore adapted the energy densities wherever possible and generated three individual protocols corresponding to high, medium and low energy densities at the individual frequency. In this study, the high energy density (50 Ws/cm^2) setting represents the threshold beyond which tissue is assumed to be damaged (Schreiber et al., 1995; Rott, 1981). Medium (25 Ws/cm^2) and low energy density (3 Ws/cm^2) was used to investigate whether ultrasound at such a low energy density still has an effect on different cells. These values range far below energy densities which have been used e.g. to produce liposomes (Kaestner, 2003) and diagnostic ultrasound. In the latter, energy densities up to 100 Ws/cm^2 can be reached (Rott, 1996).

2. Materials and methods

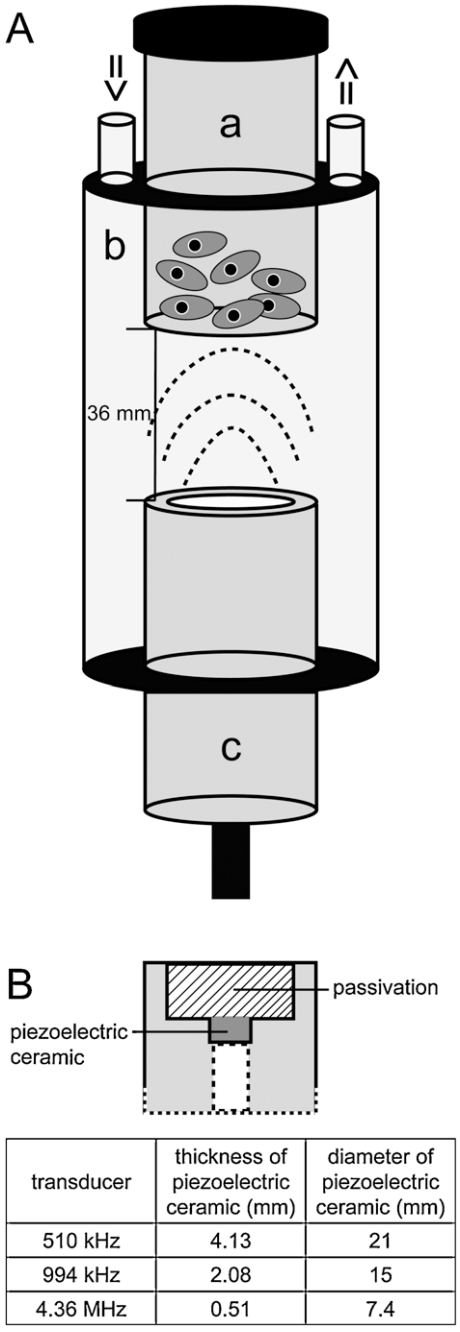
2.1. Cell lines

Four different cell lines were used in all experiments: (1) a human cardiac microvascular endothelial cell line (hcMEC), which was derived from heart ventricles from a single donor, (2) a Madin–Darby Canine kidney epithelial cell line (MDCK), which were first isolated in 1958 (Madin and Darby, 1958), (3) a mouse neuroblastoma line (Neuro2A), which was established 1967 by J.B. Olmsted from the spontaneous tumour of a strain A albino mouse (Olmsted et al., 1970) and (4) a human colon adenocarcinoma cell line (HT29), which was isolated 1964 by J. Fogh from a primary tumour of a colon adenocarcinoma (Rousset, 1986). All cell lines were cultured in Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM, Pan Biotech GmbH, Aidenbach, Germany) + 10% foetal calf serum (FCS, Pan Biotech GmbH) + 1% penicillin/streptomycin (AppliChem, Darmstadt, Germany) + 1% non-essential amino acids (NEAA, Sigma–Aldrich, Taufkirchen, Germany) at 37 °C and 5.6% CO_2 in a humidified incubator.

2.2. Ultrasound device

For the ultrasound treatment, a function generator (DDS function generator 4030, Peak Tech, Ahrensburg, Germany) was used to operate the ultrasound transducer (custom made, Fraunhofer Institute IBMT, St. Ingbert, Germany), generating a continuous wave ultrasound. Three different ultrasound transducers were used consisting of a piezoelectric ceramic. The thickness of the ceramic varies depending on the ultrasound transducer (Fig. 1). The operating focus was located in the near field – far field transition zone. An oscilloscope (TDS1012, Tektronix, Beaverton, United States) was used to monitor the frequency and the voltage. A timer was connected to the function generator to ensure a standardized ultrasound application protocol. To avoid false results by increasing media temperature due to the applied energy, all experiments were performed under standardized perfusion conditions at 37 °C, controlled by a volumetric flow rate pump (mrz-7205, HNP Mikrosysteme GmbH, Parchim, Germany). The indicated intensities were defined using a polynomial regression of single measurement points to establish intensity – voltage curve for the applied setup.

All experiments were performed under controlled, standardized and reproducible conditions. A plastic barrel with a continuous water flow (37 °C) served as experimental set-up (Fig. 1). The cells were placed in a glass tube which fitted exactly in the upper bore of the barrel. Piezo elements producing different fixed frequencies (510 kHz, 994 kHz and 4.36 MHz) were placed on the opposite side in a way that they were focused on the bottom of the glass tube and therefore on the cells which were also located at the bottom of the glass tube (Fig. 1).



transducer	thickness of piezoelectric ceramic (mm)	diameter of piezoelectric ceramic (mm)
510 kHz	4.13	21
994 kHz	2.08	15
4.36 MHz	0.51	7.4

Fig. 1. Setup of ultrasound treatment. (A) Cells were placed in a glass tube (a), whereas piezo elements (510 kHz, 994 kHz and 4.36 MHz) were focussed on its bottom and therefore on the cells (c). A continuous water flow (37 °C) was applied to the plastic barrel (b). (B) The three different ultrasound transducer applying different frequencies because of different sizes of the piezoelectric ceramic.

2.3. Ultrasound protocols

A screening protocol was established to investigate whether ultrasound has any effect on our cell lines. The cells were exposed to ultrasound for 10 min (preliminary tests showed significant results at that time interval) at a frequency of 510 kHz, 994 kHz or 4.36 MHz, respectively. Due to physical and construction-related parameters, intensities and energy densities of the fixed frequencies differ (Table 1).