J. Radiat. Res., 45, 479–489 (2004) *Review Article*

Biological Effects of Low Intensity Ultrasound: The Mechanism Involved, and its Implications on Therapy and on Biosafety of Ultrasound

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Low intensity ultrasound/Bioeffects/Apoptosis/Biosafety.

The biological effects of low intensity ultrasound (US) *in vitro*; the mechanisms involved; and the factors that can enhance or inhibit these effects are reviewed. The lowest possible US intensities required to induce cell killing or to produce free radicals were determined. Following sonication in the region of these intensities, the effects of US in combination with either hyperthermia, hypotonia, echo-contrast agents (ECA), CO_2, incubation time, high cell density or various agents were examined. The results showed that hyperthermia, hypotonia and microbubbles are good enhancers of the bioeffects, while CO_2, incubation time and high cell density are good inhibitors. Cellular membrane damage is pivotal in the events leading to cell death, with the cellular damage-and-repair mechanism as an important determinant of the fate of the damaged cells. The optimal level of apoptosis (with minimal lysis) and optimal gene transfection efficiency were attained using a pulsed low intensity US. In summary, the findings suggest that low intensity US is potentially useful in therapy, while on the other hand, they also call for further investigation of such clinical scenarios as high-grade fever, edema or use of ECA which may lead to the lowering of the threshold for bioeffects with diagnostic US.

INTRODUCTION

In medicine, ultrasound (US) is widely used for soft tissue imaging because of its perceived safety, noninvasiveness and low cost. It has also been used therapeutically in surgery, ophthalmology, physical therapy, and cancer therapy.[1,2] Because of its sufficient tissue attenuation coefficient and easy focusing manageability, ultrasound has been studied extensively in hyperthermia for cancer therapy, and tissue ablation using high-intensity focused ultrasound.[3–4] Other than the thermal effects of ultrasound, the therapeutic use of nonthermal effects such as cavitation is becoming an interesting subject in research. Acoustic cavitation, the ultrasonically induced cavitation, is known to be the primary cause of sonoluminescence, mechanical shock waves and sonochemical reactions producing reactive oxygen species. This principle of mechanical disturbance brought about by cavitation caused by US is similar to that caused by shock wave. Together with cavitation, other ultrasonic effects considered being nonthermal, and perhaps even noncavitational, offer greater potential for cancer therapy especially in combination with other agents. Promise for such combinations is evident in the outcome of some of the research works previously reviewed.[1)]

The expanding use of ultrasound (US) in medicine led to increasing demand for more research works on the mechanism by which US interacts with living cells and tissues. Studies on the biological, chemical and physical effects of US have revealed promising results.[1,5–8)] While most studies on the bioeffect of US use high intensity US, in this review we will focus and discuss various aspects of low intensity US and cellular conditions, with emphasis on the mechanism of action. Particularly we would like to search for answers to the following questions: a) how low is the US intensity that can induce its bioeffects, b) how the bioeffects are enhanced, inhibited or modulated, b) how certain mode of cell death or any desired bioeffects could possibly be optimized, and c) what will be are the ideal protocols for possible application, especially for cancer therapy. Implications of the findings towards the biosafety of diagnostic US is also discussed.

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CELL KILLING INDUCED BY ULTRASOUND

Studies have shown that low intensity US can induce cell killing even without significant temperature rise and even at

very low intensities. Some factors that enhance these effects and factors that inhibit them were identified and characterized. Several methods to investigate the mechanism of bioeffects were applied and optimization of the bioeffects was also explored.

Nonthermal ultrasound enhances hyperthermia-induced cell killing

Hyperthermia has long been recognized as a modality in anticancer therapy. US induced hyperthermia[9] was found to be more advantageous because of its manageability in terms of focusing, control and sufficient tissue attenuation coefficient for deep tumor targets. A study conducted on the low intensity nonthermal US in combination with hyperthermia showed synergistic cell lysis[10] and apoptosis[11] *in vitro*.

In a study, a human myelomonocytic lymphoma U937 cells were exposed to continuous 1 MHz US at special-average temporal average intensities (I_{SATA}) at 0.312 or 0.692 W/cm^2 considered nonthermal and sub-threshold for inertial cavitation, while in hyperthermia (40–44.0°C) for 10 min. Intensity 0.312 W/cm^2, in combination with hyperthermia, synergistically induced apoptosis. On the other hand, 0.692 W/cm^2 in combination with hyperthermia showed an augmented instant cell lysis but not apoptosis.

The study shows that the hyperthermia-induced apoptosis can be enhanced by US at intensities even below threshold for cell killing with US alone. Therefore, this may be useful when apoptosis induction is desired over instant cell killing in cancer therapy. In addition, since US is currently being used to generate heat for hyperthermia therapy, such data also help explain why US has been shown to be more effective than other technology used to generate hyperthermia (e.g. microwave).

Another study showed that apoptosis can also be induced by the combination treatment of mild hyperthermia (40°C for 30 min) and very low intensity ultrasound (0.081 W/cm^2 I_{SATA}, 1 MHz, 10% duty factor (DF) at 100 Hz pulse repetition frequency (PRF) for 1 min) when sonication was done after hyperthermic treatment. When hyperthermic treatment was done after sonication, enhanced lysis was observed rather than apoptosis. These findings suggest that hyperthermia seems to inhibit repair of membrane damage caused by sonication, while US augments the apoptosis that hyperthermia may have initiated. The value of such combination in therapy is noteworthy while considering that the mild hyperthermia is of clinical significance and that the low intensity US could simulate diagnostic US, safety of the use of US in febrile patient should be investigated.

