



# Low-frequency ultrasound induces apoptosis of rat aortic smooth muscle cells (A7r5) via the intrinsic apoptotic pathway

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Genet. Mol. Res. 13 (2): 3143-3153 (2014)

Received March 27, 2013

Accepted September 3, 2013

Published April 17, 2014

DOI <http://dx.doi.org/10.4238/2014.April.17.10>

**ABSTRACT.** Ultrasound, a non-invasive therapy method, is a potential tool for medical applications, but its biological effects on vascular smooth muscle cells (VSMCs) have not been characterized. The aim of this study was to explore the effect and possible apoptotic mechanism of VSMCs that were induced by low-frequency ultrasound (LFU). Cell viability and apoptosis of A7r5 cells were evaluated after treating A7r5 cells with a continuous 45-kHz 1.0-W/cm<sup>2</sup> ultrasound (exposure time of 0, 10, 20, 30, and 35 s) by MTT assay and flow cytometry. At the optimum ultrasound exposure condition (30 s), gene chip analysis was performed, and the apoptotic signaling pathway was confirmed by reverse transcription-polymerase chain reaction and Western blot.

As measured by flow cytometry, LFU significantly induced A7r5 cell apoptosis. Comparing the ultrasound group with the control group, the protein expression of caspase-9 and caspase-3 was increased by 50 and 57%, respectively; the caspase-3 mRNA level was increased by 37.5%. These findings indicate that an intrinsic pathway plays a major role in apoptosis that is induced by LFU and that LFU can induce A7r5 cell apoptosis via caspase-9- and caspase-3-dependent pathways.

**Key words:** A7r5 cells; Apoptosis; Caspase-9; Caspase-3; Low-frequency ultrasound

## INTRODUCTION

Vascular smooth muscle cells (VSMCs) are essential for good performance of the vasculature. By contracting and relaxing, VSMCs play an important role in maintaining an appropriate blood pressure by altering the luminal diameter, and they also perform other functions, which become progressively more important during vascular remodeling (Owens et al., 2004; Hayashi and Naiki, 2009). Unlike other muscle cells, VSMCs do not terminally differentiate. They can shift reversibly along a continuum from a contractile phenotype (also known as differentiated phenotype or quiescent phenotype) to a synthetic phenotype (also known as dedifferentiated phenotype or activated phenotype) (Beamish et al., 2010). In this process, the increased proliferation and migration of VSMCs are undoubtedly required and potent for vascular repair (Beamish et al., 2010). However, the excessive proliferation of VSMCs can aggravate vascular injury, which was reported to occur in restenosis after angioplasty (Reimers et al., 1997). Therefore, it is important to find strategies to prevent the excessive proliferation or induce the apoptosis of VSMCs to a certain extent that does not compromise the normal cellular expansion.

Ultrasound, a kind of non-ionizing energy, has been used in various therapeutic medical applications, including sonophoresis (ultrasonic transdermal drug delivery), dentistry, eye surgery, body contouring, breaking kidney stones, eliminating blood clots, and increasing blood-brain barrier permeability (Ahmadi et al., 2012; Man et al., 2012; Xia et al., 2012). Several reports have demonstrated that ultrasound can induce cell apoptosis (Fang et al., 2007; Hundt et al., 2007; Chida et al., 2009; Tsuang et al., 2011). Recently, low-frequency ultrasound (10-60 kHz) has received increased attention as a rapid and reagent-less method because of its use to enhance various biotechnological processes as a non-invasive therapy method (Rokhina et al., 2009), while the mechanism has not been fully characterized. Lower frequency acoustic waves have greater tissue penetration than higher frequency acoustic waves because of their longer wavelength (Man et al., 2012); thus, low-frequency ultrasound can easily penetrate deep tissue. Some studies have shown that low-frequency ultrasound irradiation has an antitumor ability by inhibiting tumor cell proliferation and inducing apoptosis (Lejbkiewicz and Salzberg, 1997; Jiang et al., 2012), and the antitumor effect is enhanced by combining the treatment with antitumor drugs, such as hematoporphyrin, adriamycin, endothelial monocyte-activating polypeptide II, and quercetin (Paliwal et al., 2005; Meng et al., 2008; Zhang et al., 2010).

