# CELLULAR ALTERNATIONS IN CULTURED ENDOTHELIAL CELLS UNDER THERAPEUTIC ULTRASOUND IRRADIATION

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## INTRODUCTION

Impaired tissue perfusion is a major cause of acute infarction, organ dysfunction, muscle injury and diabetic ulcers that lead to limb amputation. Restoration of blood supply depends on spontaneous or mediated angiogenesis, which involves a number of steps which include activation of endothelial cells (EC) within existing vessels, breakdown of the basement membrane, migration of EC towards a stimulus, proliferation of the EC, fusion of two sprouts to form a continuous layer of EC, tube formation, and recommencement of blood flow [1]. Medical studies of the past 50 years have shown that therapeutic ultrasound (TUS) irradiation stimulates cellular modifications in biological tissues and accelerates wound healing [2]. It has been shown that TUS sonication (i.e., frequency of 1 MHz to 3 MHz, intensity of up to 2.2 W/cm) induces cell proliferation and migration, collagen synthesis, angiogenesis, secretion of growth factors, production of cytokines, regeneration of skeletal myofibrils, and migration of smooth muscle cells [3,4]. While detailed information on the cellular processes involved in angiogenesis is lacking, it is very likely that EC proliferation is a key component in most angiogenic events. Recent studies have shown that mechanical stimuli, such as pressure and shear stress, induce cellular and biological alternations in EC [5]. Since EC proliferation is most likely to have a key component in angiogenic events, the objective of this study was to investigate the development of multiple cellular alternations within a cultured layer of EC under TUS irradiation, which may stimulate natural healing of biological tissue.

## METHODS

### Cell Culture

Bovine aortic EC were cultured in Dulbecco's modified eagle medium supplemented with L-Glutamine, antibiotics and foetal calf serum. The cells were cultured at $37^{O}C$, $5\%CO_2$ in a humidified incubator until sonication. Cells were seeded in 35mm diameter Petri dishes. Twenty-four hours after seeding the EC were exposed to TUS irradiation according to the experimental protocol. Non-irradiated EC that were seeded with the same procedure were used as control.

### The Experimental Setup

The *in vitro* setup for TUS irradiation of EC was designed for working in the far field where the intensity is uniform. For TUS probes (20 mm diameter) of different frequencies (0.5 MHz – 5 MHz, Etalon, US) the distance to the far field varies from 5 cm to 35 cm. Accordingly, the experimental system for TUS sonication of endothelial cells was built from several Perspex tubes filled with distilled water that serve as sleeve guides between the TUS transducers and the targeted cells (Fig. 1). The transducer was placed in the bottom of the Perspex tube, while the Petri dish (35 mm diameter) with medium and cultured EC was placed inside a stainless steel holder at the upper part of the tube. The Petri dish was installed partially immersed in the water in order to ensure full intensity of the ultrasonic irradiation at the cells layer. The TUS generator and control box was developed by Cardiosonix to allow for multiple variations in the sonication characteristics. It automatically recognizes the transducer frequency and allows for adjustment of the intensity, duty cycle and duration of sonication.

### Experimental Protocol

The experiments were performed with the 3.0 MHz, 1.0 MHz and 0.5 MHz transducers at maximal intensities (less than 2.2 $Wcm^{-2}$). The cultured EC were sonicated for 15 min either at a continuous mode or at a 50% duty cycle of a pulsed mode. Cellular alternations induced by the TUS irradiation were studied from viability assays using Hoechst dye and fluorescence microscopy and from confocal microscopy of variations in cytoskeleton structure and distribution of focal adhesions by implementing methods of Phalloidin and Vinculin staining. Viability was examined immediately after TUS sonication and 24 hours later. Staining for studies of structural alternations was done immediately after TUS sonication as well as after 2 and 24 hours later.

## RESULTS AND DISCUSSION

Employment of the viability assays revealed a death rate of up to 5% in both the control and the sonicated EC, which is typical in a population of cultured cells and may be related to the seeding technique. The Phalloidin stained images revealed the long stress fibers, which are assembled of actin filaments during the process of cell spreading. They stretch all over the cell and provide the main skeleton structure, which is responsible for the cell shape. The images obtained immediately and 2 hours after exposing the EC to TUS sonication clearly demonstrate the damage induced to the stress fibers (Fig. 2). The damage is usually observed by the lack of fibers at the middle of the cells and the presence of small actin spots (Figure 2A). However, in some cases the lack of stress fibers was also observed in the cortex of cells (Figure 2B). Since the stress fibers are in charge of cell spreading, the lack of actin in the cortex of the cell is an abnormal actin distribution. The damage has been observed immediately after TUS sonication, but it became more prominent after 2hours. However, the damage to actin filaments has not been observed 24 hours after TUS sonication of the EC, which may be attributed to recovery mechanisms of the cells.