Echo-contrast agents (ECA) enhance ultrasound-induced cell killing and DNA transfection

The principle behind the use of US in diagnosis is the ability of sound waves to produce echo when it hits a certain object in varying magnitude depending on the type of material. This is technically called echogenicity of material. In human body, different tissues have different characteristic responses to US. However, based on this principle alone, limitations do exist. Some tissues have similar echogenicity that delineating them is difficult; while some structures are so small that the echo from larger structures overshadows them. One method to improve echo contrasting is by making use of the Doppler effect of any moving part such as the circulating blood. This principle utilizes the concept that moving object produces a different echo pattern with respect to the stationary one. Recently, commercial development of the ECAs such as microbubbles, has improved the efficiency of these ultrasonic imaging techniques. Microbubbles are particularly useful because of the characteristics of any bubble to vibrate harmonically in response to US, thus sending characteristic echoes. Microbubbles generally localizes within the vasculature during its lifetime, hence providing a better echo-image of the vasculature and a good contrast between tissues with different levels of vascularization. This aspect is particularly important in the diagnosis of tumor tissues that have particular vascularization patterns.

The therapeutic use of US and the effects of echo-contrast agents when combined with US were also investigated. Two of the most commonly studied echo-contrast agents, Levovist™ and Optison™, are now in use clinically in many different countries. Levovist™ was said to decrease the threshold for petechiae and hemorrhages in animal model[12] though significant increase in the US-induced hemolysis was not observed. Optison™ on the other hand, was shown to augment the US-induced cell destruction[13], lysis and sonoporation (cell membrane pore formation) *in vitro*[14], induces capillary rupture in mice[15], enhances hemolysis[16] and disrupts blood-brain-barrier.[17]

A particular *in vitro* study investigated the effects of echo-contrast agents, Levovist™ and Optison™, and also including a lipid based echo-contrast agent more recently being investigated (called YM454) on US-induced apoptosis and cytolysis of U937 cells *in vitro*, under a hypothesis that these agents could be potential adjuncts in cancer therapy with US.[18]

U937 cells in suspension were exposed to 1 MHz continuous waves US for 1 min at a I_{SATA} values of 0.312, 0.692, 1.42 and 2.87 W/cm^2 with or without non-shell type ECA, Levovist™ (2 mg/ml), and shell type, Optison™ (1 μl/ml) or YM454 (1 μl/ml). Levovist™ enhanced the US-induced apoptosis at 0.692 W/cm^2 while Optison™ and YM454 did at 1.42 and 2.87 W/cm^2, as detected by flow cytometry. Cell lysis was also augmented when Levovist™ was combined with US at 1.42 W/cm^2, and when Optison™ was combined with US at 1.42 and 2.87 W/cm^2. YM454 showed the highest rate of enhanced cell lysis at 0.692 and 2.87 W/cm^2. The study concluded that Optison™ and YM454 are superior over Levovist™ in augmenting cell killing.

The results indicate that cavitation plays a role in the aug-

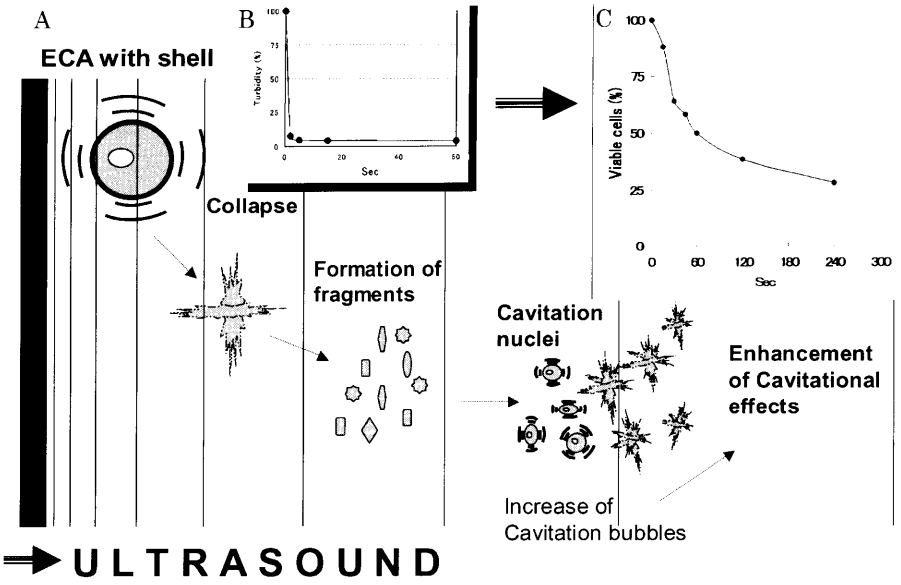

Fig. 1. Enhanced cavitation by echo-contrast agent. Rapid destruction of microbubbles in PBS by sonication based on turbidity test (B) was observed. The continued enhancement of US-induced cell killing (C) suggests that fragments from the microbubble may have served as cavitation nuclei for cavitations.

mented effects and that inertial cavitation appears necessary for Optison™ and YM454 to effect their actions. In figure 1B, rapid degradation of microbubbles by US was observed based on turbidity results, while continued enhancement of the cell killing was noted after a longer sonication period (Fig. 1C). This data suggest that fragmented primary microbubbles provided nuclei for new cavitations to be formed and thus enhancing cavitational activity within the sonicated medium (Fig. 1).