Considering the potency of low-frequency ultrasound on the proliferation and apoptosis of cells, we speculate that low-frequency ultrasound may have a similar effect. However, to date, there are few studies about the effect of ultrasound on the proliferation and apoptosis of VSMCs, and the role of the apoptotic pathway remains unclear.

In order to investigate the effect of low-frequency ultrasonic treatment on aortic smooth muscular cells (A7r5 cells) and apoptosis, this study was performed with a continuous 45-kHz 1.0-W/cm<sup>2</sup> ultrasound. The results showed the effect of ultrasound on the apoptosis of VSMCs, and they indicated that caspase-3- and caspase-9-dependent pathways play key roles in apoptosis that was induced by ultrasound, which may potentially be considered as a new therapeutic tool for restenosis after angioplasty.

## MATERIAL AND METHODS

### Cell preparation

A7r5 cells (VSMCs) were purchased from the cell bank of Chinese Academy of Science and were cultured on 100 x 20-mm tissue culture dishes (Falcon, Oxanard, CA, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% newborn calf serum, 2 M L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were harvested, resuspended, and immediately used for the experiment at a concentration of 10<sup>6</sup> cells/mL at 80% confluence in 2-mL tubes. The cells were subcultured twice per week, and cell numbers were counted by the use of a hemocytometer (Anxin Optical Equipment, Shanghai, China). The viability of the cells was determined by the trypan blue exclusion test. In all cases, cell viability was greater than 90%. Cultures were passaged upon reaching confluence by brief exposure to phosphate-buffered saline (PBS) containing 0.5 mg/mL trypsin and 0.5 mM EDTA at 37°C, and they were subcultured in DMEM supplemented with serum and antibiotics as described above. Cell growth was observed under an inverted phase contrast microscope (Carl Zeiss, Oberkochen, Germany).

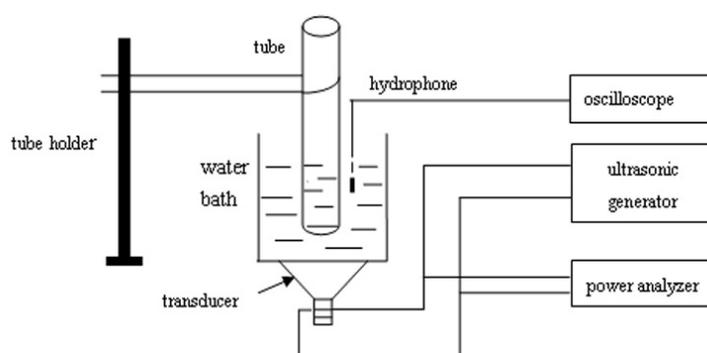
### Ultrasound apparatus

The ultrasound apparatus (model: DM-40 Shanghai Acoustics Laboratory, Chinese Academy of Sciences) was used. The experimental apparatus mainly consisted of 2 parts: an ultrasonic generator and a transducer. The ultrasonic transducer units, operating at a frequency of 45 kHz (effective radiating area, 38.04 cm<sup>2</sup>), were positioned sidewise in the bottom of a water bath. The transducer was connected to an ultrasonic generator, which worked in a continuously adjustable frequency, and its maximum output electric power was about 50 W. In addition, the output electric power was monitored by a digital electric power analyzer (model: PPA2500; N4L). By adjusting the electric power, the ultrasound intensity was set. A needle-type hydrophone (model: SPRH-S-1000; SEA), which was positioned in the tube and connected to a digital oscilloscope (model: TDS 1024B; Tektronix), was used to check the operating conditions.

