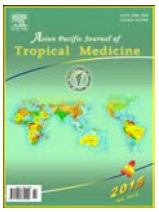
Contents lists available at ScienceDirect IF: 0.926

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60339-4

Microscopic study of ultrasound-mediated microbubble destruction effects on vascular smooth muscle cells

Bo Zhang[1*], Yi-Rong Hou[1], Tian Chen[1], Bing Hu[2]

[1]*Department of Ultrasound Medicine, East Hospital, School of Medicine, Tongji University, Shanghai 200120, China*
[2]*Department of Ultrasound, 6th People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai, China*

ARTICLE INFO

Article history:
Received15 January 2015
Received in revised form 20 February 2015
Accepted 15 March 2015
Available online 20 April 2015

Keywords:
Atomic force acoustic microscopy
Vascular smooth muscle cell
Ultrasound
Microbubble

ABSTRACT

Objective: To observe vascular smooth muscle cell morphological changes induced by ultrasound combined with microbubbles by Atomic Force Acoustic Microscopy (AFAM). **Methods:** A7r5 rat aortic smooth muscle cells were divided into groups: control group (without ultrasonic irradiation, no micro bubbles) and US+MB group (45 kHz, 0.4 W/cm^2 ultrasound irradiate for 20 seconds with a SonoVue™ concentration of [(56-140)$\times 10^5$/mL]. Cell micro-morphological changes (such as topographic and acoustic prognosis) were detected, before and after ultrasound destruction by AFAM. **Results:** In cell morphology, smooth muscle cells were spread o and connected to each another by fibers. At the center of the cell, the nuclear area had a rough surface and was significantly elevated from its surroundings. The cytoskeletal structure of the reticular nucleus and cytoplasm in the morphology of A7r5 cells (20 μ m×20 μ m) were clear before microbubble intervention. After acoustic exciting, the cell structure details of the acoustic image were improved with better resolution, showing the elasticity of different tissues. In the acoustic image, the nucleus was harder, more flexible and uneven compared with the cytoplasm. Many strong various-sized echo particles were stuck on the rough nuclear membrane's substrate surface. The nuclear membrane did not have a continuous smooth surface; there were many obstructions (pores). After ultrasound-intervention was combined with microbubbles, the dark areas of the A7r5 cell images was increased in various sizes and degrees. The dark areas showed the depth or low altitudes of the lower regions, suggesting regional depressions. However, the location and scope of the acoustic image dark areas were not similar to those found in the topographic images. Therefore, it was likely that the dark areas, both from the topographic and acoustic images, were sound-holes. In addition, some cell nuclei become round in different degrees after irradiation. **Conclusions:** Atomic force microscopy and acoustic excitation method can noninvasively and completely display a cell's structure, connections and elastic properties at a nano scale in just several minutes. The dark areas, both from the topographic and acoustic images, may be sound-holes; therefore, it would be helpful if these sound-holes were found. These findings provide a relationship between cell apoptosis after ultrasound and microbubble ultrasound irradiation, and the sound-hole effect.

1. Introduction

Coronary heart disease and cerebral vascular stenosis caused by cardiovascular and cerebrovascular stenosis has become one of the main life-threatening diseases in human health and safety[1,2]. At present, vascular stenosis treatments are divided into three categories: drug treatment, surgical operation, interventional therapy. Among these methods, interventional therapy is a new treatment that has minimum trauma effects and performs well. Percutaneous transluminal angioplasty (PTA) is a common approach for relieving stenosis, and improving cardiac and cerebral blood supply; but this approach has a recurrence rate of 30%-50%[3]. Research revealed that excessive proliferation and migration of smooth muscle cells were the main causes, which leads to restenosis[4]. Effectively preventing vascular restenosis after PTA operation has been a big problem that needs to be solved during cardiovascular and cerebrovascular disease PTA treatments. So far, there are mainly

