Application of Low Frequency Focused Ultrasound Waves Ripen the Rat Cervix During Pregnancy

by

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ABSTRACT

The object of this study is to characterize the effect of focused ultrasound stimulation (FUS) on the rat cervix which has been observed to speed its ripening during pregnancy. Cervical ripening is required for successful fetal delivery. Timed-pregnant Sprague-Dawley rats (n=36) were used. On day 14 of gestation, the FUS system was placed on the body surface of the rat over the cervix and ultrasound energy was applied to cervix for variable times up to 1 hour in the control group, the FUS system was placed on rats but no energy was applied. Daily measurement of cervix light-induced florescence (LIF, photon counts of collagen x-bridge fluorescence) were made on days 16 of gestation and daily until spontaneous delivery (day22) to estimate changes in cervical ripening. We found that pulses of 680 KHz ultrasound at 25 Hertz, 1 millisecond pulse duration at 1W/cm^2 applied for as little as 30 minutes would immediately afterwards show the cervix to have ripened to the degree seen just before delivery on day 22. Delivery times, fetal weights and viability were unaffected in the FUS-treated animals.

ACKNOWLEDGMENTS

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Chapter 1

INTRODUCTION AND LITERATURE

Cervical Ripening

Cervical ripening is necessary for successful vaginal delivery. It stays in a firm, rigid closed state during most time of gestation, once near delivery, it would switch into a soft, easy-to-open state to perform delivery. However fail to ripen the cervix will lead to protracted labor, which will cause substantial maternal and fetal morbidity. In preterm birth it is often not soft enough to insure delivery without complications. This situation can result in cervical injury, excessive uterine activity, over-distended uterus, and vaginal bleeding.

Methods of cervical ripening have historically used drugs such as steroid hormones (in particular progesterone and estrogen) (Glassman W. et al., 1995; Clark K. et al., 2006), cervical ripening also involved other hormones and mediators like dihydrotestosterone (Ji H. et al., 2008), prostaglandins (Shi L. et al., 2000), and local mediators such as platelet-activating factor (Maul H. et al., 2002) and nitric oxide (Shi L. et al., 2000).

The FDA has approved the use of progesterone in the situation where the woman has had a history of spontaneous preterm delivery. And the progesterone is the only drug that has been approved for preterm birth (Norwitz, E. et al., 2011).

Preterm Birth

Preterm birth (<37 completed weeks of gestation) is one of the major challenges and problems faced in obstetrics and gynecology. Approximately 12-13% preterm birth happened in United States (Goldenberg R, 2008; Steer P, 2005). Efforts to prevent preterm birth have mostly unsuccessful (Norwitz, E. et al., 2011), Preterm birth accounts for 85% of all prenatal morbidity and mortality (Norwitz et al., 2011). The causes for preterm births are varied such as preterm premature rupture of membranes, iatrogenic, infection, and spontaneous preterm labor (Lockwood C.J. et al., 2001; Ananth C.V. et al., 2006).

Ultrasound in Medicine

Ultrasound has been widely used in medical applications for obstetrical imaging of the fetus and provides valuable diagnostic information. Imaging systems can visualize the cervix and provide dilation measurements that can be predictive of preterm births (Iams J.D. et al., 1996; Heath V.C. et al., 1998). Ultrasound imaging has an excellent safety record. The ultrasound power levels used in obstetrics are low and far below those limitation that may cause bioeffects such as tissue heating or cavitation by vibration.

Ultrasound at much higher power levels is used therapeutically in a number of medical applications such as in tissue warming for treatment of arthritis, and injuries of tissue. These applications use different frequencies and pulse characteristics to achieve these effects.

Ultrasound is also known to evoke bioelectrical effects on excitable tissues such as nerve and brain through thermal and/or mechanical mechanism (Dalecki D. et al., 2004; Dinno M. A. et al., 1989; O'Brien, 2007; ter Haar, D. et al., 2007; Yoo S.S. et al., 2011; Tyler W.J. et al., 2008).

Ultrasound power levels are regulated by the FDA, particularly in the case of imaging systems used in obstetrics (CDRH. 2008).

This work is based on an unexpected observation that ultrasound produced cervical ripening effects on timed pregnant rats. Ultrasound has previously not been known to produce effects on the function of bodily organs or the function of the cervix. This work examines the effects of a range of ultrasound pulse characteristics such as power level; pulse durations; pulse repetition rate in an effort to understand the optimal parameters that induce cervical effects using the lowest possible ultrasound energies.

The objective of the current study is to determine whether the FUS on rat cervix could cause the cervical ripening. Such an effect could be useful in methods of promoting cervical ripening without drugs.

Chapter 2

BACKGROUND

Cervical Ripening

The cervix is a well-innervated component of the female reproductive tract, showed in figure 1. It consists mostly of connective tissue including collagen, elastin and the macromolecular components that make up the extracellular matrix (ECM). The extracellular stroma of the cervix is composed with type I and type III collagen (Kleissl HP et al., 1978) elastin (von Maillot K et al., 1981), held together by an amorphous matrix composed principally of proteoglycans (PGs), glycoproteins and water (Danforth, 1983; Yu and Leppert, 1991). Collagen in cervix is mainly provides tensile strength, elastin imparts elasticity, and the ECM contributes to the integrity of the tissue. Smooth muscle is distributed through the cervix. However the ratio of smooth muscle to collagen is widely variable across species e.g. the rat, is composed by higher proportion of smooth muscle than humans (Harkness and Harkness, 1959), the structure of the cervical extracellular matrix regulates the ability of the cervix at the beginning of gestation, it need to resist tension and firm during pregnancy. However, the cervical function is required to change to accommodate stretch and delivery (Leppert, 1992), from a firm rigid and closed state to a soft and easy-to-open state, which is necessary to successful vaginal delivery. Light and electronmicroscopy studies showed the decrease in collagen cross-link and collagen fiber disruption (Theobald

PW et al., 1982; Uldbjerg N et al., 1981) which might associate with the microstructural mechanism for cervical softening.

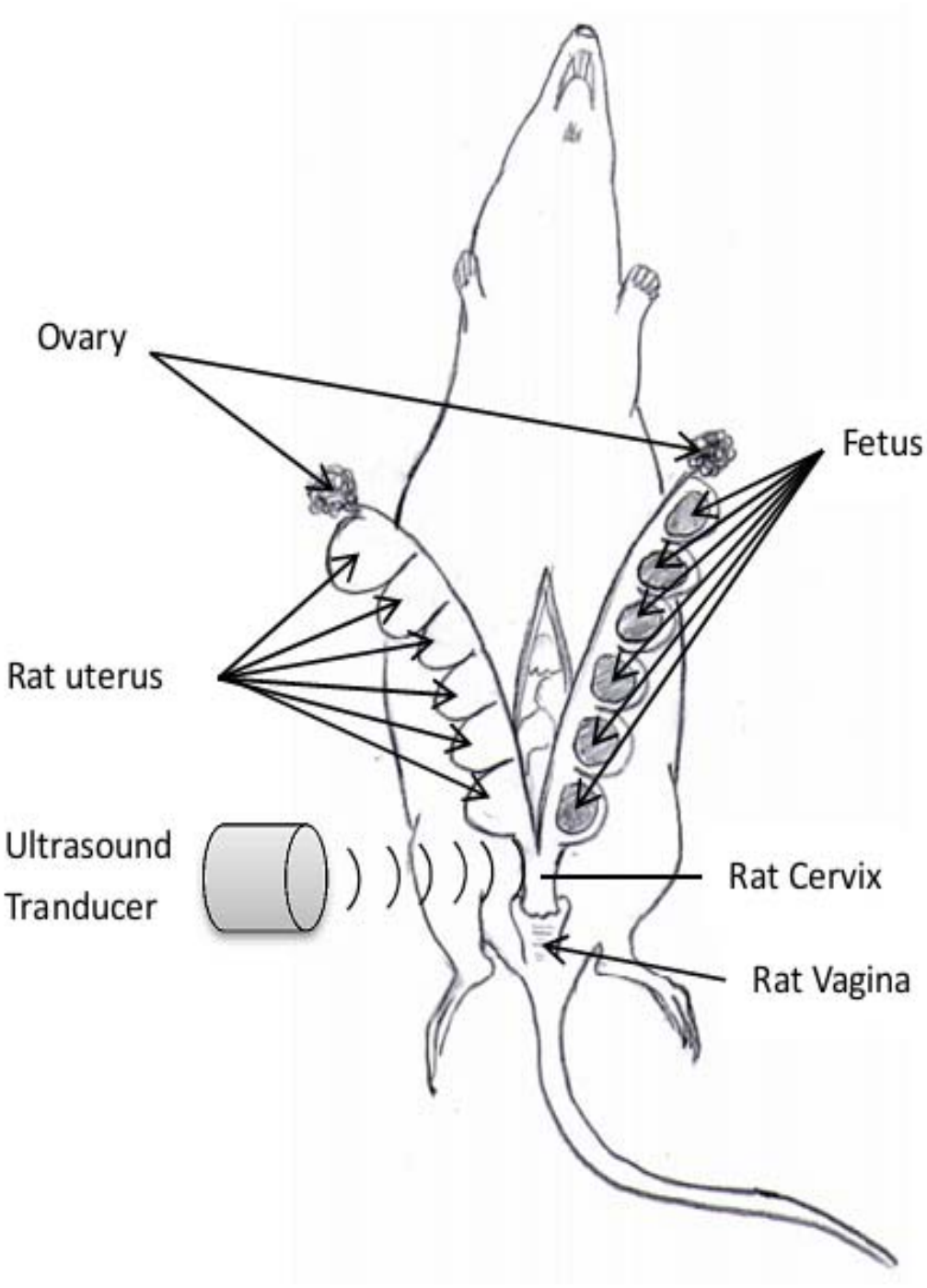


Figure 1. Reproduction system of pregnant rat.

Cervical ripening, which is necessary for delivery, is associated with a strong reorganization of collagen in extracellular matrix. The concentration decrease by 30-70%, besides, the form of collagen would switch from insoluble to more soluble collagen (Uldberg N. et al., 1983; Rechberger T. et al., 1988). However, the underling mechanism of these factors and the sequence of events that regulates cervical functions are still unknown.

Various methods have been used to evaluate this process, including measuring cervical length physically by measuring the extensibility and clinically by visual, digital or ultrasound graphing (Facchinetti F. et al., 2007; DeFranco E.A. et al., 2007).

Previous studies showed that cervical collagen content and hence degree of ripening could be determined by the intensity of fluorescence emitted from the cervix when illuminated with green light. This method has the advantage of being a noninvasive methodology of determining cervical ripening that is rapid and convenient. This is a molecular effect that depends upon the auto-fluorescent property of cross-linked collagen.

