Ultrasound in Med. & Biol., Vol. 43, No. 6, pp. 1085–1104, 2017

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2017.01.023

● *Review*

MECHANICAL AND BIOLOGICAL EFFECTS OF ULTRASOUND: A REVIEW OF PRESENT KNOWLEDGE

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(*Received* 14 *June* 2016; *revised* 26 *January* 2017; *in final form* 30 *January* 2017)

Abstract—Ultrasound is widely used for medical diagnosis and increasingly for therapeutic purposes. An understanding of the bio-effects of sonography is important for clinicians and scientists working in the field because permanent damage to biological tissues can occur at high levels of exposure. Here the underlying principles of thermal mechanisms and the physical interactions of ultrasound with biological tissues are reviewed. Adverse health effects derived from cellular studies, animal studies and clinical reports are reviewed to provide insight into the *in vitro* and *in vivo* bio-effects of ultrasound. (E-mail: z.izadifar@gmail.com or zai206@mail.usask.ca) © **2017 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Mechanical effect, Biological effect, Cavitation.**

INTRODUCTION

Ultrasound applications

Therapeutic applications of ultrasound in medicine have been accepted and advantageous for many years (Escoffre and Bouakaz 2016). Ultrasound is widely used as a therapeutic tool in physiotherapy, in lithotripsy for kidney stone destruction, in the form of high-intensity focused ultrasound (HIFU) for tissue ablation in tumor treatment and as a surgical tool. It has also been applied as a tool for drug delivery (Escoffre et al. 2013), gene delivery (Panje et al. 2013) and thrombolysis (Kandadai et al. 2015). Current research suggests promising new applications of and advancement in biomedical ultrasound in medicine. Further progress in the therapeutic applications of ultrasound requires a better understanding of the mechanisms of interaction with tissues so that this technique is safe.

The focused mode of the ultrasound beam has been extensively used in a variety of therapeutic applications of ultrasound, such as thrombolysis, gene delivery, drug delivery, surgical tools and HIFU. Reported side effects in the focused mode, especially for HIFU, are significant and need to be avoided to maximize the benefit-to-risk ratio in patients. Further progress in making therapeutic applications of ultrasound sufficiently tolerable for patients requires a greater understanding of the mechanism by which ultrasound interacts with tissues. Therefore, this review concentrates on non-thermal laboratory/clinical-based biological effects of ultrasound both *in vitro* and *in vivo*.

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HIFU AND RELATED SIDE EFFECTS

High-intensity focused ultrasound is a modality of focused ultrasound used to treat a range of disorders. In HIFU, the ultrasound beam is focused precisely on the target to deliver acoustic energy to part of the body in a non-invasive or minimally invasive manner. HIFU is used for treatment purposes in cancer therapy, surgery and enhancement of the delivery or effect of chemotherapy or immunotherapy. The intention in HIFU is to heat a target volume of tissue without affecting the tissue in the ultrasound propagation pathway. HIFU can increase the temperature of a selected area above 55°C, which results in coagulative necrosis and immediate cell death in a specific volume (the "lesion") through a focused ultrasound beam. Because the ultrasound wavelength at megahertz frequencies has a millimeter-scale

beam size and the ultrasound probe has a concave shape, the ultrasound beam can be focused into small, clinically relevant volumes of tissue. The energy absorption raises the temperature at the focus point but increases it only to non-cytotoxic levels outside the region (Haar and Coussios 2007).

HIFU is applied from sources placed either outside the body for treatment of liver, kidney, breast, uterus, pancreas and bone cancer or inside the body through the rectum for treatment of prostate cancer (Haar and Coussios 2007). HIFU is gaining rapid clinical acceptance for non-invasive tissue heating and ablation for various applications. However, there are complications and side effects of the HIFU treatment of tumors. For example, second-degree skin burns have been reported in all patients (Jung et al. 2011) and third-degree skin burn in 3% of patients (Xiong et al. 2009) during HIFU treatment of pancreatic tumors. Adverse effects such as tumor or vessel rupture during HIFU can lead to metastasis *via* the bloodstream. Serious adverse effects such as intrahepatic metastasis (Xin 2008), lung embolism, deterioration of liver function, renal failure and death can result from HIFU treatment (Yu and Luo 2011). The rates of adverse events in both malignant and benign lesions during HIFU depend largely on the disease type and the HIFU device used (Yu and Luo 2011). HIFU therapy involves both thermal effects and cavitation, which may lead to adverse effects and lesions. Skin burn is considered a thermal lesion and arises from the thermal effects of HIFU. Adverse effects such as tumor or vessel rupture and bleeding, ectopic embolism and metastasis arise from cavitation during HIFU. The mechanical effect of cavitation can rupture vessel walls at the primary tumor site and at the same time detach cancerous cells/emboli and lead to their release into circulation, where they may cause a metastasis or embolism (Miller and Dou 2006; Yi et al. 2005). For example, intrahepatic metastasis (Xin 2008) and rupture of esophageal varices have been reported (Wang et al. 2004; Yongjian et al. 2002) after HIFU treatment. Cavitation gives rise to sensitivity of tissue to heat and causes lesions to extend beyond the HIFU focal point (Yu et al. 2006), which can lead to severe events if the lesion is in the vicinity of vital structures (Yu and Luo 2011). Peripheral nerve injuries after bone cancer treatment (reversible or irreversible) (Chen et al. 2004), ischiadic or sacral nerve damage and hematuria during uterine fibroid treatment (potentially reversible) (Ren et al. 2009) are some other adverse effects of HIFU treatment. Adverse effects of HIFU frequently occur in tissues adjacent to the target focus as well as in the pathway of the HIFU beam. Therefore, selecting the appropriate delivery pathway for the HIFU beam (Yu and Luo 2011) and, more importantly, improving cavitation monitoring techniques toward upgrading the accuracy of HIFU devices are necessary to improve the safety profile of this technique.

BASIC PRINCIPLES OF THERMAL AND NON-THERMAL INTERACTIONS OF ULTRASOUND WITH CELLS, TISSUES AND ORGANS

Thermal and non-thermal physical and biological effects of ultrasound in tissues are the basis of various therapeutic applications. The thermal effects of ultrasound that arise from the absorption of ultrasonic energy and creation of heat depend on ultrasound exposure parameters, tissue properties and beam configuration (Dalecki 2004). Acoustic radiation force, radiation torque, acoustic streaming, shock waves and cavitation are considered non-thermal effects of ultrasound. Radiation force that results from a transfer of momentum from the ultrasound field to the object (Rooney and Nyborg 1972) is the cause of contrast agent displacement to the walls of blood vessels in laboratory animals (Dayton et al. 1999). Radiation force is itself an underlying mechanism of radiation torque and acoustic streaming effects. Acoustic streaming occurs when acoustic field propagation in a fluid causes a rise in fluid flow. Acoustic streaming has been used as a diagnostic method to identify cysts non-invasively (Nightingale et al. 1995). Cavitation is perhaps the most widely studied non-thermal mechanism of ultrasound and is often the basis for new therapeutic applications (Kooiman et al. 2014). Considering that the therapeutic and imaging mechanisms of ultrasound are based on the interaction of sound waves with tissue, cavitation could also have hazardous bio-effects on tissues. The cavitation that occurs is largely unpredictable, and the bio-effects of ultrasound could be hazardous to healthy tissues. Cavitation is recognized as a major cause of ultrasound-induced mechanical and thermal effects and can be accentuated by the presence of ultrasound contrast agents in the tissue.

CAVITATION PHENOMENON

Ultrasound cavitation is described as the formation and oscillation of a gas bubble. Such bubbles can form from a pre-existing stabilized gas body or nuclei. Gas nuclei can be stabilized in crevices of impurities in the liquid, and as the pressure in the liquid drops, the gas in the crevice expands and forms a microbubble (Dalecki 2004). A variety of biological effects, both *in vivo* and *in vitro*, can be attributed to acoustic cavitation. The non-thermal effects of ultrasound, including cavitation, may play a more important role in treatment of soft tissue lesions than thermal effects (Speed 2001), but this strongly depends on the type of cavitation. Non-inertial cavitation occurs when a bubble is exposed to an acoustic field and goes through repetitive oscillations around its

equilibrium radius over many acoustic cycles. The oscillation of the bubble can result in heat generation, microstreaming of nearby fluid and localized shear stresses (Dalecki 2004). Inertial cavitation, known as microbubble formation and collapse, is induced by ultrasound waves traveling through tissue fluids during ultrasound therapy. When the acoustic field is at higher amplitudes, the radius of a bubble may grow to a maximum and then collapse. This kind of bubble is called an inertial (transient) cavitation bubble. Inertial cavitation bubbles can expand and collapse violently during a single ultrasound exposure on the order of 1 μs (Flynn 1982). Extremely high pressure and temperature, high-speed microstreaming (Doinikov and Bouakaz 2010; Wu 2002) and high-speed liquid microjets (Ohl et al. 2006; Postema et al. 2005) are induced as a result of cavitation processes. The high pressure and temperature generated are localized at the minimum radius of the inertial bubble collapse and are temporally limited to the duration of collapse (Dalecki 2004). The motion of the bubble wall during inertial collapse produces a spherically diverging shock wave in the liquid surrounding the collapsing bubble. Inertial cavitation close to a solid surface (such as metal, tissue, cell wall and stone) generates a high-speed liquid microjet that drives into the solid surface and results in pitting of the surface (Bekeredjian et al. 2007). This mechanical outcome of cavitation is used for fragmentation of kidney stones during lithotripsy.