*Corresponding author: Bo Zhang, M.D., Chief Physician, Department of Ultrasound Medicine, East Hospital, School of Medicine, Tongji University, Shanghai 200120, China.
Tel: 86-15800620806
E-mail: zhangbodongfang@qq.com
Foundation project: It is supported by Shanghai Pudong New Area Health Plan Committee Of Academic Leaders Project (NO. PWRd2013-02), National Natural Fund (NO. 81401428).

four methods used for reducing restenosis; but the effects are not ideal[3]. Among those methods, anticoagulants does not achieve high local drug concentrations surrounding the stent. Radiation can easily cause hemal brittleness, increasing the rate of cancer. At present, gene therapy is still not a safe, effective and stable method for enabling the objective gene to express in the target organs. The long-term efficacy of drug-eluting stents has not yet been determined; however, the risk of inducing thrombosis has already been proven[4]. Therefore, there is an urgent clinical need for establishing a method that has a long-term efficacy and can conveniently, safely, and efficiently inhibit intimal hyperplasia; as well as preventive methods for vascular restenosis.

In recent years, it has been found that ultrasonic irradiation can inhibit proliferation and promote vascular smooth muscle cell apoptosis[5]. This method can be used for treating vascular restenosis, which promises positive results in clinical practice. However, the efficiency of inhibiting cells' proliferation is rather low. Study of Zhang *et al* revealed that cell apoptosis is only around 3%[6] after ultrasonic irradiation. Consequently, researchers are studying the effect of the combination of ultrasonic irradiation and microbubbles. And it turned out that the combination method performed better than using ultrasonic irradiation purely, which could led 20% cells' apoptosis after s ultrasonic irradiation for 24 hour[7]. What's more, with the same irradiation frequency and intensity, different types of microbubbles can have a direct impact on ultrasonic intervention, which may be associated with the number of microbubbles, the expansion size at a certain frequency and radiation intensity, and the jets and shock waves that occurs when a bubble bursts. Atomic force acoustic microscopy (AFAM) has been developed to observe the morphology and internal information of a cell. AFAM can produce high-resolution images by atomic force microscopy (AFM) and provide a non-destructive imaging method by acoustic microscopy. These features can help observe atomic force micrographs and acoustic microscopy in situ, simultaneously. AFAM can obtain the internal information of cells through acoustic waves at a nanometer-level resolution. Once the ultrafine images and elastic coefficients of the same internal and surface areas are acquired, a 3D image of the sample can be easily obtained. The elasticity information of cells can also be observed by AFAM, which has become a powerful tool for observing cell morphology. The research aims to observe the morphological changes of vascular smooth muscle cells (A7r5 cells) after ultrasound, and microbubble ultrasound irradiation by AFAM. We hope to explain the effects of microbubbles on single vascular smooth muscle cells and to explore the mechanisms of vascular smooth muscle cell apoptosis. Providing experimental basis and reference would be helpful in exploring the most suitable frequency and microbubble dose for further studies.

2. Materials and methods

2.1. Cell preparation method and cell samples

A7r5 rat aortic smooth muscle cells were purchased from the cell bank of the Chinese Academy of Sciences. Cells were cultured with a high glucose DMEM medium, containing 10% fetal bovine serum and 1% penicillin and streptomycin, in a cultivating box with 5% CO_2 at 37 ℃. A borosilicate glass was used as a cell adhesion substrate. To make the polylysine adhere on the glass coverslips, the coverslips were immersed and soaked in ethanol for 15 minutes, and 100 μg/mL of polylysine diluted with PBS solution 0.25 mL was dribbled on the coverslips; after preparation, the coverslips kept at 4 ℃ overnight. On the next day, the excess polylysine was absorbed and the coverslips were washed with sterile water, twice[5]. Then, the coverslips were air-dried and irradiated in ultraviolet light for 15 minutes[5].