These findings suggest that these ECAs have potential to be adjuncts in cases wherein augmented US-induced cell killing is needed such as in cancer therapy with US. On the other hand, potential lowering of the threshold for bioeffects with diagnostic US when used with ECA is also implied. A review on the safety considerations of ultrasonic contrast agents cited studies which affirmed that indeed there is such risk in the clinical standpoint.[19]

Ultrasound-induced cell killing enhanced by some chemical agents

Synergistic effects between US and some agents, especially anticancer drugs, were previously reviewed[1] and the possible cellular mechanisms identified were: 1) Increased permeabilization that is characterized by increased cellular uptake of the agent, 2) Increased sensitivity of the cells to the agent, 3) Potentiation of the agent, also called sonodynamic effect, 4) Partial damage made irreversible and 5) Thermal effect. However, it was suggested that these mechanisms greatly overlap each other in a proportion that varies depending on the many factors which includes the type of agent being used.

Inspired by the positive results of the combined treatment with US, a different type of agent called thermal sensitizers[20–22] was investigated. Thermal sensitizers are agents that may not have much value in therapy when used alone, but can enhance the therapeutic effect of hyperthermia if used in conjunction with it. These agents are now drawing attention not only in the field of hyperthermia, but also in free radical research and especially on research related to apoptosis.

Among the heat sensitizers, 2,2' azobis (2-amidinopropane) dihydrochloride (AAPH) and 2,2' azobis (2,4-dimethylvaleronitrile) (AMVN) are top in the researchers' list. A water soluble temperature-dependent free radical initiator, AAPH, has been shown to sensitize Chinese hamster V79 cells to thermal killing[21] and enhance hyperthermia-induced apoptosis of U937[23], CaSki and Hela cells.[24] This was attributed to the ability of this agent to generate carbon-centered free radicals which is temperature-dependent. Although free radicals are generated by AAPH at 37°C, it is not toxic to most cells even at concentrations up to 50 mM. However, at hyperthermia (42–45°C), cells were found to be sensitized to this agent. Hypothetically, increased cell membrane permeability to AAPH under hyperthermic conditions, is an important factor in the enhancement of hyperthermia-induced cell killing. This is also supported by the finding that a lipophilic temperature-dependent free radical initiator, AMVN, which readily enters into the cells compared to AAPH, has more enhancing ability than AAPH, even at milder hyperthermia.[22]

A study showed that US-induced cell lysis and apoptosis

can be enhanced in the presence of AAPH regardless of the temperature at the time of sonication.[25] Although free radicals were increased in the combined treatment, this increase did not correlate well with the cell killing. The mechanism of enhancement points to the increased uptake of the agent during sonication rather than potentiation by AAPH (Fig. 2). The increased uptake hypothesis was affirmed by the result showing that more free radical could be detected in the lysates of sonicated cells than the unsonicated ones. These findings suggest the potential of temperature-dependent free radical generators in cancer therapy with therapeutic US.

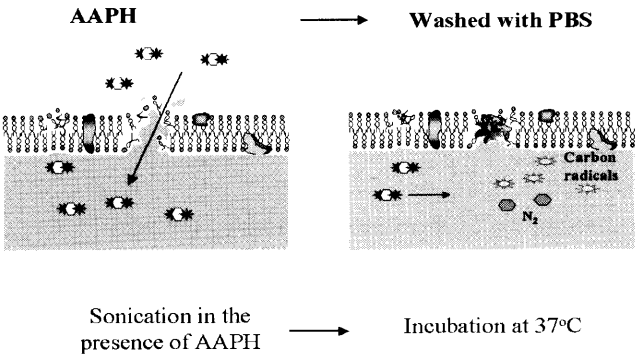

Fig. 2. Mechanism of enhanced US-induced apoptosis by AAPH. Sonication results to cell membrane damage resulting to increased uptake of AAPH. Membrane repair is expected if cell survives, trapping the AAPH inside the cells. Incubating the cells at 37°C or higher leads to intracellular production of carbon-centered radicals which will eventually induce apoptosis.

For future advanced study, *in vivo* trial to verify if use of a nontoxic level of AAPH in combination with hyperthermia induced by US is more advantageous than hyperthermia induced by other methods (e.g. microwave) is particularly of interest.

Hypotonia enhances ultrasound-induced cell killing

A study has shown that nonlethal osmotic cell swelling induced by hypotonia (146 mOsm) can enhance US-induced cell killing.[7] Although change in fluid viscosity can modify acoustic cavitation formation, the data showed that it did not play an important role in the enhancement but rather the increased mechanical susceptibility of the swollen cells.