### Protocol for ultrasound treatment

A7r5 cells of confluent cultures were trypsinized and were suspended at a concentration of 5 x 10<sup>5</sup> cells/mL DMEM in a culture tube measuring 16 x 125 mm (Corning, Staffordshire, UK). The tubes were then positioned in the tube holder so that the cell suspension was

in the middle of the transducer at a distance of 10 mm from its surface within the “near field” of the ultrasound beam (Figure 1). Then, they were exposed to ultrasound irradiation using the ultrasound apparatus described above. The frequency of the ultrasound was 45 kHz. The ultrasound intensity of 1.0 W/cm<sup>2</sup> was achieved by adjusting the electric power. The exposure time to ultrasound was set at 0, 10, 20, 30, and 35 s for each tube of cells. To avoid the thermal effect of ultrasound, the culture tube was put in a thermostatic water bath (precision:  $\pm 0.05^{\circ}\text{C}$ ), and the temperature was measured by thermocouple in the test tube and the bath. To diminish the effect of standing waves, the tube was shaken slightly during ultrasound irradiation.



**Figure 1.** Schematic diagram of sonication set-up.

### Viability of ultrasound-treated cells by tetrazolium (MTT) assay

In the MTT assay, aliquots of A7r5 cells (at 10,000 cells per well) were trypsinized, suspended, treated by ultrasound, and transferred to a fibronectin (25 ng/mm<sup>2</sup>) precoated flat-bottomed 96-microwell plate (0.1 mL/well). Briefly, the MTT assay was performed according to the manufacturer recommendation. The absorption was determined at 492 nm using the microplate reader (Kontron SLT-210-Thermo Fisher Scientific, Waltham, MA, USA). Tetrazolium dissolved in dimethyl sulfoxide and diluted in PBS to a final concentration of 7 mM was added (10 mL MTT/well) to each well. After incubation for 4 h, 100 mL 0.004 N HCl-isopropanol was added to each well, and the contents were vigorously mixed by repeated pipetting. After 5 min, the plates were read by an enzyme-linked immunosorbent assay reader (Kontron SLT-210) at a wavelength of 492 nm, and the results are reported as optical density (OD).

### Detection of apoptotic cells by flow cytometry

To observe cells undergoing apoptosis, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection kit according to the manufacturer recommendation (KeyGEN, Nanjing, China). Briefly, the cells were harvested and washed twice with PBS, resuspended in 500  $\mu\text{L}$  binding buffer, and incubated with 5  $\mu\text{L}$  annexin V and 5  $\mu\text{L}$  PI for 15 min in the dark at room temperature. Then,  $1 \times 10^4$  cells were measured with a flow cytometer using the Cell Quest software. The percentage of apoptotic cells was determined using a flow cytometer (BD FACSCalibur; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

## Gene display analysis

To determine the effect of ultrasound on the gene expression in A7r5 cells, the supernatants from control and ultrasound-treated (30 s) cells were collected. Invitrogen Trizol was used to extract total RNA according to manufacturer instructions. Reverse transcription, cDNA synthesis, and biotinylated cDNA were prepared from 100 ng total RNA according to the standard Roche-NimbleGen protocol. Briefly, 5.5 µg cDNA was hybridized for 16 h at 45°C on GeneChip Human Gene 1.0ST Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450 and were scanned using the GeneChip Scanner 3000 7G. The data were analyzed with Partek Genomics Suite version 6.5 using Robust Multichip Analysis settings and global scaling as the normalization method. Then, the Significance Analysis of Microarrays software was used for data analysis (Tusher et al., 2001). Usually, the selection standard of gene difference is to control the false-discovery rate to less than 5% and the fold-change is over 1.5 or 2.