Instruments are commercially available that employ an optical fiber probe to both illuminate and detect the resulting fluorescence. Figure 2 shows a photograph of the SureTouch Collascope (Reproductive Research Technologies, Houston, TX) used in this work.



Figure 2. SureTouch Collascope

We used light-induced fluorescent (LIF) to evaluate changes in cervical collagen in whether treated groups or non-treated groups, which has been used to analyze cervical changing during pregnancy before (Glassman et al., 1995; Garfield et al., 1998; Shi et al., 1999). However the biochemical mechanisms underlying the changing in the cervix is still unknown. Besides, we also used mechanical test to confirm changes of the cervical resistance and the treatment results.

Hypothesis of cervical sensitivity to ultrasound

1. Direct mechanical softening of cervix through vibration. There is a direct breaking of collagen bonds by mechanical forces of shear stretching and compression induced by ultrasound waves to cause the cervix softening.

2. Bioelectrical mediated activation through its ultrasound (Yoo S.S et al., 2011; Tyler W.J. et al., 2008) effects on ion channel. Ultrasound effects on nerve and ganglion innervating the cervix may relate to that. The sensitivity of nerves to ultrasound, which has been showed recently, that it may leads to neurotransmitter activated of tissue/ biochemistry related to enable collagen decomposition.

3. Activation of stretch sensitive cervical fibroblast cells. A reported effect of cyclic mechanical stretch is to cause cervical ripening (Takemura M. et al., 2005). We suggest that the hypnosis that cellular metabolic shifts in cellular metabolism induced by stretch triggers the alteration in tissue collagen biochemistry.

Ultrasound Bioeffects

Ultrasound is a high frequency mechanical wave with frequency exceeding the range of human hearing (>20 KHz). It can propagate through tissue to a depth and penetration that depends upon its frequency.

Diagnostic imaging US frequency is ranged from 1 to 15 MHz. Therapeutic US tends to near 1MHz (O'Brien, 2007). The FDA has tightly regulates power level of clinical ultrasound imaging systems.

80 years ago, ultrasound was first found to have an interactive effect on nerve tissue of frog and turtle (Harvey E.N. 1929).

Ultrasound for noninvasive local stimulation has been investigated for effects on humans (Gavrilov et al., 1995). These applications of ultrasound have been used to induce different motor response and somatic, hearing and other sensations. Recently, ultrasound has been reported to affect the brain tissue and parts of the nervous system (Tyler W.J. et al., 2008; Yoo S.S et al., 2011; Bystricksy,) and has modulation effects on cell membrane kinetics (Colucci V. et al,. 2009). Therefore, the possible mechanism for cervical ripening may be related to the interaction of ultrasound with cervical neural electrical sensitivity. TABLE 1 provided a comprehensive summary of many empirical studies of ultrasound evoked bioeffects.

Table 1. Summary of studies demonstrating ultrasound evoked bioeffects

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
Frog and turtle in vitro	Frog and turtle muscle and heart	Continuous wave	0.34 MHz	Unknown	Nerve and muscle stimulation	(Harvey E.N. 1929)
Dogs in vivo	Nerve	10 minute exposures	8 KHz	5W/cm2 dose;	Suppression of CAPs	(Anderson et al.195

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
						1)
Rats in vivo	Sciatic Nerve	0.38-.78 cm diameter exposure beam	1MHz	0.18-6W for 1 to 10 seconds	Evoked nerve responses cause toe movement; phenomena presented in terms of classical nerve excitability in terms of ultrasound intensity and time; rheobase and chronaxie established	(Mazoue et al. 1976)
Mice	Spine lumbar	Unfocused ultrasound 2.6 mm beam width; 1-100 second exposures	0.2-1.2 MHz	30-170 W/cm2	Paralysis	(Fry and Dunn 1955)
Frog in vitro	Muscle bicep	40 second exposures	1MHz	60-170 W/cm2	Suppression of muscle CAPS; dependent upon number of exposures	(Welkowitz and Fry 1956)
Cat in vivo	Brain Visual Cortex	Not given		Not given	Suppression of visually evoked	(Fry et al.195

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
					responses	8)
Frog in vitro	Sciatic Nerve	Focused ultrasound; focal half-power spot of 5 mm;	1-3MHz;	Intensities of 16-35 W/cm2	Enhancement and suppression of evoked compound action potential events; modifications dependent upon ultrasound intensity	(Takagi et al.1959)
Toad in vitro	Spinal cord dorsal and ventral roots	Focused ultrasound; focal half-power spot of 5 mm; exposures of 1-20 seconds	1-3 MHz;	intensities of 3-70 W/cm2	Enhancement and suppression spontaneous dis charges and latency changes produced by ultrasound stimuli; modifications dependent upon ultrasound intensity and exposure times	(Takagi et al.1959)
Frog Tadpole in vitro	Brain	Focused ultrasound; focal half-	1-3 MHz	Intensity of 9.3 W/cm2	Enhancements and suppression of brain activity; modifications	(Takagi et al.1959)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
		power spot of 5mm			dependent	
Cat in vivo	Spinal cord Lumbar Region	Single bursts of focused ultrasound of 300 ms duration every 1-2 seconds; focal spot 1-3mm; Exposure of 60 seconds;	2.7 MHz;	Intensity unknown	Enhancement and suppression of evoked monosynaptic and polysynaptic spinal reflex recordings; Effect reversible.	(Sheally and Henneman 1962)
Awake Animal	Brain visual cortex	Focused ultrasound			Reversible block of the visual cortex	(Adrianov et al.1960)
Cat in vitro	Nerve	Not given		Not given	Suppression of evoked compound action potential components; Differential effects on A,B and C fibers; Effects	(Young R. and Henneman E. 1961)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
					dependent upon total ultrasound dosage	
Frog in vitro	Sciatic Nerve Preparation	Focused Ultrasound pulses for 0.14-1.2 seconds; single pulse to 20 consecutive pulse employed; focal spot of 0.8mm2;	2.7 MHz	Maximum intensity of 1150 W/cm2	Suppression of evoked compound action potential components; Differential effects on A,B and C fibers; Effects dependent upon total ultrasound dosage; Large dosages produced irreversible effects	Young and Henneman 1961
Worm Cat Monkey Man in vitro	Nerve	Focused Ultrasound	5-3 MHz ultrasound;	Average Intensities of 2-42 W/cm2	Enhancement and suppression of action potential events; Dose dependent	(Lele PP., 1963)
Human in vivo	Ulnar Nerve	7cm2 exposure area	870 kHz	0.5 to 3.0 W/cm2	Nerve conduction velocity	(Farmer 1970)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
					increase and decrease; intensity dependent	
Monkey in vivo	Brain parietal lobe	Continuous wave exposure or 2 us bursts at 1kHz repetition; exposure area of 0.7-2.4 cm2	2.25 - 5 MHz	average acoustic intensities of 3-900 mW/cm2	Produced evoked potentials in EEG	(Hu and Ulrih 1976)
Human in vivo	Hand	1-100ms pulse;	0.48 -3.0 MHz	10-17000 W/cm2 peak intensities	Induction of pain sensations; Hot and Cold Sensations	(Gavrilov 1974)
Snail in vitro	Hypopharyngeal Ganglion/statocyst	Single bursts; focused ultrasound; 1 ms pulse; focal spot of 1.6mm; Exposure of 60 seconds;	2.25 MHz	Peak intensities of 2000 W/cm2	Increase and decrease in discharge activity of ganglion and statocyst cells;	(Gavrilov 1978)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
Cat in vivo	Brain auditory cortex	0.6cm diameter exposure beam;	5MHz frequency	70 us second pluses at 30 W/cm2	Evoked responses in auditory nerve	(Foster et al., 1978)
Human in vivo	Spinal roots	5cm2 exposure area; 20 second exposures	1 MHz	2 W/cm2	Produces pain in buttocks	(Cole and Douglas 1980)
Human in vivo	Ulnar Nerve	Flat transducer; 4.1 cm diameter beam;	870 kHz	0.5 to 2.0 W/cm2	Nerve conduction velocity increase	(Kramer 1985)
Aplysia in vitro	Central Ganglia	2 ms Ultrasound bursts; .16 cm2 irradiation area; 3Hz repetition for 30 seconds	2.4 MHz	60 mW/cm2 power density;	Increase in the firing rate of ganglia neurons; Effect reversible after 150 seconds	(Mori et al.1987)
Cat in vivo	Brain visual cortex	2.5cm diameter exposure beam;	1MHz frequency	Spatial and temporal average intensities of 1.0 W/cm2	Ultrasound prevents depression of visually evoked potentials in hypoxic cats	(Foster et al. 1987)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
Rabbit in vivo Cat in vivo	Brain cortex	Focused beam; 0.1-100 ms pulses are 1-20 pulses/sec; exposures 1 to 60 seconds		Intensities of 1 uW/cm2 to 1400 W/cm2	Enhancement and suppression alterations in ECoG recordings; Effect intensity dependent	(Velling and Shklyaruk 1988)
Rat in vitro	Heart	5.4mm exposure area	0.543 MHz	3 W/cm2 continuous wave	Depolarization of membranes; loss of excitability and resting tension	(Zakharov et al.1989)
Frog in vitro	Sciatic Nerve	Single bursts of focused ultrasound for 5ms; focal half-power spot of 3mm	1-7MHz	Pulse average intensities of 50-800 W/cm2	A fiber enhancement and B fiber suppression in evoked compound action potential(CAP)recordings; Effect reversible; Excitability changes occur in 0-50 ms window following exposure	(Mihran et al.1990a;1990b)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
Lobster in vitro	Ventral Cord	Single bursts of focused ultrasound .5ms;focal half-power spot of 3mm	2 MHz	pulse average intensities of 100-800 W/cm2	Changes in membrane rest potential and conductance recordings;reversible;changes occur in 0-35ms window following exposure;	(Mihran et al.1990c)
Frog in vitro	Nerve-muscle	Continuous wave exposure	1.5 MHz	250 mW/cm2 spatial-average temporal-average intensity	Increase in postsynaptic motor end-plate potential discharges; Effects dependent upon exposure conditions	(Revell and Roberts 1990
Rat in vitro	Hippo-campus (Brain slices)	Pulsed, focused ultrasound; 1 mm spot size; train of pulses at 20% duty cycles	0.5 - 5 MHz	Spatial peak temporal average intensity of 40-110W/cm2;	Enhancement of orthodromic cell population spikes;Suppression of antidromic cell population spikes; Reduction in fiber volley; Enhancement of dendritic responses	(Rinaldi et al.1991; Bachtold et al.1998)