It is known that cells swell in response to hypotonia, with the cell membrane as the major player of this event. Nyborg[26,27] extensively described tension on a cell membrane under the influence of internal and external pressures. For a membrane of thickness h the quantity of tension σ is defined by,

$$\sigma = S_0 h,$$

where S_0 is the stress in the function of pressure (P) acting on the membrane. Furthermore, if membrane thickness h is much less than the cell radius R (h<<R), tension can be expressed by,

$$2\sigma = R (P-P_{ext}),$$

where P is the pressure on the interior of the cell and P_{ext} the pressure outside, while $P-P_{ext}$ is denoted by ΔP. In the case of hypotonic cell swelling, R increases as ΔP increases due to increase in P, thus increasing tension on the membrane. In addition, in the strained membrane, an increased spacing between particles along the axis parallel to the membrane is expected. Changes in cell shape and size may occur as ΔP increases; the cell may burst at some critical value of ΔP.

Enhancement of US-induced cell lysis was observed at all I_{SATA} values (0.312, 0.692, 1.42 and 2.87 W/cm^2), and most prominently at 1.42 W/cm^2, while apoptosis induction was significantly enhanced at intensities of 0.312 and 0.692 W/cm^2 but not at 1.42 W/cm^2(Table 1). The enhanced cell lysis is attributed to the increased susceptibility of the cells to mechanical damage. This is consistent with the previous reports describing the effects of mechanical stresses on cell

Table 1. Average US-induced cell killing with or without hypotonia

Ultrasound exposure I_{SATA} (W/cm^2)		Lysis + loss of viability (%)	Apoptosis (%)	Total cell killing (%)
0.31 (10 min)	Isotonia	1.1	2.1	**3.2**
	Hypotonia	7.2	3.53	**10.73**
0.69 (10 min)	Isotonia	16	3.03	**19.03**
	Hypotonia	28.2	6.78	**34.98**
1.43 (1 min)	Isotonia	24.7	16.77	**41.47**
	Hypotonia	48.5	11.66	**60.16**

Note: The data is based on the cell count before treatment (taken as 100%).

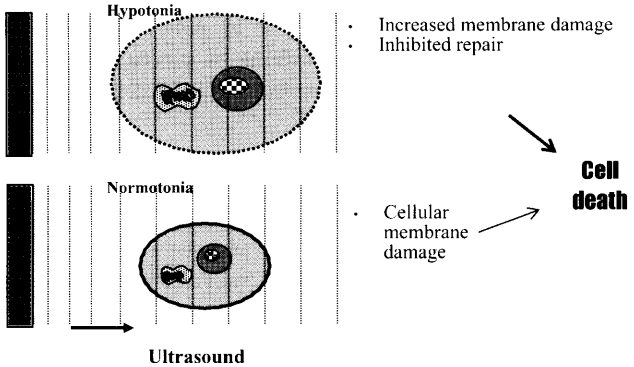

Fig. 3. Mechanism of enhanced US-induced cell killing by hypotonia. Hypotonia swells the cells making the cells more susceptible to the sonomechanical effects leading to increased cell killing by ultrasound. Cellular ion images also suggested inhibited repair of membrane damage as a contributing factor in the enhanced cell death.

membranes. Cellular ion scanning images also suggest that hypotonia has an effect on membrane damage-and-repair mechanism of the cells. Figure 3 illustrates how increased membrane damage in hypotonia-treated cells associated with poor membrane repair ability as supported by ion images increased the cell killing.

This finding might be helpful in elucidating the mechanical nature of the US-induced biological effects, and the cellular response to these effects. Eventually, this may also be useful clinically when infusion of hypotonic fluid to the target tissue is applied in conjunction with US therapy, especially in cancer therapy.

Ultrasound-induced cell killing is inhibited by carbon dioxide

Dose dependent inhibition of US effects (both bioeffects and chemical effects such as free radical production) was observed when we used equal doses of HCl and H_2CO_3 to generate measurable concentration of CO_2 in the medium used (Fig. 4).[28] It is known that CO_2 lowers the final temperature of collapsing bubbles since it has a low gamma ($\gamma = C_p/C_v$) value.[29] For the adiabatic collapse of a cavitation bubble, the final intracavity temperature at the end of the collapse, T_f is given by

$$T_f = T_i\,(R_{max}/R_{min})^{3(\gamma-1)}$$

Where T_i is the initial temperature, γ is the specific heat ratio (C_p/C_v) of the gas inside the bubble; R_{max} is the initial radius of a bubble which collapses to a final radius of R_{min}. Figure 5 illustrates this concept.

This finding implies how handling of cell samples is important in experiments related to US effects, while also guides researches to consider CO_2 concentration in the living body when doing *in vivo* studies.

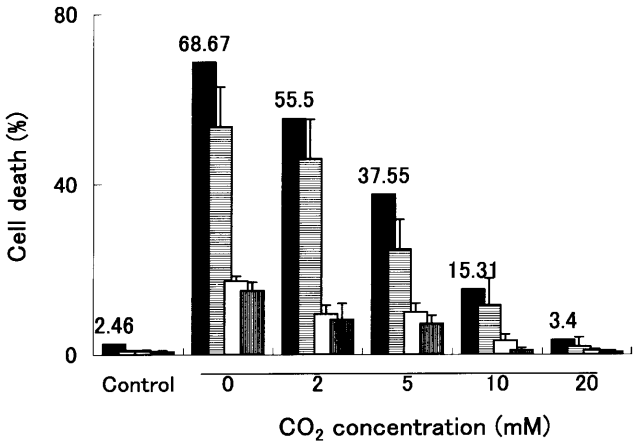

Fig. 4. Effect of CO_2 on US-indueed cell killing. Average total cell killing (black); fraction of cells killed immediately after sonication (horizontal lines); early apoptosis (white); secondary necrosis (vertical lines).