One parameter that can directly change the bubble response from non-inertial to inertial cavitation is the acoustic pressure amplitude. The acoustic pressure at which this transition occurs is called the threshold for inertial cavitation (Dalecki 2004). Another parameter used to determine the likelihood of cavitation is the mechanical index (MI). The MI is based on the derated peak rarefactional pressure and defined as the maximum value of the negative peak pressure divided by the root square of the acoustic center frequency (Nelson et al. 2009).

The mechanical effect of cavitation can cause substantial injury to cells when ultrasound-induced microbubbles expand and then collapse (micro-explosion) close to them. Non-inertial cavitation (or stable cavitation in which the microbubble does not violently collapse and instead oscillates for many cycles around its resonance size) is considered more beneficial to injured tissue, whereas inertial cavitation (transient) causes tissue damage (Sun et al. 2015; Wells 1977). Many therapeutic applications of ultrasound rely on the ability of ultrasound to focus within tissues at the focal point where the beam converges and to use the energy for non-invasive thermal or mechanical effects. A quantitative analysis of the amount of heat deposited by ultrasound revealed that inertial cavitation is key in addressing some of the major challenges of high-intensity focused ultrasound (Coussios and Roy 2008). In the context of drug delivery, both inertial and non-inertial cavitation bubbles play roles. When ultrasound is applied *in vivo*, cavitation can occur anywhere that appropriate microbubbles are present, such as the lung, intestine or tissue containing ultrasound contrast agents.

MICROBUBBLE CONTRAST AGENTS

Ultrasound contrast agents are gas-filled microbubbles encapsulated by a protein, lipid or polymer shell, stabilized from dissolution and injected intravenously (Lammertink et al. 2015). These ultrasound contrast agents are used clinically to enhance diagnostic images and have new applications in the areas of molecular imaging, drug delivery and gene therapy (Klibanov 1999; Unger et al. 2002). The application of ultrasound contrast agents facilitates drug delivery because of the formation of temporary pores in the cell membrane by ultrasound. Deng et al. (2004) report that ultrasound raises the transmembrane current as a direct result of pore formation, which leads to a decline in membrane resistance (Deng et al. 2004).

Microbubbles have also been used as a new therapeutic method for direct deposition of stem cells at the site of injury after acute myocardial infarction (Woudstra et al. 2016). In this new stem cell therapy technique, stem cell–microbubble complexes (StemBells) are assembled by binding dual-targeted microbubbles to adipose-derived stem cells. These complexes target the myocardial infarct area of the heart *via* microbubbles; specifically, the StemBells were injected into the body of a rat acute myocardial infarction–reperfusion model and unloaded *via* ultrasound. The effect of ultrasound on directing StemBells to the vessel wall was illustrated in an *in vitro* flow model. The feasibility of improving cardiac function was successfully illustrated in a rat model (Woudstra et al. 2016).

Another efficient use of microbubbles and ultrasound is therapeutic gas delivery through microbubbles and liposomes. Gaseous molecules of nitric oxide (NO), carbon monoxide (CO), xenon (Xe), oxygen (O_2) and hydrogen sulfide (H_2S) mediate cell signaling pathways, play an important role is physiology and biological responses and have great therapeutic potential. However, controlled delivery is a significant challenge for therapeutic techniques using these gases. Researchers are using microbubbles and liposomes in novel therapeutic gas delivery methods (Fix et al. 2015). Extensive studies have been performed on microbubble gaseous delivery in microbubble oxygenation therapy of hypoxic tumors (McEwan et al. 2015) in a rabbit model of hypoxemia (Legband et al. 2015), in rats with acute respiratory

distress syndrome (Legband et al. 2016) and for pancreatic cancer (McEwan et al. 2016). Tissues that naturally contain gas, such as the lung and intestine, as well as vessels containing ultrasound contrast agents are more susceptible to ultrasound bio-effects. Mechanical damage (related to cavitation) to the microvasculature in the lung and intestine has been observed in several mammalian laboratory studies (Child et al. 1990; Dalecki et al. 1996).

Application of an ultrasound contrast agent (suspensions of stabilized bubbles) seems to give rise to biological effects through gas–body activation (Miller 2007). Brayman et al. (1999) observed that the contrast agent in suspension adjacent to orientation of the monolayer (*i.e.,* simulating the sites of ultrasound entry or exit from a blood vessel) gave rise to damage and erosion of cells. They modeled the endothelial layer of blood vessels using fibroblast monolayers *in vitro* and conducted ultrasound treatment at 1.0, 2.1 and 3.5 MHz. The lysis or breaking open of erythrocytes leads to the release of hemoglobin into the surrounding fluid. The frequency dependence of hemolysis with a first-generation ultrasound contrast agent (Albunex; Mallinckrodt Medical, St. Louis, MO, USA) in whole blood and with a second-generation perfluorocarbon-based ultrasound contrast agent (Optison; Molecular Biosystems Inc., San Diego, CA) was studied by Miller et al. (Miller and Gies 1998a, 1998b, 1998c; Miller et al. 1997). Miller and Gies (1998a, 1998b, 1998c) reported that Optison caused more hemolysis than Albunex, especially when ultrasound exposure was in pulsed mode. The ultrasound contrast agent gas bodies can nucleate inertial cavitation (Miller and Thomas 1995a; 1995b) as there is good correlation between the amount of hemolysis induced by ultrasound and inertial cavitation activity (Chen et al. 2003a, 2003b; Everbach et al. 1997; Miller et al. 2001a, 2001b). Ultrasonically induced hemolysis strongly depends on the frequency (Brayman et al. 1997; Miller et al. 2001a, 2001b, 2003), with overall biological effects decreasing with frequency (Health Protection Agency 2010).

Contrast agent injection may enhance the risk of capillary rupture by diagnostic ultrasound (Health Protection Agency 2010). Miller and Quddus (2000) anaesthetized hairless mice, injected them with Optison (5 mL kg^{-1}) and scanned them using a 2.5-MHz transducer (610-ns pulses with 3.6-kHz repetition frequency and 61-Hz frame rate); this resulted in an increasing number of petechiae (capillary ruptures with erythrocyte extravasation) in the intestine and abdominal muscle (Miller and Quddus 2000). The increase in petechiae was considerable above 0.64 MPa for muscle and 1 MPa for intestine (Health Protection Agency 2010). Li et al. (2003, 2004) used the rat heart as a model system to examine microvascular injury caused by ultrasound. Rats were examined in a water bath using a 1.7-MHz diagnostic ultrasound system and bolus doses of three different ultrasound contrast agents (Optison; Mallinckrodt Inc., St. Louis, MO), Imagent (Alliance Pharmaceutical Corp., San Diego, CA) and Definity (Bristol-Myers Squibb Medical Imaging, Inc., N. Billerica, MA). Li et al. detected petechiae on the heart surface and microvascular leakage by injecting Evans blue dye before scanning. The results of their study indicate that the contrast agent delivery mode and dose, as well as the ultrasound parameters, have a considerable effect on cardiomyocytes. Vascular damage might be physiologic and not accompanied by irreversible cellular injury (Zachary et al. 2006). In fact, the dynamics of microbubbles both *in vitro* and *in vivo* reveal that the oscillation of microbubbles increases vascular permeability and even locally injured vasculature (Hwang et al. 2005; Stieger et al. 2007). Depending on the degree to which temperature rises during ultrasound application, the effects on the vasculature can be either reversible/repairable or irreversible. In reversible cases, the effects are temporary, and no permanent vascular occlusion is produced. In irreversible cases, the energy deposition is sufficient to produce long-term damage with persistent effects of vascular spasm, obstructed blood flow and increasing endothelial destruction that lead to a loss of vascular relaxation responses (Shaw et al. 2014). Therefore, appropriate strategies need to be designed to minimize irreversible damage to capillaries (Stieger et al. 2007).

