In vitro modulation of intracellular oxidative stress of endothelial cells by diagnostic cardiac ultrasound

Giuseppina Basta*, Lucia Venneri, Guido Lazzerini, Emilio Pasanisi, Mascia Pianelli, Nicoletta Vesentini, Serena Del Turco, Claudia Kusmic, Eugenio Picano

CNR, Institute of Clinical Physiology, Pisa, Italy

Abstract

Diagnostic cardiac ultrasound are commonly assumed to pose no hazard to the patient—but this is not synonymous with being biologically inert. The production of intracellular reactive oxygen species (ROS) on endothelial cells is a key modulator of atheroprotective (at low level) and atherogenic (at high levels) actions. The aim of the study was to evaluate in vitro the effects on intracellular ROS of endothelial cells after ultrasound exposure of variable duration with commercially available cardiac imaging systems. Endothelial cells fluorescence was evaluated in vitro after sham (transducer off) exposure to ultrasound and after 5′, 15′ and 30′ of ultrasound irradiation with second harmonic 1.3/2.6 MHz cardiac ultrasound scan (mechanical index 1.5). Intracellular ROS were 83 at baseline, and rose to 86, 112 and 122 fluorescence intensity at 1 h incubation after 5′, 15′ and 30′ of ultrasound exposure respectively ($P < 0.01$ for *30′ versus baseline and 5′* comparison). There were microscopic signs of endothelial damage only following 30′ stage. Ultrasound exposure induced significant DNA laddering and LDH leakage after 15′ of ultrasound exposure. Effects on endothelial cells could be reproduced by adding exposed extracellular medium to unexposed cells, and could be prevented removing exposed medium from cell culture or pretreating the medium with catalase. Cardiac ultrasound of current clinical diagnostic use increases intracellular oxidative stress on endothelial cells in vitro. This increase is accompanied by morphological evidence of endothelial damage only after longer exposure times, persists 1 h after withdrawal of ultrasound, and can be modulated over a wide range according to the duration of ultrasound exposure. Free radical production in the extracellular medium is the likely mediator of ultrasound effect.
© 2003 European Society of Cardiology. Published by Elsevier Science B.V. All rights reserved.

Keywords: Endothelial function; Free radicals; Ultrasound

1. Introduction

One of the principal reasons for the popularity of echocardiography is that the examination is presumed to pose no hazard to the patient [1]. The question of the safety of this external energy source, however, must still be constantly reviewed since there are obvious biological effects of ultrasound and large gaps exist in the knowledge base concerning the effects of ultrasound and living cells [1]. The effects that occur on a gross scale are easily identified and, hence, appreciated, but more subtle effects, which are not readily evident, may be retained within the system and go clinically undetected. Therefore, it is necessary to explore more subtle cellular functions of key biological relevance. Among these cellular functions, intracellular reactive oxygen species (ROS) generation of endothelial cells is obviously important, since it may have athero-protective (at low level) and atherogenic (at high levels) actions [2], because large amounts of ROS are known to induce apoptotic endothelial cell death [3], and may contribute to the initial endothelial injury which promotes atherosclerotic lesion formation [4,5].

Free radical formation by ultrasound is due to inertial cavitation and is strongly dependent on its threshold

---

*Corresponding author. Tel.: +39-50-315-2661; fax: +39-50-315-2166.

E-mail address: lapina@ifc.cnr.it (G. Basta).

acoustic pressure at specific frequencies [6]. When cavitation occurs in response to the passage of ultrasonic waves through water, H$^.$ and OH$^.$ are produced initially by water pyrolysis inside cavitation bubbles. H$^.$ and OH$^.$ either combine to form H_2, H_2O_2 and H_2O or attack solute molecules that are reduced or oxidized [7].

Our study hypothesis was that diagnostic cardiac ultrasound can modulate intracellular oxidative stress of endothelial cells in vitro.

Aim of this study was to evaluate in vitro the effects of ultrasound exposure of variable duration (from 5 up to 30') with commercially available imaging systems commonly used for transthoracic cardiac imaging (second harmonic, 1.3/2.6 MHz, mechanical index=1.5) on intracellular free radical production of endothelial cells monolayer. DNA laddering and LDH leakage were used to investigate the presence of apoptosis and cell membrane damage respectively, in cell cultures following ultrasound exposure.