The coverslips were placed in a 6-well culture plate, placed in 1 $\times 10^6$/mL cells 1 mL, then the cells were cultured according to the above conditions. It was removed when 70%-80% of the coverslip was covered with cells.

2.2. Experimental equipment

Instruments used for the experiment included: carbon dioxide incubator (BPH-9042, Shanghai Yiheng Scientific Instrument Co. Ltd.); HiRox7700 optical microscope (for observing cells in 6-well culture plate); ultrasonic transducer connected to an ultrasonic generator (model: dm-40; Acoustic Laboratory of Shanghai Academy of Sciences, China), the output power was monitored by a digital power analyzer (model: ppa2500; N4L); needle hydrophone (model: SPRH -S -1000; SEA) connected to a digital display (model: TDS 1024B Tech); Atomic force acoustic microscopy was used for monitoring ultrasound (AFAM, SPM by Veeco DI, Santa Barbara, CA, USA production). Two AFAM modes, contact mode and vibration mode were used.

2.3. Analysis method

The cells were divided into 2 groups: control group (treated without ultrasonic irradiation, no microbubbles) and US+MB group [45 kHz, 0.4 W/cm^2 by ultrasound-mediated microbubble destruction for 20 seconds with microbubble concentration: (56-140)$\times 10^5$ microbubbles per milliliter]. The coverslips that were covered with cells were placed into culture plates with a single hole. Saline, SonoVue™, and a microbubble concentration of (56-140)$\times 10^5$ microbubbles per milliliter were added; then, exposed to ultrasonic irradiation at 45 kHz, 0.3 W/cm^2.

Cells were first observed under an optical microscope. Cells in the 6-well culture plate were observed by a HiRox7700 optical microscope; different sizes of spindle-shaped cells attached to the bottom of culture plate and several round cells floating above could be observed[5]. Then, the cells were observed by AFAM. Experimental probe model DNP-10; specific parameters were k=0.113 4N/M, f=22.26 kHz[5].

Cover glass A was removed; a dropper was used to wash the cover glass with distilled water, 3 times; and posted in the AFM sample table after drying. Parameters were adjusted to contact mode and needle scanning; then, cell surface topographic imaging was carried out. Acoustic excitation was applied to the sample in incentive mode, and similar acoustic results were acquired. The acoustic

excitation frequency of the image was multiplicatively decreased by 10 kHz from 112 kHz (for sweep frequency values); vibration and alternating voltage amplitude was 10 V[5]. During the experiment, the acoustic driving frequency was constantly changed to observe the acoustic imaging results. The obtained images became satisfactory when the vibration frequency reached 30 kHz. After ultrasound intervention combined with microbubble irradiation, the cells were immediately immersed in 2% glutaraldehyde. After 30 minutes, the same detection method above was used.

AFAM images provided cell surface information; different image brightness reflected the cell's surfaces at various elevations-convex surfaces were brighter and concave surfaces were darker in the topographic image. In the AFAM acoustic images, the difference in brightness reflected the cell's surface and the sub-surface elasticity was different. Bright sections showed hard textures and elastic modulus; dim points showed soft textures and the elastic modulus was small. The obtained image became satisfactory when the vibration frequency was set to 30 kHz, which could be used for frequency analysis.

3. Results

3.1. Morphological similarities in A7r5 smooth muscle cells

Various smooth muscle cell spindle sizes had a transverse diameter of 15-30 μM. Cell dimensions from the AFAM images were the same with dimensions under a light microscope. Based on the topographic image, smooth muscle cells were spread on the glass surface and cells were connected by fibers (Figure 1, white arrow). The nuclear area was located at the center of the cell, which had a rough surface and significantly elevated than its surrounding areas; the maximum height of the nuclear area was 600 nm (Figure 1).