Kobayashi et al. (2002, 2003) studied the microvascular injury in rat mesentery caused by applying a phased array ultrasound system at a frequency of 1.8 MHz. Endothelial cells were damaged in capillaries and venules under all conditions at 0.82 MPa. The influence of contrast-enhanced diagnostic ultrasound (Optison and several experimental agents) in kidneys of rat at three different frequencies of 1.8, 4 and 6 MHz was studied by Wible et al. (2002). Glomerular capillary hemorrhage was driven from the glomerular tuft into Bowman's capsule and proximal convoluted tubules. Shigeta et al. (2004) reported that platelet aggregation in the liver sinusoids of rats occurred after exposure of the liver to diagnostic ultrasound at 8 and 12 MHz with an ultrasound contrast agent (Levovist). They also observed endothelial cell damage in samples taken 5 h after acoustic exposure. On the basis of experiments by Stroick et al. (2006) and Hardig et al. (2003) in an animal model, the extent of intracerebral hemorrhage was found not to be enhanced by ultrasound exposure in the presence of an ultrasound contrast agent. The effect of ultrasound exposure on rat heart was detected using diagnostic imaging with an experimental ultrasound contrast agent by Vancraeynest et al. (2006). Findings of

histologically definable injuries in rat hearts were confirmed by Miller et al. (2005a, 2005b) and indicate that elevating the parameters for therapeutic efficacy results in severe microscale injury and functional impairment of the heart (Vancraeynest et al. 2006).

New techniques have been developed with respect to the use of ultrasound, and especially designed contrast agents, to aid drug delivery across the blood–brain barrier (BBB) (Health Protection Agency 2010). It was first thought that opening of the BBB was induced by inertial cavitation, although disruption in the absence of indicators of inertial cavitation has been observed (Health Protection Agency 2010). In almost every study featuring a combination of ultrasound and ultrasound contrast agent, some BBB injury has been observed (Health Protection Agency 2010). Mesiwala et al. (2002) reported that HIFU resulted in selective and non-destructive disruption of the BBB in a rat model. It is possible that the BBB is opened at the focal point without sharp neuronal damage if microbubbles are introduced into the bloodstream before exposure to focused ultrasound (Hynynen et al. 2001). Therefore, limiting the effect of ultrasound on the vasculature and decreasing the intensity required to produce BBB opening can be achieved by introduction of cavitation nuclei into the bloodstream. This also reduces the risk of damage to tissue (Health Protection Agency 2010). BBB disruption was also detected by Hynynen et al. (2001) and Kinoshita et al. (2006) after using contrast-enhanced magnetic resonance imaging (MRI) at the desired location, as well as by Mesiwala et al. (2002) and Kinoshita et al. (2006) in post-mortem histology. Hynynen et al. (2005) studied the localized effects of ultrasound exposure on rabbit brains using contrast-enhanced MRI. Their study indicated BBB disruption for pressure amplitudes above 0.4 MPa, with 10 ms exposure at a frequency level of 690 kHz, a repetition frequency of 1 Hz and total exposure time of 20 s. The results of a histologic study 4 h after exposure revealed about 70% to 80% brain tissue necrosis at pressure amplitude levels greater than 2.3 MPa. Small areas of erythrocyte extravasation were also observed at lower pressure amplitude levels. Another study reported that when rabbit brain was exposed to ultrasound at 1.63 MHz, a pulse length of 100 ms and a pulse repetition frequency of 1 Hz at 0.7 to 1.0 MPa for 20 s, only a few cells in some of the sonicated areas underwent ischemia or apoptosis, but no ischemic region that would indicate a compromised blood supply was observed. MRI or histology up to 4 wk after sonication revealed no delayed effect (McDannold et al. 2005). Therefore, it is possible that BBB disruption after ultrasound may occur without any basic vascular damage; however, red blood cell extravasation into tissue indicates BBB injury has occurred, and as such, the method can be harmful especially in therapeutic applications for brain disease (Health Protection Agency 2010).

Hynynen et al. (2003) studied the effect on brain tissue of the burst mode of ultrasound in the presence of an ultrasound contrast using contrast-enhanced MRI and histology. Brain tissue damage, including vascular wall damage, hemorrhage and sometimes necrosis, was induced at a pressure amplitude of 6.3 MPa (exposure conditions: 1.5 MHz, 10-μs bursts repeated at a frequency of 1 kHz for 20 s). At all pressures tested, occasional smooth vascular damage in almost 50% of the sonicated locations was observed without any signs of ischemia.

Fatar et al. (2005) studied the effects of ultrasound (at 2 MHz, peak negative pressure of 1052 kPa and temporal intensity of 37.3 W cm^{-2}) and microbubbles (SonoVue) on brain infarct volume, apoptosis, interleukin-6 and tumor necrosis factor α levels and disruption of the BBB in a middle cerebral artery occlusion model in rats. They observed a reduction in infarct volume in treated samples compared with controls. The results indicated no additional BBB disruption and no rise in apoptosis outside the infarction area. Another study found that exposure of rabbit brain to low-intensity ultrasound (at 1.7 MHz for 30 s) and close to the threshold for tissue damage gave rise to apoptotic cells over 48 h, but the lesions were dominated by necrosis (Vykhodtseva et al. 2001). Addition of an ultrasound contrast agent (Optison) to the treatment process (at 1.5 MHz, 1.4–8.8 MPa, and for 20 s) resulted in domination of lesions by apoptosis, with the number of apoptotic cells approximately six times that of necrotic cells (Vykhodtseva et al. 2006). Another study on the short-term tolerability of BBB opening using focused ultrasound and an ultrasound contrast agent (Baseri 2010) reported the feasibility of tolerable BBB opening under a specific set of sonication parameters. In this study, a short-term (30-min or 5-h survival) histologic assessment was performed on 49 mice with an intravenously injected ultrasound contrast agent. Mice were exposed to ultrasound at a frequency of 1.525 MHz, pulse length of 20 ms, pulse repetition frequency of 10 Hz, peak rare factional acoustic pressure of 0.15–0.98 MPa and two 30-s sonication intervals with an intermediate 30-s delay. The BBB opening threshold and the most tolerable acoustic pressure were reported to be 0.15–0.3 and 0.3–0.46 MPa, respectively (Baseri 2010). Another study indicated that repeated opening of the BBB through FUS and ultrasound contrast agents at the basal ganglia of non-human primates is safe for up to 20 mo with no long-term negative physiologic or neurologic effects (Downs et al. 2015). This study was conducted using ultrasound parameters of 500 kHz, 200–400 kPa, administration of 4- to 5-μm microbubbles and 2 min sonication, resulting in repeated opening of the

BBB. These results showed promise for clinical and basic scientific applications (Downs et al. 2015).

ULTRASOUND BIOLOGICAL EFFECTS

Discussion of cavitation behavior usually assumes the pre-existence of bubbles or bubble nuclei with the potential to grow in a medium propagated by the ultrasound beam. Although liquids can be saturated with gas, suitable cavitation nuclei might not always be present. In some cases, an ultrasound contrast agent is used and injected into the body to improve diagnosis *via* ultrasound. It is very doubtful if either inertial or non-inertial cavitation occurs at diagnostic levels of ultrasound within soft tissues or fluids in the body in the absence of contrast agents (Health Protection Agency 2010). A number of studies of ultrasound biological effects related to the presence of cavitation/microbubbles during ultrasound exposure in different organs, in body fluid and microvasculature, in cells and prenatally are reviewed below.

