THE USE OF ULTRASOUND TO FACILITATE THE DELIVERY OF GENETIC-BASED THERAPEUTIC STRATEGIES

BY

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The Use of Ultrasound to Facilitate the Delivery

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ABSTRACT

Gene therapy is the introduction of exogenous genes to a cell to "fool" the cell into producing the coding protein. There are ongoing studies in cancer-killing gene therapies, kidney cell regeneration and many genetic disorders such as cystic fibrosis and severe combined immunodeficiency (SCID). However, it has been discovered that developing the therapy is not enough. A method to deliver the drugs is equally important.

The objective of this work was to explore two potential uses of ultrasound for gene delivery. The first is to determine if ultrasound-induced cavitation can be used to enhance *in vivo* transfection of gene therapy. Second, to use non-invasive ultrasonic heating to spatially control gene therapy expression *in vivo* after delivery.

In the first phase of this project, feasibility experiments were performed to show an increase in transfection efficiency occurred when using microbubbles, ultrasound, plasmid and genes compared to plasmid and genes alone. An ultrasound transducer was then designed to optimize experimental conditions for invasive in vivo experiments taking into consideration optimal transducer size, focal distance, and frequency. When testing the transducer, a potential side effect to using ultrasound to increase transfection was discovered.

In the second phase of exploration, the use of MRI-monitored ultrasound to induce enhanced expression of luciferase after local injection of the gene construct Ad-HSP-Luc was explored. Using a construct that includes an hsp promoter allows the activation of the associated transgene only in areas that are subsequently heated after injection. Increased luciferase expression was observed in areas that were exposed to ultrasonic heating.

It is hoped that the positive results of these preliminary experiments will eventually lead to a clinical methodology where ultrasound-enhanced gene therapy transfection would be followed with ultrasound-directed spatial control of gene expression.

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Finally, I dedicate this thesis to my mother and father who believed in me every step of the way through my years of education and were the first people to teach me to question the world. Thank you.

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1. INTRODUCTION TO GENE THERAPY

1.1. WHAT IS GENE THERAPY?

Gene therapy is the introduction of exogenous DNA into a cell that then causes the cell to produce a therapeutically beneficial protein. Although initially gene therapy was thought best suited to cure inherited genetic diseases, technical difficulties such as targeting specific cell types *in vivo*, regulating expression of the therapeutic genes, and controlling vector immunogenicity (Nabel 2003) has shifted research focus to acquired diseases such as cancer, cardiovascular disease and HIV. GeMCRIS (http://www.gemcris.od.nih.gov), the NIH database listing all ongoing clinical trials involving gene therapy, lists 442 of the 1005 current trials as cancer-related while ongoing gene therapy studies in the cardiovascular field explore clinical problems such as prevention of restenosis after angioplasty or stent implantation, prevention of venous graft failures, and therapeutic angiogenesis (Gruchala et al. 2004).

1.2. HOW GENE THERAPY WORKS

1.2.1. DNA INCORPORATION

For gene therapy to be effective, the gene must be delivered past both the cell and nuclear membranes into the cell nucleus. Once in the nucleus, the DNA segment can be incorporated in one of three ways: incorporating randomly into the cell DNA, cutting out and replacing a defective segment of the cell DNA, or remaining separate in the nuclear material and using various enhancers and promoters to encourage translation by the host cell messenger RNA. Incorporation into the cell DNA is essential for stable expression of the therapeutic gene, a requirement in long-term treatment of inherited genetic disorders. If the therapeutic gene remains separate from

the host DNA, expression is lost over time. This property can be used to advantage in the treatment of many acquired diseases, for example when using a "suicide gene" to treat malignant tumors (Colosimo et al. 2000).

1.2.2. IN VIVO VERSUS IN VITRO

Gene therapy can be delivered to target cells by both *in vitro* and *in vivo* methods. There are advantages and disadvantages to each method (Culver 1996). *In vitro* delivery requires obtaining cells from the patient, delivering the gene therapy, and implanting the cells back into the patient. This has the advantages of both high efficiency, defined as the ratio of gene expression to DNA inputted, and selective delivery of the therapeutic gene to the cell types desired to be transfected. The significant limitation to the *in vitro* method is that the target cells must be removable from the body. This generally limits *in vitro* gene delivery to hematopoietic, endothelial, and tumor cells. In contrast, *in vivo* delivery does not require cells to be removed from the body, but the delivery efficiency is generally much lower and the methods developed to increase this efficiency have additional side effects. In addition, selectively transfecting only certain cell types *in vivo* is challenging.

1.2.3. VIRAL VECTORS

Vectors were created to increase the efficiency of *in vivo* gene therapy. Modified viruses are the most commonly used and most effective vectors. Viral vectors are classified into categories based on the original viral type, for example retroviruses, adenoviruses and adeno-associated viruses.

Retroviruses, such Moloney Murine Leukemia Virus (MMLV), were the first viruses modified into viral vectors (Guild et al. 1988; Miller 1992; Miller et al. 1993; Riviere et al. 1995; Robbins et al. 1994) and are still the most common delivery vector used (McTaggart and Al-Rubeai 2002; Andreadis et al. 1999). To make a viral vector the virus is first disabled of its disease function and made incapable of replication. The therapeutic gene desired for transfer is then grafted onto the stripped virus. Retroviral vectors retain the ability to reverse transcribe into the target cells' DNA, thus integrating into the host DNA and allowing the therapeutic gene to divide with the cell (McTaggart and Al-Rubeai 2002). This property can be advantageously exploited when treating disease states that require lifelong therapeutic effects but render retroviruses inappropriate for disease states that only need temporary therapy. Another disadvantage of using the majority of retroviral vectors is that only retroviral vectors derived from the lentivirus subgroup can infect cells that divide slowly or that are quiescent (Lewis and Emerman 1994; Federico 1999). The best-characterized lentivirus for vector delivery is the HIV virus but patient acceptability may limit its clinical use. Other concerns with retroviral vectors involve long-term side effects as well as fears that the virus may mutate back into a replication-capable form or integrate into an oncogene site and promote cancerous growth. This last fear has heightened under the recent discovery that several children treated with a retrovirus-delivered gene therapy agent have developed a leukemia-like disease (Hacein et al. 2003). Because of this, all human clinical studies involving gene therapy delivered with a retrovirus in the US were halted early in 2003 (Stolberg 2002) and are currently being reviewed on a case-by-case basis (Fox 2003). However, research has

recently suggested that the adverse result in this case was due to an unlikely coincidence particular to the type of promoter/gene sequence inserted and the location of insertion (Dave et al. 2004; McCormack and Rabbitts 2004) and should not occur with other therapeutic genes.

Adenoviruses are a class of viruses most commonly associated with causing upper respiratory infections but can also cause pharyngitis, conjunctivitis, gastroenteritis and pneumonia. Unlike retroviruses, these vectors do not integrate into the host cell DNA, which causes the therapeutic function to be lost over time. Adenoviruses have the ability to transfect a wide variety of cell types and can transfect non-dividing as well dividing cell types. The smaller size of adenoviral vectors allow delivery in higher titer volumes than retroviruses (10^{12} to 10^{13} viral particles/mL) (Volpers and Kochanek 2004) and efficiency is high meaning large amounts of therapeutic protein can be produced from a single injection. The biggest concern in using adenoviruses is the danger demonstrated by the highly publicized death of Jesse Gelsinger in September 1999, which was caused by an immune response to administration of the adenoviral vector. Adenoviral vectors express low levels of viral antigens following infection. This often stimulates an immune response to the infected cells and results in a loss of therapeutic gene expression 1–2 weeks after injection (Yang and Wilson 1995; Yang et al. 1996). In the Gelsinger case, the amount of virus given to him caused such an immense and immediate immune reaction that several of his organs went into failure. Strict control over dosage and concurrent immunosuppressive therapy can be used to control this side effect but may still ultimately limit adenoviral

vector usefulness. So-called "second generation" and "gutless" adenoviruses are currently in development to help solve these problems (Volpers and Kochanek 2004).

The adeno-associated virus (AAV) is not technically a virus but rather a symbiotic organism found with adenoviruses. Because AAVs are not known to cause any diseases in humans and no immune response is caused by the introduction of AAVs, these vectors are considered very safe. The disadvantages of using AAV vectors are mostly practical: the small size of the vector restricts the size of the therapeutic gene that can be attached to less then 5.2kB and these vectors are very expensive to produce. Also, recent research has found that AAV vectors integrate preferentially into the gene-rich areas of the host chromosomes (Nakai et al. 2003). This means that AAV vectors could potentially share some of the risks now being associated with retroviruses, but since AAV vectors only integrate into cell chromosomes rarely, the risk is much reduced.

As a means of incorporating the benefits of a range of viral vectors, chimeric vectors are also under development. These vectors try to combine the desirable features of two or more virus types to eliminate the undesirable effects (Robbins and Ghivizzani 1998; Caplen et al. 1999; Duisit et al. 1999).

1.2.4. NON-VIRAL VECTORS

Due to ongoing concerns about the safety of viral vectors, non-viral vectors have also been explored: liposome transport, naked DNA injection, electroporation and ultrasound among others. In the 1990s it was shown that naked plasmid DNA

injection can induce gene expression in skeletal muscle (Wolff et al. 1990), liver (Hickman et al. 1994), thyroid (Sikes et al. 1994), heart muscle (Ardehali et al. 1995), brain (Schwartz et al. 1996), and urological organs (Yoo et al. 1999). Although naked DNA injection usually does not cause integration into the chromosome, expression can persist for several months (Acsadi et al. 1991; Manthorpe et al. 1993). However, transfection efficiencies are much lower than with viral vector. By transporting genes on liposomes, the negative charge of the DNA is decreased, facilitating interactions with cell membranes and increasing transfection efficiency (although not up to viral vector levels). Liposomes also protect the DNA from damage, cause no immunogenic response and can be designed to target specific cells and tissues. Electroporation, the application of short and intense DC electric pulses that can reversibly increase cell membrane permeability (Mir et al. 1988), is an effective tool for delivering gene therapy *in vitro* but is limited *in vivo*. Ultrasound, however, has the advantage of being able to be used in conjunction with other vectors and can also direct the gene therapy to target tissues/cells.