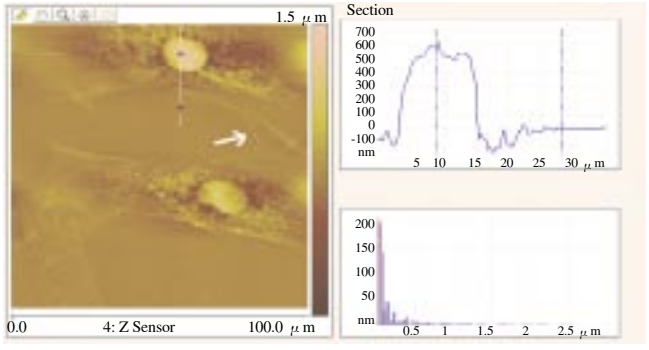


Figure 1. Morphological similarities in A7r5 smooth muscle cells.
The arrow indicated the fibrous connections between A7r5 rat aortic smooth muscle cells; the height difference between the nucleus and cover glass was approximately 800 nm.

3.2. Detection of A7r5 cells combined with microbubbles before ultrasound intervention

3.2.1. Topography

Combined with microbubbles before intervention, AFAM displayed the local A7r5 cell's (20 μm×20 μm) morphology, as shown in Figure 2A; where A was the nucleus and B is the cytoskeleton network distributed in the cytoplasm.

3.3.2. Acoustic image

The acoustic image of local A7r5 cells (20 μm×20 μm) in Figure 2B was achieved by applying acoustic excitation. The acoustic image showed different morphological features, since the details of the cell's structure were displayed more clearly, providing more extensive information, improved contrast, better resolution, and displayed different flexibility. As the acoustic excitation frequency was decreased from 112 kHz to 10k Hz, the image of the cell's structure became more detailed, particularly when the acoustic excitation frequency reached approximately 30 kHz. In the acoustic image, the texture of the nucleus region was more visible than the cytoplasm, which was slightly hard, had an elastic modulus, and had a less uniformed distribution. The membrane substrate surface was unsmooth, which had strong echo particle sizes (Figure 2B, refer to the arrow), which may be ribosomes. The continuous incomplete nuclear membranes with visible multiple interruptions were the pores of the nucleus.

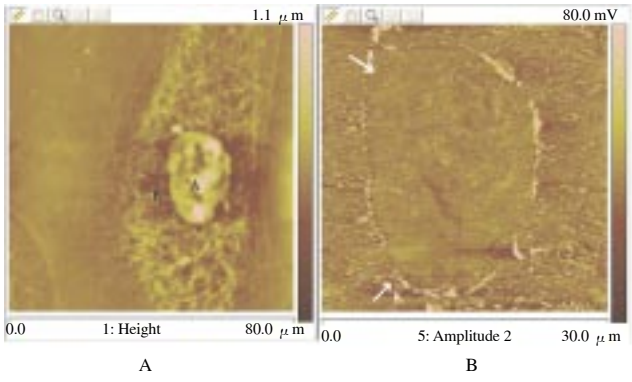


Figure 2. A7r5 cells combined with microbubbles before ultrasound intervention.
A: partial topographic image of A7r5 cells (20 μm×20 μm), B: A7r5 nucleus acoustic image. The nuclear membrane substrate surface as seen from acoustic image with strong echoes, the different sized particles may be ribosomes. A continuously incomplete nuclear membrane showing multiple continuous interruptions nuclear pores.

3.3. Detection of A7r5 cells combined with microbubbles after microbubble intervention

3.3.1. Topography

In the US+MB group, the dark areas of the cells were increased in different sizes and degrees (Figure 3A). The dim areas revealed deep areas, suggesting depressed regions.

3.3.2. Acoustic image

In the US+MB group, there were many dark areas with different sizes and degrees; the locations and scopes were not equal to the dark areas in the topographic images (Figure 3B). It is likely that the dark areas observed from the topographic and acoustic images represent sound-holes. The cell surface was depressed by jets and

shock waves, but only a small part was penetrated. Cell membrane elasticity was significantly greater than the cytoplasm; however, the elasticity of the penetrated area was obviously lower than the surrounding areas. AFAM detection revealed that ultrasound combined with microbubble irradiation can cause sound-holes on A7r5 cells. After radiation, part of the cell's nucleus had different levels of roundness. The strong echo granules that were attached to the nuclear membrane's substrate surface disappeared.