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
Frog Mice in vivo	Heart	Single 1-5ms pulse	1.2-3.7 MHz	Spatial peak averages of 350-1000W/cm2	Premature ventricular contractions	(Dalecki et al.1993;1996a;1996b)
Mouse in vitro	Sciatic Nerve	Single bursts; focused ultrasound for 5ms;focal spot of 3mm	2 MHz	Pulse peak intensities of 50-800 W/cm2	A fiber suppression of evoked CAP recordings; suppression of myocardium strips; Effect reversible; Excitablility changes occur in 0-30 ms window following exposure;	(Mihran et al.1996)
Human in vivo	Ulnar Nerve	3ms pulses; 1Hz repetition	5 MHz;	.153 W/cm2;	No effect	(Rutten et al.1996)
Rat in vitro	Nerve-Sciatic	Single bursts of focused ultrasound for 5 ms;	1-7 MHz;	Pulse average intensities of 4.5 W/cm2	Enhancement of compound action potentials	(Rutten et al.1996)
Frog in vitro	Sciatic Nerve	Ultrasound pulses .5-75ms second	10-100 MHz	Peak intensities of 200 W/cm2	Suppression and Enhancement of evoked CAPs	(Phillips 2002a;2002b;200

Summary of studies demonstrating ultrasound evoked bioeffects						
Species	Tissue	Protocol	Frequency	Intensity	Tissue Response	Reference
		s; 1-20 Hz repetition rates;				2d)
Model	Nerve	Single pulses of 10 us ultrasound; frequency of 5 MHz;			Evoked action potential in presence of a magnetic field	(Edrich and Zhang 1993)
Mouse brain in vitro	hippocampal slice cultures	Tone burst	0.44 - 0.67 MHz	2.9 W/cm2	Evoke action potentials and neurotransmitter release	(Tyler W.J. et al., 2008)
Mouse in vivo	Mouse cortex and hippocampus	Pulse mode	0.25 -0.5 MHz	0.75-0.229 W/cm2	Evoke action potentials and synchronous oscillation. Neurotransmitter release	(Tufail Y. et al., 2010)
Rabbit	Craniotomized rabbit cortex	Continous wave	0.69 MHz	3.3-12.6 W/cm2	Stimulation of motor cortex; visually evoked	(Yoo S.S et al., 2011)

Chapter 3
MATERIALS AND METHODS

Acoustic Intensity Calculation

The pulse intensity integral (PII) is defined as:

$$\mathrm{PII} = \int \frac{p^2(t)}{Z_0} dt$$

Where p is the instantaneous peak pressure, Z_0 is the characteristic acoustic impedance in Pa·s m-1 defined as ρc, where ρ is the density of the medium and c is the speed of sound in the medium. We estimate ρ to be 1028 kg m-3 and c to be 1515 m s-1 for brain tissue on the basis of previous reports (Lugwig G.D. 1950).

Spatial-peak pulse-average intensity (I_{SPPA}) is defined as:

$$\mathrm{I_{SPPA}} = \frac{PII}{PD}$$

Where PD is the pulse duration defined as (t) (0.9PII—0.1PII) 1.25 as outlined by technical standards, which is established by the American Institute for Ultrasound in Medicina and National Electronics Manufacturers Administration (NEMA, 2004).

Spatial-peak temporal-average intensity (I_{SPTA}) is defined as:

$$\mathrm{I_{SPTA}} = PII(PRF)$$

PRF is pulse repetition frequency, which is represented in Hertz.

The Mechanical Index was defined as: $\mathrm{MI} = \frac{p_r}{\sqrt{f}}$ where p_r is the peak rare-factional pressure and f is the acoustic frequency.

Ultrasound I_{SPPA} was measured by a force balance as shown in figure 3.

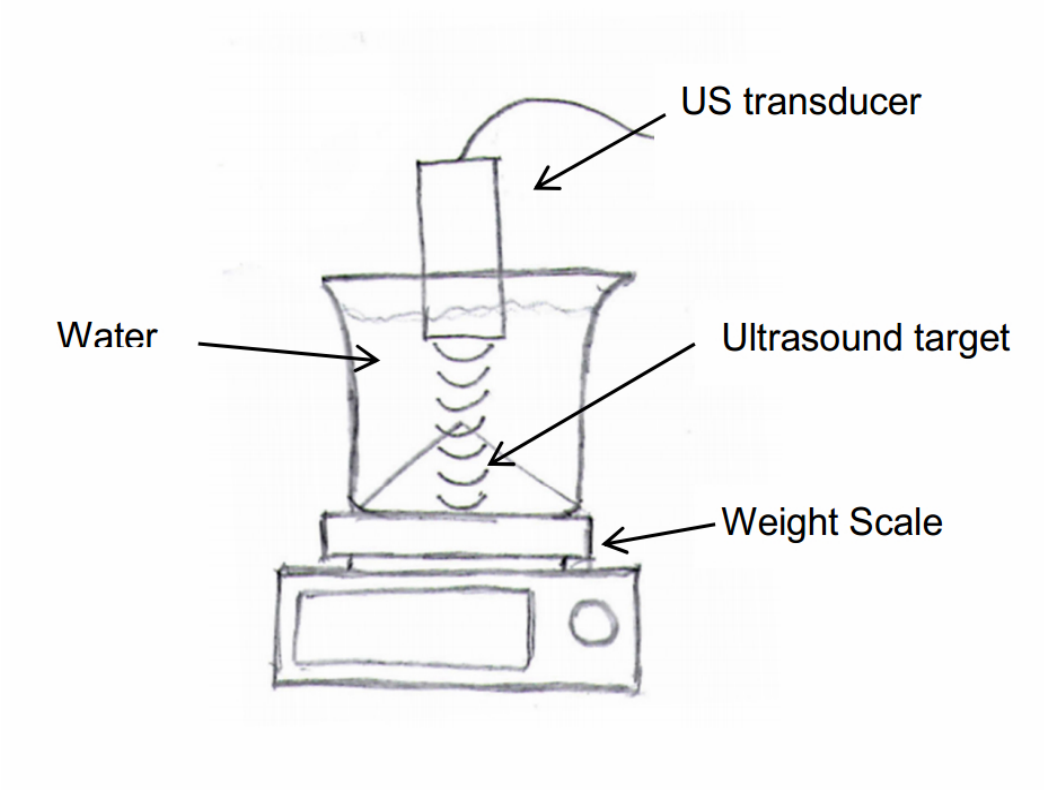


Figure 3. Method of measuring ultrasound power level

The downward force from ultrasound transducer will be showed on a weight scale (AE 240, METTLER, Inc.).

Ideally when measuring ultrasound radiation pressure, we would run continuous wave (CW) mode on transducer. The downward force could be directly showed in mass value (in grams) on weight scale. Once the radiation force of sound beam from transducer is observed, this power of sound beam (in watts) can be calculated:

$$W_{peak}(\text{watts}) = \text{Force (Newton)} * c \text{ (m/s)}$$

Where c is the velocity of sound in the medium, for water c = 1500 m/s. Force is a calculated according to Newton's law, the acceleration in equal to gravity, which is 9.8 m/s.

Force = mass * acceleration

For example, in this method, the scale reading in grams in converted to watts by multiplying 15.

Once the peak power is known, the average sound intensity at the face of the emitter can be calculated by dividing surface area of the transducer's emitter.

Intensity = W_{peak} / Area

The reflectivity coefficient for a normally incident sound beam between two media is given by:

$$\mathrm{R} = \frac{Z_1 - Z_2}{Z_1 + Z_2}$$

Where Z_1 and $Z_2$are the acoustic impedances of two different propagating media.

Another sound property is attenuation. The following equation described the exponentially decaying nature of sound in tissue, which is related to sound pressure by its attenuation coefficient (α):

$$\mathrm{I} = I_0 e^{-x\alpha}$$

Where I is the decayed value at some distance x in the medium, and I_0 is the initial sound intensity.

Experimental set-up

Focused Ultrasound (FUS) stimulation system was set up based on Figure 5: a Stanford Research Systems DS-345 function generator provided the excitation waveform as showed in Figure 8, a customized ultrasound power amplifier was built to match low transducer impedance, and an ultrasound transducer (Figure 6) was made from a 5 cm PZT disk (Steminc Inc.) with a fundamental frequency of 0.682MHz. The transducer was coupled to a custom made spherically focused epoxy lens (West System Inc. part A: 105+part B: 206), molded around a sphere having a 6.5 cm radius. The resulting acoustic lens thus concentrated the power of the ultrasound into a focal spot, in this case assumed the roughly Gaussian with FWHM of approximately 5 mm diameter. Figure 4 showed the focal zone power distribution profiled by a Precision Acoustics hydrophone (Devonshire, UK) connected to an oscilloscope (TDS 2024 Tektronix, Inc.). Its output power was measured in a CW mode using the method presented before.

The ultrasound transducer was applied through a water coupling column and acoustic coupling gel to the rat's abdominal skin surface over the cervix region as determined by palpation of an internal probe placed at the cervical entrance. The ultrasound beam passed through the annulus of the cervix in a parallel incidence to the plane of the cervix. At 680 KHz the ultrasound energy passes substantially though the tissue with minimal

attenuation. A thermocouple was inserted into the vagina to measure temperature rise.