2. INTRODUCTION TO ULTRASOUND

2.1. DEFINING ULTRASOUND

Sound is a form of mechanical vibrational energy; it moves and propagates by vibrating particles into compressions and expansions, resulting in a mechanical wave. By definition any sound wave over 20,000 Hz (the upper range of human hearing) is considered ultrasound. Application of ultrasound has been known to bring about changes in tissue structure and biological processes given high enough intensities since the late 1920s (Wood 1927; Harvey 1928). Early investigations suggested the

possibility of ultrasound energy inducing a therapeutic effect in biological tissue (Horvath 1944) by mechanisms that are now divided generally into thermal (Barnett 1992) and non-thermal/mechanical effects (Dunn 1978; Barnett et al. 1994; Barnett et al. 1997). Research into the application of ultrasound has been conducted for such varied disciplines such as neurosurgery (Fry et al. 1954), ophthalmology (Lizzi et al. 1978), urology (Gelet et al. 1993; Foster et al. 1993), oncology (Chapelon et al. 1992; Yang et al. 1991; Prat et al. 1995) and physiopathology (Delon-Martin et al. 1995; Vaezy et al. 1997; Vaezy et al. 1999). Clinical treatments have targeted the eye (Coleman et al. 1985), breast (Hynynen et al. 1996; Hynynen et al. 2001), kidney (Vallancien et al. 1993), liver (ter Haar et al. 1998a), bladder (Vallancien et al. 1996), and prostate (Bihrle 1985; Vallancien et al. 1992; Gelet et al. 1993; Madersbacher et al. 1993; Foster et al. 1994; Sanghvi et al. 1996; Nakamura et al. 1997; Mulligan et al. 1997).

2.2. BIOEFFECTS OF ULTRASOUND

When tissue temperature is elevated (especially when induced artificially), the condition is called hyperthermia. Ultrasound is able to induce hyperthermia because the longitudinal propagating sound waves cause any particles in their path to begin to oscillate. These oscillations cause density changes in the medium thru which the sound is traveling. The medium resists the density change and energy is absorbed into the medium in the form of heat. Different mediums are able resist this particle oscillation to different degrees, which is quantified in a parameter called the acoustical impedance. Acoustical impedance quantified as the product of sound velocity and density of the material. As ultrasound travels from one material to

another, the relative transmission and reflection at the interface are governed in part by the acoustic impedances of the materials on each side of the interface. A large difference in acoustic impedance will cause a greater fraction of the ultrasound to reflect back into the material from which it came.

Recently man-made bubbles have been developed for bolus use as imaging contrast agents. If ultrasonic sound waves hit bubbles at sufficient energy and frequency, bubble diameter increases and decreases in a fashion inversely proportional to the acoustic pressure, a process known as cavitation. This process absorbs more acoustic energy than normal passage through tissues and can damage cells due to shear stresses caused by microstreaming around the bubble. The resonance size of the oscillating bubbles decreases with increasing frequency; at 1 MHz the resonance size of an air bubble is 3.5μm in free fluid. Smaller bubbles will grow to resonance size by rectified diffusion. When acoustic intensity is increased, inertial cavitation rather than stable cavitation occurs. In the transient state, bubbles expand and then rapidly contract and implode. This causes local pressures exceeding thousands of atmospheres, temperatures in the thousands Kelvin and the formation of free radicals (Flynn 1982). The pressure and temperature within the collapsing bubble hydrolyze water vapor within the bubble, producing free radicals (Makino et al. 1983). Bubble collapse also generates shock waves and shear forces within the medium that may cause high velocity fluid jets capable of puncturing surrounding structures (Blake et al. 1986; Zhang et al. 1993; Zhang and Duncan 1994; Sato et al. 1994; Krasovitski and Kimmel 2001; Chahine 1982; Vogel et al. 1989; Kodama and Takayama 1998).

2.3. MODES OF ULTRASOUND

Ultrasound is created using piezoelectric materials that convert (or transduce) electrical energy into vibration. In the 1880s, Jacques and Pierre Currie found that this is a result of the anisotropic structure of the material, because the lattice structure that makes up piezoelectric material does not have a center of electrical symmetry. When an electric charge is introduced across the crystal the particles expand or contract (depending on the charge configuration) until elastic forces between the particles counteract the electric forces (Mason 1950; Richardson 1962).

Therapeutic ultrasound is commonly delivered in three different modalities: shock wave, continuous wave and pulsed continuous wave. The modalities are distinguished by the different electrical signals through an ultrasonic transducer that produces them as well as the bioeffects resulting from treatment.

Sending a voltage spike into an ultrasonic transducer produces a shockwave. These signals are generally of very short duration (~5μs) and high amplitude. Shockwaves are most often used clinically in lithotripsy of kidney stones and gallstones and stimulation of bone growth and tendon healing. The mechanism at work in these therapies is widely believed to be cavitation as the pulses are too short for any significant heating to occur.

Sending only a single frequency electrical input through an ultrasonic transducer produces continuous wave ultrasound. CW ultrasound is both historically and most often used clinically for various types of hyperthermia treatments. Pulsing continuous

wave ultrasound, which separates segments of continuous wave ultrasound with periods of time where no signal is inputted, can be used to cause cavitation with minimal heating.

2.4. ULTRASONIC CONTRAST AGENT

Ultrasonic contrast agents (USCAs) were first developed in the 1960s, but only in the last decade have they been available for use in humans. Most commonly, USCAs are microbubbles filled with gas such as air or perfluorocarbon stabilized by external shells made of galactose, albumin, lipids, or polymers. These shells slow the degradation of microbubbles in the bloodstream, thus allowing contrast agents to be injected systemically. Correas *et al.* in their 2001 review of ultrasonic contrast agents defined USCAs as "an exogenous substance...administered, either in the blood pool or in a cavity, to enhance ultrasonic signals." How the signals are enhanced depends on local acoustic power (Powers 1997). Depending on the output power of the ultrasound system, the ultrasonic frequency and the attenuation of the ultrasonic signal, USCAs act as cavitation nuclei for either stable or inertial/transient cavitation.

This ability to cause cavitation events may be why multiple studies have shown that several brands of USCAs enhance gene therapy uptake both *in vitro* and *in vivo*. Li *et al.* (2003) performed an *in vivo* study in rat muscles comparing the gene efficiency increase using ultrasound and each of the three commercially available USCAs: Optison (human albumin; Nycomed-Amersham, Oslo, Norway), Levovist (SH U 508A; Schering, Berlin, Germany), and Albunex (Molecular Biosystems, San Diego, CA). They found Optison, which is filled with perfluorocarbon and covered with a

shell made of human albumin, increased gene expression more than its air-filled competitors. They hypothesized this may be because Optison is more stable in the bloodstream so there may be a higher bubble concentration at the cavitation site during sonication. The next section of this paper covers the history of ultrasound-mediated gene transfer (USMGT) in more detail.

3. COMBINING GENE THERAPY AND ULTRASOUND

3.1. CAVITATION EXPERIMENTS

Fechheimer *et al* in 1987 was the first to use ultrasound to transfect mammalian cells with plasmid DNA using a method called sonication loading. The mechanism by which the procedure worked was not explored in the article, although the process was described as "gentle" to the cells. Wasan *et al.* used ultrasound in conjunction with gene therapy in 1996 when it was observed that liposomes carrying DNA had a tendency to form large complexes (Wasan et al. 1996). Ultrasound was used to break up these complexes into uniform size. During this experiment, it was shown that the liposomes protect the plasmid DNA from destruction by ultrasound-induced cavitations.

Also in 1996, Kim *et al* compared 1 and 3.5 MHz continuous wave ultrasound against pulsing wave ultrasound in vitro on primary fibroblasts, using variable sonication times and pressures. By using β-galactose to calculate transient expression and pMClneo poly(A) to calculate stable expression, they found the most efficient stable transfection rates using 30 sec, 4 MPa continuous wave. With those settings a transfection rate of 0.34% of the surviving cells was measured. Transient transfection

rates were maximized to 2.4% using 60 sec, 3 MPa continuous wave sonications. In both instances the survival rate for the cells was 50% (Kim et al. 1996). The authors believed the mechanism of increased transfection was stable cavitation.

Greenleaf *et al.* first used cavitation bubbles in 1998 to increase transfection. A linear relationship was found between transfection efficiency and average peak ultrasound pressure (threshold 0.12MPa) with the peak transfection rate being 50%. They hypothesized that bubbles violently collapse (transient cavitation) in first second of sonication and permeabilize the cell.

Shockwave versus pulsed continuous wave sonications were compared by Huber *et al* (1999) in HeLa cells using β-galactosidase and luciferase plasmid DNA reporter. The various modalities used were 60-360 shockwave pulses at 1Hz at magnitudes of 13, 16 and 19 kV, and 1.18MHz pulsed continuous wave with a 40% duty cycle and pulse frequencies ranging from 1 to 500Hz. The optimal shockwave modality was shown to have 0.08% efficiency (8 times greater than DNA alone) and 5% cell viability while sinusoidal sonications were shown to have 3% efficiency (80 times greater than DNA alone) with 45% cell viability.

Studies using ultrasound to facilitate gene therapy delivery *in vivo* have also been performed. MR-guided focused ultrasound was used to direct delivery of plasmid DNA into the thigh of an anesthetized rabbit (Bednarski et al. 1997). The experiment showed a high level of transfection in the muscle parenchyma of the sonicated rabbit

thigh and a much lower level in the control animal's muscle, although these results may be partially explained by the experimental setup and the known ability of ultrasound to disrupt endothelial barriers. Most other *in vivo* studies have involved the cardiovascular system, either myocardium or vasculature. Taniyama et al. reported that using ultrasound and microbubbles with naked plasmid wild type p53 gene injection significantly decreased restenosis compared to controls in blood vessels after balloon angioplasty injury (Taniyama et al. 2002b). Ultrasound was also used to increase angiogenic gene expression delivered with naked DNA injection in ischemic pig myocardium, although in this case no microbubbles were used (Schratzberger 2001).

But the big question still remains, how does ultrasound work with contrast agent to increase uptake of gene therapy drugs? Taniyama et al. recently showed that plasmid DNA could be delivered in clinically significant doses using ultrasound and the contrast agent Optison with no apparent toxicity and speculated on the mechanism of increased gene therapy transfection efficiency (Taniyama et al. 2002a). They believe the ultrasound causes transient holes in the cell membrane. Another mechanism that has been brought forth for this increased transfection rate is that the experimental conditions are causing a type of cavitation where the microbubbles begin to vibrate, getting bigger and smaller, instead of exploding. The vibration action causes reversible mechanical stress on the cells, facilitating diffusion of the gene therapy into the cells. Although these studies clearly demonstrate the feasibility of enhancing transfection efficiency using ultrasound, there is little information about the local ultrasound

conditions (frequency, power density, etc), and little understanding of the physical mechanism(s) responsible for enhanced efficiency.