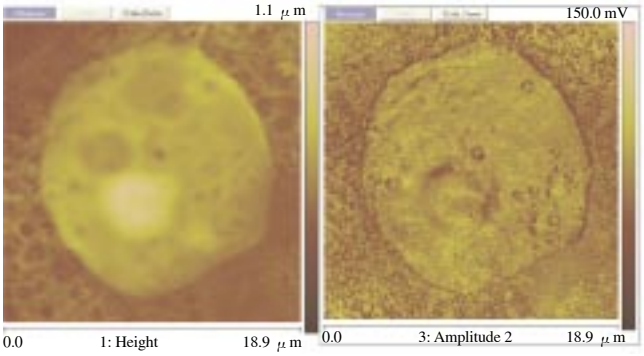


Figure 3. Detection of A7r5 cells combined with microbubbles after microbubble intervention.
A: US+MB group, A7r5 cell morphology image; B: 2 US+MB group, A7r5 cells acoustics image.

4. Discussion

This study used AFAM to observe the morphological changes of vascular smooth muscle cells through ultrasound combined with microbubbles, in order to explore the effects of ultrasound-mediated microbubble destruction on single vascular smooth muscle cells and to reveal the principle or mechanism of vascular smooth muscle cell apoptosis. At the same time, it also provides an experimental basis and reference for further studies.

Based on ultrasonic wavelength, the scale used for a general acoustic microscope is in millimeters[8,9]. It was not able to realize a finer scaled analysis and observation of the sample properties by microscopy. Based on atomic force microscopy, AFAM amplified its acoustic detection technology function, which helped analyze the structure and elastic properties of the cell's surface and internal ultrastructure at a nanoscale[10]. In addition, it is easy to make an AFAM sample using atomic force microscopy as a platform combined with the acoustic detection technique without damaging the cells. The technology can be used to analyze the surface and internal structure of a cell at a nanometer-scale; to observe the cell's shape, connections, membrane, cytoplasm, arrangement structure, distribution and three-dimensional imaging of nucleus, and cytoskeleton in real-time; and to analyze the mechanical properties of the samples, which has unique advantages[11].

In this experiment, it is one of the most important factor to recognize the cell surface Ebert reported that[12], cavitation can make the permeability of cell membrane increased, and then porosity may appear on the surface of the cell membrane, which could be observed only by the atomic force microscope. Atomic force microscopy could tell the pore from 500 nm to 16 μm[13]. The microbubble cavitation is the main mechanism of the inhibition of cell proliferation and induction of cell apoptosis, which is actually a fact that the bubbles transiently cavitate under ultrasound irradiation. In the process of the transient cavitationin, the micro bubble expands rapidly and then collapse quickly under the ultrasound with negative pressure and positive pressure, generating strong shock waves and micro jet. It resulted in the formation of many small holes in cell membrane, which damage the cell membrane, DNA and other cell structures, and even cause cell apoptosis. Ashush research shows that the cavitation effect of ultrasonic can be reported that can induce tumor cell apoptosis and DNA fragments in apoptotic cells were found[14]. In this research, AFAM could collect the ultrastructure and elastic coefficient of the cells on both internal and surface levels without causing any damage to the cells.

In the experiment, it only took a few minutes to get a topographic and acoustic image of the arterial smooth muscle cells, without incurring any damage. The smooth muscle cell's nucleus, nuclear membrane, cytoskeletal structure, cell membrane, cytoplasm, and connecting structure between cells can be clearly displayed, as well as reflecting the elastic distribution of smooth muscle cells.