2. Methods

2.1. Ultrasound irradiation

Ultrasound irradiation was performed in the second harmonic mode with an HP Sonos 5500 System (Philips, Andover, Mass) equipped with an S3 transducer emitting at 1.6 MHz and receiving at 3.2 MHz (mechanical index 1.5). The tip of the transducer was carefully positioned at 5 cm from cell surface, a distance corresponding to the focal distance of the transducer in the z axis. The mechanical index indicates the potential for mechanical bioeffects and is the default display with 2D/B-mode imaging. The mechanical index is calculated using two variables: $MI =$ Peak rarefactional pressure (derated)$/\sqrt{Fc}$. The denominator Fc is the center frequency of the transmitted field.

2.2. Endothelial cell culture

Human umbilical vein endothelial cells were harvested and isolated by enzymatic digestion in the presence of type II collagenase (0.1%) as previously described [8]. Isolated cells were maintained in Medium 199 (Life Sciences, Grand Island, NY) containing fetal bovine serum (5%) and growth factors (heparin, 50 U/ml; and endothelial cell growth factor, 50 μg/ml). Once grown to confluence, cells were replated onto gelatin-coated flasks (1.5%) at a density of 20 000 cells/cm^2. Cultured endothelial cells were characterized as endothelium as previously described [9]. Assessment of cell number was performed by direct cell counting of adherent cells after detachment by trypsin using a hemocytometer. Cell viability was assessed by exclusion of trypan blue.

2.3. Detection of intracellular ROS generation

Generation of ROS in endothelial cells was measured with the fluorescent dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate bis(acetoxymethyl)-ester (C-DCDHF-DA) (Molecular Probes), which is a carboxy derivative of 2′,7′-diclorofluorescein diacetate (DCF-DA) that exhibits much better retention in living cells than DCF-DA [10]. C-DCDHF-DA, being nonpolar, diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases and forms carboxydichlorofluorescein, which is polar and thereby trapped within the cell. In this status, C-DCDHF-DA, provides a substrate for oxidation by ROS, resulting in the production of a highly fluorescent intracellular product emitting fluorescence with an intensity proportional to the level of intracellular oxidative stress. The assay is especially sensitive to the increased production of hydrogen peroxide or some of its downstream [11]. Generation of ROS was measured in unstimulated cells and in cells exposed to ultrasound, after 60 min of incubation on ice.

Briefly, endothelial cell monolayers, grown in 6-well plates, were washed with Hanks' buffered saline phenol red-free and loaded with C-DCDHF-DA (10 μmol/l) in Hanks' buffer and incubated for 30 min at 37 °C. After this step, cells were washed once and exposed to ultrasound for 5, 15 and 30 min. In a separate experimental set, endothelial cells was cotreated with ultrasound and catalase (Sigma) (500–1000 U/ml). The fluorescence microscopy was performed to qualitatively assess the formation of intracellular ROS. Using an inverted microscope (×10 objective) the samples were epi-illuminated with 100 W Hg lamp and photographed using 490 nm excitation and 520 nm emission filters, respectively. Since illumination causes increased fluorescence emission because of the oxidation of the fluorescent dye, each field was exposed to light for exactly the same time (20 s). Images were processed with a custom-made software to measure the fluorescence of each cell in the field. Fluorescence intensity was expressed in arbitrary units.

2.4. DNA laddering

DNA fragmentation was measured by agarose gel electrophoresis using previously reported procedures [12]. Briefly, 1×10^6 cells were exposed to ultrasound at 37 °C for 5, 15 and 30 min in Hanks' buffer.

DNA was extracted according to nucleon extraction kit (Amersham Biosciences, Uppsala, Sweden). The amount of DNA was measured by the absorbance at 260 nm, using a spectrophotometer. Samples showing a 260:280 absorbance ratio of ≥1.8, which ascertained the purity of the isolated DNA, were analyzed by gel electrophoresis.