ORGAN STUDY

Lung

Tissues naturally containing gas bodies, such as the lung and intestine, are more sensitive to the bio-effects of ultrasound exposure because of the presence of gas. Because fetal lungs are gas free, they do not exhibit any sign of the lung damage evident in air-filled adult lungs (Hartman et al. 1990). The trauma at the surface of the lung and in the intestines has been interpreted to result from cavitation-like processes in the body (Dalecki et al. 1996; Hartman et al. 1990). To explore the hypothesis of cavitation-based bio-effects of diagnostic ultrasound on the lungs of mammals, rat lung was exposed to 4.0-MHz (the threshold of lung damage in rat) pulsed Doppler and color Doppler ultrasound; then, by use of a 30-MHz active cavitation detection scheme, the first *in vivo* evidence of cavitation from diagnostic ultrasound pulses was reported (Holland et al. 1996). Damage to the microvasculature of the lung was characterized by extravasation of red blood cells from capillaries into the alveolar space (Penney et al. 1993). Although this extravasation of red blood cells was reversible, and apparently occurred during exposure without rise in severity in the subsequent 5 min, exposure of the lung to pulsed ultrasound was deleterious to the lung (Penney et al. 1993). Lung damage was first reported *in situ* and under exposure conditions of about 1 MPa peak positive pressure, 2 MHz frequency, 10 μs pulse duration, for 3 min (Child et al. 1990) and has since been reported in mice, monkey, pigs, rabbits and rats (Baggs et al. 1996; Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e; Holland et al. 1996; O'Brien and Zachary 1997; Tarantal and Canfield 1994; Zachary and O'Brien 1995). A study in humans reported a lack of lung damage after intra-operative transesophageal echocardiography with ultrasound exposure (Meltzer et al. 1998). Lung hemorrhage may result from the thermal, mechanical or cavitational effects of ultrasound. Ultrasound-related lung hemorrhage is a function of frequency (Child et al. 1990; Zachary et al. 2001), pulse duration (Child et al. 1990; O'Brien et al. 2003a, 2003b), pulse repetition frequency (Child et al. 1990; O'Brien et al. 2001) and duration of exposure (Hartman et al. 1990; O'Brien et al. 2001). Cavitation-related bio-effects are more dependent on frequency (Child et al. 1990; Zachary et al. 2001).

Child et al. (1990) observed hemorrhage in mouse lung tissue after ultrasound exposure (at 1.2 MHz, pulse average intensity 1 mW cm^{-2}, 10-μs pulse, peak positive pressure 0.7 MPa, 3-min exposure) (Child et al. 1990). Since then, ultrasound-induced lung hemorrhage has been reported *in vivo* in mice (Child et al. 1990; Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e; O'Brien et al. 2000, 2001; Zachary et al. 2001), rat (Kramer et al. 2001, O'Brien et al. 2001, 2003a, 2003b, 2005), rabbits (O'Brien and Zachary 1997; Zachary and O'Brien 1995) and pigs (Harrison et al. 1995; O'Brien and Zachary 1997; O'Brien et al. 2003a, 2003b, Zachary and O'Brien 1995).

Lung damage manifests as localized lesions on the lung surface, but there has been no report of damage to adult or neonatal human lungs so far (Health Protection Agency 2010). The reason for this effect of ultrasound on the lung surface is not fully understood and is still under investigation. The presence of gas in the lung and intestines is thought to result in mechanical trauma to adjacent soft tissues as a result of the cavitation process.

Intestines

Acoustic cavitation can be generated in a wide range of intestinal environments, as these contain gas bodies located in a fluid-like medium (Dalecki 2004). Cavitation-related damage is more certain in the intestines and microvasculature with the presence of microbubbles than in the lung (Dalecki 2004). Mammalian studies reveal the occurrence of intestinal hemorrhage when the thermal effects of ultrasound have been minimized (Dalecki et al. 1995a, 1995b, 1995c, 1996; Miller and Thomas 1995a, 1995b). Petechial hemorrhage in the intestines of laboratory animals exposed to a lithotripter field has been reported in several studies (Dalecki et al. 1995a, 1995b, 1995c; Miller and Thomas 1995a, 1995b; Raeman et al. 1994). The threshold of pressure for intestinal hemorrhage in mice ranges from 1 to 3 MPa (Dalecki et al. 1995a, 1995b, 1995c; Miller and Thomas 1995a, 1995b).

Further evidence revealing cavitation as a mechanism underlying the effects of ultrasound on the intestines relies on studies using ultrasound contrast agents. The area of murine intestinal hemorrhage significantly increased when the vasculature was filled with ultrasound contrast agents and exposed to ultrasound (Miller and Gies 1998a, 1998b, 1998c 2000). The threshold of intestinal damage in the presence of a contrast agent was about 3 MPa at 2.4 MHz with a pulse duration of 10 μs (Miller and Gies 2000). The effect of ultrasound on intestinal damage increases with increasing frequency and decreasing pulse duration (Miller and Gies 1998a, 1998b, 1998c, 2000). The response of microbubbles to negative pressure is greater than the response to positive pressure. More damage was noted in the intestines as well as other tissues of mice exposed to negative versus positive pressure of a lithotripter field in the presence of microbubbles (Dalecki et al. 2000). Lehmann and Herrick (1953) observed vascular damage in the wall of the intestines; this was followed by an investigation by Dalecki et al. (1995a, 1995b, 1995c) that noted areas of hemorrhage at several abdominal sites of mice exposed to pulsed ultrasound (10 μs, 100 Hz) at 0.7–3.6 MHz. The hemorrhage occurred at a level of exposure above the threshold of about 1 MPa; lower frequency levels are more effective at producing hemorrhage than higher frequency levels. Using a piezoelectric lithotripter, Dalecki et al. (1995a, 1995b, 1995c) determined the threshold of ultrasonically-induced hemorrhage to be about 1–3 MPa. In addition, they found gas-body activation to be important by observing a very extensive hemorrhage in the (gas-containing) intestines of adult mice and almost no effect in the (gas-free) intestines of their fetuses (at a pressure amplitude of 10 MPa) (Dalecki et al. 1996). Petechiae and hemorrhage in the intestines were also observed by Miller and Gies (1998a, 1998b, 1998c). They found the threshold level for petechiae to be 0.28 MPa (spatial average, temporal average intensity [I_{SATA}] = 2.6 W cm^{-2}) and that for hemorrhage to be 0.65 MPa (I_{SATA} = 14.2 W cm^{-2}) for the longest exposure (up to 1000 s) of the sample (hairless mice) to 0.4-MHz continuous ultrasound at 37°C. Pulsed exposures or higher bath temperatures affect the threshold level of hemorrhage and particularly petechiae. The threshold level of petechiae increased with pulse exposure. In addition, application of ultrasound contrast agent, in both continuous and pulsed modes of ultrasound exposure, increased the number of petechiae and hemorrhages (Miller and Gies 1998a, 1998b, 1998c, 2000). Stanton et al. (2001) reported the effect of diagnostic ultrasound on the progression of epithelial cells in the crypts of the small intestine through the cell cycle. Histologic examination of the distal portion of the intestines of adult CD 1 mice whose anterior abdomen had been exposed to ultrasound (at 8 MHz for 15 min; spatial peak temporal average intensity [I_{SPTA}] = 1120 mW/cm^2; P_{MAX} = 420 mW) revealed that the number of cells undergoing mitosis was considerably decreased 4.5 h after exposure, and the number of apoptotic cells was significantly increased (Stanton et al. 2001). Overall, investigations on ultrasound-related intestinal damage indicate that cavitation is the mechanism responsible.

Urinary tract system

The application of lithotripsy as a clinical treatment for urinary calculosis, or kidney stones, has revolutionized the non-invasive treatment of this disease. During a lithotripsy procedure, high-amplitude acoustic pulses are generated at the site of the kidney stone using short pulses of high-acoustic-pressure ultrasound. The lithotripter shock wave is a short pulse of about 5- μs duration with a near-instantaneous jump to a peak positive pressure that typically varies between 30 and 110 MPa. This fast transition in the waveform is called "shock." The pressure then falls to zero about 1 μs thereafter and is followed by a negative pressure between −5 and −15 MPa. This negative pressure drives the cavitation bubbles that are critical for stone destruction. Most of the energy in the shock wave is between 100 kHz and 1 MHz (Cleveland and McAteer 2007).

Cavitation induced by lithotripters behaves as a cluster of bubbles rather than individual bubbles, and the coherent collapse of the cluster may give rise to its destructive power (Pishchalnikov et al. 2003; Pittomvils et al. 1995; Tanguay and Colonius 2002). Almost all patients who receive at least an average dose of shock waves (2000 shock waves at midrange or higher power) experience some form of tissue trauma, and some patients can experience severe, even catastrophic, adverse effects (Tuteja et al. 1997), including capillary damage and bleeding around the outside of the kidney (Evan et al. 1998). The clinical implications of such side effects are still under investigation. Mechanisms that may contribute to tissue injury include shear stress and cavitation. In lithotripsy, cavitation is more likely to create injury within blood vessels and to cause mechanical damage to organs such as the kidney. However, severe full skin burns after extracorporeal shock wave lithotripsy for renal calculi (Rao et al. 2014) or second-degree burns after shock wave lithotripsy (Rangarajan et al. 2012) are often reported. Some patients also face post-operative side effects such as pain, vomiting and skin wounds.

Experiments with lithotripsy indicate that damage to *in vitro* cells and *in vivo* tissue is considerably decreased when cavitation is reduced or eliminated (Evan et al.