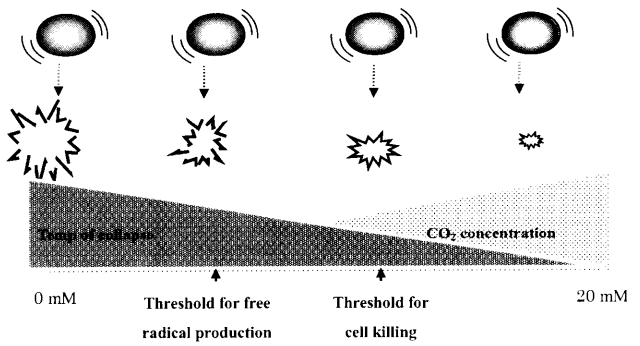

Fig. 5. Dose dependent inhibition of US-induced cell killing and free radical production. As CO_2 concentration increases, energy of cavitation collapse decreases resulting to decreased chemical and biological effects. Total inhibition was observed at CO_2 concentration 20 mM and above.

Ultrasound enhances liposome-mediated gene transfection

The discovery that most human diseases (e.g. genetic disorders, cancers and metabolic disorders) are somehow linked to a particular gene or genes[30] has brought unprecedented progress in the science of therapy. Gene therapy, in particular, is formulated for treating such ailments and is carried out by introducing recombinant genes into the somatic cells to alter the course of a disease process. Several strategies have been designed to attain transfection and eventual integration into the nucleus of the target cells. Viral-mediated gene transfer is efficient to the task, but cytotoxicity, cytopathy, and antigenicity are among the drawbacks that limit its use in therapy. Nonviral methods are considered relatively safer and those include electroporation and liposome-mediated transfection. However, the inability of nonviral methods to act beyond facilitating cellular uptake of the therapeutic gene, leads to a poor transfection rate. The search for a method or combination of methods that could improve the general outcome of therapy remains a big challenge to workers in this field.

Use of US in therapy and also in gene transfection[31–33] has been investigated both *in vitro* and *in vivo*. Poor transfection rates remained a problem for which several combined methods were applied to improve the outcome. So far the mechanism remains generally unknown, but the leading belief is that US increases DNA uptake by the cells. A study investigated the effects of US on liposome-mediated transfection using 3 different types of liposomes,[34] which have been previously shown to mediate transfection with different degrees of efficiency, showed that US significantly increased luciferase expression when combined with liposomes.[35] Optimal enhancement was observed when US was given 2 hr after incubation of the cells with the liposome-DNA complexes, suggesting that US works to enhance transfection only after cells had enough time to interact with the DNA.

This finding suggests that US could be useful in gene therapy in combination with liposome-mediated transfection.

Optimization of ultrasound-induced apoptosis

Based on the above findings and underlying hypothesis on the mechanism of cell killing induced by US, it is considered that certain conditions would optimize killing on a desired mode of cell death, e.g. apoptosis.

Apoptosis induction has been used as a gauge for an effective cancer therapy by many different modalities including radiation, hyperthermia and anticancer drugs. Therapeutic US has been used in cancer therapy as a heating device for hyperthermic cancer therapy; recent study showed that nonthermal low intensity US is also capable of generating apoptosis both *in vitro* and *in vivo* at certain conditions.[36,37] Some major problems however are their low yields compared with other modalities and predominance of cell lysis (*in vivo*) rather than apoptosis as the form of cell killing in most instances. To search for an optimal condition to generate apoptosis (highest possible ratio of apoptosis over cell lysis) of cancer cells *in vitro*, different US conditions by varying the intensity (0.1–1.0 W/cm^2), the DF (5–100% at pulse frequency of 100 Hz), the PRF (0.5 to 100 Hz at 50% DF) and the duration of exposure (1–10 min) were applied.[38] Sonicated U937 cells were incubated at different time intervals (1–24 hr) before measuring the apoptosis and its signal transduction.

Optimal apoptosis (70.0 ± 13.8%) was attained in a 1 MHz setup at intensity 0.3 W/cm^2 with 10% DF of 100 Hz PRF. These findings showed that high level of apoptosis, comparable to apoptosis induced by X-irradiation at 20 Gy or 44°C for 20 min on U937 cells, can be attained by low intensity therapeutic US if appropriate parameters and conditions are observed; thus suggesting that it is potentially competitive to the other modalities of cancer therapy in terms of apoptosis induction. On the aspect of biosafety of diagnostic ultrasound, intensity I_{SATA} of 0.3 W/cm^2 is apparently much lower than that of the maximum limit set by the British Medical Ultrasound Society, which is 0.72 W/cm^2.[39] Preliminary studies show that similar concept also works in optimizing ultrasound-mediated gene transfection. In both studies, the role of heme oxygenase-1, a molecular indicator of oxidative stress,[40,41] was also investigated.