Equal amounts of DNA (2 μg) of each sample were loaded on a 1% agarose gel containing 0.5 mg/ml ethidium bromide, and run at 100 V for 45 min in 1×Tris–
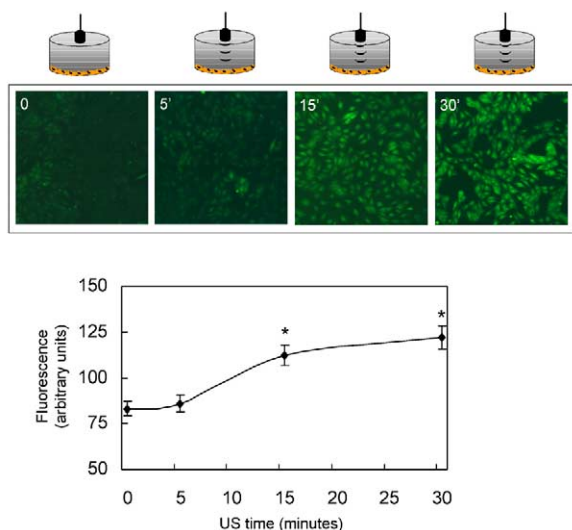
Fig. 1. Time course of ROS production in endothelial cells after ultrasound exposure. Upper panel. Schematic representation of the experimental design, with (from left to right) sham and ultrasound irradiation of 5', 15' and 30'. Middle panel. Representative photomicrographs of intracellular ROS production (proportional to the intracellular fluorescence) and endothelial cells damage (detectable as confluent dark zone in the endothelial cells monolayer) at baseline and following ultrasound irradiation for the indicated time periods. Lower panel. Kinetics of quantitated ROS production induced by ultrasound; results are mean±SD of 3 separate experiments, with each experimental field comprising 150 to 200 cells. *$P<0.001$, compared with corresponding baseline values.

acetate/EDTA electrophoresis buffer. The DNA laddering pattern was visualized by UV transillumination and photographed.

2.5. LDH leakage analysis

Lactate dehydrogenase (LDH) leakage, as an indicator of cytotoxicity, from endothelial cells exposed to ultrasound for 5, 15 and 30 min in Hanks' buffer at 37 °C, was measured in the culture supernatants, according to the method described by Wroblewski and La Due [13] and optimized to the present conditions.

The principle of this procedure is the pyruvate reduction

to lactate accompanied with the oxidation of NADH. The reduction of absorbance due to the NADH oxidation has been spectrophotometrically read at 340 nm. The reaction was performed at 25 °C and each value obtained indicated the LDH activity, expressed as U/l.

2.6. Experimental protocol

In a first set of experiments, detection of intracellular ROS production, DNA laddering, LDH leakage was evaluated in vitro after sham (transducer off) and after 5', 15' and 30' of ultrasound irradiation (Fig. 1, upper panel).

In a second set of experiments, endothelial cells were exposed to ultrasound for 30' and the medium was removed, and transferred to a second cell culture and intracellular ROS production was evaluated.

2.7. Statistical analysis

All values were expressed as mean±SD. Differences between different groups were evaluated by analysis of variance and intergroup testing by Bonferroni test. Statistical significance was established at a value of $P<0.05$.

3. Results

Intracellular radical activation after sham (transducer off), 5', 15' and 30' of ultrasound exposure is displayed in Fig. 1 (lower panel), at 60' after the end of the ultrasound exposure. A representative example of intracellular fluorescence for each of the time points of the study is shown in Fig. 1 (upper panel). There is an obvious, time-dependent increase in intracellular radical production, which increases of 35% after 15' and 47% after 30' irradiation (Fig. 1, lower panel).

The temperature of the medium exposed to ultrasound, monitored during the treatment, was maintained at 37 °C and no increase grater than 0.5 °C was recorded. Endothelial monolayer appeared markedly damaged after 30' of ultrasound exposure at focal region and all around (Fig. 2).