The damage to the fibers could cause a high concentration of the Actin fiber's monomer G-actin. High concentration gradient of G-actin will cause a high polymerization rate of the actin fibers resulting in many lamellipodia and fillipodia, which we have noticed in many cells (Figs. 2C, 2D). This lamellipodia formation is part of the procedure of cell spreading that may lead to a fast recovery of the cells. Such a recovery mechanism may explain the observation of an undamaged cytoskeleton in the EC 24 hours after TUS sonication. Analysis of Vinculin stained images revealed that the damage may first be induced to the stress fibers and later to the focal adhesions. In a normal healthy cell, the focal adhesions appear to be at the end of the stress fiber that maintain its mechanical characteristics. In some cases the damage to the stress fibers was observed while the focal adhesions were intact (Fig. 3).

## CONCLUSIONS

This study clearly demonstrated that TUS sonication of a layer of cultured EC provokes an observable damage to the stress fibers of the cytoskeleton, which may recover within 24 hours. The EC constitute the inner layer in *in vivo* blood vessels, and thus, induction of a reversible damage to the stress fibers could lead to a fenestrated endothelium that stimulates an immune response required to accelerate cell migration. Cell migration is an important feature in angiogenesis, which is an impotent process in healing. Thus, the temporarily damage induced to the stress fibers by TUS sonication may explain the healing effects of TUS.

## REFERENCE


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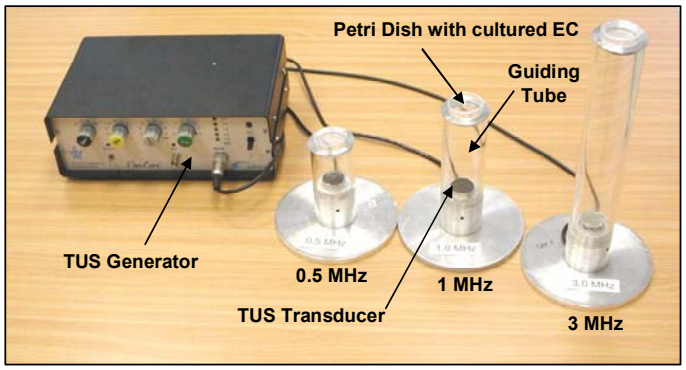


**Figure 1.** The *in vitro* setup for TUS sonication of EC.

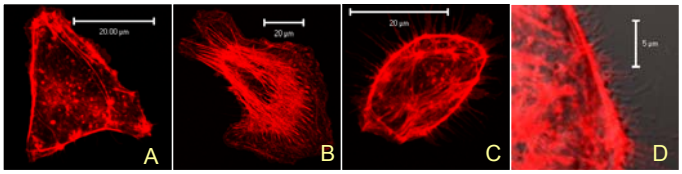


**Figure 2.** The Actin filaments of EC 2hr after the TUS sonication.

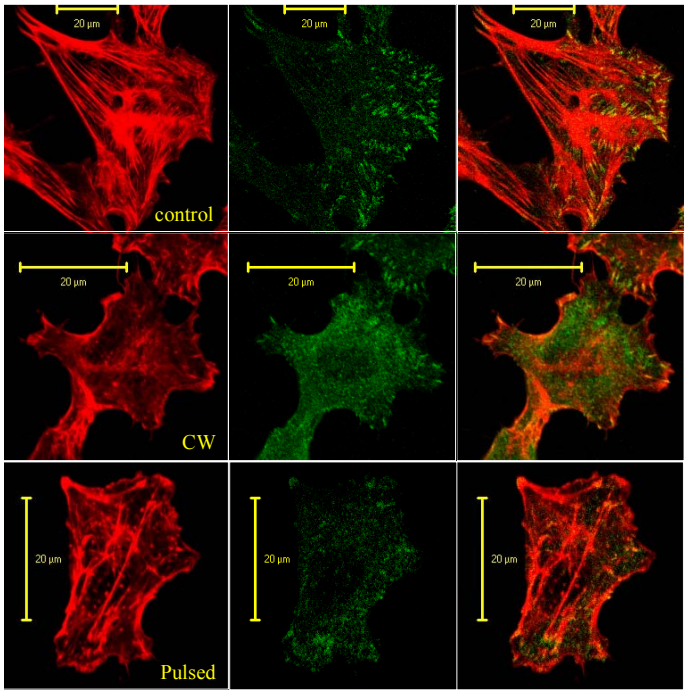


**Figure 3.** Phalloidine staining of stress fibers (left), Vinculin staining of focal adhesions (middle), and super-position of stress fibers and focal adhesions 2 hours after TUS sonication of cultured EC (right).