## Detection of caspase-3 and caspase-9 expression in A7r5 cells

Real-time quantitative PCR was used to detect apoptotic gene expression in A7r5 cells. The templates were reverse-transcription products of the A7r5 cell line, whose RNA was extracted by the RNA extraction kit of TIANGEN and reverse transcribed by the MBI RevertAid™ First-Strand cDNA Synthesis Kit. The primers of the target genes were caspase-9 (forward: 5'-AAGACCATGGCTTTGAGGTG-3' and reverse: 5'-CAGGAACCGCTCTTCTTGTC-3') and caspase-3 (forward: 5'-TGTCGATGCAGCTAACCTCAGA-3' and reverse: 5'-CCACAGGTCCGTTCCGTTCAA-3'). The reaction was set up according to the TAKARA SYBR Premix Ex TaqII (Tli RNaseH Plus) KIT, and an ABI 7500 Fast Real-Time PCR System was used to perform PCR under the following conditions: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 3 s and 60°C for 30 s; and a final melt curve. Amplified products were separated on 1% agarose gels and photographed with a Kodak Gel Logic 100 Imaging System. The densities of the bands were determined using the Gel-Pro Analyzer 4.0 software.

## Western blot analysis

Treated cells were harvested and lysed in nondenaturing lysis buffer (Applygen Technologies, Beijing, China). Samples containing 30 µg protein per lane were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were blocked in 5% skim milk-TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) for 1 h. Then, the respective primary antibodies for caspase-3 and -9 were added in the same milk and incubated overnight at 4°C, and the membrane was then incubated with horseradish peroxidase-conjugated secondary antibody in TBST for 2 h at room temperature. The blot was developed with LAS3000 chemiluminescence system (Fujifilm, Tokyo, Japan), and the densities of the bands were determined using the Gel-Pro Analyzer 4.0 software.

## Data acquisition and statistical analysis

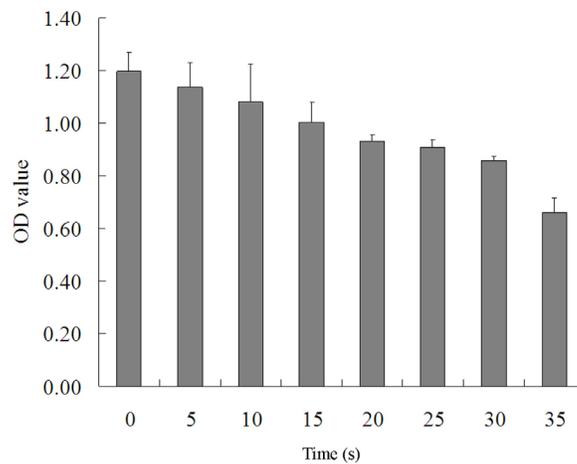
At least 3 independent experiments were performed per data point. The results are

reported as means and standard deviations of these ratios. The differences of the means were analyzed by the Student *t*-test. P values that were  $>0.05$  were not considered statistically significant.

## RESULTS

### Cell viability

To analyze whether the ultrasound treatment was toxic to the A7r5 cell line and to determine the optimal time course, the MTT assay was used to measure changes in cell viability. The results are reported as OD. Results showed that with longer exposure time (over 35 s), the cell viability decreased sharply, as shown in Figure 2. Therefore, we chose less than 30 s as the optimal time point for apoptosis analysis by flow cytometry and 30 s for gene chip analysis to avoid too much cell death in the samples.



**Figure 2.** Viability of ultrasound treated A7r5 cells by MTT assay. The results represent one of three individual experiments. OD values of ultrasound-treated and -untreated A7r5 cells were measured by filter 492.