As showed by DNA agarose gel electrophoresis, sham

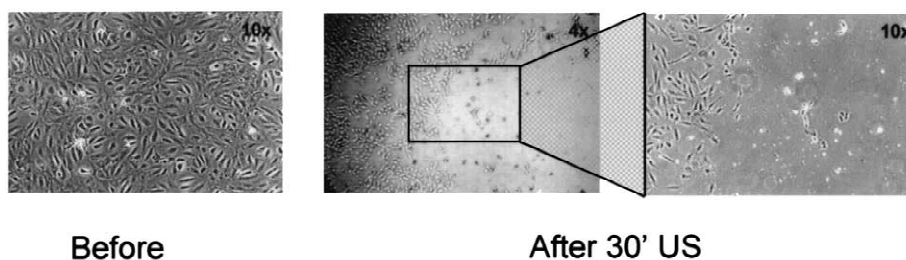
Before   **After 30' US**

Fig. 2. Microscopic appearance of endothelial monolayers before and after ultrasound exposure. (Left panel) Confluent monolayer with normal morphology of the cells before ultrasound irradiation. (Middle panel) Monolayer following 30 min of ultrasound irradiation; in the focal region of exposure, formation of a central hole essentially without cells and around this region the monolayer appeared markedly fissured (magnification of detail area in right panel).
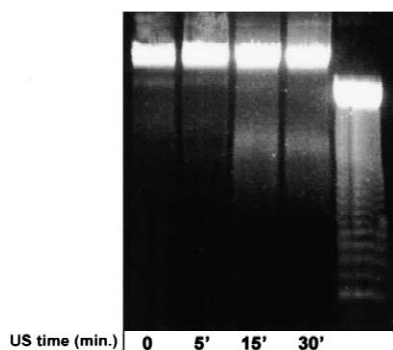
Fig. 3. DNA laddering pattern in endothelial cells as a function of the time of ultrasound exposure. Unexposed cells (lane 1) did not show measurable DNA fragmentation, whereas the cells exposed to ultrasound showed detectable DNA laddering after 15 and 30 min of exposure (lane 3 and 4 respectively). Lane 5: 100 bp molecular weight marker, from 100 to 2000 bp.
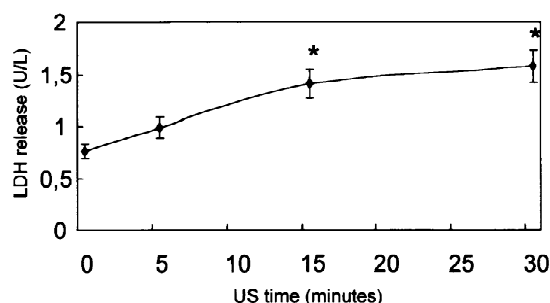

Fig. 4. Time course of LDH leakage from endothelial cells after ultrasound exposure. Kinetics of LDH release induced by ultrasound; results are the means±SD of triplicate assay from 3 separate experiments. *$P < 0.01$, compared with corresponding baseline values.
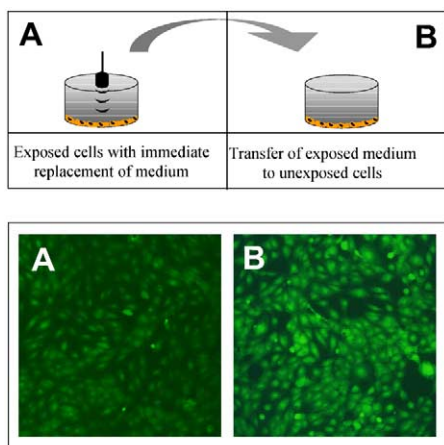

Fig. 5. Upper panel. Schematic representation of the experimental design, with 30' ultrasound irradiation followed by removal of the medium and transfer of exposed medium to unexposed cells. Lower panel. The effects of the medium removal ablates the effect of 30' of ultrasound exposure to the endothelial cells (A); the transfer of exposed medium to unexposed cells fully reproduces the effects of 30' of ultrasound exposure (B). The entry of extracellular H_2O_2 into the cells occur mostly during postexposure incubation.

(transducer off), 5 min of ultrasound exposure did not produce DNA ladders (Fig. 3, lane 1–2), whereas 15 and 30 min of ultrasound exposure did produce DNA ladders (Fig. 3, lane 3–4).

In the same conditions, LDH release increased of 85% after 15' and 107% after 30' irradiation (Fig. 4).