2002; Williams et al. 1999). The cavitation cycle time (time for a bubble to grow and collapse) is on the order of 300 μs in a free field and about 600 μs on the surface of the stone (Bailey et al. 1999). On the basis of recent studies, it has been found that the cavitation bubbles produced by one lithotripsy pulse can be manipulated by a second pulse (Sokolov et al. 2001, 2003).

Heart

Premature ventricular contractions are another effect that results from exposure to a lithotripter ultrasound field (Dalecki et al. 1991; Delius et al. 1994); cardiac rhythm can be affected by even a single high-amplitude pulse. The threshold required to generate premature cardiac contractions is below the pressure amplitudes applied in clinical lithotripsy (positive pressure between 30 and 110 MPa and negative pressure between −5 and −15 MPa). To avoid affecting the cardiac rhythm during lithotripsy, the clinical delivery of lithotripter pulses is synchronized with an electrocardiogram (Dalecki 2004). To cause premature contractions, long ultrasound pulse durations and high-pressure amplitudes (*e.g.,* a 5-ms pulse and 2–5 MPa, which is the threshold to cause a premature contraction in mice and frogs at 1.2 MHz) are required, but these are not the exposure characteristics of diagnostic ultrasound (Dalecki 2004). Therefore, premature ventricular contractions should not occur during diagnostic ultrasound imaging.

Investigations using ultrasound contrast agents suggest that cavitation may be responsible for ultrasound-related premature cardiac contractions. The threshold for this effect of ultrasound is considerably lower in the presence of ultrasound contrast agents and at shorter pulse durations (*e.g.,* the threshold for production of a premature contraction in the presence of contrast agents in mice exposed to a single 10-μs ultrasound pulse at 1.2 MHz was about 1 MPa [Dalecki et al. 2005]) (Dalecki et al. 2005; Li et al. 2003; van der Wouw et al. 2000; Zachary et al. 2002). Premature ventricular contractions have been reported in humans with ultrasound contrast agents in their blood and exposed to diagnostic ultrasound (van der Wouw et al. 2000). The threshold conditions for such an effect in laboratory animals is a 10-μs pulse of 1-MHz ultrasound and peak pressure amplitudes (positive and negative) on the order of 1 MPa (Dalecki et al. 2005; Li et al. 2003). Microvasculature damage has also been reported in hearts exposed to ultrasound in the presence of ultrasound contrast agents, but without any certain relationship to the generation of arrhythmia (Li et al. 2003; Zachary et al. 2002).

The other mechanical bio-effect of ultrasound on the heart is cardiac contractility, which can be generated by a single pulse of high-amplitude ultrasound (peak positive pressure of about 1 MPa) (Dalecki et al. 1993, 1997a, 1997b, 1997c, 1997d, 1997e). A series of experimental studies suggest that radiation force is responsible for this effect (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e). Animal studies (frog) revealed that the direct aortic pressure effect of ultrasound is related to the magnitude of the radiation force exerted on the heart (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e). The role of radiation force alone in this effect was confirmed by a study in which an acoustic reflector was placed on the surface of the heart to preclude the possibility of heating and cavitation and instead maximize the radiation force delivered (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e).

Bone

Low-intensity pulsed ultrasound (*e.g.*, 0.5–50 mW/cm^2) has a favorable effect on bone fracture healing (Busse et al. 2002). Several studies have found that low-intensity ultrasound increases the rate of tissue repair after injuries, especially those associated with bone fracture (Dijkman et al. 2009; Mundi et al. 2009). The reasons why ultrasound can give rise to tissue repair and the functional significance of changes in neuronal migration in the developing brains of mice are not known.

In physiotherapy, hyperthermia treatment and pulsed Doppler exposure at its maximum output power, one of the main concerns and problems has been pain induction caused by heating of the highly innervated periosteum. Biologically relevant temperature increases (>2°C) in the skull bone of laboratory animals have been recorded during ultrasound exposure (Health Protection Agency 2010). Smith et al. (2001) reported that ultrasound exposure at high intensities ($>I_{SATA}$ = 40 W cm^{-2}) in thermal ablation therapy can lead to osteocyte damage and thermal necrosis. Evidence indicates that very low intensity ultrasound (I_{SATA} = 12–100 mW cm^{-2}) can influence bone regeneration (Claes and Willie 2007) and, for this reason, is used for treatment of fractures. These effects are due predominantly to a non-thermal mechanism that critically depends on the intensity applied (Health Protection Agency 2010). The temperature increase for I_{SATA} = 20–50 mW cm^{-2} was below 1°C (Chang et al. 2002). Duarte (1983) also reported negligible increases in temperature (0.01 ± 0.005°C) in rabbit fibula osteotomies after treatment at I_{SATA} = 50 mW cm^{-2} for 15 min per day. However, even a small rise in temperature (<1°C) can influence some enzymes, such as matrix metalloproteinase 1, which has been reported to be very sensitive to temperature (Welgus et al. 1981, 1985).

The biophysical process behind bone regeneration stimulation remains unknown. However, several studies suggest that low-intensity ultrasound can affect cell membrane permeability (Dyson and Brookes 1983; Chen et al. 2013a, 2013b; Dinno et al. 1989; Hu et al. 2014; Lim et al.

2013; Montalti et al. 2013; Mortimer and Dyson 1988; Rawool et al. 2003; Ryaby 1991; Ryaby et al. 1989; Sun et al. 2001) and increase hydrostatic pressure (Rawool et al. 2003) or can induce mechanical stimulation of micromotion (Wolff 1892) and cause acceleration of fracture healing. Relative to an untreated control, high-intensity ultrasound (I_{SATA} = 0.2–3 W cm^{-2}) gives rise to callus formation and accelerates fracture healing in rabbit radii (Corradi and Cozzolino 1953; De Nunno 1952) and tibias (Klug et al. 1986) and guinea pig ulnas (Murolo and Claudio 1952). High-intensity ultrasound treatment (I_{SATA} = 0.5 W cm^{-2}) of limbs resulted in a 36% increase in new bone formation and an 80% increase in torsional stiffness compared with controls (Chang et al. 2002).

MICROVASCULATURE, BLOOD VESSELS AND BODY FLUID

The rupture or destruction of blood cells in the presence of ultrasound contrast agents at diagnostic levels of ultrasound has occurred in *in vivo* studies with laboratory animals (Miller et al. 2008). Fetal red blood cells are even more susceptible to lysis from exposure to ultrasound in the presence of contrast agents *in vitro* (Miller et al. 2001a, 2001b). However, this is not known to occur in mammals at diagnostic levels of ultrasound in the absence of ultrasound contrast agents (Dalecki 2004). In an *in vivo* study, hemolysis occurred when mice were exposed to ultrasound at a frequency of 1.1 MHz (10-μs pulse duration) and ~2-MPa negative pressure with ultrasound contrast agents (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e).

To study the effects of ultrasound and contrast agents on microvasculature, isolated rabbit hearts were treated using a cardiac ultrasound system at 1.8 MHz with 1-Hz triggering of imaging frames (Ay et al. 2001). Capillary damage and red blood cell (erythrocyte) extravasation were observed at an MI of 1.6 when the treated heart was examined. Chen et al. (2002) examined the injurious effects of ultrasound contrast agents (Optison or Definity) in rat heart *in vivo* and observed elevation of troponin T in blood plasma as evidence of myocardial damage.

Cavitation is more likely to cause injury within blood vessels than in the surrounding tissue because a bubble surrounded by tissue is constrained and cannot undergo the violent growth and collapse cycle compared with a bubble in a fluid environment such as a blood vessel. Bubbles can cause mechanical damage to organs such as the kidney by at least two mechanisms: collapse and expansion. The asymmetric collapse of cavitation bubbles forms high-velocity microjets of fluid that travel at speeds close to 111 m/s (Chemat et al. 2004), as well as the emission of secondary shock waves that are radiated into the bubble and have an amplitude comparable to that of the focused shock wave (Cleveland and James 2007). The liquid microjets are forceful enough to easily puncture the fragile wall of a capillary or other blood vessel. Vessel walls may also rupture during the expansion phase of the bubble cavitation cycle (Cleveland and James 2007). When a bubble undergoes explosive growth in the vessel, it pushes the vessel outward and ruptures it. Experiments using capillary phantoms in an *in vitro* system confirm this hypothesis (Zhong et al. 1998, 2001).

The bubble expansion mechanism may also lead to other tissue damage. When blood vessels rupture and blood is collected in pools (*e.g.*, in a hematoma), the potential for cavitation increases because the pooling of blood provides a fluid-filled space in which cavitation can occur (Cleveland and James 2007). Although cavitation is the primary mechanism of tissue injury studied, much more investigation is still required (Cleveland and James 2007).