Based on the images obtained, the acoustic image had a more ideal topographic contrast and resolution, which can also provide cell surface elastic differences. The bright regions of the topographic image did not show up in the acoustic image, which demonstrates that these images are based on different tip-sample interaction modes. The position of the nuclei can be clearly observed from the morphology diagram; however, the nuclear membrane, nuclear pore, and the elasticity distribution of the nuclear content could not be seen. These structural details can be clearly displayed in the acoustic image after acoustic excitation.

The possible mechanisms of ultrasound combined with a microbubble contrast agent to induce apoptosis are as follow: The microbubbles with air in them could lead compression firstly and then inflation under ultrasound, which resulted in the microbubble deformations. And then the threshold for perforation effect with ultrasound was decreased, but the perforation effect was increased[15]. The perforation could lead to cell permeability increased[16] Research suggests that the concentration of the contrast agent is low under the same acoustic radiation conditions; which means that the distance between the bubble and the cell is large, and that the sound-hole is repairable[14]. However, when irradiation time is prolonged or the contrast agent concentration is increased, the distance between bubble and cell becomes narrow; the sound-hole on the cell membrane cannot be repaired and is converted to the lethal effect[16,17]. The sound-hole effect can cause "temporary" or "permanent" pores on the cell membrane to appear; leading to increased tissue cell permeability and capillary injury[14]. Prentice used contrast optical tweezers that could capture shelled microbubbles in the vicinity of single cells. By shock wave, microbubble cavitation and cell surface holes appeared; atomic force microscopy found cells with sound-holes.

In this experiment, AFAM detection revealed that the depressed area in the topographic image is not equal to the less elastic zones in acoustic image; suggesting that the dim areas in both the topographic and acoustic images may probably be sound-holes. Depressions were formed on the cell's surface due to the external force of jets and shock waves; but only a very small part had perforations. Because the elastic modulus of the cell membrane was significantly higher than the cytoplasm, the elasticity of the perforated parts were obviously lower than nearby areas, and the acoustic image showed less elastic zones. AFAM detection provides morphological evidence that ultrasound combined with microbubble-irradiated A7r5 cells can cause sound-holes. In addition, the study revealed that some cells became round after ultrasound combined with microbubble treatments, which may be related to cell apoptosis or necrosis. Because of cell shrinkage, nuclear roundness is one of the morphological features of apoptotic and necrotic cells.

Ultrasound combined with microbubble Technology (UTMD) has become one of the important means of transporting specific gene products or drugs *in vivo*[18.19]. When transporting the target gene contained in microbubble, ultrasound combined with microbubble technology could inject the purpose gene into target cells. At the same time, high amplitude of the microbubbles can increase the capillary and the permeability of the cell membrane, and make tissue or cells more prone to the uptake the released gene .Therefore, the efficiency of gene therapy can be significantly improved. So far, UTMD has been successful in many diseases research, and has made remarkable progress in the past twenty years[20].

Atomic force microscopy and acoustic excitation method can noninvasively display a cell's nano-scale structures, connections and elastic properties. In cells irradiated by ultrasound combined with microbubbles, the dim region in both the morphologic and acoustic images may represent sound-holes. Therefore, evaluating these sound-holes would be helpful in providing evidence that cell apoptosis is associated with sonoporation.

Conflict of interest statememt

We declare that we have no conflict of interests.

References

[1] Lopez AD. Assessing the burden of mortaIity from cardiovascuIar diseases. *WorId Health Star Q* 1993; **46**(2): 91-96.

[2] Angelo A, Dickstein K, Michael B, Anker D, Stamatis A, Kenneth D, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2008: the Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2008 of the European Society of Cardiology. *Eur Heart J* 2008; **29**(19): 2388-442

[3] Williams DO, Holubkov R, Yeh W. Percutaneous coronary intervention in the current era compared with 1985-1986: The National Heart, Lung, and Blood Institute Registries. *Circulation* 2000; **102**(24): 2945-2951.