The exposed cells with immediately replacement of medium did not show significant damage or increase of ROS production (Fig. 5A). Cells that were not exposed to ultrasound, but only to the exposed medium which had been insonated for 30', showed signs of ROS production and endothelial cells damage fairly comparable to cells exposed to ultrasounds for 30' directly (Fig. 5). Further, fluorescence induced by medium insonated for 30' in unexposed cells, was reduced in a dose dependent manner when catalase was added (Fig. 6).

4. Discussion

Cardiac ultrasound of current diagnostic use increases intracellular oxidative stress in endothelial cells monolayers in vitro. This increase is time-dependent, persists up to 1 h following withdrawal of ultrasound energy, and can be very pronounced—up to 47% the baseline values for 30' ultrasound exposure.

4.1. Ultrasound-induced endothelial damage

It has long been recognized that sound waves may contain sufficient mechanical energy to damage or to destroy biological tissue [14]. It is now well established that in organs containing air or in the presence of strong cavitation nuclei, such as contrast agents, ultrasound exposure may induce significant tissue damage, particularly to the microvasculature [15–18]. Endothelial cell damage occurs after ultrasound exposure to cultured cells and organs containing air, such as the lungs [19] or the intestine, or the heart after contrast injection [20]. In particular, simultaneous exposure of isolated rabbit hearts to ultrasound and contrast agents results in limited capillary ruptures dependent of mechanical index, with a mean of 3.6% of capillaries ruptured at a mechanical index of 1.5. Ultrasound alone, at a mechanical index of 1.5, induced virtually no damage of capillaries; however, the exposure time was only of 5'—which also in our experimental setting was unable to induce signs of morphological damage of endothelial cells. Our study extend these previous observations, showing that ultrasound alone, with high mechanical index, may modulate the production of reactive oxygen species in the endothelial cell and may eventually determine a damage of endothelial cells. The ultrasound energy that we employed fall fully in the range of diagnostic energy used for cardiac ultrasound. However, in cross-sectional cardiac imaging, the actual exposure at any point within the heart is further affected by the
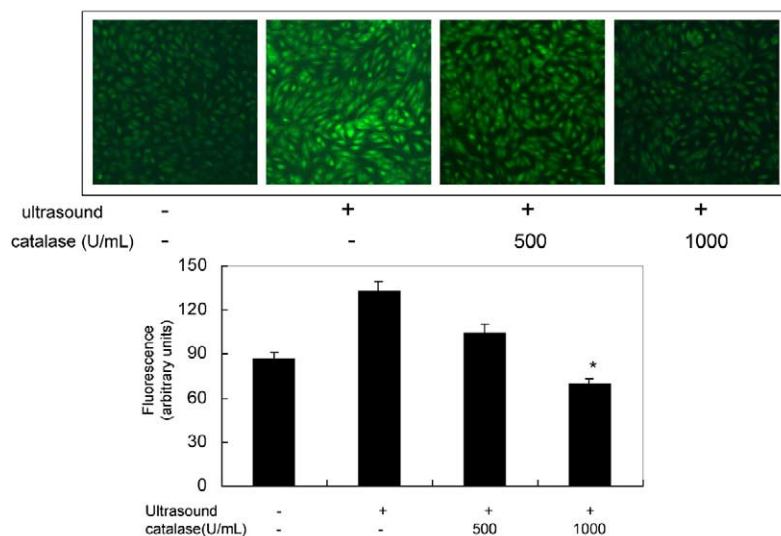
Fig. 6. ROS production was markedly attenuated when catalase was added. Upper panel. Qualitative photomicrographs of ROS production in endothelial cells induced by medium exposed for 30' of ultrasounds in the absence and presence of catalase (500–1000 U/ml). Lower panel. Bar histogram showing ROS values; results are mean±SD of 4 separate experiments, with each experimental field comprising 150 to 200 cells. °$P<0.001$ versus control counterpart, **$P<0.001$ versus ultrasound counterpart.

attenuating properties of the tissues that the sound energies must pass through to reach the heart.