Damage to the microvasculature in tissues such as the kidney and liver in laboratory animals after exposure to lithotripter high-amplitude ultrasound has been reported in several studies (Delius et al. 1990a, 1990b; Mayer et al. 1990; Raeman et al. 1994). Damage to the microvasculature is considerably increased in the presence of ultrasound contrast agents in the vasculature (Dalecki et al. 1997a, 1997b 1997c, 1997d, 1997e; Miller and Gies 1999). In the presence of ultrasound contrast agents and an amplitude of only 2 MPa, reversible microvasculature damage has been observed in several soft tissues of mice, including muscle, mesentery, kidney, stomach, bladder and fat, with persistent sensitivity to lithotripter exposure for several hours (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e). In the absence of ultrasound contrast agents, minimal damage occurs at amplitudes up to 40 MPa. High-amplitude-pressure lithotripter pulses can cause cavitation *in vivo,* and in the presence of contrast agents, inertial cavitation can cause microvasculature damage. This understanding relies on the fact that hemorrhage in tissue (as a sign of response of microbubbles) at negative pressures is much greater than at positive pressures, as reported in different studies (Dalecki 2004; Dalecki et al. 2000).

Evidence indicates that pulsed ultrasound can also produce capillary damage in the presence of ultrasound contrast agents in the blood. Capillary rupture has been observed in muscle (Miller and Quddus 2000; Price et al. 1998), kidney (Wible et al. 2002) and cardiac tissues (Li et al. 2003) of laboratory animals exposed to diagnostic levels of ultrasound. Considering that no damage has been reported in these tissues from exposure to ultrasound in the absence of an ultrasound contrast agent, it can be concluded that normal tissue may contain no cavitation nuclei (Dalecki 2004).

The effect of ultrasound on tissues in the presence of ultrasound contrast agents can be decreased by increasing the applied frequency, as well as decreasing pressure amplitudes and the amount of contrast agent in the tissue (Dalecki 2004). In general, the mechanical effect of ultrasound increases in the presence of contrast agent microbubbles, which have potential utility in future therapeutic applications of ultrasound. For example, the capillary damage effect of ultrasound in the presence of ultrasound contrast agents facilitates the process of microbubble drug or gene delivery, in which microbubbles are loaded with a drug or gene and then unloaded in a specific localized area of body (Aryal et al. 2015; Chu et al. 2016; Meairs and Alonso 2007; Price et al. 1998; Song et al. 2002a, 2002b); it also facilitates arteriogenesis (Chen et al. 2013a, 2013b; Song et al. 2002a, 2002b) and tumor therapy (Aryal et al. 2015; Ji et al. 2016; Simon et al. 1993; Zhang et al. 2016).

VanBavel (2007) reviewed the effects of ultrasonically induced shear stresses on endothelial cells and noted that a major stimulus for many endothelial responses is provided by the shear stress associated with normal blood flow. For example, normal shear stress found in large arteries away from branches is on the order of 2–4 Pa (Davies 1995) and very rarely exceeds 8 Pa. The shear stress that veins experience is around 0.1–0.6 Pa (VanBavel 2007). Microstreaming associated with an ultrasonic field induces shear stresses that may be expected to increase biological effects. These shear stresses are higher than normal physiologic levels and can occur on a membrane, rupture it or alter its permeability (Health Protection Agency 2010).

Studies on the effect of ultrasound on blood have focused on platelets, the most fragile component. *In vitro* experiments indicate that ultrasound exposure can lead to platelet activation. In the presence of stable bubbles (those that do not collapse violently and instead oscillate for several cycles around their resonance size), a pressure amplitude of 10 MPa and low average ultrasound intensity of $I_{SATA} = 0.8$ W cm^{-2} can cause platelet disruption (Miller et al. 1979). Although erythrocytes seem to be more resistant than platelets to ultrasound damage, hemolysis has been reported when inertial cavitation occurs (Rooney 1970; Williams et al. 1970; Wong and Watmough 1980). Williams and Miller (1980) suggest that adenosine-5′-triphosphate may be released at lower intensities when inertial cavitation occurs.

Because of the continual filtration of impurities in whole blood *in vivo*, the probability of cavitation nuclei is reduced, and therefore, under normal conditions, cavitation is unlikely to occur (Health Protection Agency 2010). However, Brayman et al. (1996) reported that cavitation may occur at sufficiently high pressures. Damage to blood components *in vivo* has not been clearly illustrated (Dalecki et al. 1997a, 1997b, 1997c, 1997d, 1997e; Deng et al. 1996; Poliachik et al. 1999; Williams et al. 1977). In experiments by Dalecki et al. (1997a, 1997b, 1997c, 1997d, 1997e), a clinically insignificant level of hemolysis (0.46%) was detected in mice exposed to ultrasound through the chest wall at a frequency of 2.35 MHz and a peak positive pressure amplitude of 10 MPa.

PRENATAL EXPOSURE TO ULTRASOUND

At lower levels of ultrasound, such as for diagnostic purposes, ultrasound does not induce cavitation in the absence of pre-existing gas bubbles and does not generally cause heating beyond the normal physiologic range (Health Protection Agency 2010). Prenatal exposure to a diagnostic level of ultrasound produces changes in neuronal migration in the developing brains of mice (Ang et al. 2006). Significant concerns in the study of side effects of ultrasound in humans are related to *in utero* exposure to diagnostic ultrasound. Based on available evidence, there are no effects on perinatal mortality and childhood malignancies; however, some observational studies have found an increased prevalence of non-right-handedness in males with prenatal ultrasound exposure (Health Protection Agency 2010). This might reflect confounding effects rather than causation; however, random comparisons of individuals who had prenatal ultrasound exposure with those who did not provides weak evidence for an ultrasound effect on non-right-handedness (Health Protection Agency 2010). At high levels of exposure, ultrasound is capable of causing permanent damage to biological tissue as a result of heating, acoustic cavitation and radiation force (Health Protection Agency 2010), which can disturb the development of an embryo or fetus (*i.e.,* teratogenic effects).

In work by Williams et al. (1978), therapeutic ultrasound was found to decrease the recalcification time of anticoagulated whole blood *in vitro*. Therapeutic ultrasound has also been found to be capable of inducing platelet aggregation and releasing the platelet-specific protein β-thromboglobulin (β-TG), indicating that the platelet is the probable site of interaction and damage (Williams et al. 1978). Their study also suggested that ultrasound interacts with blood platelets (the exceptionally fragile cells that play an important role in the early stages of clot formation) possibly *via* the occurrence of cavitation processes at megahertz frequency levels. Furthermore, their *in vivo* study reported the production of platelet thrombi and true clots within the intact vascular system of mice as a result of acoustic microstreaming, similar to that developed around oscillating gas bubbles (Williams et al. 1978).

Concerns regarding the effect of ultrasound on fetal and embryonic development (teratogenic effects) have generated a large number of studies using different animal species and various exposure conditions. The probability of adverse effects of ultrasound on fetal and embryonic development has been reviewed by Ziskin and Barnett (2001), Miller et al. (2002) and Church and Miller (2007). Non-thermal interactions of pulsed ultrasound, especially cavitational mechanisms in the presence of an ultrasound contrast agent, may have adverse effects on the integrity of maternal and developing tissue. However, there is a low possibility of occurrence of these interactions in the fetus (Abramowicz 2005; Sienkiewicz 2007). In addition, the teratogenic effects of heat as a result of probable localized hyperthermia during pregnancy ultrasound scans have been extensively reviewed by Miller and Ziskin (1989), Miller et al. (2002) and Edwards et al. (2003). High maternal or fetal temperatures can have adverse effects on many developing tissues, particularly the brain and nervous system (Miller et al. 2002).

The teratogenic effect of heat on mammals is well accepted, and among all organs and tissues, developing central nervous systems manifest the greatest sensitivity (Health Protection Agency 2010). Miller and Ziskin (1989), Miller et al. (2002) and Edwards et al. (2003) conducted animal studies on the possible pathogenic mechanisms and thermal effects on the embryo and fetus. Embryo death, growth retardation, internal and external abnormalities, developmental deficits and behavioral changes that persist into adulthood are some of consequences of fetal hyperthermia (Edwards et al. 2003). The occurrence of these thermal effects depends on three main parameters: the grade of normal core temperature promotion, the duration of the elevation in temperature and the specific phase of development (pre-implantation, organogenesis or fetal period) in which the heating occurred (Health Protection Agency 2010). Edwards et al. (2003) reported that the sensitivity of the embryo and fetus to heat changes considerably during development and depends on the particular cell cycle, for example, cell proliferation, differentiation, or migration. They also described the teratogenic or biological effects of heat at different steps of development. Higher temperatures for a shorter time may raise the risk of a certain defect; the best estimate by Miller et al. (2002) is a threshold of 1.5°C to 2.5°C above normal body temperature for approximately an hour in pregnant animals, acknowledging that each type of defect and species has its own temperature threshold.