3.2. HEAT SHOCK PROTEIN EXPERIMENTS

Ultrasound has also been explored as a non-invasive heat source to use for activation of a gene therapy/heat shock protein promoter complex. It has been suggested that heat shock proteins (hsp) promoters could be used to spatially and temporally control gene therapy delivery (Madio et al. 1998; Arai et al. 1999; Brade et al. 2000; Braiden et al. 2000; Huang et al. 2000; Lohr et al. 2000; Vekris et al. 2000; Borrelli et al. 2001). Heat shock proteins, also called *stress proteins*, are a group of proteins that are expressed when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. This induction is controlled by the activation of the gene promoter by mechanisms that are not completely understood. The well-characterized hsp70B promoter has been shown to be activated mainly through heating and is useful due to the magnitude of difference between induced protein expression versus the basal expression. Previous work using hsp promoter gene therapy constructs has shown that *in vitro* gene expression increased linearly with heating duration after reaching a threshold temperature (Smith et al. 2002). Borrelli *et al.* (2001) also demonstrated significant induction *in vitro* after exposure to temperatures greater than 40°C and the magnitude of the induction increased from 41 to 43°C and varied with the duration of heating. Others have obtained similar results (Brade et al. 2000; Gerner et al. 2000; Vekris et al. 2000) *in vitro* using higher temperatures, up to 48°C, with reduced exposure times of 30 to 180 seconds.

This methodology could be especially useful in delivering cytotoxic genes to malignant tumors, where concern about gene expression in non-targeted areas is paramount. Hsp70B-mediated gene therapy has been used *in vivo* for the expression of a cytotoxic transgene to reduce tumor size in animal models of melanoma, breast cancer, and glioma (Braiden et al. 2000; Huang et al. 2000; Lohr et al. 2000; Vekris et al. 2000). Smith *et al.* (2002) have shown the utility of this system for targeted induction using an adenovirus containing the cytotoxic Fas ligand gene under the control of the hsp70B promoter. In that study the systemic delivery of the Fas ligand adenoviral construct did not lead to liver toxicity unless the animals were exposed to ultrasound-mediated hyperthermia. Ultrasound-induced hyperthermia is an optimal choice for inducing gene expression because of short heating times, the ability to heat deep tissue non-invasively, and its compatibility with MRI temperature monitoring. Previous gene therapy experiments making use of this ultrasonic heating methodology were able to show heat-induced gene expression in both rats, after a 3 minute exposure at 40-50°C, and mice, after a 25 minute exposure at 43°C (Guilhon et al. 2003).

4. FEASIBILITY EXPERIMENTS

4.1. EXERIMENTAL PURPOSE

In the following chapters, we seek to determine both the methodology in which therapeutic focused ultrasound can be used to increase gene therapy uptake and some of the risks associated with using ultrasound in this manner.

To begin, we sought to test the effect of focused ultrasound with albumin-coated microbubbles on an *in vivo* myocardial gene transfer of a plasmid/reporter gene construct using a large animal model.

4.2. Materials and Methods

Five Yorkshire pigs were used in the feasibility experiments. Inhalation anesthesia using Isofluorane 1-2% was followed by intramuscular injections of Telazol (4.4mg/kg) and xylazine (2.2mg/kg) to cause general anesthesia and intravenous application of lidocaine (100mg) and Ampicillin (1g) to prevent cardiac arrhythmias and infection. A left thoracotomy exposing the lateral free wall of the left ventricle was performed. Four areas on the exposed ventricle sized 1cm square and spaced 1 cm apart were marked using 5/0 prolene suture thread. Each area was treated in one of the following ways: (a) 2mL intramyocardial injection via 30G needle of 500ug naked DNA plasmid encoding β-galactosidase mixed in TE buffer, (b) 2mL intramyocardial injection via 30G needle of 500ug naked DNA plasmid encoding β-galactosidase mixed in TE buffer and subsequent ultrasound application as described below, (c) 2mL intramyocardial injection via 30G needle of 500ug naked DNA plasmid encoding β-galactosidase and 0.3 mL albumin-coated microbubbles (Optison, Mallinckrodt Inc; St Louis, MO) mixed in TE buffer and subsequent ultrasound application as described below, or (d) 2 mL of TE buffer solution.

The ultrasound application was generated with a spherically curved air-backed piezo-electric transducer with a 7 cm diameter, a 7 cm radius of curvature and a resonant frequency of 0.891 MHz resulting in a focal width of approximately 2.5 mm and a

focal length of 12 mm. The acoustic pressure was measured in water using a hydrophone (Sonic Industries, Hatboro, PA). The peak pressure amplitude was 0.75 MPa, with a time average power of 1.26 W. The sonication time was 1 minute, consisting of a 50ms sinusoidal burst every 1s (5% duty cycle). The transducer was fixed inside a cone-shaped applicator (built in-house) to ease targeting of the ultrasound field that was filled with degassed water to increase ultrasonic coupling (Figure 4.1). The outside of the flexible coupling surface was coated with sterile ultrasound gel (Aquasonic 100; Parker, Orange, NJ) for the same purpose.

After each of the four areas was treated, the thoracotomy was closed and the animals recovered over the next 72 hours. Euthanasia was performed by intra-cardiac injection of potassium chloride after general aesthetic was given. The hearts were harvested, and the marked areas were sectioned and flash frozen in liquid nitrogen.

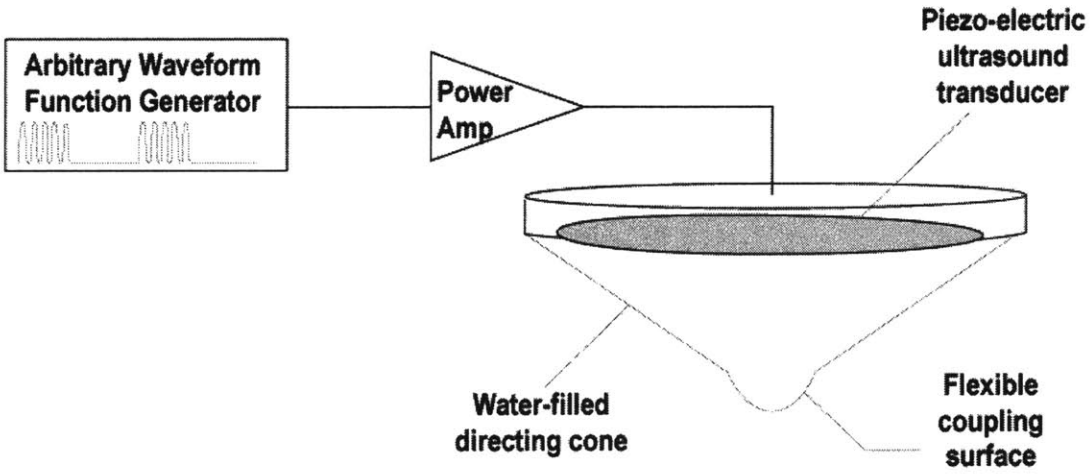

Figure 4.1: Experimental set-up. *An arbitrary waveform generator emitted 50ms bursts of 0.891 MHz sinusoidal electrical energy every second. This energy was amplified and then transformed to ultrasound by the transducer. The ultrasound was focused through the water and into the tissue.*

An initial experiment in one animal was performed using the same protocol as above to determine an appropriate power level to use in the experiments. Each of the four areas of the heart was injected with 0.3 mL albumin-coated microbubbles mixed in 2mL TE buffer via a 30G needle. Each area was then exposed to ultrasound of increasing power: 0W (as a control), 1.86 W time average power (peak pressure 4.8 MPa), 2.68 W time average power (peak pressure 5.7 MPa) and 3.68 W time average power (peak pressure 6.7 MPa). Twenty-four hours after exposure, the animal was sacrificed and the myocardium was sectioned and flash frozen in liquid nitrogen.

The frozen sections were ground to powder and tissue debris was pelleted by centrifugation at 14000g. Cell lysates were prepared using lysis buffer and β-galactosidase protein concentration was quantified using an ELISA (Boehringer Manheim, Manheim, Germany) according to manufacturer's instructions. Total protein concentration was determined using the Bradford assay (Biorad Laboratories, Richmond CA). Absorbance values were quantitated using an ELISA plate reader at a wavelength of 405nm. Gene expression was quantified as the amount of β-galactosidase relative to the total protein amount. All tissue samples were measured in duplicate. A paired t-test was used to compare means and all analyses were performed using the Sigma Stat software program (SPSS Inc, Chicago IL) with p values ≤ 0.05 considered significant.

4.3. RESULTS

The damage to myocardium after exposure to increasing focused ultrasound power

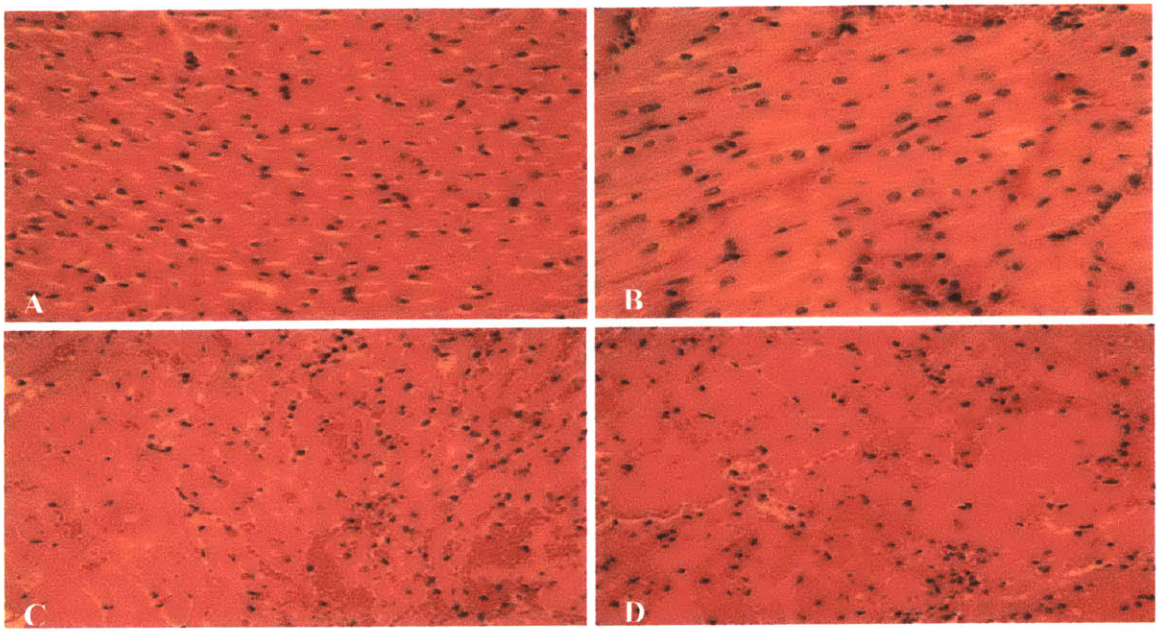

Figure 4.2: Myocardial sections of areas exposed to increasing ultrasonic power; *(a) 0W - control, (b) 1.86 W time average power (peak pressure 4.8 MPa), (c) 2.68 W time average power (peak pressure 5.7 MPa) and (d) 3.68 W time average power (peak pressure 6.7 MPa). Damage ranges from local hemorrhage at the lowest power to necrosis at the highest power.*

was examined to determine the power level to be used in this experiment (Figure 4.2). The lowest power (1.88W) reveals only focal hemorrhage with no myocyte necrosis, while the 2.71W setting shows moderate hemorrhage with focal areas of necrosis. At 3.68W, there was marked hemorrhage with confluent zones of necrosis up to 0.5cm. The hemorrhage and necrosis was most pronounced in the middle of the ventricular wall, where the ultrasound power was focused. Based on these results, a time average power level of 1.26 W (peak pressure 1.6 MPa) was chosen for the subsequent experiments involving injection of genes.