4.2. Mechanisms of ultrasound-endothelium interaction

The ultrasound effects can be either thermal and non-thermal; and the latter may involve cavitation or non-cavitation phenomena [21,22]. Our study design was unable to describe the underlying mechanisms. However, a thermal effect was unlikely, since experiments were conducted in a thermostatic setting with constant temperature, and the thermal effect are negligible with the employed frequency [14]. The biological effects were transferred by exporting, and prevented by removing, the extracellular medium, suggesting that the extracellular formation of H_2O_2 might have played a major role. Since catalase remains extracellularly, these results suggest that H_2O_2 production, induced by ultrasound, occur essentially outside the cells.

Production of free radicals after ultrasound exposure has been previously observed [22,23]. Free radical formation is due to inertial cavitation and has been observed in vitro in aqueous solutions and biological fluids following exposure of ultrasound at acoustic pressures similar to those that may be encountered during medical applications [6,24,25]. Free radicals are associated with cell killing in vitro, and can cause intracellular DNA damage in vitro [26,27]—a reasonable molecular basis for cell damage up to death [28,29]. Also in vivo, local concentration of ultrasound-induced free radicals production may be comparable to the concentrations that can be biologically significant, if found

at a biochemically sensitive site. In addition, the potential for inertial cavitation to occur in vivo greatly increases with the use of stabilized microbubbles, now widely used for diagnostic ultrasound [21,30].

4.3. Study limitations

Our results obtained in vitro cannot be directly transferred to the in vivo setting for a number of reasons. In the real world clinical practice, one can hardly imagine that the cardiac echo probe is fixed in the same position for more than 5 min. It is true however that a 'difficult' echo study and a dynamic stress echo study may require up to 30 min of continuous echocardiographic irradiation [31], although through different transducer positions.

The results of our experiments suggest that the critical place for ultrasound-evoked reactive oxygen species generation is the supernatant. Human plasma or full blood contains several systems for maintaining antioxidant status (gluthatione, ascorbate, catalase). It is still possible that ultrasound-evoked reactive oxygen species production may be physiologically negligible in-vivo due to the rapid inactivation of H· and OH· radicals antioxidant defence systems of blood [32]. Another factor that may limit the in vivo role of ultrasound-derived reactive oxygen species is rapid dissolution of reactive intermediates in blood flow. In this respect, it seems less probable that in vessels with rapid blood flow like arteries ultrasound-derived ROS hardly affect endothelial function. Conversely, some effects may be seen in capillary network, where blood flow is slow.

4.4. Possible implications for the clinical use of ultrasound

These data challenge the commonly accepted concept that ultrasound energy currently employed in diagnostic cardiac imaging is biologically inert. It is true that there are no known morphological and macroscopic functional effects. However, biological effects are marked and persistent at the cellular level even for short term exposures currently used in the clinical arena, and obvious cellular damage was apparent after 30' ultrasound exposure. For instance, second harmonic, low frequency imaging and prolonged imaging time are currently adopted with state of art technology in stress echocardiography. Until more exhaustive data obtained in vivo become available, a prudent extension to ultrasound imaging of the universal ALARA principle—by which radiation exposure should be kept ''As low as reasonably achievable''—is probably warranted.

On the other side, the 'fine tuning' of intracellular radical stress offers a clue to novel applications of ultrasound energy outside the diagnostic domain. Ultrasound can be focused upon almost any organ and so represents—at least theoretically—an intriguing mean for non-pharmacologic modulation of endothelial function.

References

[1] Nyborg WL. Biological effects of ultrasound: development of safety guidelines. Part II: general review. Ultrasound Med Biol 2001;27:301–333.

[2] Kunsch C, Medford RM. Oxidative stress as a regulator of gene expression in the vasculature. Circ Res 1999;85:753–756.

[3] Jacobson MD. Reactive oxygen species and programmed cell death. TIBS 1996;21:83–86.

[4] MacLellan WR, Schneider MD. Death by design: programmed cell death in cardiovascular biology and disease. Circ Res 1997;81:137–144.

[5] Dimmeler S, Hermann C, Zeiher AM. Apoptosis of endothelial cells—contribution to the pathophysiology of atherosclerosis. Eur Cytokine Netw 1998;9:697–698.