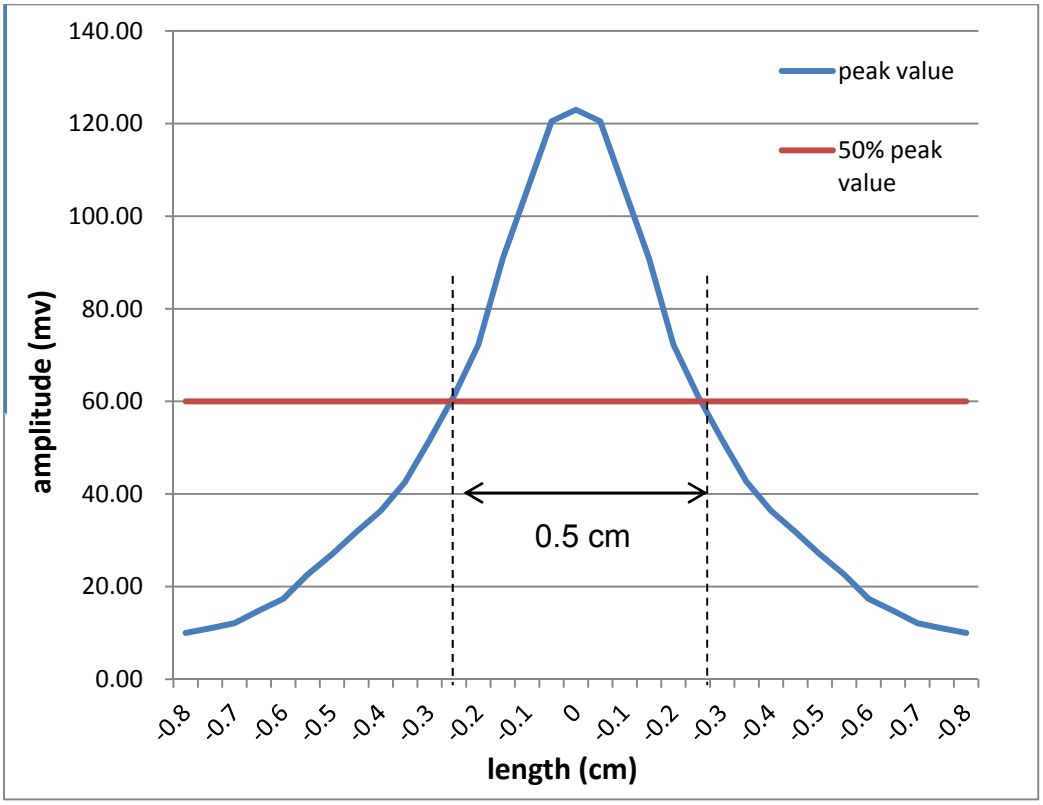


Figure 4. Focal zone power distribution profile

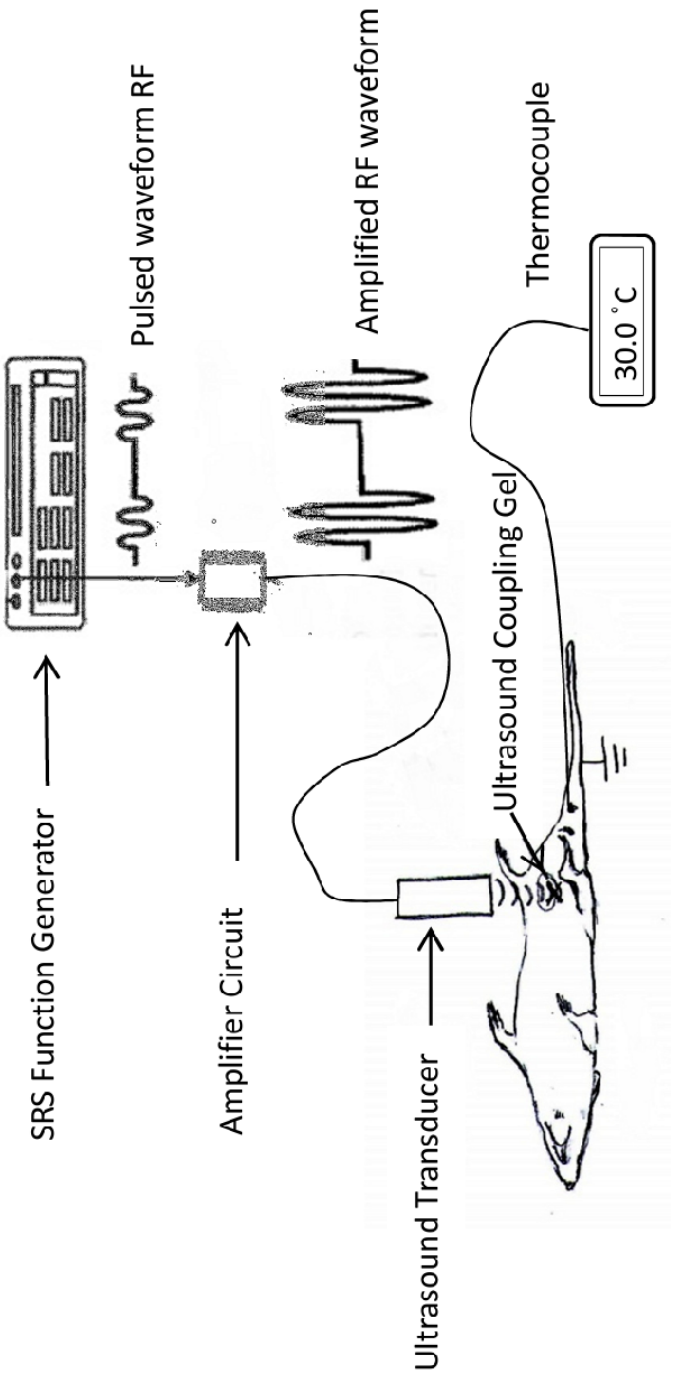


Figure 5. Experiment set-up

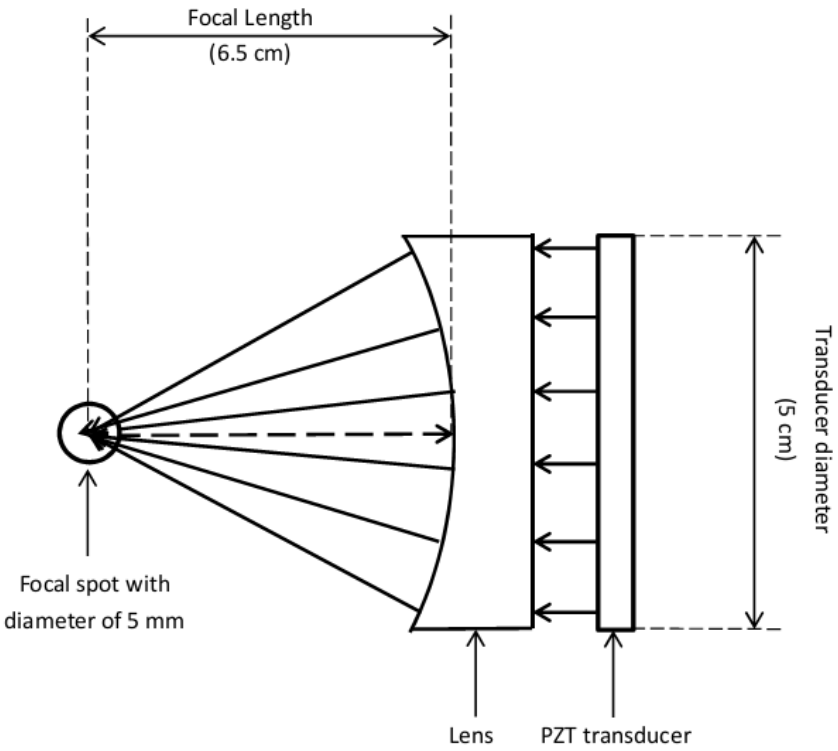


Figure 6. Structure of PZT transducer

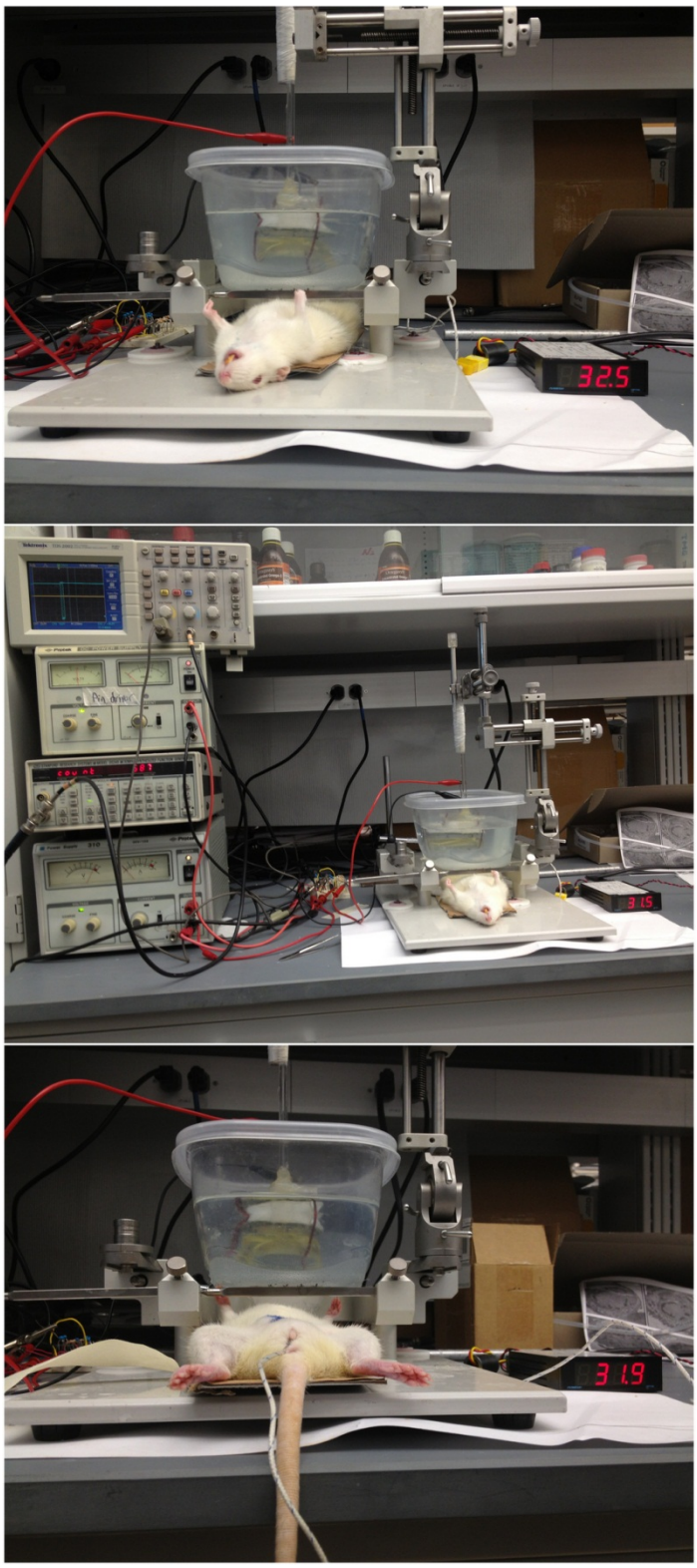


Figure 7. Photos of ultrasound stimulation in vivo

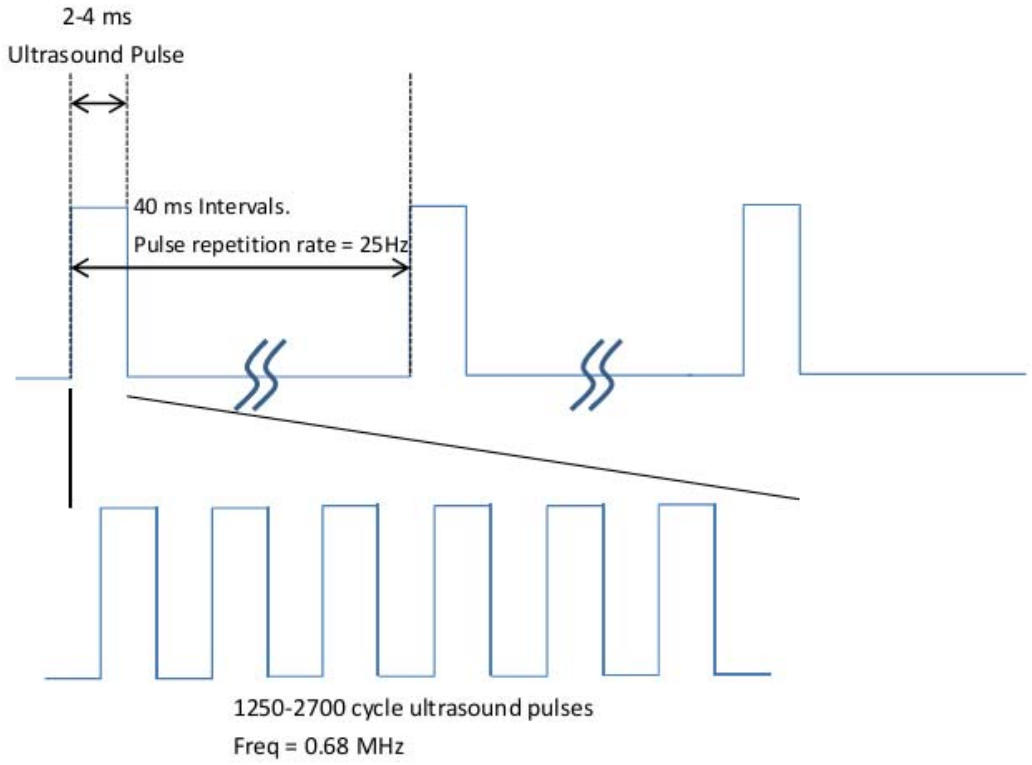


Figure 8. Ultrasound waveform

Ultrasound stimulation parameters

The experiment tested a range of ultrasound pulse parameters. All experiments used pulses of 680 KHz ultrasound at a rate of 25 Hertz of pulse repetition. The pulse durations varied from 1 millisecond to 4 milliseconds. Ultrasound power levels were calculated to be 1W/cm^2 to 4 W/cm^2 respectively with approximately 80 volts on the transducer. The duration of ultrasound application tested was in the range of 30 minutes to 1 hour.

Animals

Timed-pregnant Sprague-Dawley rats (Charles River Laboratoried, Wilminton, MA, USA) were housed separately. The rats were maintained

on a constant 12 hour light and 12 hour dark cycle. The pregnant rats have a 22 days gestation cycle, day 1 being the day on which the sperm plug was observed. While doing Focused ultrasound stimulation, the animals were anaesthetized with a combination of xylazine (Germin, Burns Veterinary Supply Inc, Rockville Center, NY, USA) and ketamine HCl (Ketaset; Fort Dodge Laboratories Inc, Fort Dodge, IO, USA) based on their weight 1µl/g. They were killed by surgical dislocation for cervical tissue collection. All procedures were approved by the Animal Care and Use Committee of the St. Joseph's Hospital and Medical Center in Phoenix.

Procedure

The cervixes of (n) rats were exposed to ultrasound according to the Table 2.

Table 2. Number of rats for experiments

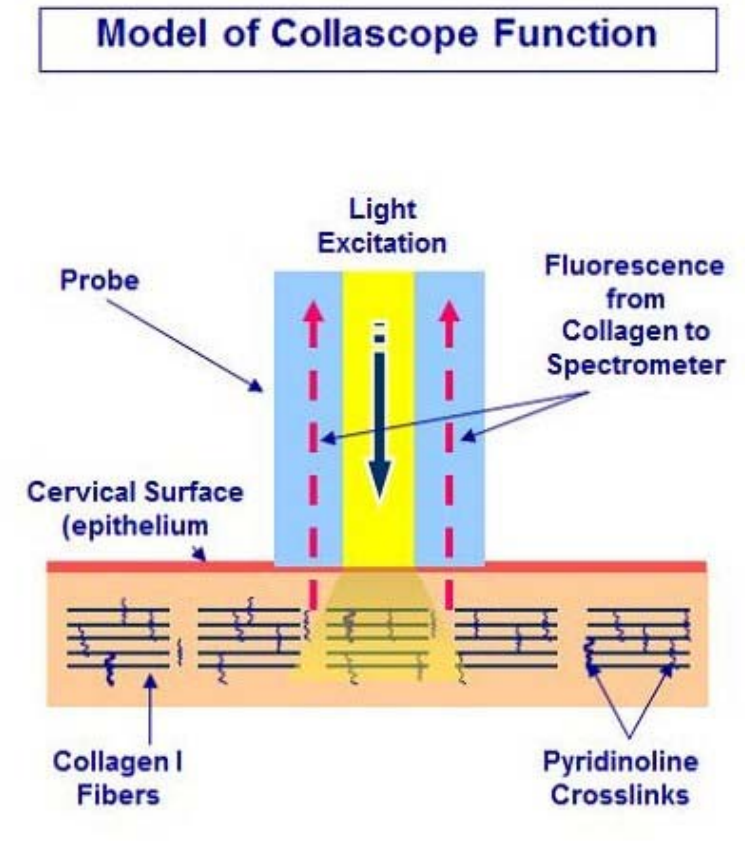
Day of gestation	Stimulation time	Frequency	Power level	Number of rats
D15_Control	NR	NR	NR	10
D15	1 hour	680 KHz	4 Watts/cm^2	9
D15	0.5 hour	680 KHz	4 Watts/cm^2	2
D14_Control	NR	NR	NR	5
D14	0.5 hour	680 KHz	4 Watts/cm^2	4

Day of gestation	Stimulation time	Frequency	Power level	Number of rats
D14	0.5 hour	680 KHz	2 Watts/cm^2	4
D14	0.5 hour	680 KHz	1 Watts/cm^2	2
TOTAL				36

Collagen Test

In this work we use light induced fluorescent to evaluate the changes in the elasticity of the cervix over time during gestation (Maul H. et al., 2002; Fittkow CT. et al., 2001). Before FUS ultrasound treatment, LIF measurements were tested for both treating and control group. 