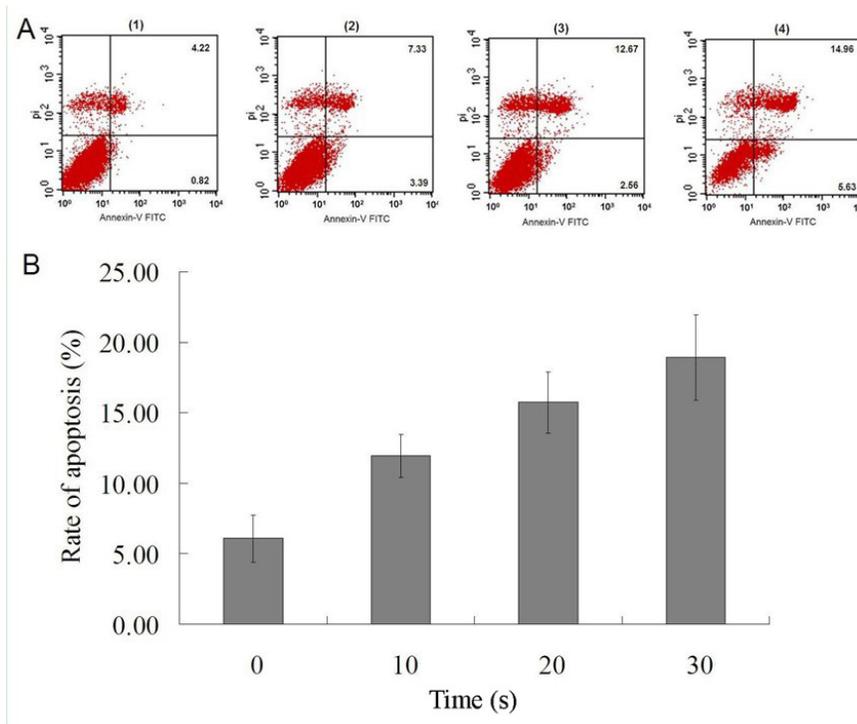
### Apoptotic rates detected by flow cytometry

The A7r5 cell apoptosis that was induced by low-frequency ultrasound was evaluated with flow cytometry. We found that the apoptosis rate increased with increasing ultrasound exposure time. Compared with the control group ( $6.09 \pm 1.67$ ), the apoptosis rate in treated groups (10, 20, and 30 s) was significantly higher ( $11.96 \pm 1.52$ ,  $15.74 \pm 2.16$ , and  $18.94 \pm 3.04$ ;  $P < 0.001$ ) (Figure 3). These results indicated that a significant amount of apoptosis could be induced by low-frequency ultrasound.

### Gene display analysis

At the optimum exposure condition (30 s), gene chip analysis was performed on the

ultrasound irradiation-treated and control group. There were significant changes in the gene expression of the caspase signaling pathways. The expression of caspase-3 and caspase-9 was upregulated over 1 time, as shown in Table 1. These results indicate that the intrinsic pathway may play a key role in apoptosis that is induced by low-frequency ultrasound. Further studies on the detailed mechanism should also be performed.