In addition to the thermal effect of ultrasound, pulsed and continuous-wave ultrasound can affect reproduction and prenatal development of the embryo and fetus (Jensh and Brent 1999; Sikov 1986). Notable ultrasound effects such as increased malformation rates and weight changes have been observed in some studies, whereas others do not report any firm exposure-related effects in either dam or child (Brown et al. 2004; Carnes et al. 1991; Devi et al. 1995; Fisher et al. 1994, 1996; Gu et al. 2002; Hande and Devi 1992, 1995; Jia et al. 2005; Karagöz et al. 2007; Norton et al. 1991; Oh et al. 1999; Ryo et al. 2001; Tarantal et al. 1993). These studies used different endpoints, pregnancy ages, species and ultrasonic exposure conditions, making direct comparisons of results problematic. No considerable treatment-related effects were observed on reproductive outcome or maternal weight during gestation, on viability or weight of offspring or on the morbidity of skeletal or visceral malformations when rats were exposed to 3-MHz continuous wave (Vorhees et al. 1991, 1994) or pulsed (Fisher et al. 1994, 1996) ultrasound up to 30 W cm^{-2} (I_{SPTA}). Statistically considerable decreases in weight of offspring were observed after frequent exposure of cynomolgus macaques to ultrasound (at 7.5 MHz and I_{SATA} = 0.28–12 mW cm^{-2}) (Tarantal and Hendrickx 1989a, 1989b; Tarantal et al. 1993); this effect occurred during the first 3 mo of life and not the subsequent 9 mo.

In a study in which contrast-enhanced ultrasound imaging and Doppler were used to quantitatively monitor uteroplacental perfusion in rat pregnancies, Arthuis et al. (2013) detected no microbubbles in the umbilical vein or fetal components. The absence of contrast agents in the fetal compartment would suggest the innocuity of contrast-enhanced ultrasound imaging on fetal development (Arthuis et al. 2013). Overall, there may be no real tolerability concerns with respect to common clinical use of sonography; however, caution must be exercised when high-output regimes such as pulsed Doppler are applied in obstetrics (ter Haar 2010). Considering the difficulty in establishing the thresholds for biological effects, it is suggested that as-low-as-reasonably-possible scanner outputs be used to collect the required diagnostic information. Unnecessary examinations (non-medical sonography) during pregnancy are not advised because of the large number of remaining unknowns (ter Haar 2010).

ULTRASOUND EFFECT ON CELLS

To study the interaction of ultrasound with biological systems, experimental studies with cells and animals have been performed under a variety of exposure conditions. Studies that describe ultrasonically induced biological changes in cells *in vivo* and *in vitro* have been reviewed by Feril and Kondo (2004a, 2004b), Miller (2007), ter Haar (2007), the National Council on Radiation Protection and Measurements (Nyborg et al. 2002) and the American Institute of Ultrasound in Medicine (Holland 2000). The most important representative examples of potential adverse effects on cells are cell

lysis, changes in cell division capability, ultrastructural changes, chromosomal and cytogenetic effects and functional changes (Health Protection Agency 2010). The effects of ultrasound on cells fall into two categories: gross effects, such as lysis, effects on cell division capability and damage to cellular ultrastructure; and subtle effects, such as chromosomal changes, functional changes and altered growth patterns (Health Protection Agency 2010).

There is extensive and unequivocal evidence that exposure of cells in suspension to ultrasound leads to cell lysis (Health Protection Agency 2010). Several studies, including, for example, Kaufman et al. (1977), Morton et al. (1982), Hallow et al. (2006) and Lai et al. (2006), have found that cavitation is a major mechanism resulting in this sort of complete cellular disruption; however, it is not clear if ultrasound is able to produce lysis in the absence of cavitation effects (Health Protection Agency 2010). Apart from the ultrasound exposure conditions, the amount of cell lysis can depend on the concentration of cells in suspension (Brayman et al. 1996; Ellwart et al. 1988) as well as cell size (Brayman and Miller 1993; Nyborg and Miller 1982). Lysis appears to be an immediate consequence of ultrasound exposure effects on cells and may affect cells in mitosis more than other cell cycle stages (Clarke and Hill 1969). Colonogenic assays enable assessment of cell division capability after a specific insult. Based on studies performed by Bleaney et al. (1972) and Morton et al. (1982), cells that survive ultrasound exposure and stay intact will continue producing progeny in the same way as their untreated counterparts. However, there are exceptions for ultrasound-exposed cells that remain at elevated temperatures (Feril and Kondo 2004a, 2004b; ter Haar et al. 1980).

The interaction of ultrasound with the cell membrane has been of interest with respect to ultrasound-mediated drug delivery and sonoporation (de Jong et al. 2000; Escoffre et al. 2010; ter Haar 2007) and extraction of medicinal compounds from biological resources (Izadifar 2013). Change in permeability to ions is one of the usual changes that occurs in cells exposed to ultrasound. Research by Chapman (1974) indicated that acoustic exposure at 1.8 MHz and $I_{SATA} = 1$ W cm^{-2} *in vitro* resulted in sublethal alteration in the thymocyte plasma membrane, which leads to a decrease in potassium content. A reversible rise in calcium ion uptake in fibroblasts was observed by Mortimer and Dyson (1988) at an ultrasound frequency of 1 MHz and I_{SATA} of 0.5–1 W cm^{-2}.

Results of electron microscopy of cells and tissues after exposure to ultrasound reveal damage to a variety of subcellular organelles, primarily mitochondria, and damage to lysosomes with consequent release of lysosomal enzymes in tissue (Dvorak and Hrazdira 1966; Hrazdira 1971; Taylor and Pond 1972). In addition to the membrane and mitochondrial damage caused by cavitation, Harvey et al. (1975) observed dilated rough endoplasmic reticulum and some irregular lesions. Generally, the cell nucleus seems unaffected by ultrasound exposure; the only type of lesion observed is slit-like vacuoles at the nuclear membrane (Ter Haar et al. 1979). Watmough et al. (1977) hypothesized that ultrasound may produce cavitation microbubbles within cells and that nuclear, mitochondrial and granular endoplasmic reticulum membranes act as nucleation sites; thus, when these organelles are affected, damage might manifest as lesions next to the membrane.

Cytogenetic studies on the effect of ultrasound on chromosomes clearly indicate that high-intensity ultrasound can cause degradation of DNA in solution. Damage appears to be caused by hydrodynamic shear stresses, free radical formation or excessive heating resulting from cavitation (Miller and Thomas 1995a, 1995b, 1996; Thacker 1973). A large amount of evidence indicates that high-intensity ultrasound up to $I_{SATA} = 100$ W cm^{-2} does not produce chromosomal damage (European Federation of Societies for Ultrasound in Medicine and Biology 1994; Rott 1981). However, when exposure to ultrasound at an intensity of 3 W cm^{-2} and frequency of 810 kHz is followed by (not preceded by) X-irradiation to 1 Gy, some synergistic interactions may result in chromosomal aberrations (Kunze-Mühl 1981). Diagnostic ultrasound, even up to $I_{SATA} = 3.0$ W cm^{-2} (3.15 MHz), does not produce sister chromatid exchanges *in vitro* (Liebeskind et al. 1979). Whether ultrasound may result in chromosomal damage is not certain; however, the most carefully documented studies have returned negative results. It must also be considered that these were *in vitro* studies, and the interaction mechanism *in vitro* may be different from that in intact tissue *in vivo*.

Overall, ultrasound may cause epigenetic changes, such as modification of histone protein structure, that can have a long-term influence on gene expression (Health Protection Agency 2010). Ultrasound may also lead to stimulation of cellular functions that mostly involve interactions at the cell membrane level (Health Protection Agency 2010). Taylor and Newman (1972) reported that ultrasound exposure under treatment conditions of 1 MHz, $I_{SATA} = 10$ W cm^{-2}, pulses of 20 μs to 10 ms, for more than 2.5 min influenced the electrophoretic mobility of cells, which reflects a cell surface charge density change as a result of volume changes (Mummery 1978). The Time-lapse photomicrography of cellular movements *in vitro* revealed ultrasonically induced changes that may last for several generations of cells (Liebeskind et al. 1982); however, such results are far from clear *in vivo* (Health Protection Agency 2010).