Inhibitory effects of cell density or incubation time on ultrasound-induced free radical production and cell killing and the effects of ECAs

Studies have showed that echo contrast agents (ECA) can enhance US-induced apoptosis and lysis,[18] while other studies showed that CO_2 can inhibit US-induced cell killing dose dependently.[28]

More recently, it has been shown that CO_2 generated by the cells in culture is effective in inhibiting the bio-effects of US. This is influenced by time of incubation and cell density.[38] Another study showed that in such inhibited condition, ECA can restore the US-induced cell killing dose dependently.[42] Comparable cell killing with that of US in the absence of CO_2 are attained at doses 1 μl/ml for YM 454 and 2 mg/ml for Levovist; and twice these doses, ECA can restore US-induced cell killing even at inhibitory cell densities. These findings suggest that ECA are potent inducer of US bioeffect even *in vivo* where cell density is high and CO_2 are present, suggesting its value in cancer therapy with therapeutic US.

The inhibition showed by high cell density and carbon dioxide are the two factors that are likely to protect a living body against the bioeffects of US *in vivo*, especially with the use of diagnostic US where bioeffects is not desired. However, these two factors may not work to protect the body if US is used with microbubbles.

MECHANISM OF BIOEFFECTS

The sonomechanical effects

Some actual and potential medical applications of low intensity ultrasound were cited in studies related to orthopedics,[43–52] vascular system,[53,54] nervous system[55] and ophthalmology.[56] These applications were focused on the healing potential of ultrasound by stimulating tissue repair.[57] Such bioeffects utilizes the mechanical nature of ultrasound that may induce potential damage to cell membranes that may also be translated as a potential therapeutic potential for cancer therapy.

With low intensity US in which bulk temperature is not significant and free radical production is minimal, if at all present, mechanical damage on the cells is the likely mechanism involved in the biological effects. Here we used the term "sonomechanical effects"[7] in contrast to sonodynamic effects and the thermal effects. Sonodynamic effects are usually associated with the free radical production while the thermal effect is associated with bulk temperature rise of the sample within a medium. The following findings support the hypothesis on the sonomechanical effects:

1. In the studies cited involving low intensity US, temperature rise due to sonication were to a non-significant level, generally less than 1°C;
2. Cell damage can be observed at very low intensity, which usually does not cause cell killing, if susceptibility of the cells to mechanical damage is increased such as in hypotonia and hyperthermia.
3. Using CO_2 to inhibit free radical production and cell killing,[28] it was shown that the threshold for free radical production is one order higher magnitude than the threshold for cell killing. This simply means that cell killing precedes the free radical production. Although when free radicals are present, this may augment the cell killing.

Cellular membrane damage

Convinced that the mechanical effect is the one responsible in the bioeffects, we then propose that membrane damage is pivotal in all the cellular damage considering it to be the most susceptible structure and exposed to any gross external mechanical stress—that is the US. Depending on the degree of membrane damage and the ability of the cells to repair the damage,[58,59] determine the mode of cell death; it may be instant lysis, necrosis or apoptosis (Fig. 6). Although damaged cells which are able to successfully repair the damage may eventually survive, some of these cells will die by apoptosis or necrosis. Apoptosis is a natural form of cell death that occurs during the development of organs and tissues, and in response to specific types of cellular stress in order to delete irreversibly damaged and/or undesirable cells. In contrast, necrosis is always inappropriate or accidental, and usually occurs under extremely adverse environmental conditions. Apoptosis and necrosis are traditionally defined by morphological features shown in Table 2.[60]

While apoptotic cells will eventually undergo secondary necrosis, also known as late apoptosis, any disruption of the membrane may abort the process and kill the cell by necrosis or lysis. In such instance, inhibition of the end points of apoptosis such as DNA fragmentation may be observed. Our findings show that in US-induced apoptosis, necrosis is an unavoidable occurrence. Such condition will also likely to result in some cells dying in a type of cell death that combines some of the features of apoptosis (requirement for protein synthesis) and necrosis (cytoplasmic vacuolation)—called paraptosis. Electron micrograph of sonicated cells, identified as "apoptotic" also showed some cytoplasmic vacuolations.[61] In addition, unusually early appearance of secondary necrotic cells (both FITC and PI) and low yield in DNA fragmentation compared to the level of apoptosis detected by flow cytometry and by microscopy could also be explained by this concept.

Failure to repair the membrane damage will likely results to lysis and in cases when damage is not so extensive, necrosis is expected. Any necrotic cells will eventually somehow undergo lysis.

The above concept is supported by the following findings:

1. At the lowest possible intensity that cell killing was observed, apoptosis to lysis ratio is high. As the intensity is increased, lysis becomes predominant over apoptosis as a form of cell death.
2. If a higher intensity than the minimum to cause cell death is chosen, effective induction of apoptosis is obtained if pulsed modulation is used with a longer pause than the irradiation time. The strong intensity guarantees inducing membrane damage while the longer pause allows membrane time to do repair, thus shifting the mode of death to apoptosis.
3. Considering that by definition apoptosis requires intact membrane to work properly until the hallmark endpoint is attained, that is DNA fragmentation, portion of the cells will end up necrotic before such event is completed. This is shown in our data that necrosis proportionally increases with apoptosis and eventually predominates as time of incubation is increased.