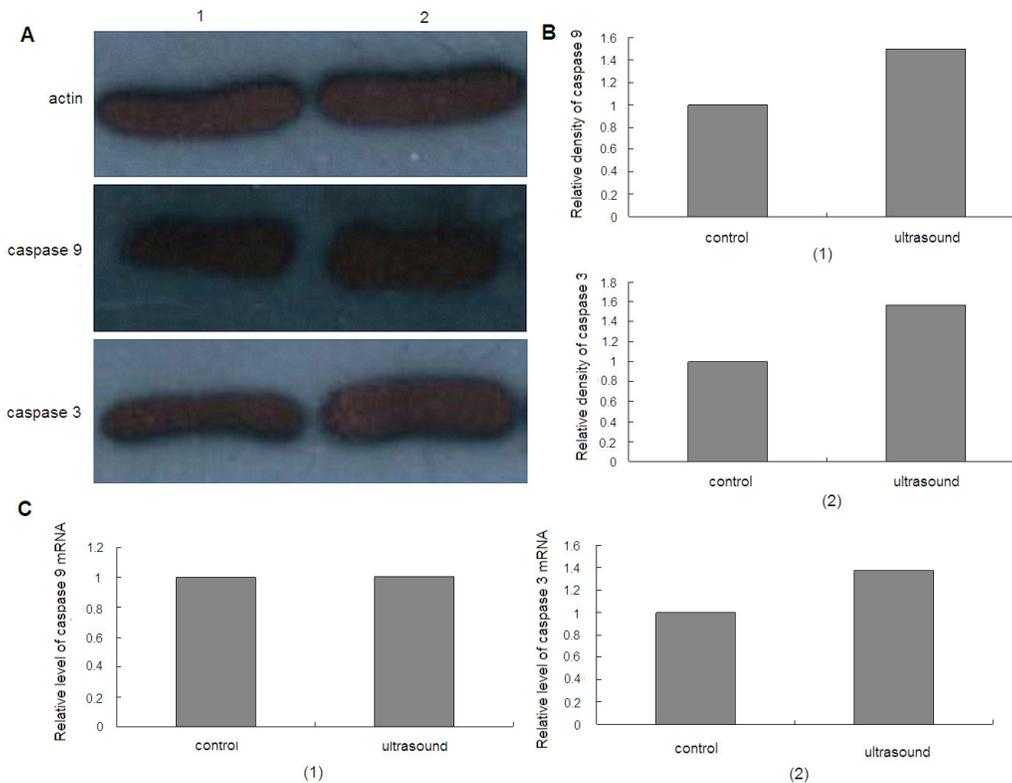
**Figure 3.** Detection of apoptotic cells by flow cytometry. **A.** Detection of apoptotic cells by flow cytometry at different exposure time to low-frequency ultrasound. A (1), (2), (3) and (4) represents exposure times 0, 10, 20, and 30 s, respectively. **B.** Histograms show that there are significant higher rates of apoptosis in ultrasound irradiation groups compared with the control group (10 vs 0 s,  $P < 0.05$ ; 20 and 30 vs 0 s,  $P < 0.001$ ,  $N = 4$ ).

**Table 1.** Differential expressed genes involved in caspase signaling pathways.

Name	Fold-change (overexpressed HSP27 group vs A7r5 group)
CASP8AP2	1.45887 up
CASP3	1.01182 up
CASP6	-1.31528 down
CASP8	-1.13575 down
CASP10	1.24914 up
CASP14	-1.00248 down
CASP1	1.43117 up
CASP5	1.33765 up
CASP4	1.04137 up
CASP12	-1.15627 down
CASP7	1.01752 up
CASP9	-1.03515 down

### Confirmation of RT-PCR and Western blot

In order to confirm if the gene expression results were correct, RT-PCR and Western blot analysis of caspase-9 and caspase-3 were performed (Figure 4). The results showed that caspase-9 and caspase-3 proteins were expressed higher in the ultrasound irradiation group than in the control group, as shown in Figure 4A and B, which is consistent with the results of the differential gene display. In the ultrasound irradiation group, the protein expression of caspase-9 and caspase-3 was increased by 50 and 57%, respectively, compared with that in the control group. Additionally, the caspase-3 mRNA level was increased by 37.5%, but the caspase-9 mRNA level did not change, as shown in Figure 4C. The reason that no change was detected for the caspase-9 mRNA level may be related to the late time course; caspase-9 mRNA might be expressed in cultured cells in the very early stage after exposure to ultrasound. These results confirmed that the intrinsic apoptotic pathway played a very important role in cultured rat aortic smooth muscular cells (A7r5 cells) that was induced by low-frequency ultrasound.



**Figure 4.** Western blot and RT-PCR analysis of caspase-9 and caspase-3. **A.** Immunoblotting assay for the inactive precursor of caspase-9 and caspase-3. **B.** Histograms showing the densitometric analysis of caspase-9 and caspase-3 respectively; expression level of both caspase-9 and caspase-3 proteins was increased by 50 and 57%, respectively. **C.** RT-PCR assay for the inactive precursor of caspase-9 and caspase-3 mRNA level respectively; caspase-3 mRNA level in ultrasound-treated group was increased by 37.5%, compared with the control group; but caspase-9 mRNA level had no change; data represent one of three independent experiments.