To obtain a clearer indication of the biological effects of ultrasound, various studies focusing on biological systems such as bone, blood, vasculature and lung, as

well as effects on the fetus and embryo, have been conducted using small animal models.

LIMITATIONS AND CONSIDERATIONS OF CURRENT ULTRASOUND BIOLOGICAL STUDIES

The interaction of ultrasound with biological systems has been the subject of a considerable body of research. The potential biological changes associated with clinical applications of ultrasound, especially in obstetrics and gynecology, have been studied in a wide variety of *in vitro* and *in vivo* models and ultrasound exposure conditions. However, sufficient caution must be exercised when extrapolating *in vitro* results to *in vivo* conditions. For example, the mechanism of interaction of sound waves with cells *in vitro* (in the liquid environment), where cell cultures are in suspension in a nutrient liquid medium or attached to a coated surface, may be very different from that in the *in vivo* environment, which features more solid structures such as tissue and bone (Health Protection Agency 2010).

The effect of ultrasound in cell suspensions is also different from that in monolayers of cells attached to a surface (Health Protection Agency 2010). In the liquid environment, the most dominant physical mechanisms for biological effects of ultrasound are acoustic cavitation and streaming; substantial heating is unlikely because of the low acoustic absorption coefficient (Health Protection Agency 2010). When tissue is exposed to ultrasound *in vivo*, high acoustic absorption energy in tissue causing thermal effects becomes more important compared with *in vitro* systems, whereas the probability of cavitation occurring in tissue is less than that in liquid. However, the probability of cavitation occurring in intact tissue depends on temperature, the tissue state and gas content (Health Protection Agency 2010).

Acoustic exposure conditions, as well as the mode of energy deposition, are also very important for studying the biological effects of ultrasound. Two acoustic exposure conditions with the same exposure time and energy but different modes of energy deposition (one in continuous mode and the other in short pulses at a low repetition rate) may result in very different effects in tissue. Cavitation activity and its associated characteristic cell damage are more probable with short pulses at a low repetition rate, whereas thermal effects are more likely in continuous mode (Health Protection Agency 2010). Therefore, in diagnostic ultrasound that features short exposures and relatively low temporal average intensities, thermal effects may not be very important in soft tissue; heating is more likely in tone burst and continuous exposure in therapeutic applications. However, high-pressure amplitude with even short pulse mode exposure, such as used in lithotripsy, may promote acoustic cavitation in liquid media (Health Protection Agency 2010).

Passive cavitation detection has been used in several studies of ultrasound ablation (Coussios et al. 2007; Farny et al. 2009; Hoerig et al. 2014; Li et al. 2014; Mast et al. 2008; McLaughlan et al. 2010) and lithotripsy-induced cavitation detection (Cleveland et al. 2000). Measurements using passive cavitation detection in both human and pig have revealed the presence of bubbles in the perirenal fat, the collecting system, the parenchyma and subcapsular hematomas. The onset of detectable cavitation in the parenchyma of a pig model required delivery of approximately 1000 shock waves with a Dornier HM3 lithotripter (Cleveland and James 2007). In addition to acoustic exposure conditions, the maturity of the individuals being exposed is an important consideration, as biological damage to a few cells of the developing embryo is much more significant than that to a small volume of adult cells. The acoustic properties of the early embryo are very similar to those of water. Therefore, bulk heating effects may be unimportant in the early embryo, whereas heating at the bone surface may occur in a third trimester fetus in which bone mineralization has occurred (Health Protection Agency 2010).

DISCUSSION AND CONCLUSIONS

Ultrasound interacts with tissue through both thermal and non-thermal mechanisms (mostly attributed to cavitation and radiation force) and generates a variety of biological effects at the cellular or intact tissue level (structural or functional changes). Three main factors can result in bio-effects caused by ultrasound: heating, radiation pressure and the presence of gas (ter Haar 2010). The ultrasound beam's energy and frequency, as well as the properties of the medium through which the ultrasound beam passes, play important roles in the biological effects. Heating is related mostly to absorption of ultrasound energy by tissue. The mechanical effects that arise from cavitation are related primarily to bubbles created during the rarefactional cycle of acoustic pressure; the presence of gas in the solution that turns into microbubbles *via* the negative pressure of ultrasound; naturally gaseous body tissues, such as lung alveoli and intestines; or introduction of stabilized gas-filled microbubbles (ultrasound contrast agent) into the bloodstream by extravasation.

Evidence from cellular and animal studies has indicated that high-power devices used in therapeutic and surgical applications, the purpose of which is to deliver high-intensity ultrasound to a target tissue, can clearly cause potential biological effects in the body. However, these biological effects have brought about unique opportunities for non-invasive ultrasound therapy; apart from

tolerability, the main concerns relate to accurate targeting of ultrasound in the desired target volume without damaging other tissues.

At lower levels of exposure, such as diagnostic ultrasound, there is no established evidence of any specific harmful effect; however, too few research data are available to draw firm conclusions, especially with respect to the long-term use of ultrasound. The subtle effects of diagnostic ultrasound, such as neuronal migration or changes in membrane permeability, are not completely understood.

Although the application of ultrasound in fetal imaging has evolved beyond medical practice to commercial souvenir scans, detailed 3-D facial imaging and recording of the baby's movement in the womb *via* 4-D sonography (in which the image is continuously updated with time and becomes like a movie), which require prolonged and more intense ultrasound exposure, there are unconfirmed indications from the biological and epidemiologic literature of possible neurologic effects on *in utero* ultrasound exposure. Therefore, diagnostic ultrasound should be used wisely, especially with respect to newer equipment, which can have higher acoustic output levels than earlier models. Furthermore, for continued safety, there is a great need to further study the long-term hazards of exposure, especially *in utero* exposure, to diagnostic ultrasound. In addition, a better understanding of the direct physical effect of ultrasound (acoustic cavitation) is required to determine the cause of any biological effects in humans (*e.g.,* trauma to lung or intestines, capability to improve healing of bone and soft tissue, platelet damage caused by cavitation) as well as improve the quality of ultrasound applications in many clinical practices (*e.g.,* drug delivery, tumor ablation).

A literature review by ter Haar (2010) presented from the viewpoint of diagnostic safety revealed that most bio-effects from clinically relevant practices arise from short, high-amplitude ultrasonic pulses at high repetition rates. However, until recently, most studies have focused on continuous-wave (long-tone-burst) exposures. Furthermore, most *in vitro* studies have investigated the effect of ultrasound on either suspended culture cells or monolayers, yet the way ultrasound interacts with cells in an aqueous environment is different from the way it interacts with intact tissues (ter Haar 2010). Because thermal effects can be better observed through energy absorption of tissue, cultured cells may not properly reflect thermal effects and instead may more manifest more cavitation effects. When an *in vivo* study is performed, the body's physiologic response can be studied. Also, the size of the animal (and, consequently, the attenuation of the beam in its body) in most *in vivo* studies is much smaller than in a human. In small animal studies, it is usual for most of the body to be exposed to the ultrasound beam (ter Haar 2010). Therefore, having the more appropriate model size and measuring the attenuation caused by the intervening tissue can ensure the findings are relevant with respect to effects in humans. Although ultrasound bio-effects reported in animal models occur under conditions similar to those used in humans, we cannot confidently relate these results to humans. The absence of human studies on the bi-effects of diagnostic ultrasound does not necessarily mean there are no effects, but, rather, there is a lack of techniques to detect them. Cavitation thresholds are usually determined experimentally through acoustic emission, broadband noise and subharmonic signals from bubbles. Because of the attenuation of signals in tissue, detection of signals arising from deep in tissue is more difficult than detection of those arising from the surface. This makes the detection of cavitation in tissue very difficult. Detection and visualization of cavitation responses in the body under different operating conditions would be very helpful in threshold determination, as well as characterization and analysis of cavitation in the human body.

A complete understanding of the interaction of ultrasound with ultrasound contrast agents is required to develop the full potential of ultrasound contrast agents in biomedical ultrasound applications such as drug/gene delivery, tumor therapy and arteriogenesis. More information regarding the location of microbubbles in the body, as well the area where microbubbles are in contact with tissue and cells are subjected to ultrasound exposure, is necessary.

Further advances in the use of ultrasound in medicine require better knowledge of the cellular and molecular events interfering with physical mechanisms of ultrasound combined with their related biological effects. In addition, more effort is required to visualize, detect and monitor ultrasound-induced cavitation bubbles combined with ultrasound contrast agents deep inside the body to have more control over the biological effects of ultrasound.

Acknowledgments—This work was supported in part by a Natural Sciences and Engineering Research Council (NSERC) Discovery grant, the Canada Research Chairs program and the University of Saskatchewan.

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