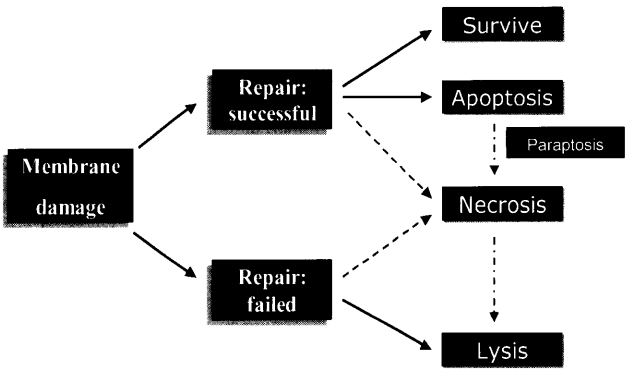

Fig. 6. Schematic presentation showing that membrane damage-and-repair. The degree of membrane damage caused by the mechanical effects of ultrasound and the degree of repair by the fate of the cells and will also determine the type of cell death the cell will die.

Table 2. Morphological features of apoptosis and necrosis

	APOPTOSIS	NECROSIS
DNA	Internucleosomal cleavage	Random degradation ("ladder")
Nucleus	Chromatin margination	Pyknosis
Membrane integrity	Persists until late	Compromised early
Mitochondria	Appear normal	Swollen, increased Ca^{2+}
Pattern	Individual cells affected	Multiple cells affected
Cell volume	Decreases	Increases early
Cell fragmentation	Yes (apoptotic bodies)	No (cell lysis)

Table 3. Cell classifications and their indicators

	Light microscopy	Trypan blue dye exclusion test	Flow cytometry	DNA fragmentation
Apoptosis	Intact	–	Annexin V-FITC (+)	+
	Visible		PI (–)	
Necrosis	Apparently intact	+	Annexin V-FITC (+)	–
	Visible		PI (+)	
Lysis	Fragmented	NA	NA	NA
	Not visible			
Live/normal	Intact	–	Annexin V-FITC (–)	–
	Visible		PI (–)	

Total in vitro cell killing

In the *in vitro* works presented here, total killing is generally defined as the total loss of cell after a given time, usually 6 hrs. This would include cell lysis, loss of cell viability, necrosis and apoptosis. But complexity in the delineation of one type of death to another, total cell killing is here grouped into two. One is the immediate cell killing and the other is the late cell killing. After sonication Trypan blue dye exclusion test is done and microscopy is performed. Immediately killed cells included the non-visible and fragmented cells, which are considered instantly lysed cells, and Trypan blue positive cells (stained blue), which are nonviable cells. The nonviable but apparently intact cells are also classified as necrotic cells. The fraction of cells killed late (usually 6 hr after the treatment) includes the apoptotic (Annexin V-FITC positive cells by flow cytometry) and the necrotic (PI positive cells by flow cytometry) cells (Table 3)

The sonodynamic effect and the role of free radicals in the bioeffects

Previous works have cited sonodynamic effect of US as the mechanism behind the enhancement of bioeffects when some anticancer drugs and other agents were combined with sonication.[1] The ability of some agents to generate free radical resulted to the potentiation of the bioeffects and as such, free radical is considered the major player in the sonodynamic effects. In the present works however, the results downplayed the role of free radicals in the bioeffects of US and its enhancement by physical (hyperthermia or hypotonia) and chemical (AAPH) factors. Use of free radical scavengers (e.g. histidine, mannitol, ascorbic acid) did not significantly inhibit the cell killing. However, it could not be ruled out that free radical may contribute to the cell killing whenever it is present in a significant amount. This could be shown by the possible correlation of free radical scavenging activity of the ECAs used and the apoptosis induction.[18] Another study showed that free radical scavenger NAC inhibited apoptosis induced by US whether it is added to the cells during or after sonication.[43] The later show that free radical scavenging may work against the free radical within the internal intracellular mechanism of apoptosis itself rather than by scavenging the free radical generated by US.

Because H_2O_2, a stable reactive oxygen species (ROS), could be produced as a byproduct of the sonolysis of water, we tried to detect H_2O_2 in most US set-ups. However with the US set-ups used in the studies reviewed here, H_2O_2 was detected only in a relatively small amount at a higher intensity and a longer duration of sonication. To verify its bioeffects, we sonicated a cell-free medium and added it to freshly collected cells. No cell killing, including apoptosis, was observed even after 12 hr of incubation. Considering that such US condition can instantly kill almost all cells in a suspension, the role of stable radicals, in this case H_2O_2, that may persist after sonication may not be significant to induce any observable bioeffects.

SUMMARY, IMPLICATIONS AND FUTURE DIRECTION

Summary and implications

The factors that enhance the effects may be useful in cases where effects of US are limited; while inhibitory factors may find their use as modulator in the therapeutic process. Inhibitory factors may also provide protection to the normal tissues to avoid damage, thus limiting and localizing the effect on the target tissue. The information also gave us better understanding of the mechanism on how US works and the possible problems that may be encountered when applied *in vivo* or clinically.