## DISCUSSION

Apoptosis, or programmed cell death, is a normal physiologic process that contributes to the homeostasis of tissue and maintains the balance between cell proliferation and cell death. Apoptosis is always a special cellular response to stress stimuli, such as exposure to chemotherapeutic drugs, oxidative stress, free radicals, X-rays, ultraviolet radiation, and physical stress including ultrasonic irradiation (Feng et al., 2008; Vlad et al., 2011). Apoptosis occurs through 2 main pathways: the extrinsic (death ligand) and intrinsic (mitochondrial) pathway (Salvesen, 2002; Harrington et al., 2008; Ghavami et al., 2009). This extrinsic activation then triggers the hallmark caspase cascade that is characteristic of the apoptotic pathway, in which caspase-3 plays a dominant role (Perry et al., 1997; Stennicke et al., 2000). In intrinsic activation, cytochrome c from the mitochondria works in combination with caspase-9, apoptosis-activating factor 1, and ATP to process caspase-3 (Katunuma et al., 2001; Li et al., 2004), which subsequently activates the rest of the caspase cascade and leads to apoptosis.

A new non-invasive method to detect DNA damage using mid- to high-frequency ultrasound (10-60 MHz) has been developed for future investigations regarding the use of ultrasound in preclinical and clinical settings to non-invasively monitor tumor responses to specific interventions that induce cell death (Vlad et al., 2011). Although reports showed that tumor cell death could be induced by low-frequency, low-intensity ultrasound and that the apoptotic pathways were caspase-3-dependent with or without the involvement of bax (a pro-apoptotic protein) and bcl-2 (an anti-apoptotic protein) (Bai et al., 2012; Zhang et al., 2012), we did not know if VSMCs respond like tumor cells. Besides, there are few reports about the effect of low-frequency ultrasound irradiation on apoptosis of VSMCs. Thus, we were prompted to investigate the effect and possible mechanism of VSMCs that were exposed to low-frequency ultrasound.

The exposure time is an important index in this study. The MTT results revealed that the cell viability decreased sharply with longer exposure time (over 35 s) (Figure 2). Therefore, we chose less than 30 s as the optimal time point for apoptosis analysis by flow cytometry and 30 s for gene chip analysis to avoid too much cell death in the samples.

The apoptosis of this cell line was evaluated at the optimal time point in our lab for cell death (<30 s) because exposure to ultrasound alone resulted in minimal cell death (Figure 2). We found that apoptosis could be induced in A7r5 cells by continuous 45-kHz 1.0-W/cm<sup>2</sup> ultrasound treatment.

Our results indicated that when treated with low-frequency ultrasound, the protein expression of caspase-9 and caspase-3 was increased by 50 and 57%, respectively. Additionally, the caspase-3 mRNA level was increased by 37.5%, but the caspase-9 mRNA level did not change. These results suggested that the intrinsic apoptotic pathway was induced in VSMCs that were exposed to ultrasound. The proliferation and apoptosis of VSMCs play important roles in the pathologic restenosis of blood vessels after percutaneous transluminal angioplasty and stenting (Reimers et al., 1997). Therefore, ultrasound treatment may potentially be considered as a new therapeutic tool for intimal hyperplasia. However, further investigations are needed to clear the following questions:

- 1) How does ultrasound induce the intrinsic apoptotic process?
- 2) Does the extrinsic (death ligand) pathway play any role in the apoptotic process?
- 3) Is a new pathway involved in the apoptotic process that is induced by low-frequency ultrasound?

Taken together, our results showed the remarkable activation of caspase-9 and caspase-3 in aortic smooth muscle cells, indicating that the intrinsic pathway (caspase-9- and caspase-3-dependent) plays a major role in the A7r5 cell apoptotic process that is induced by continuous 45-kHz 1.0-W/cm<sup>2</sup> ultrasound.

## ACKNOWLEDGMENTS

Research supported by the Shanghai Pujiang Project of China (Grant #09PJ1408800), the Academic Leaders Training Program of Pudong Health Bureau of Shanghai (Grant #PWRd2013-02), and the National Natural Science Foundation of China (Grant #81271597).

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