1h after the beginning the FUS ultrasound treatment, we performed the LIF test again. From the day we applied the treatment, we did the LIF measurement every 24h until their spontaneous delivery. The total number of cervical collagen was measured in vivo by an instrument, termed collascope (Reproductive Research Technologies, Houston, TX), it could measure the auto-fluorescent properties of cross-linked collagen (). A speculum with optical probe was inserted into the vagina of the anesthetized animal, once the optical probe touched the surface of the exit of cervix, the main unit connected through an optical fiber cable delivers excitation light (wavelength, 339 nm) onto the cervix. The reflection light carries the fluorescent light (mainly caused by pyridinoline cross-links of collagen with a maximum wavelength peak at 390 nm) back the

instrument to a charge-coupled device camera to display the full spectrum of the reflection and the number of photon count (fluorescent intensity) that are emitted by cervix as showed in figure 9. In our study, the exposure time for excitation was 100 milliseconds. The average of 16 measurement of fluorescent intensity at 390 nm wavelength was used to evaluate collagen data for each animal at any time.



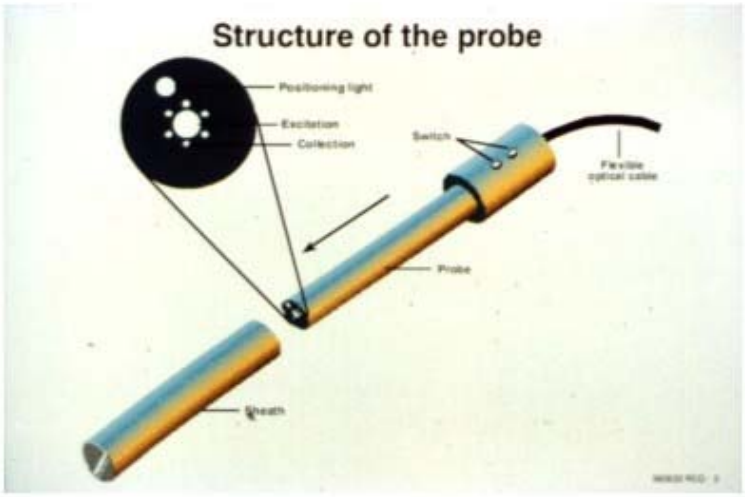


Figure 9 Function of collascope

Mechanical Test

In order to confirm the effects of focused ultrasound on the cervix mechanical compliance we conducted extensibility testing based on Figure 10. All tests were conducted on a universal Tissue Organ Bath System (750TOBS, DMT, Inc.) (Figure 11). Force data were collected with Organ Bath Software. The variation in strain is due to noise on force transducer. The whole cervix is defined as the least vascular tissue with two parallel lumina between the uterine horns and the vagina. Connective tissue and fat were removed and the cervix was suspended with its longitudinal axis vertically in a tissue bath chamber for tension recording. The chamber was filled with physiological Kreb's solution, bubbled with a mixture of 95% O_2 and 5% CO_2, and maintained at 37 ˚C. As showed in Figure 12 each canal of the cervix was inserted a hook. The lower side of the cervix was stable on the bottom of the chamber frame, while the upper side was connected to a force transducer, which was recorded to a computer. The isolated cervix was elongated incrementally at the rate of 0.015 mm/s. All the tension force was continuously recorded. As all elastic tissues have, each stretch resulted in a sharp rise in tension followed by an exponential decrease to a stable tension that was higher than the plateau at the previous length. The result curve will have a saw-tooth shape. The slope of the regression line through the linear portion of the length-tension curve was derived and defined as the indication of the

cervical extensibility. The slope of the length-tension curve is related to cervical resistance.