[4] Smith DA1, Galin I. Statin therapy for native and peri-interventional coronary heart disease. *Curr Mol Med* 2006; **6**(5): 589-602.

[5] Zhang B, Zhou H, Cheng Q, Lei L, Hu B. Low-frequency low energy ultrasound combined with microbubbles induces distinct apoptosis of A7r5 cells. *Mol Med Rep* 2014; **10**(6): 3282-3288.

[6] Wei C, Bai WK, Wang Y, Hu B. Combined treatment of PC-3 cells with ultrasound and microbubbles suppresses invasion and migration. *Oncol Lett* 2014; **8**(3): 1372-1376.

[7] Ebert AM, Du JK, Wang XW. The elastic properties of hamster kidney cells evaluated by ultrasonic atomic force microscopy. *IEEE Ultrason Symp* 2004; 689-692.

[8] Hammer MU, Brauser A, Olak C, Brezesinski G, Goldmann T. Lipopolysaccharide interaction is decisive for the activity of the antimicrobial peptide NK-2 against *Escherichia coli* and *Proteus mirabilis*. *Biochem J* 2010; **427**(3): 477-488.

[9] Certin B, Sthal F. Scanning microdeformation microscopy. *Appl Phys Lett* 1993; **62**(8): 829 -831.

[10]Ebert AM, Du JK, Wang XW. The elastic properties of hamster kidney cells evaluated by ultrasonic atomic force microscopy. *IEEE Ultrason Symp* 2004; 689-692.

[11]Nowakowski R, Lucklam P. Imaging the surface details of red blood cells with atomic force microscopy. *Surf Interface Anal* 2002; **33**: 118-121.1.2013.08.020.

[12]Ebert A, Tittmann BR, Du J, Scheuchenzuber W. Technique for rapid in vitro single-cell elastography. *Ultrasound Med Biol* 2006; **32**(11): 1687-1702.

[13]Wu G, Mikhailovsky A, Khant HA, Fu C, Chiu W, Zasadzinski JA. Membrane disruption by optically controlled microbubble cavitation. *Nature Physics* 2005; **1**(2): 107-110.

[14]Ashush H , Rozenszajn LA , Blass M , Barda-Saad M , Azimov D, Radnay J, et al. Apoptosis induction of human myeloid leukemia cells by ultrasound exposure. *Cancer Res* 2000; **60**(4): 1014-1020.

[15]Shen ZY. The effects of low-frequency ultrasound and microbubbles on rabbit hepatic tumors . *Exp Biol Med (Maywood)* 2014; **239**(6): 747-757.

[16]Ebert AM, Du JK, Wang XW. The elastic properties of hamster kidney cells evaluated by ultrasonic atomic force microscopy. *IEEE Ultrason Symp* 2004; 689-692.

[17]Ma J. Ultrasound contrast-enhanced imaging and *in vitro* antitumor effect of paclitaxel-poly (lactic-co-glycolic acid)-monomethoxypoly (ethylene glycol) nanocapsules with ultrasound-targeted microbubble destruction. *Mol Med Rep* 2014; 311-317.

[18]Ashush H, Rozenszajn LA, Blass M, Barda-Saad M, Azimov D, Radnay J, et al, A study of the ultrasound-targeted microbubble destruction based triplex-forming oligodexinucleotide delivery system to inhibit tissue factor expression. *Mol Med Rep* 2015; **11**(2): 903-909.

[19]Zhou XL, Shi YL, Li X. Inhibitory effects of the ultrasound-targeted microbubble destruction-mediated herpes simplex virus-thymidine kinase/ganciclovir system on ovarian cancer in mice. *Exp Ther Med* 2014; **8**(4):1159-1163.

[20]Chen ZY, Lin Y, Yang F, Jiang L, Ge SP, Gene therapy for cardiovascular disease mediated by ultrasound and microbubbles. *Cardiovascular Ultrasound* 2013; **11**(2): 111-117.