ß-galactosidase expression, as measured 24 hours after injection and sonciation, was statistically different only between territories with plasmid alone and territories

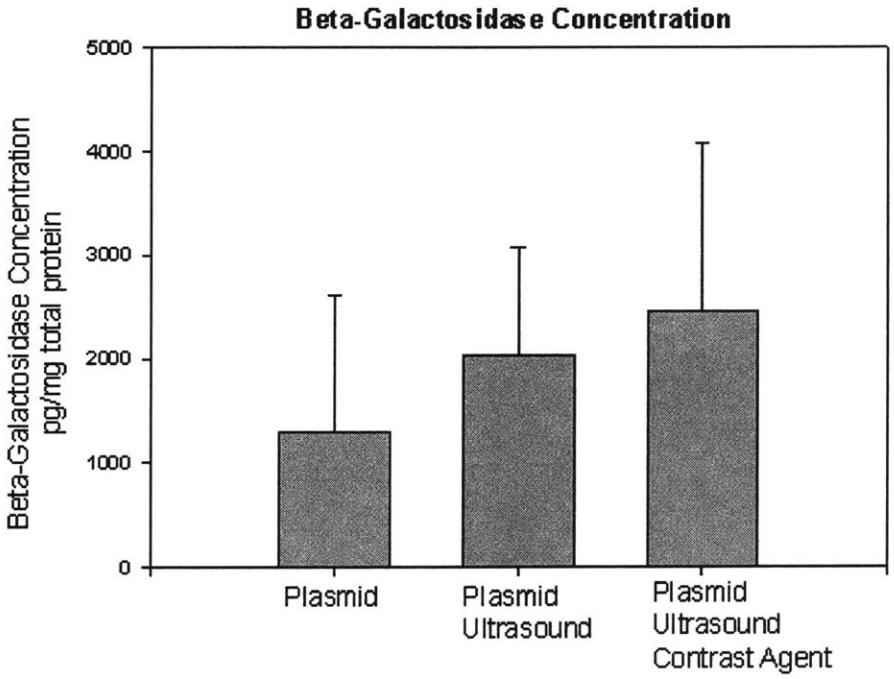

***Figure 4.2: ß-galactosidase expression**, as measured 24 hours after injection and sonication, in areas injected with plasmid alone, injected with plasmid and then exposed to ultrasound, and injected with plasmid and contrast agent and then exposed to ultrasound.*

exposed to plasmid, microbubbles and ultrasound (Figure 4.3). The mean beta-galactosidase concentration in the plasmid-alone treatment territory was 1301 pg/mg total protein versus 2451 pg/mg total protein in the ultrasound, microbubbles and plasmid; a 88% increase ($p = 0.044$). In territories that had ultrasound exposure without microbubbles, the mean was 1.5 fold higher than plasmid alone (2038 pg/mg total protein); however this was not statistically significant. No beta-gal expression was found in control injection territories.

4.4. DISCUSSION

Our first study confirms the ability of using focused ultrasound with microbubbles to increase gene therapy uptake in a plasmid construct. In the areas that were injected with plasmid and microbubbles before being treated with focused ultrasound, a statistically significant 88% increase was found over plasmid alone. This result agrees with a previous *in vivo* study in which focused ultrasound and microbubbles were found to increase the gene uptake of an adenoviral gene therapy construct 2-fold (Shohet et al. 2000). Based on the pathology of the porcine myocardium after ultrasound exposure, there should be little to no necrosis, although localized hemorrhage should be expected. The increase in efficiency using contrast agent and ultrasound was not as high as other reports previously published, which found as much as a 20-fold increase in gene expression (Schratzberger et al. 2002). Differences are to be expected since experiments differ in gene type, animal model, cell type, and measurement methodology. However, differences could also be directly attributed to the lack of knowledge of optimal ultrasound parameters. To date, there has not been a published study that characterizes the optimal ultrasound frequency, modality, timing and power for most effective gene transfer *in vivo*. Two recent papers have started this process. Pislaru *et al* (2003) compared diagnostic versus continuous wave ultrasound in gene transfer in rat muscle and found gene expression almost doubled using the diagnostic imager. However, this was using only a single frequency and power, and pulsed continuous wave was not compared. Chen *et al.* (2003) examined the optimal modality, frequency, and power parameters using a diagnostic ultrasound scanner only, and found that using pulsed wave versus continuous wave ultrasound increased gene expression in rat myocardium significantly *in vivo*. Increased gene

expression was also found with increasing power and decreasing frequency, with the lowest frequency tested being 1.3 MHz. A similar systematic study characterizing the optimal parameters for continuous wave and pulsed continuous wave will be invaluable to decisions about the ability of to use ultrasound mediated gene transfer to replace viral vectors without sacrificing transfer efficiency or if instead ultrasound will be a helper-vector used only in conjunction with viral vectors to increase efficiency farther.

5. BUILDING AND TESTING AN OPTIMIZED TRANSDUCER

5.1. BUILDING A NEW TRANSDUCER

Using the experience of the first feasibility tests, the need to optimize experimental conditions for invasive *in vivo* experiments taking into consideration optimal transducer size, focal distance, and frequency generation was recognized as a necessary step in the final design of a minimally invasive ultrasound system for gene therapy delivery.

Size was the first factor considered. The large size of the transducer used in the feasibility experiments described in chapter 4 was inconvenient as it limited our choice of experimental model as well as being bulky and hard to use. The new transducer was designed to again be an spherically curved air-backed piezoelectric transducer but this time smaller, with only a 2 cm diameter.

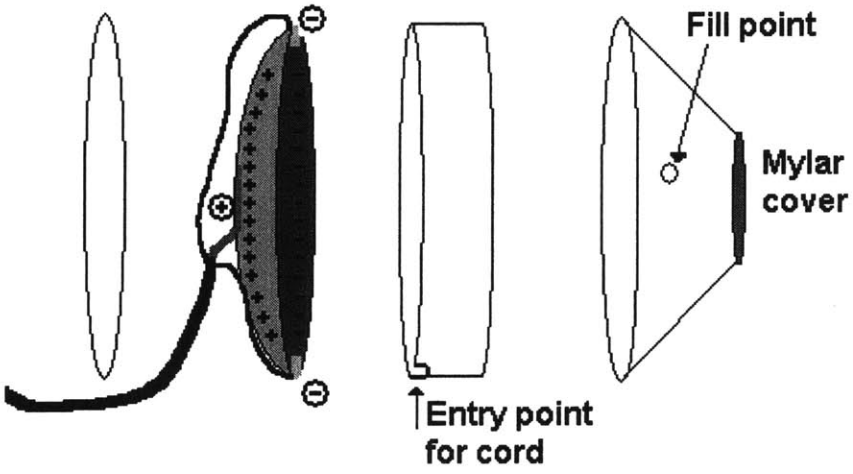

Figure 5.1: Construction schematic of a single element transducer and holder. *The electrically asymmetric piezoelectric transducer is wired and placed in a Plexiglas holder designed to hold the transducer by the perimeter edge only as well as maintaining an air backing to reflect backward ultrasound propagation. A cone shaped water-bath is attached to the front to increase ease of experimental use.*

By choosing a transducer with a resonant frequency of 1.33 MHz to be used as the "low" frequency, the same transducer can be used to create the "high" frequency by using the transducer's 5th harmonic at 7.379 MHz. This comparison will determine if the future clinical system should be designed as an external non-invasive device or a minimally invasive catheter device.

The transducer was constructed from a 2cm diameter spherically shaped piece of piezoelectric material (Staveley Sensors Inc, East Hartford CT) with a 2 cm radius of curvature. Piezoelectric materials, as discussed in Section 2.3, convert electrical energy into mechanical energy as a result of the electrical asymmetry of the crystalline structure. When an electric charge is introduced across the crystal the particles expand and contract to counteract the electric forces (Hynynen, 1990). A 28-

gauge coaxial cable (Belden, St. Louis MO) is wired to the crystal by soldering the conducting wire to the convex side of the crystal and then flipping the ground wires over to the concave side and soldering them into place. To ensure proper conductivity, the ground wires are split and soldered onto the crystal in two places, 180 degrees apart.

The crystal is then placed in a plexiglass cylinder whose inner opening was milled to the size of the transducer. Using VIL-SIL silicone (Rhodia Silicones VSI, Troy NY) mixed to a ratio of 40:1 compound (Part A): hardener (Part B), the crystal is attached to the Plexiglas cylinder only around the outer rim to allow the free-hanging crystal maximal freedom of movement. The coaxial cable and the back disk are then attached to the bottom of the cylinder with silicone to create a watertight seal. The resultant air backing of the transducer holder is important because the ultrasound waves created by the movement of piezoelectric materials are bi-directional. When the ultrasound waves traveling backwards (i.e. away from the desired location) encounter the crystal/air interface, the acoustical impedance mismatch causes nearly all the energy to be reflected back into the crystal where it adds to the ultrasound signal moving in the desired direction.

To facilitate the use of the transducer, a cone shaped water basin was attached to the front of the holder. First, another piece of Plexiglas was milled into a 1 cm long cone shape. The larger opening matched the size of the transducer holder, while the smaller opening tapered down to a 1 cm diameter. The smaller opening was capped with

acoustically translucent Mylar and the cone was attached to the transducer holder with silicone, carefully avoiding the crystal. A hole drilled in the side of the transducer allows for filling the water bath with degassed water and a small screw seals the cone for use.

Finally, the transducer must be electrically matched to 50 Ohms to match the output impedance of the driving amplifier system. This is accomplished through a first-order LC circuit.

5.2. TESTING AND CHARACTERIZING THE TRANSDUCER

A transducer is fully characterized by performing efficiency tests and measuring the pressure field around the focus. Characterizations were performed at the 1st and 5th harmonics.