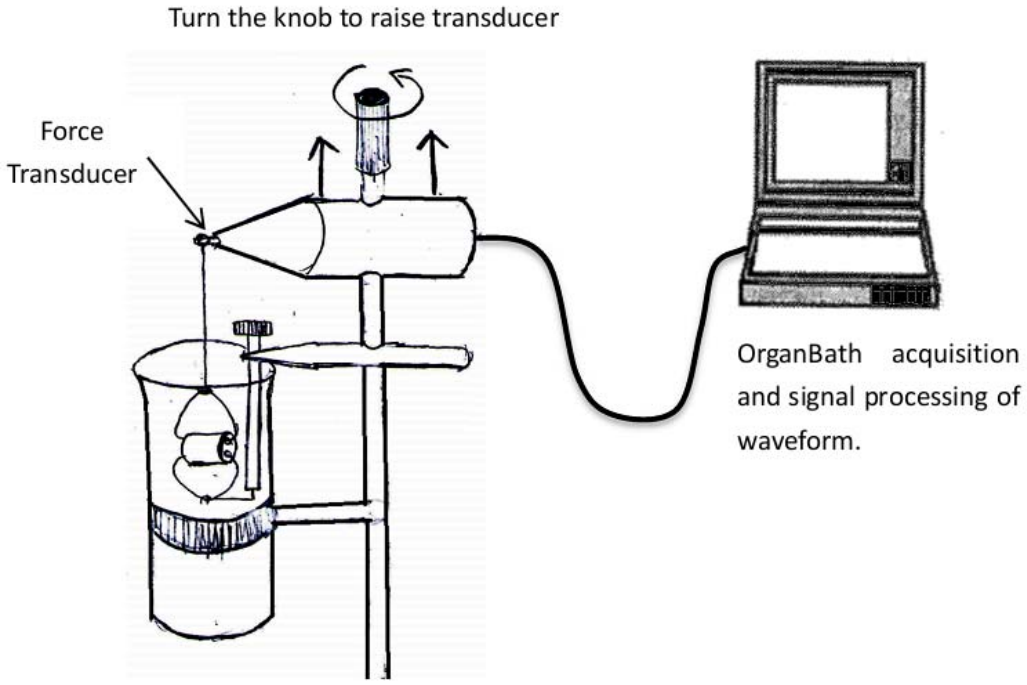


Figure 10. Mechanical test experiment set-up

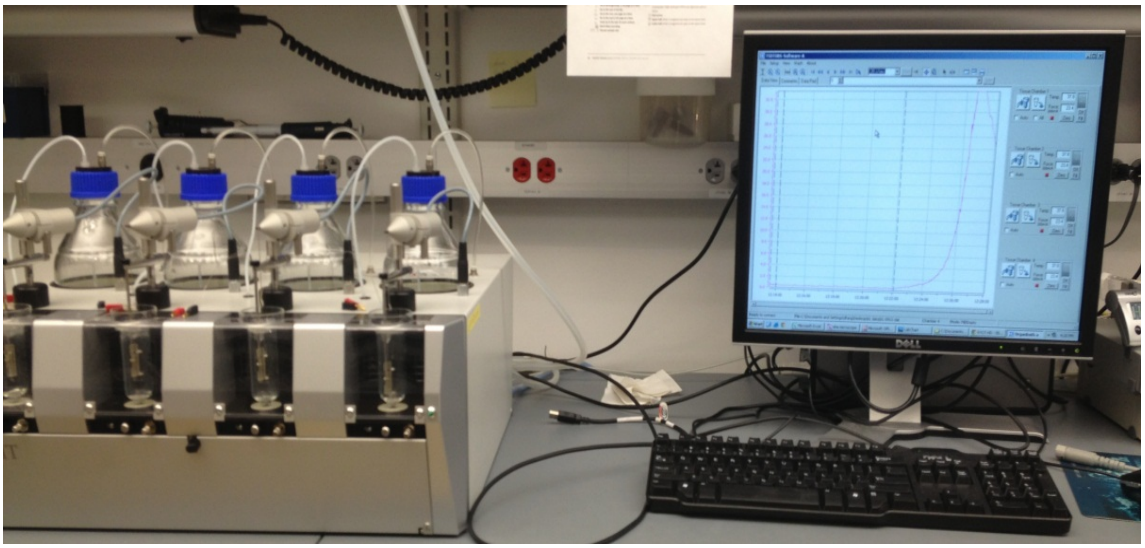


Figure 11. Tissue bath system



Figure 12. Photos of cervix mechanical test in vitro

Statistical Analyses

Statistical comparisons between two group data were estimated by unpaired student's t-test analysis. A 2-tailed probability value of $P<0.05$ was considered to be statistically significant different. Results are expressed as means ± SEM.

Determining the changes in delivery time

The time of delivery was plotted. Controls and treated groups were determined as hours after 8 AM of day 22 of gestation. The expulsion of 1 pup was defined as delivery.

Chapter 4

RESULT

Figure 13 plots the collagen percent as determined by the LIF for ultrasound treated (n=9) animals and for untreated control (n=10) animals for treatment duration of 1 hour at ultrasound pulse width for 4 milliseconds. This plot starts from gestation day 15 when FUS stimulation was applied till the day the rats spontaneously delivered on day 22.

The data, presented as mean ± SEM, showed treated group on day16 and d17 has a significant difference compared to controlled group. In control animals the cervical LIF values normally follow a decline from day 15 of gestation to day 22. There is then we observe a statistical variation in LIF values of that vary from 2064 ± 116 from day 15 to 22, reached the lowest levels during delivery (637 ± 133). However, LIF values are significantly lower in FUS animals on days 16 and 17 of gestation as compared to controls (day 16: 700 ± 237 VS control 1319 ± 241, day 17 503 ±231 VS control 1000 ± 178) and remain low until delivery.

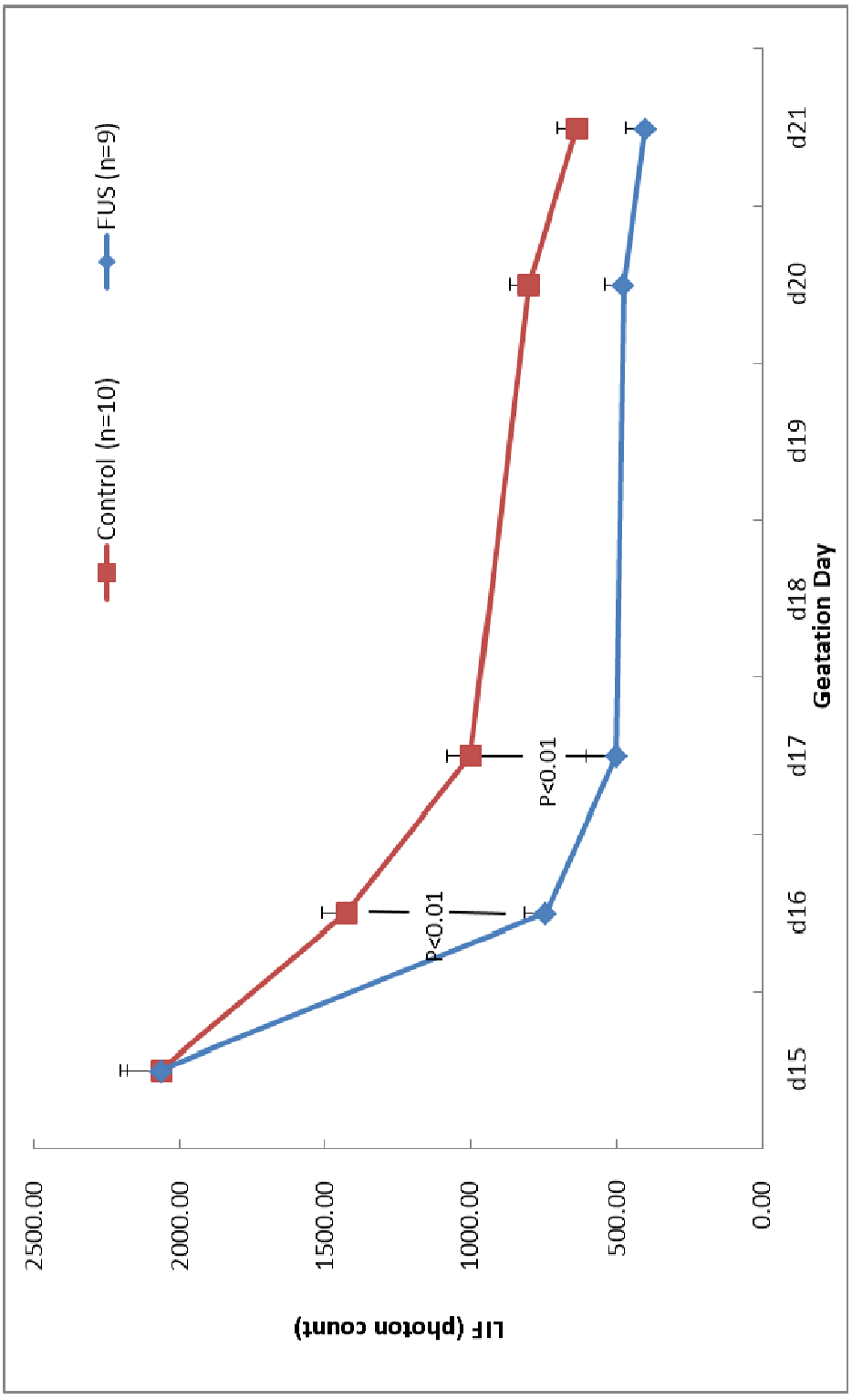


Figure 13. Daily LIF measurement during gestation in treated and control group

The 30 minutes and 1 hour FUS stimulation was applied on different day of gestation (d14, d15) with different power level (1W/cm^2 - 4W/cm^2). The collagen data was measured before stimulation, 1 hour after stimulation and 24h after stimulation. Control group was only treated without power. Cervix was collected after all collagen tests. Individual mechanical test about cervical resistance was performed. Figure 14 plotted out that the effect of 1W/cm^2 -4W/cm^2 FUS energy applied on pregnant rat of day 14. Significant difference was demonstrated 1 hour after stimulation. (LIF value (photon count): 1W/cm^2: 1726±330, 2W/cm^2: 1458 ± 103, 4w/cm^2: 1119 ± 89 compared to control: 2467 ±126). Figure 15 potted out the result of different stimulation duration on gestation day 15 with same power level of 4W/cm^2. (1hour: 806 ± 90, 0.5h: 897 ± 99 compared to control: 2059 ± 122). Figure 16 shows that the mean value of cervical resistance for 2 groups. Similar to the result above, with FUS treatment, the stimulation showed result on increasing cervical extensibility while the treated group significantly decreased cervical resistance

Compared to control group, FUS application to the cervix on day 14 and 15 of gestation we observe that collagen data and cervical resistance are all significantly reduced 1h after stimulation and remain low until 24h after stimulation, which is similar to the daily measurement result we showed before.