[6] Riesz P, Kondo T. Free radical formation induced by ultrasound and its biologiacal implications. Free Radical Biol Med 1992;13:247–270.

[7] Suslick KS. Sonochemistry. Science 1990;247:1439–1445.

[8] Basta G, Lazzerini G, Massaro M et al. Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 2002;105:816–822.

[9] De Caterina R, Libby P, Peng HB et al. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J Clin Invest 1995;96:60–68.

[10] De Clerck LS, Bridts CH, Mertens AM, Moens MM, Stevens WJ. Use of fluorescent dyes in the determination of adherence of human leucocytes to endothelial cells and the effect of fluorochromes on cellular function. J Immunol Meth 1994;172:115–124.

[11] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227–231.

[12] Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.

[13] Wroblewski F, La Due J. Lactic dehydrogenase activity in the blood. Proc Soc Exp Biol Med 1955;90:210.

[14] Baker KG, Robertson VJ, Duck FA. Therapeutic ultrasound has traditionally been regarded as having a strong biophysical basis. Physical Ther 2001;81:1352–1358.

[15] Holland CK, Apfel RE. Thresholds foe transient cavitation produced by pulsed ultrasound in a controlled nuclei environment. J Acoust Soc Am 1990;88:2059–2069.

[16] Holland CK, Deng C, Apfel RE. Direct evidence of cavitation in vivo. Ultrasound Med Biol 1996;22:917–925.

[17] Wei K, Skyba DM, Firschnke C. Interactions between microbubbles and ultrasound: in vitro and in vivo observations. J Am Coll Cardiol 1997;29:1081–1088.

[18] Skyba DM, Price RJ, Linka AZ. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and it local effect on tissue. Circulation 1998;98:290–293.

[19] Dalecki D, Child SZ, Reaman CH. Age-dependence of ultrasonically-induced lung haemorrhage in mice. Ultrasound Med Biol 1996;46:220–232.

[20] Ay T, Havaux X, Van Camp G et al. Destruction of contrast microbubbles by ultrasound. Effects on myocardial function, coronary perfusion pressure, and microvascular integrity. Circulation 2001;104:461–466.

[21] Barnett SB, Rott HD, ter Haar GR, Ziskin MC, Maeda K. The sensitivity of biological tissue to ultrasound. Ultrasound Med Biol 1997;23:805–812.

[22] Miller MW, Miller DL, Brayman AA. A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. Ultrasound Med Biol 1996;22:1131–1154.

[23] Edmonds PD, Sancier KM. Evidence for free radical production by ultrasonic cavitation in biological media. Ultrasound Med Biol 1983;9:635–639.

[24] Misik V, Riesz P. EPR characterization of free radical intermediates formed during ultrasound exposure of cell culture media. Free Radical Biol Med 1999;26:936–943.

[25] Misik V, Miyoshi N, Riesz P. Effects of cysteamine and cystamina on the sonochemical accumulation of hydrogen peroxide-implication for their mechanisms of action in ultrasound-exposed cells. Free Radical Biol Med 1999;26:961–967.

[26] Miller DL, Thomas RM, Buschbom RL. Comet assay reveals DNA strand breaks induced by ultrasonic cavitation in vitro. Ultrasound Med Biol 1995;21:841–848.

[27] Kondo T, Kodaira T, Kano E. Free radical formation induced by ultrasound and its effects on strand breaks in DNA of cultured FM3A cells. Free Radical Res Commun 1993;19:S193–200, Suppl. 1.

[28] Irani K. Oxidant signaling in vascular cell growth, death, and survival. A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 2000;87:179–183.

[29] Li AE, Ito H, Rovira HI, Kim KS. A role for reactive oxygen species in endothelial cell anoikis. Circ Res 1999;85:304–310.

[30] Carstensen EL, Gracewski S, Dalecki D. The search for cavitation in vivo. Ultrasound Med Biol 2000;29:1377–1385.

[31] Picano E. Stress echocardiography. From pathophysiological toy to diagnostic tool. Circulation 1992;85:1604–1612.

[32] Misik V, Riesz P. Free radical intermediates in sonodynamic therapy. Ann NY Acad Sci 2000;899:335–348.