Efficiency was computed as the percentage of acoustic power delivered over electrical power inputted. Acoustic power was measured with a radiation force system; where an ultrasound beam is directed towards a perfectly absorbing target hung from a laboratory balance (AE 200, Mettler Toledo, Columbus, OH) (Stewart 1982; IEEE 1990). Pressure exertion of the ultrasonic wave on the target will cause the balance to measure a change of mass and the following equation can be used to calculate acoustic power (P_A)

$$P_A = \Delta m * g * c(T) / \cos(\theta)$$

where g is the gravitational constant, c(T) is the temperature dependant speed of sound, and θ accounts for the curvature of the transducer. Transmitted electrical power was measured with a commercial power meter and a dual-directional coupler (HP 438A, Agilent Technologies, Palo Alto, CA).

Table 5.1: Efficiency Measurements at the 1st, 3rd and 5th harmonics.

First Harmonic 1.33 MHz

Voltage (V)	Electric Power (W)	Acoustic Power (W)	Standard Error (W)	Efficiency
0.07	1.10	0.759	0.004	69 %
0.08	1.43	0.983	0.001	69 %
0.09	1.83	1.27	0.003	69 %
0.10	2.26	1.56	0.017	69 %
0.11	2.77	1.89	0.002	68 %
			Average	**69 %**

Third Harmonic 4.421 MHz

Voltage (V)	Electric Power (W)	Acoustic Power (W)	Standard Error (W)	Efficiency
0.07	0.955	0.675	0.005	71 %
0.08	1.24	0.869	0.010	70 %
0.09	1.57	1.10	0.007	70 %
0.10	1.95	1.35	0.003	69 %
0.11	2.36	1.63	0.004	69 %
			Average	**70 %**

Fifth Harmonic 7.379 MHz

Voltage (V)	Electric Power (W)	Acoustic Power (W)	Standard Error (W)	Efficiency
0.07	0.883	0.387	0.002	44 %
0.08	1.15	0.505	0.004	44 %
0.09	1.44	0.638	0.004	44 %
0.10	1.79	0.783	0.002	44 %
0.11	2.16	0.941	0.003	44 %
			Average	**44 %**

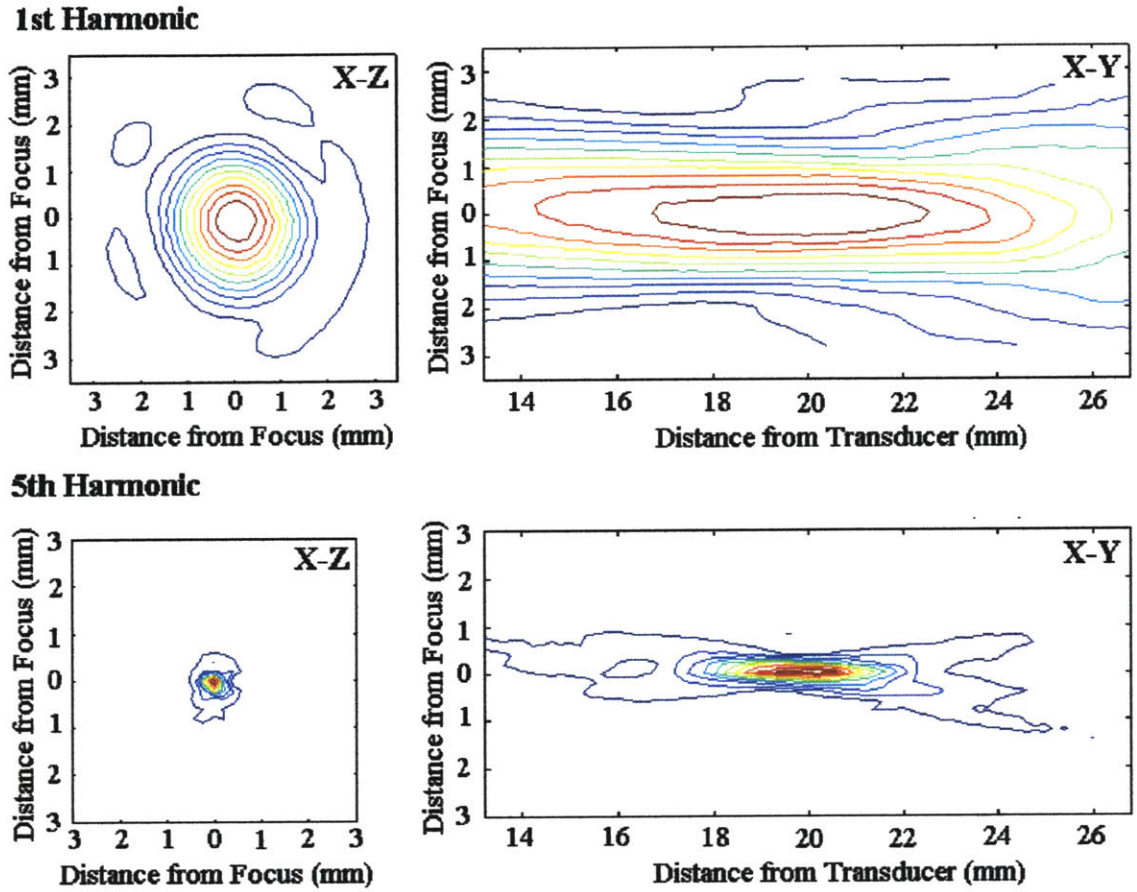

Figure 5.2: Relative Pressure Intensity Field Scans. *The scans show the relative pressure field around the focal point of the transducer. The X-Z plane is taken 20mm from the transducer face and oriented parallel to it while the X-Y plane runs along the ultrasound pathway.*

Results from the efficiency tests are shown in Table 5.1. At each harmonic frequency the transducer was tested at a variety of driving voltage levels. The acoustic power was calculated using the equation above and the efficiency at that voltage was determined. The efficiencies are then averaged at each frequency. The average efficiency for this transducer was 69%, 70%, and 44% at the first, third and fifth harmonics, respectively, well within the normal range.

The ultrasound field was characterized using automated stepper-motor scans (VP 9000, Velmex, Bloomfield, NY) in a degassed water bath (Figure 5.2). The acoustic intensity was sampled using a PVDF needle hydrophone (0.075 mm diameter, Precision Acoustic, England) connected an oscilloscope. The relative intensity measurement was obtained by measuring the peak amplitude of the hydrophone output signal. Beam profile measurements were made in a raster pattern with the transducer driven in pulsing continuous-wave mode. The measurement tank dimensions (0.25 x 0.33 x 0.75 m) were much larger than the beam profile and the tank walls were lined with absorbing rubber to minimize acoustic reflection and standing wave effects.

6. Effects of Ultrasonic Gene Therapy

6.1. Motivation

After characterizing the newly built transducer, the next step was an investigation that would test not just the efficiency of getting marker genes into cells using ultrasound and microbubbles, but also how an actual therapeutic gene therapy would work after being delivered using ultrasound. The Heme Oxygenase-1 gene (HO-1) codes for an oxidative stress enzyme, seen in many types of cells and tissues, thought to protect cells from ischemia. After incorporating this gene into *in vivo* rat kidneys and microbubbles, we deprive the kidneys of oxygen and compare the protective function in kidneys that incorporated the gene due to an adeno-associated viral (AAV) vector alone to those that incorporated the gene with a combination of AAV vector, ultrasound and microbubbles.

6.2. MATERIALS AND METHODS

6.2.1. ULTRASOUND EXPOSURE

In these experiments, the same 1.33 MHz transducer designed and tested above was again used at the first harmonic. Twenty-six Wister rats were anesthetized, secured in a dorsal recumbent position and incised down the midline. A kidney was pulled forward with blunt forceps until completely exposed, the renal artery and veins clamped and a catheter was inserted into the renal artery. The transducer was placed over the exposed kidney and the sonication started; after 10 seconds, the injection would be pushed in over a 15 second interval, and the sonication would continue for the last 35 seconds; totaling 1 minute of ultrasound exposure time. The kidney was then tucked back into proper position and the other kidney was exposed and the procedure repeated. A time average power of 0.13 W (peak pressure 0.33 kPa) was used for the sonication, a value reduced from the previous work due to visible damage on the kidney at higher powers. For the same reason, the duty cycle was also reduced to 2.5% of the 200ms burst period. The injection consisted of a solution of an adeno-associated viral vector/gene construct (Harvard Gene Initiative, Boston MA) mixed with buffer and Optison equaling 10% of the solution.

6.2.2. BILATERAL ISCHEMIA INDUCTION

Three months after the gene therapy was injected, bilateral renal ischemia was induced. Animals were anesthetized and both renal pedicles (artery and vein) were exposed using a midline incision and clamped for 30 minutes followed by 24 hour reperfusion. Sham animals had an incision made and kept open for 30 minutes of

waiting time without clamping the renal arteries. After ischemia or sham treatment, the muscle layer incisions were sutured and the skin incisions closed. Saline solution was supplied to the rats during and after surgery to prevent dehydration.

6.2.3. RENAL FUNCTION MEASUREMENTS

Acute renal failure (ARF) is characterized by rapid decline in glomerular filtration rate (GFR), estimated using measurements of creatinine levels in the blood and in the urine. Usually decreases in GFR, increases in plasma creatinine, and increases in blood urea nitrogen are indications of deterioration of the kidney. One month after bilateral clamping, the experimental and control rats were housed in metabolic cages for collecting urine. Blood samples were also drawn at this time. The rats were then sacrificed and the kidneys were removed and snap-frozen in liquid nitrogen and stored at -80°C. A total of 26 animals survived to the end of the study; 7 receiving the AAV/gene construct injection without ultrasound with subsequent bilateral clamping on 4, 6 receiving the AAV/gene construct injection with ultrasound and Optison with subsequent bilateral clamping on 4, 6 receiving a buffer injection with ultrasound and Optison with subsequent bilateral clamping on 3, and 7 animals using a buffer injection without ultrasound with 4 receiving subsequent bilateral clamping.

6.3. RESULTS

After the experiments were concluded, it was communicated to us that the gene constructs used in this experiment were mistakenly made with the HO-1 gene in the reverse configuration. Although the reverse-configured gene did seem to show a protective function from ischemia, the reasons for this are unexplained. To simplify results only the data from injections of the buffer solution will be discussed.

Creatinine concentration in blood serum, elevation of which is an indicator of renal failure, was measured (Figure 6.1) as milligrams of creatinine per deciliter blood over 24 hours. The mean creatinine concentration in each of the four experimental conditions was as follows: in the animals treated with ultrasound, microbubbles and buffer and then clamped to induce ischemia the creatinine concentration was 2.9±0.7mg/dL, while the animals treated with ultrasound, microbubbles and buffer but not clamped had a mean of 0.3±0.1mg/dL. The group injected with buffer alone and then clamped had a mean creatinine of 1.4±0.3mg/dL, while the animals injected with buffer and not clamped had a mean creatinine of 0.4±0.1mg/dL.