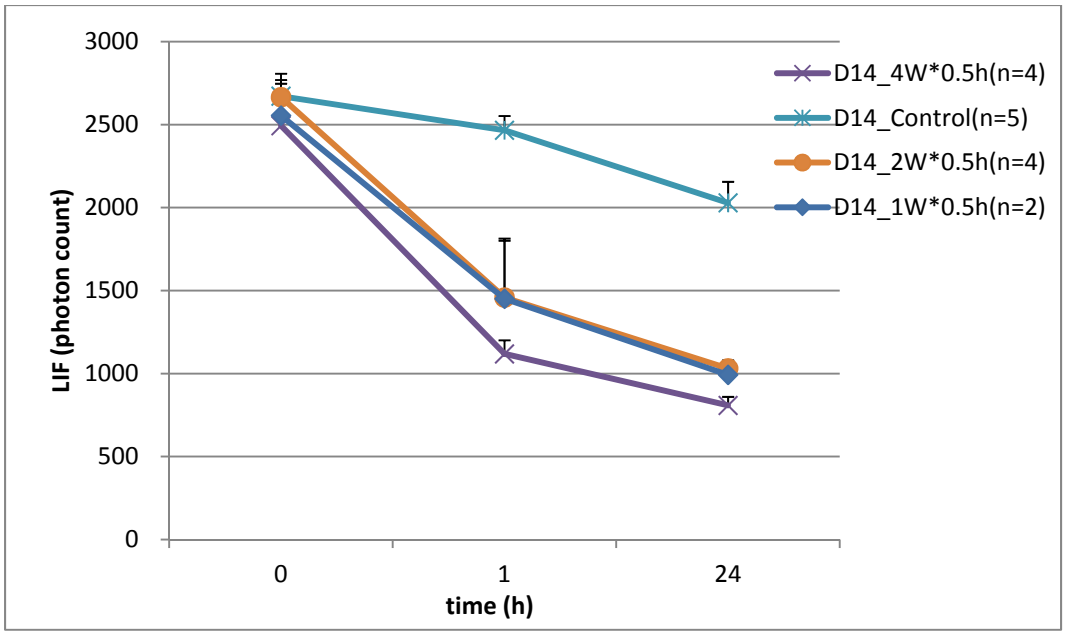


Figure 14. Changes in LIF after stimulation, measurements were performed 1 and 24 hours after stimulation

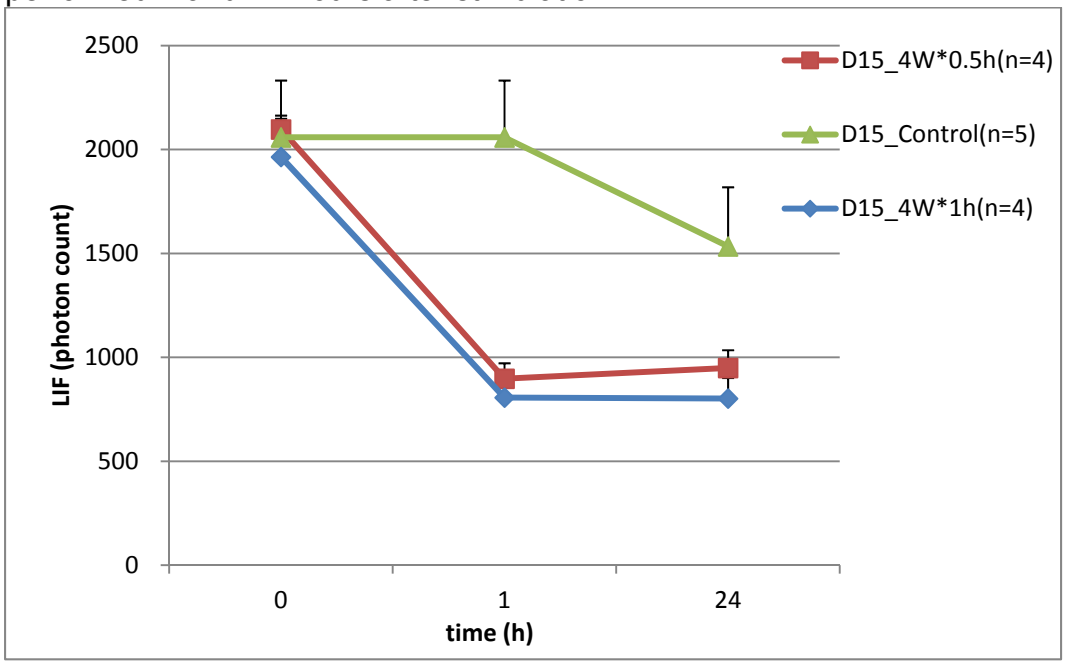


Figure 15. Changes in LIF after stimulation, measurements were performed 1 and 24 hours after stimulation

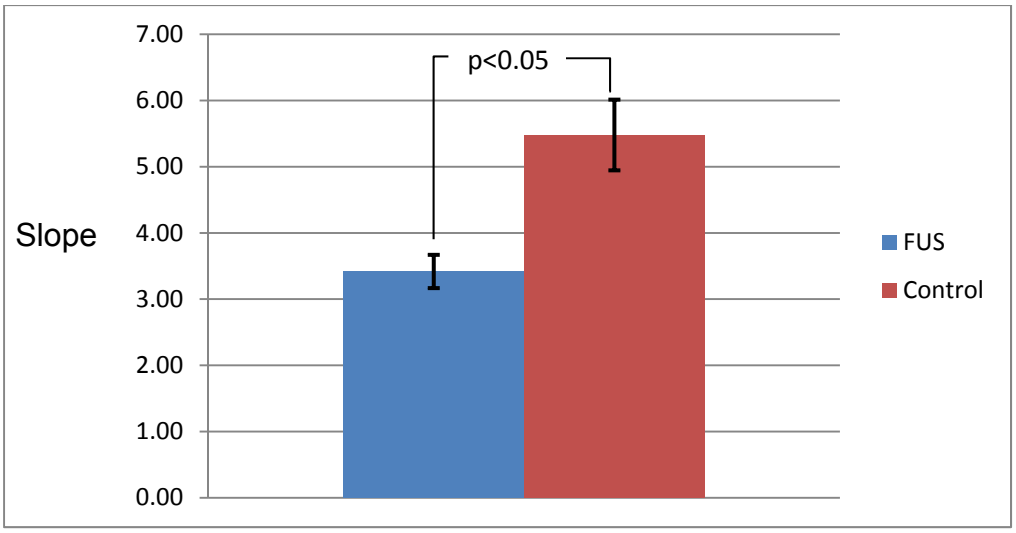


Figure 16. Cervical resistance for FUS and control group after stimulation

Figure 18 showed the average weight (grams) of fetus of control and treated groups. There is no significant difference between 2 groups (FUS: 5.81± 0.43 VS Control: 5.41± 0.25). The FUS energy was considered to apply just on the part of cervix, and has no influence on fetus. The photos of cervix were taken before and after FUS (Figure 19). There is no visual difference between them. No preterm births happen, control group and FUS treated group. All fetuses were delivered within 30 hours after 8 am of day 22. Even the cervix has been showed above to be softening already after stimulation. No hormone change during stimulation. This supports the safety of the ultrasound application.



Figure 17. Photo of fetus

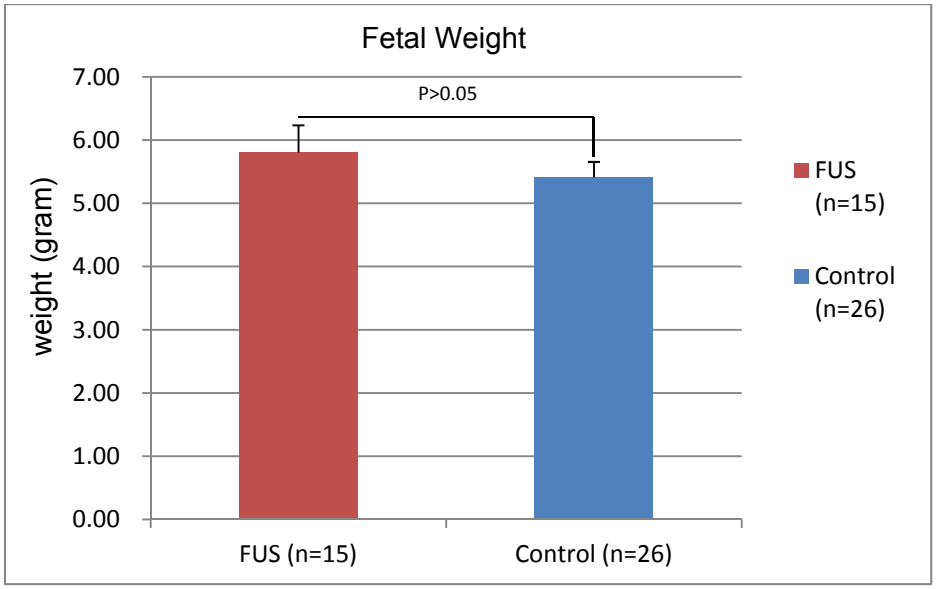


Figure 18. Fetal weight in FUS stimulated and control rats

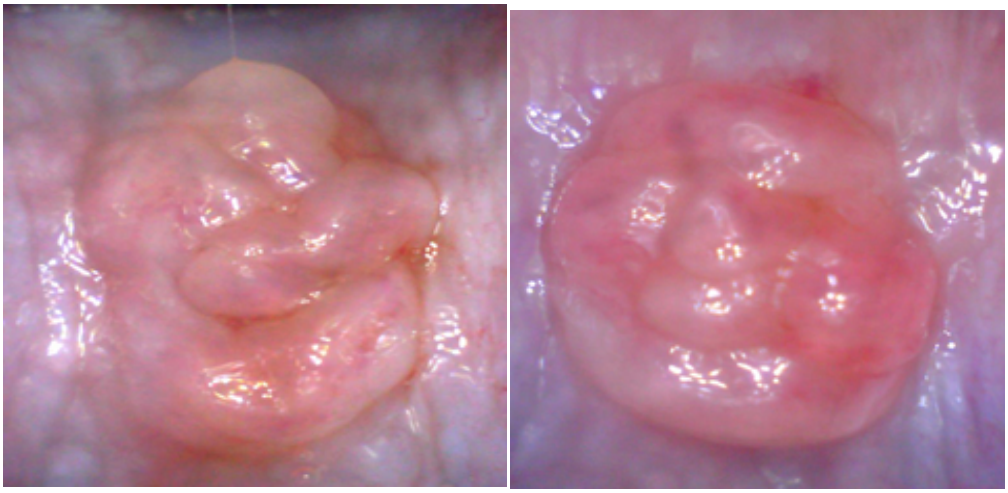


Figure 19 Photos of cervixs before(Left) and after(Right) FUS stimulation on day 14 of gestation

Chapter 5

DISCUSSION AND CONCLUSION

These studies show that focused ultrasound has an unexpected and significant effect on rat cervical ripening.