Use of high intensity US for hyperthermia and for thermal ablation therapy has been in clinical application in conjunction with diagnostic US. A similar set-up can be used to treat localized tissue or organ with low intensity US in combination with enhancing or delivery agent such as ECAs (Fig. 7). For a systemic hematological problem such as leukemia, an ex vivo type of therapy could be useful. Figure 8 shows a

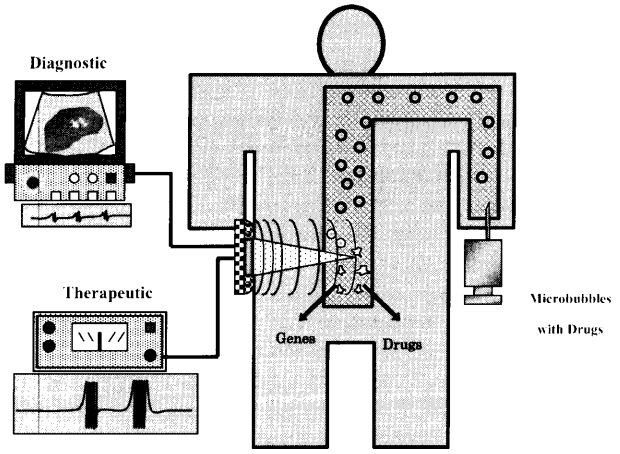

Fig. 7. A schematic diagram of a simultaneous use of therapeutic and diagnostic ultrasound clinically. Low intensity ultrasound may be used with the similar set-up when combined with some enhancer or carrier such as ECA. Diagnostic ultrasound will be used to identify a localized target and monitor the treatment. On the other hand, therapeutic low intensity ultrasound will facilitate the release of agent from the carrier (e.g. ECA) or potentiate the effect of drugs by inducing mild bioeffects on the localized target tissue.

schematic diagram of how low-intensity US can be applied to treat leukemia using a devise similar to a hemodialysis machine. The selective killing of cells has been suggested by previous studies showing that cancer cells are more susceptible to low intensity ultrasound over the healthy ones.[36,60] Such selective killing makes the above concept theoretically feasible.

In summary, fine-tuning of the US parameters and other factors involving the cells and its micro-environment, are important to attain the desired bio-effects of US. Among the factors affecting the *in vitro* effects are temperature, osmotic pressure, presence of some agents, cell density and incubation time were identified in the studies. Presence of microbubbles, dissolved gasses, and some agents during sonication are other important condition that could modulate the effects of US on the cells. The high cell density and the presence of carbon dioxide *in vivo* are factors that can potentially inhibit the bio-effects while other enhancing factors can compensate to attain the desired US effects when applied for therapy. Though, no direct implications of these findings that would put into question the safety of diagnostic US, considering the low intensity used in our experiments and the nature of enhancing factors which could be simulated *in vivo*, e.g. mild hyperthermia (fever at 40°C), hypotonia (cell swelling by edema), and presence of some agents (anticancer drugs during chemotherapy and use of ECA during diagnosis); these are real clinical scenarios that could potentially lower the threshold for bioeffects of diagnostic US. Other than following the advice to allow sensible assessment of risk over benefit and the practical implementations of the ALARA (as low as reasonably achievable) principle,[62] other particular risk factors that could be identified to every individual patient should also be carefully studied and noted when using ultrasound.

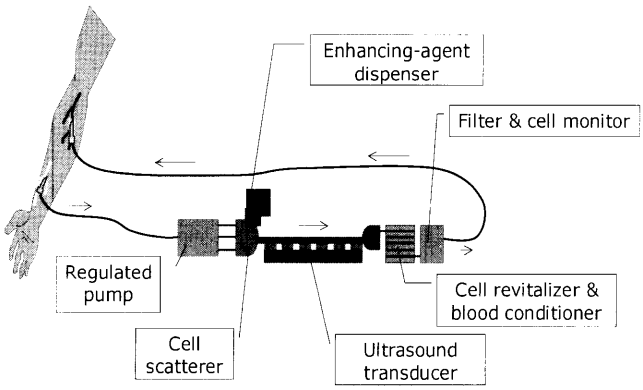

Fig. 8. A schematic diagram on possible application of low intensity pulsed ultrasound ex vivo. A sonication set-up designed similar to a hemodialysis machine may be used to sonicate blood to selectively eliminate leukemia cells ex vivo. The system may also incorporate agent that could selectively increase susceptibility of cancer cells to ultrasound.

Future direction

To design future studies, various US parameters (e.g. frequency, intensity, PRF, DF, duration) and US in combination with other physical factors (e.g. hyperthermia, hypotonia, X-rays) and other agents (e.g. ECAs, AAPH) are among the possible aspects to investigate. They seem limitless in number, but a greater challenge for the future is to verify if the *in vitro* findings hold true *in vivo* and if they do, to what extent.

ACKNOWLEDGEMENT

This study is supported in part by the Grant in Aid for Scientific Research on Priority Areas © (12217049) from the Ministry of Education, Culture, Sports, Sciences and Technology; in part by the 21st Century COE Program of the Toyama Medical and Pharmaceutical University, Japan; and also in part by Research and Development Committee Program of the Japan Society of Ultrasonics in Medicine.

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Received on July 13, 2004
1st Revision on August 26, 2004
Accepted on September 16, 2004