Blood urea nitrogen (BUN) in the urine over 24 hours was also measured as another indication of renal failure (Figure 6.1). The mean BUN concentration of the animals treated with ultrasound, microbubbles and buffer after clamping was 74.7±1.8mg/dL while the animals treated with ultrasound, microbubbles and buffer that were not clamped had a BUN concentration of 18.9±14.6mg/dL. The group injected with buffer alone and clamped had a mean BUN of 56.3±1.4mg/dL versus 25.7±7.8mg/dL in the unclamped group.

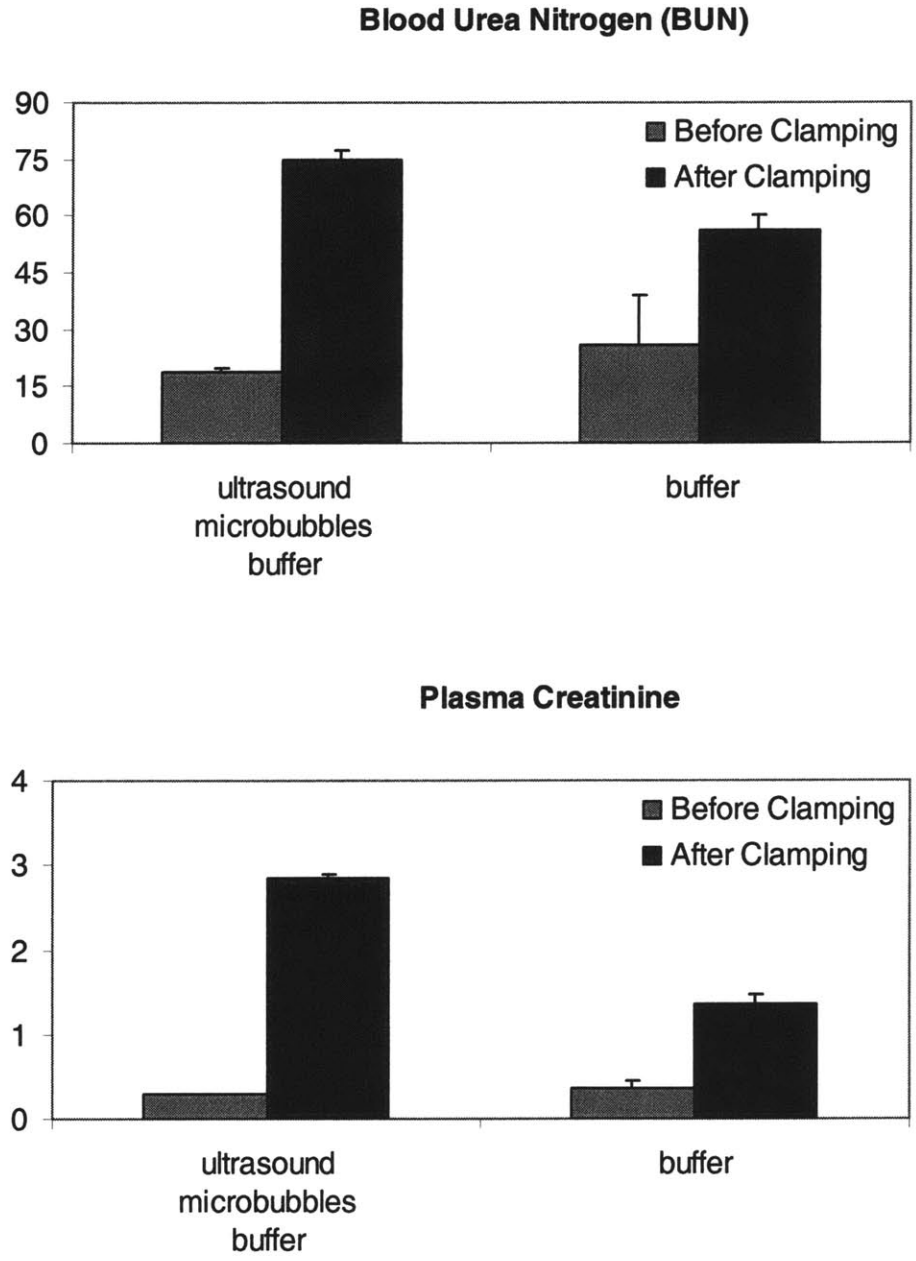

Figure 6.1: Kidney function measurements 24 hours after bilateral clamping to induce ischemia. *Animals were previously subjected to ultrasound application to the kidney with concurrent microbubble injection 4 weeks prior to the clamping. In both measurements the animals exposed to ultrasound previously showed more functional kidney damage after the ischemic event than the control animals.*

6.4. DISCUSSION

Despite not using the gene expression data from the experiments, important information about the usability of this method of gene delivery for therapies meant to protect against ischemia can be gleaned from this study. The animals that were not subjected to bilateral clamping had normal levels of BUN and creatinine, while the animals that were clamped bilaterally had significantly elevated levels, indicative of renal failure presumably due to ischemic damage. Interestingly, the animals that were subject to injections of microbubbles and ultrasound application had higher levels BUN and creatinine, meaning that the ultrasound treatment seems to have preconditioned the renal system for ischemic damage. This could be because the known bioeffect of ultrasonic cavitation in which capillaries transiently collapse, blocking oxygen delivery to the immediate area. This may be damaging kidney cells enough that the cells are less able to withstand the later prolonged oxygen deprivation, even though the initial damage is not functionally significant. A study with careful optimization of the lowest power levels still able to increase transfection efficiency is needed to evaluate if this effect is inevitable. If so, this may have significant implications when using ultrasonic cavitation mediated gene transfer to protect against ischemic damage, a major area of gene therapy research, or in areas already prone toward ischemic damage.

7. SPATIAL CONTROL OF GENE THERAPY DELIVERY

7.1. MOTIVATION

In this study, ultrasound is examined for facilitation of gene therapy delivery in the context of spatially controlling therapeutic protein production. By attaching the gene of interest to a heat shock protein promoter before placing it in the vector construct

the gene is prevented from being transcribed into RNA. The cell will not produce the therapeutic protein until the cell is heated to the point that the hsp promoter allows transcription of the gene of interest. Ultrasound can be utilized to heat the gene minimally invasively or even non-invasively after delivery into the cells. In the following experiment we attempt to restrict protein production to one lobe of the canine prostate after injecting gene therapy to the entire prostate.

7.2. MATERIALS AND METHODS

7.2.1. DEVELOPMENT OF GENE VECTOR

Recombinant adenoviral vectors were generated by standard techniques using the shuttle plasmid pACCMV-pLpA and pJM17 as described by Becker et al. (1994), which uses recombination in 293 cells to generate a replication-defective serotype 5 adenovirus. In these constructs, an expression cassette containing a minimal human hsp70B promoter and RNA leader sequences upstream of the transgene with a 3'rabbit β-globin splice site and poly-adenylation sequences replacing the CMV promoter and SV40 polyadenylation signal sequences from pACCMV-pLpA, as described previously by Smith *et al.* (2002). The viral construct (Ad-HSP-Luc) contains the transgene, firefly luciferase, under transcriptional control of the hsp70B promoter.

7.2.2. GENE TRANSFER INTO PROSTATE

Three beagles ranging in size from 13-17 kg were anesthetized using a mixture of 200mg of ketamine hydrochloride (Abbott Laboratories, North Chicago, IL) and 20 mg of sodium xylazine (Xyla-ject, Phoenix Pharmaceuticals, St. Joseph, MO) injected intramuscularly. Using a transrectal ultrasound imager, the prostate was visualized

and catheters were inserted either just below the rectum or through the abdomen and into each lobe of the prostate. The purified virus was then injected into each lobe of the prostate (1 ml of solution in each lobe, consisting of 1 x 10^9pfu in saline), followed by a saline wash. The animals were then allowed to wake naturally and pain was controlled using twice-daily 0.07mL/kg intramuscular injections of buprenorphine hydrochloride (Buprenex, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA).

7.2.3. HEAT INDUCTION

48 hours after the virus injection, the animals were again anesthetized with the same mixture of ketamine and xylamine; then intubated. Hydration was maintained with a saline IV drip. The procedure was monitored with a 1.5 T clinical MRI unit (GE Medical Systems, Milwaukee, WI). The ultrasound field was created using an

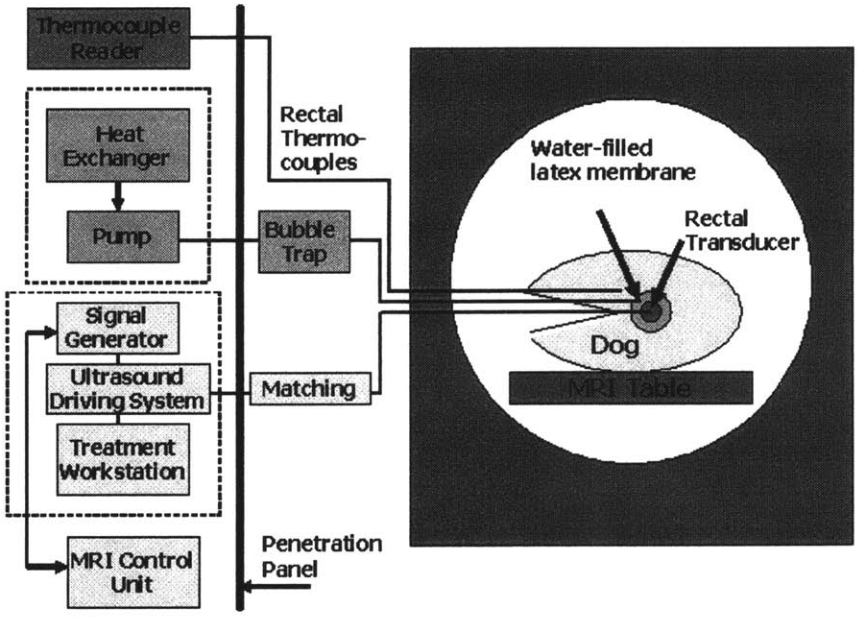

Figure 7.1: Experimental set-up for heat induction. *A transrectal ultrasound transducer surrounded by a water-filled latex membrane was placed in the rectum of the animal. The sonication was driven by an RF system while MRI and thermocouples monitored temperature.*