The softening of cervix normally is a gradual and progressive process occurring during the last half of gestation cycle, as showed in Figure 20 (Garfield, 1999; Shi, 1999). In this study, we find that the process of cervical ripening can be substantially speeded up to a level comparable to that of fetal delivery but nearly seven days early. The plot in Figure 20 shows that much high value of LIF occurring in early gestation. The ultrasound induced change is greater in day 14 than day 15, suggesting that the ultrasound application can cause a greater effect on collagen that is not fully ripened. Although the study has not investigated the effect of ultrasound earlier in gestation than day 14, further studies should look at effects at a much earlier in gestation. This would provide insight into whether ultrasound has similar effects on non-pregnant cervix or when ultrasound becomes first effective in the gestation cycle. Two trials (not shown) suggest the ultrasound effects on non-pregnant rats to be not a large enough to discriminate from the normal range of variability.

The mechanism for the physiologic effect of ultrasound is not clear. This effect occurs after application of sound for as little as 30 minutes and at ultrasound power levels as low as $1W/cm^2$. An immediate change supports the any of the three hypothesis presented above.

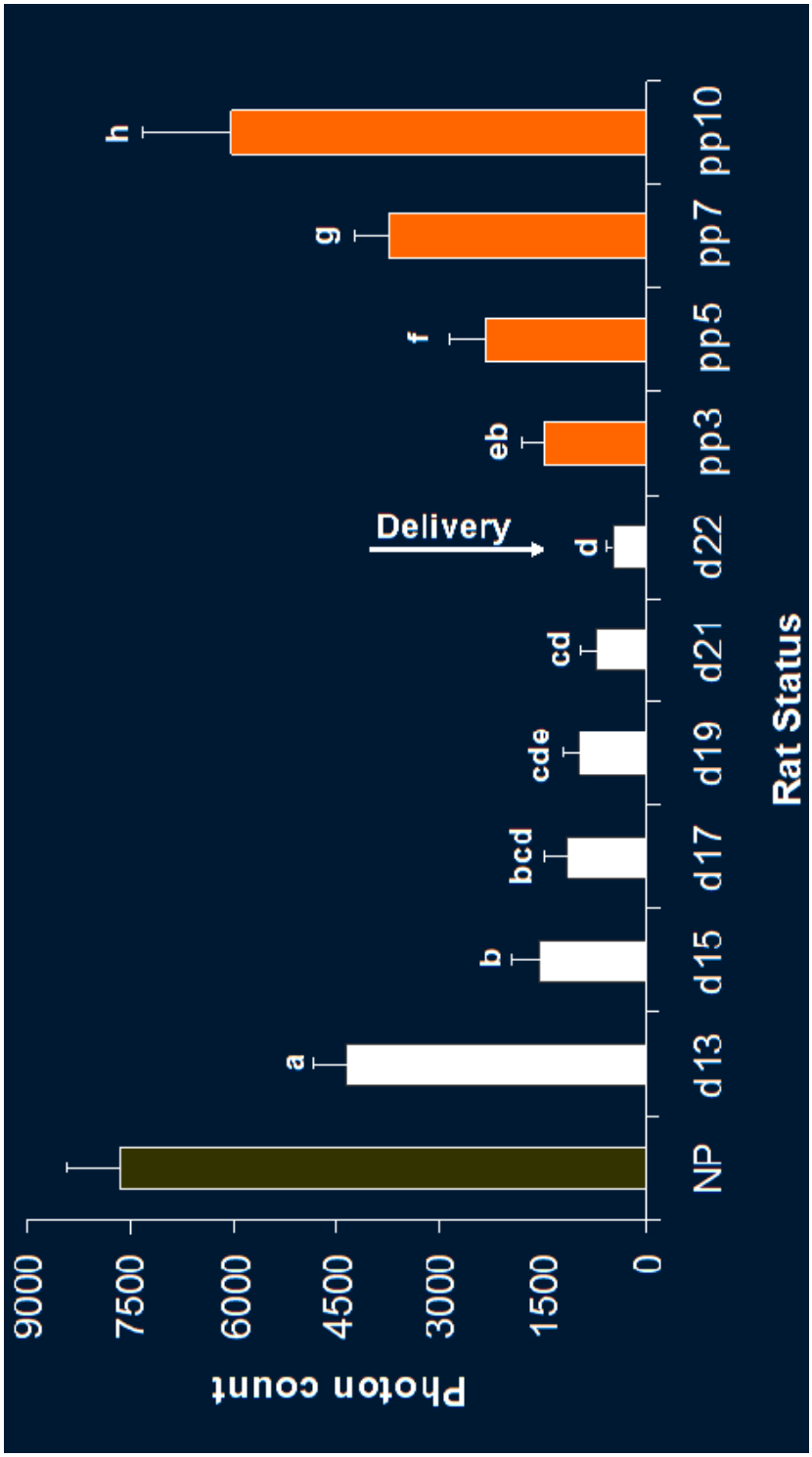


Figure 20. Cervical LIF detected in non-pregnant, pregnant and postpartum rats (Ruben J.K. et al., 2010).

The time course of the ultrasound physiologic effect is relatively rapid and most of the physiologic change can be seen to occur within an hour. This is contrast to the much slower process of ripening occurring over seven to eight days that occurs during normal pregnancy. Previous studies have showed that the use of collascope could detect LIF in vivo (rats, guinea pigs and humans), which is effective and useful to assess quantitative change in cervical ripening (Fittkow C.T. et al., 2001; Fittkow C.T. et al., 2005; Shi L. et al., 1999). With the insoluble fraction of collagen in cervix become soluble, the LIF value would show an estimate result of changing in pyyridinoline cross-link within those collagen fibers (Ruben J.K. et al., 2010). The LIF result was verified by a mechanical test of cervical extensibility that also indicated cervical changes by FUS stimulation.

FUS with frequency of 0.68 MHz used to stimulate the cervix of rat could produce fast and early ripening during pregnancy. The mechanism underlying this effect is still unknown. Through daily LIF measurement from day 15 to 22 when the rats spontaneously performed the delivery, we could watch a statistically significant difference ($p<0.001$) 24 hours after stimulation compared to the control group. And the LIF value will maintain at low level which is no significant difference compared to LIF value of d22 for treated and control groups. No preterm birth or delay of birth was observed, however the cervix of treated group has been showed to be soft enough to produce delivery. This demonstrated that the FUS stimulation

has strongly speeding the process of softening cervix, no biochemical change related to hormone was happened.

The relatively rapid ripening of the cervix by ultrasound is much faster than the typical application of prostaglandins over 12 hours intervals as currently clinical practice to promote cervical ripening (Norwitz E. R. et al., 2011). This could be a substantial advantage of the ultrasound technique in that it could be applied once to achieve ripening before delivery.

The ultrasound dose is a function of both its intensity and time of application. In this work the dose was in the range of 2.88 kJ to 14.4 kJ. We did not see a reduction of effect at the lowest energy used. We speculate that the peak pulse power is important factor that defines the dose level.

In contrast to the effects of certain drugs, ultrasound induced cervical ripening has not been observed to affect delivery times. Thus the ultrasound effect appears to be highly localized to the cervix and suggests there are no alterations in the other aspects of the gestation process. Measurement of fetal weights in the cases where gestation was progressed to delivery as shown in Figure 18 reveal no change. Photos additionally support the conclusion that there is no deleterious physical effect of the ultrasound application. This supports the potential safety of the ultrasound technique.

Safety of ultrasound employed

The ultrasound power level applied in this study appears to be close to the safety limits as defined by FDA. The Isppa is 40 W /cm^2 while the limit is 190 watts/cm^2 for both the body periphery as well as the fetus. The mechanical index (MI) is 0.2 according to above calculation, within the FDA limitation of 1.9 (ODRH, 2008) (Table 3). This value suggests the safety of the technique since they are within regulatory guidelines for diagnostic imaging systems.

Table 3. FDA regulation on imaging systems (CDRH. 2008)

Use	Ispta.3 (mW/cm^2)	Isppa.3 (W/cm^2)	M.I.
Peripheral Vessel	720	190	1.9
Cardiac	430	190	1.9
Fetal Imaging	94	190	1.9
Cervical Stimulation	<1000	40	0.2

The Ispta of this work was on the order of 1W/cm^2 delivered to a power measurement system (radiation force balance), which is considered to be emitted power. This power is attenuated as its passes through the skin interface and tissues of the rat body. There are losses through ultrasound absorption along the path to the cervix and through the cervix itself, according to reflection equation and attenuation equation before.

FDA regulations define ultrasound power levels in terms of derated power at the target organ Isppa.3 where the .3 indicate the derated power at target. For example, the Isppa.3 regulatory limit on diagnostic imaging systems to organs in the body periphery such as the blood vessels is

presently 0.72 W/cm^2. Although rigorous studies of ultrasound to the rat cervix have not been conducted, it seems fair to say based on known tissue reflection and attenuation coefficients of ultrasound at 680 kHz that as much as 25% of the applied ultrasound power could be lost in the coupling through the rat body surface and passage through the tissue to the cervix.

These studies have not determined the lower limit on the effective amount of ultrasound power. FDA regulations do not define how long ultrasound at the regulated power levels can be applied. However the physiologic effect may be related to exposure time. Longer durations at correspondingly lower ultrasound power levels may be effective. This would allow further decrease in applied ultrasound power levels.

There are thus many variables that could be optimized in an effort to achieve lower ultrasound powers. The pulse parameters chosen in this study were somewhat arbitrary and used only as a starting point. Multiple variables of Isppa, pulse repetition rate, and pulse width that can be optimized to achieve the specific physiologic effect required. This work did not seek optimum values for any of these parameters and hence tradeoffs in these parameters appear possible as a means of minimizing ultrasound exposure while maximizing physiologic effect. For example, increasing the Isppa.3 by factor of 4.7 to 190 W/cm^2 would be possible to increase the ultrasound radiation pressure by this factor and so possibly

the physiologic effect if stretch activation occurs while decreasing the pulse width to achieve the same Ispta.3.

Thermal Effects

Rises in tissue temperature were measured by the thermocouple placed inside the vagina on the order of 1.4 to 1.8 degree C. The accuracy of this measurement is unclear since measured temperature rises were almost immediate and not rising exponentially as would be expected as a result of energy delivery to tissue. Moving the steak in and out of the focal spot showed a temperature change on the order of 1.0 C and so it may be that at least part of the recorded temperature rise is an artifact of coupling of electric fields from the ultrasound transducer into the thermocouple.

Conclusion

This effect of ultrasound on the cervix is unexpected in view of the literature to date and suggests of a primary mechanism whereby ultrasound affects it. The basis for this effect is unclear. This work presents 3 theories of function which may explain the result.

Further study should focus on optimize the parameters of ultrasound effective in cervical ripening. And in further, reducing ultrasound power, there is a significant possibility ultrasound power level effective in cervical ripening within regulatory limits in case of safety. Current result show that sound power creates cervical ripening would be acceptable if they not impact on fetus. Further studies are needed to see if ultrasound

powers can be effective on human cervix tissue to see if similar result will happen by using comparable power level.

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