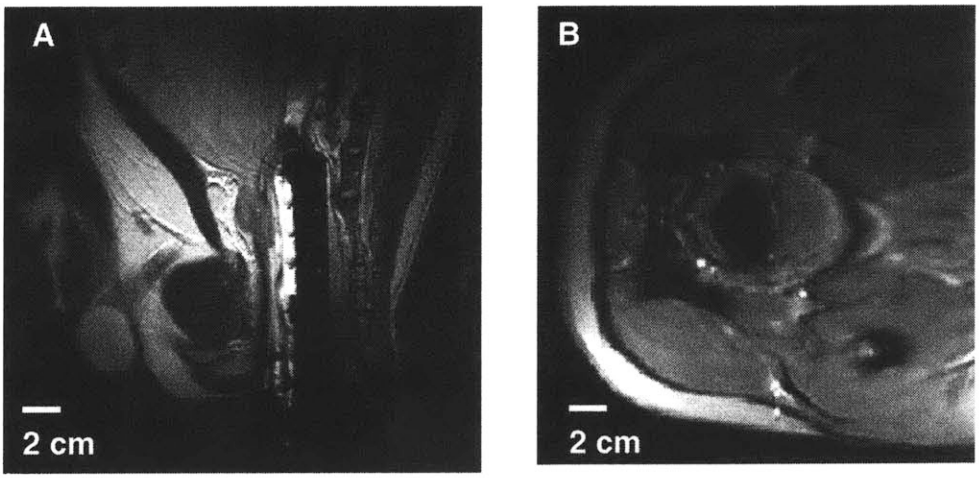

Figure 2: MR images of the transducer inserted into the rectum and positioned next to the prostate. *A latex membrane surrounding the transducer is expanded using heated degassed water and covered with ultrasound gel to remove air pockets between the transducer and rectum.* ***(a)*** *Coronal image (fast spin echo T2 weighted image TR/TE 2000/75ms, echo train length 8, field of view 24 cm, number of excitations 2)* ***(b)*** *Axial image (the magnitude of fast spoiled gradient-echo imaging described in text).*

unfocused, 1.5MHz, air-backed transrectal transducer (Smith et al. 1999). The transducer was made of four 120 degree sections of cylindrical PZT-8 material (lead zirconate–titanate, EC-69, EDO, Salt Lake City, UT) with a length of 15mm. Each of these pieces were placed along the primary axis of the transducer shaft and scored on the inner electrode surface. This divided each piece of PZT into four individually powered and controllable sections, creating a total of 16 independent channels. A multi-channel RF driving system, constructed in-house (Daum et al. 1998), was used to control power delivery (Figure 7.1).

The prostate was found through palpitation and MR imaging and thermocouples were inserted into each lobe. MR imaging confirmed the correct positioning (Figure 7.2).

The transducer was then inserted into the rectum and the latex membrane (Civco, Kalona, IA) surrounding the transducer was then inflated to fit snugly against the rectal wall using degassed water continually circulated at body temperature using a heat exchanger and pump. Ultrasound coupling gel (Aquasonic 100; Parker, Orange, NJ) was used to eliminate air pockets between the latex membrane and rectal wall. A heating pad was placed around the animal to maintain its body temperature.

Temperature was monitored using both MR thermometry and four copper-constantan thermocouples placed in each lobe of the prostate, between the rectal wall and the heated balloon, and externally. The thermocouples were constructed in house of 50μm diameter copper and constantan wire (California Fine Wire Co., Grover Beach, CA) connected by soldering. The wires were incased in a fused silica sleeve used to guide the thermocouple through a catheter to the prostate. The bare junction extended outside of the tubing approximately 5mm to minimize viscous heating artifacts (Fry and Fry 1954, Hynynen and Edwards 1989). As the temperature changes at the solder joint, a voltage is generated that can be measured and converted to temperature by a commercially available thermocouple reader (TC1000, Sable Systems, Henderson, NV).

MR thermometry was performed as described elsewhere (Smith et al. 1999). Briefly, temperatures were measured by exploiting the temperature dependence of the proton-resonant frequency (Ishihara et al. 1995). Changes in the proton-resonant frequency were estimated by measuring changes in phase and dividing by 2π times the time in

which the phase developed (the echo time of the image). A fast spoiled gradient-echo sequence was used to acquire the phase maps (Chung et al. 1996). The following parameters were used: 39.5/19.3 (repetition time msec/echo time msec); flip angle, 30°; bandwidth, 3.57 kHz; field of view, 20 cm; section thickness, 5 mm; matrix size, 256 x 128; imaging time, 5.2 seconds. The temperature dependence of the proton-resonant frequency shift was assumed to be 0.010 ppm/°C, the value used by Chen *et al.* (2000) when monitoring experiments in the human prostate. A set of four axial images were taken approximately every 40 seconds to average out noise, then imaging was paused to allow a thermocouple measurement without MR interference, and then the next set of four images were acquired.

By using only the ultrasound channels that lined up with the left lobe of the prostate, we were able to limit the hyperthermia exposure to that area in 2 of the 3 dogs. RF powers up to 70 W were used to heat and maintain the sonicated area at the desired temperature. The procedure continued until the temperature of the left lobe was at or above 42°C for 25 minutes, as measured by the thermocouple.

7.2.4. SACRIFICE

Animals were sacrificed 15-20 hours after the heating using a 0.13 ml/kg intravenous injection of Euthasol (Delmarva Laboratories, Midlothian, VA, USA). The prostate was removed, sectioned and flash-frozen using liquid nitrogen and stored at -80°C for processing.

7.2.5. ANALYSIS

The prostate was sectioned by four axial cuts into five pieces, labeled 1-5 from most superior to most inferior. Sections 1, 3 and 5 were ground with a mortar and pestle under liquid nitrogen. The resulting powder was dissolved in Promega reporter lysis buffer (Promega Corporation, Madison, WI) containing protease inhibitors and assayed (2 μl) using the Promega luciferase assay reagent (100 μl). Light measurements were made with a luminometer (Berthold Lumat LB9501, Nashua, NH) over 10 seconds and measured as light units per microgram of protein. Protein was measured using the bicinchoninic acid (BCA) method first described by Smith *et al.* (1985) (Pierce Chemical Co, Rockford, IL) with BSA as a standard.

Thermal dose was calculated from temperatures from the middle of each lobe in dogs 1 and 2 using the equation set forth by Sapareto and Dewey (Sapareto and Dewey 1984). This equation is used to equate the range of actual temperatures during the experiment with an "equivalent" time at a reference temperature of 43°C that would have the same cellular response.

7.3. RESULTS

The temperature progressions during the experiments in the left (treated) and right (untreated) lobes of the animals' prostates are shown in Figure 7.3 alongside the electrical power delivered. The temperature data from dogs 1 and 3 was extracted from the thermocouples placed in the left and right lobes of the prostate during the experiment. With the first dog, a temperature difference was maintained throughout the experiment, with the left lobe measuring 42°C or greater for 29 minutes and the right lobe measuring less than 42°C throughout the experiment. Note that power was

continually increased to compensate for temperature decreases, likely due to increased blood flow resulting from the homeostatic vasodilation response to heat. In Dog 3, because of the small size of the prostate, it was not possible to limit heating to only one lobe with the experimental set-up used. Thus, the left and right lobe temperature measurements were equal to or greater than 42°C for 24 and 23 minutes, respectively, during 35 minutes of heating.

In dog 2, the left thermocouple was dislodged mid-experiment, which necessitated the use of the MR thermometry data to review the temperature progression during the

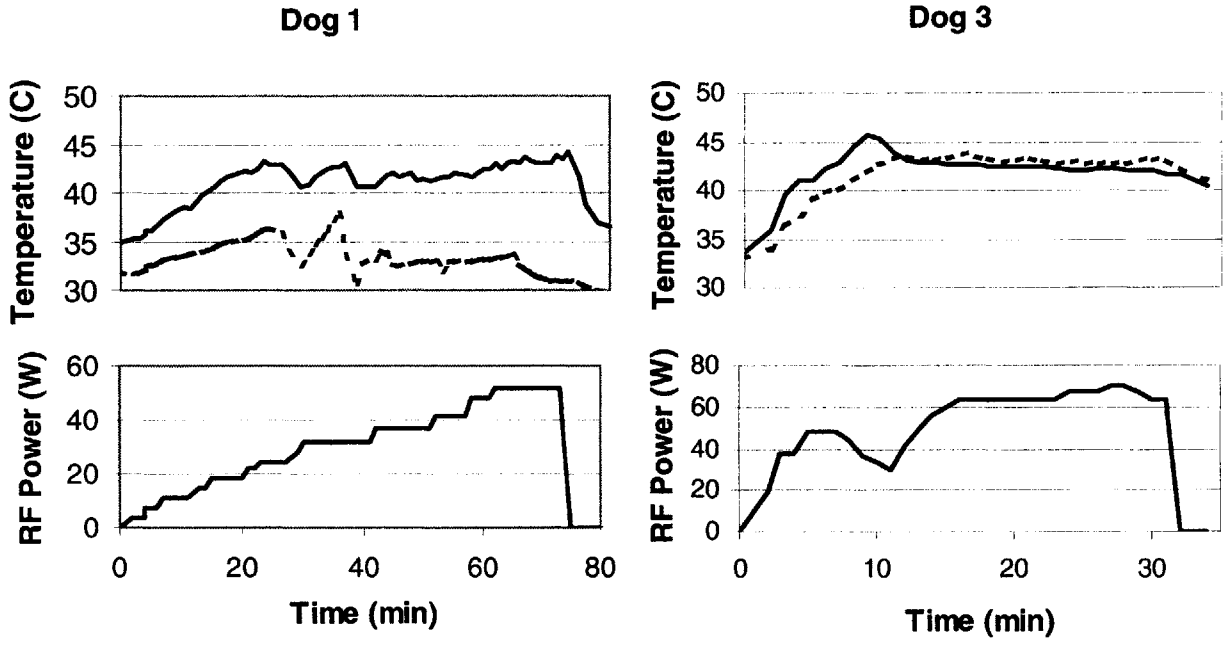

Figure 7.3: Temperature maps of the prostate lobes during heat induction in dog 1 and dog 3. *The left lobe temperatures are marked with a solid black line and the right lobe temperatures are marked with a dashed black line. Temperature data is from thermocouples placed in the middle of each lobe. The lower graph shows the RF power delivered to the transducer at each point during the experiment.*

104 minute long experiment. The majority of heating occurred between 12 and 20 minutes, between 75 and 85 minutes and between 90 and 97 minutes into the experiment. Figure 7.4 shows the temperature delineation of the left and right lobes during the heating, as measured by MRI, in dog 2. Signal intensity increases with temperature and the lower temperature in the right lobe of the prostate is clearly distinguished from the higher temperature in the left lobe at 12 minutes and 79 minutes into the experiment.

Thermal dose was calculated using temperatures from the middle of each lobe using thermocouple data in dogs 1 and 3 and MR thermometry in dog 2. Dog 1 had a

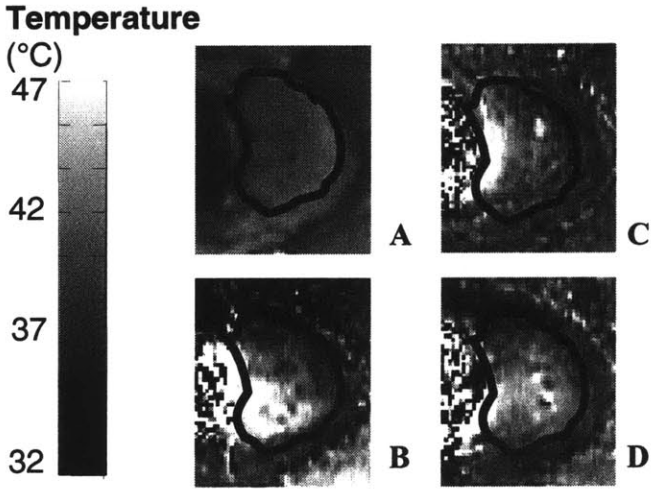

Figure 7.4: MRI temperature maps of Dog 2. ***(a)*** *An MRI image (the magnitude of fast spoiled gradient-echo imaging described in text) of Dog 2 before heat induction with the prostate outlined in black. The next three images are temperature maps (MR parameters described in text) of the same area at* ***(b)*** *12,* ***(c)*** *39 and* ***(d)*** *79 minutes into the experiment. Note that signal intensity increases with temperature and that the lower temperature of the upper (right) lobe of the prostate is clearly delineated from the higher temperature of the bottom (left) lobe in (a) and (c).*

thermal dose of 29.7 minutes in the left lobe and less than 0.01 minutes in the right lobe while dog 2 had a thermal dose of 71.9 minutes in the left lobe versus 3.7 minutes in the right lobe. The third dog received 27.1 equivalent heating minutes in the left lobe and 22.3 minutes in the right.

The luciferase activity observed in each lobe of the prostate for the three animals is shown in Figure 5. Dog 1 shows a 31-fold increase in activity in the left (treated) versus the right (untreated) lobe, while dog 2 shows a 14-fold increase and dog 3 shows no significant difference in luciferase activity between the two lobes.

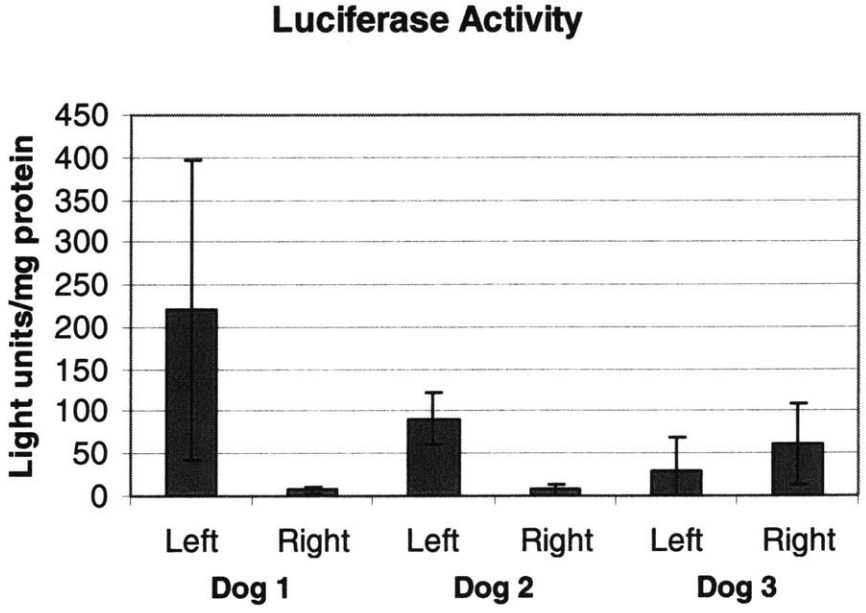

Figure 7.5: Luciferase activity in the three animals, *separated into each lobe and the axial sections numbered 1, 3, and 5 from superior to inferior. Dog 1 shows a 31-fold increase in activity from the left to the right lobe, while dog 2 shows a 14-fold increase and dog 3 shows none.*

7.4. DISCUSSION

We have successfully used MRI-guided, ultrasound-induced hyperthermia to spatially control gene expression in an animal model relatively close to human in size. The luciferase expression in dogs one and two was 31 and 14-fold higher, respectively, in the heated lobe versus the control lobe. Dog three, in which both lobes were heated, showed no significant difference in luciferase expression between the lobes.

The temperatures in these experiments were monitored in two ways, by MRI thermometry and by thermocouples inserted into the animal. In the clinic, MR would be the preferred temperature monitoring method due to its non-invasive nature and for its ability to allow continuous monitoring in multiple planes, allowing any unwanted heating to be identified and corrected. There are limitations when using MR thermometry (as reviewed by Quesson *et al.,* 2000) since temperature measurement is based on the changes in phase relative to an image acquired before heating, any movement by the subject disrupts the alignment between the images and the continuity of the temperature measurements is lost. Fortunately in a clinical situation both the situations can be controlled either by pausing the experiment to regain temperature integrity or by using MRI thermometry sequences less sensitive to motion (Kuroda et al., 2000). In addition, blood flow in large vessels can also cause artifacts in the MRI-derived temperature maps.

Smith et al (2002) showed that gene expression increased linearly with the duration of heating. In this work, dog 1 received the longest duration of heating above 42°C and the highest level of luciferase activity was observed in the left lobe of this animal. In

contrast, both lobes of the dog 3 prostate and the left lobe of the dog 2 prostate were heated above the 42°C threshold temperature for 4-5 minutes less and all had a comparably lower level of luciferase activity than the left lobe of dog 1. Madio *et al* (1998) examined intrinsic heat shock protein expression *in vivo* with MR-monitored ultrasonic heating of the rat leg to temperatures ranging from 42-45°C for forty-five minutes. They found that hsp70 expression increased from 3 to 67 fold compared to unheated muscle tissue. This agrees well with the 14 and 31 fold induction of luciferase expression reported in this paper, despite the difference in tissue type.

A point of considerable interest is whether the induction of the hsp promoter is simply the result of reaching a threshold temperature or if the level of expression is related to a more complicated relationship between heating duration and temperature, as has been found in numerous hyperthermia experiments. It is interesting that comparable levels of induction have been obtained in vitro by Borrelli *et al.* (2001) and Vekris *et al.* (2000) at temperatures ranging from 41°C for 4 hours to 48°C for 30 seconds, conditions which result in thermal doses that are approximately equal. The small number of animals in the current experiment does not allow any conclusions to be drawn regarding this question, partially due to the pattern of heating in dog 2. In that animal, the thirty minute long heat treatment was performed in two phases with significant cooling between the phases. This discontinuity of heating may be important since it has been shown in vitro that cells develop thermotolerance after exposure to non-lethal heating that can inhibit heat shock protein induction after a second heat treatment (Mizzen and Welch 1988, Li and Mak 1989). During ultrasonic

heating, animals are periodically able to regain temperature homeostasis through vasodilation, which increases blood circulation to the heated tissue to cool the area (Lehmann and de Lateur 1990). When the power is increased to return to the hyperthermic temperature range, periods of heating are interspersed between intervals at lower temperatures. Although the time intervals are much shorter then those generally used when investigating thermotolerance, the inability to correlate reporter expression with thermal dose in this study suggests the possibility of thermotolerance. Further studies are needed to determine how thermal dose and thermotolerance function in determining the extent of transgene expression from the hsp70B promoter.

This preliminary study supports the concept that transgene constructs under control of the hsp70B promoter can be used in conjunction with MRI-guided ultrasound heating to spatially control gene expression. It should be noted that in the current study an unfocused 16-element array was used which allows only limited control of the field being heated. The next step is to utilize a focused transducer to determine the extent of spatial resolution that can be achieved with ultrasound mediated control of gene expression. More sophisticated phased array applicators could provide greater control of temperature and volume (Hutchinson and Hynynen 1998), which would enhance the utility of this method. In addition the mechanism of activation remains to be resolved, an issue which can be addressed by varying thermal conditions and the duration of treatment, to determine if a simple threshold temperature is the principal factor *in vivo,* or if a more complicated relationship exists.

8. FUTURE WORK

Ultrasound-mediated gene transfer has the ability to make gene therapy a clinical reality. The increase in gene uptake and the spatial control demonstrated in this work is crucial for allowing clinically significant dosage restricted to tissues desired for expression. More work is needed to optimize the methodology of ultrasound mediated gene transfer and understanding how USMGT will be used in conjunction with other vectors. Future work should include optimization of ultrasonic parameters, including a final determination of the best ultrasound modality. Optimization of contrast agents, the decision to attach genes to contrast agents or not, and accompanying treatment such as lidocaine or heat treatment will also need to be included into a final protocol to deliver gene therapy. Most interesting is the possibility of combining the two systems described in this work. Experiments can be designed combining ultrasonic cavitation to increase gene uptake into cells with ultrasonic heating to spatially control expression. In the brain, ultrasonic cavitation could be used first to open the blood/brain barrier in a selected site and then to increase gene uptake. Ultrasonic heating could then be used to precisely control gene expression.

In the course of the experimental work outlined above, work by other groups has progressed in some of these goals. As mentioned in section 4.4, ultrasonic parameter optimization studies have begun comparing diagnostic versus continuous wave ultrasound (Pislaru et al. 2003) and by examining the optimal modality, frequency, and power parameters using a diagnostic ultrasound scanner (Chen et al. 2003). There has also been progress in trying to understand the mechanism behind the increase in

gene expression in USMGT. Lawrie *et al* (2000) examined the effects of free radicals on gene expression, as cavitation is known to increase free radical formation. However, gene expression was statistically unchanged when the intensity of ultrasound was reduced until free radical production, as measured by hydrogen peroxide production, was no longer detectable. They conclude that the mechanism is likely to be purely mechanical. However, Bekeredjian *et al.* (2003) showed that when an area was injected with microbubbles, sonicated, and then immediately infused with plasmid solution, gene expression was consistent with areas of no ultrasound exposure. This would suggest that any increase in cell membrane permeability occurs only during the sonication, which is inconsistent with the theory that microstreaming punches holes in the cell membrane. If ultrasound-induced cavitation increases the "fluidity" of cell membranes rather then actually damaging it then you would expect the permeability to end with the ultrasound application. Nokazi *et al.* (2003) used lidocaine and heat, both known to increase membrane fluidity, in conjunction with USMGT *in vitro* and found that increased membrane fluidity did increase gene expression. They also found that increasing dosages of lidocaine increased cell viability, possibly by facilitating membrane repair of any shear stress damage that did occur. It is clear that further work is needed to elucidate the mechanism behind ultrasound-mediated gene transfer.

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