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The effect of low-intensity pulsed ultrasound on chondrocyte migration and its potential for the repair of articular cartilage

Kee Woong Jang
University of Iowa

THE EFFECT OF LOW-INTENSITY PULSED ULTRASOUND ON CHONDROCYTE MIGRATION AND ITS POTENTIAL FOR THE REPAIR OF ARTICULAR CARTILAGE

by

Kee Woong Jang

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Thesis Supervisors: Assistant Professor James A. Martin
Professor Tae-Hong Lim

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Kee Woong Jang

has been approved by the Examining Committee
for the thesis requirement for the Master of
Science degree in Biomedical Engineering at the July 2011
graduation.

Thesis Committee: _____
James A. Martin, Thesis Supervisor

_____
Tae-Hong Lim, Thesis Supervisor

_____
Edwin L. Dove

_____
Joseph M. Reinhardt

_____
Prem S. Ramakrishnan

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ABSTRACT

Articular cartilage, also called shock absorber, is a complex living soft tissue that covers gliding surfaces of joint and enables the joint to withstand weight bearing from human. Since there is no direct blood supply in the articular cartilage, it is generally hard to be repaired itself when it is injured. Although there have been several approaches to the repair of injured articular cartilage, current medical treatment is not able to give patients satisfactory treatment.

Ultrasound has been used as one of physical therapy tools. Recently, there have been frequent reports that ultrasound has beneficial effect on the repair of bone fracture and soft tissue healing including articular cartilage. Although there have been appreciation of beneficial effect of ultrasound therapeutically, its mechanism is not fully understood and under investigation.

From literature review, several researches tried to find optimal conditions of ultrasound such as intensity, frequency and duration on the repair of articular cartilage and it was reported that more effective ultrasound dose was found. However, different reports have different optimized ultrasound dose. It might be due to the variations of the type of ultrasound wave, intensity, frequency and duration as well as the different condition of experimental samples.

Therefore, low intensity pulsed ultrasound (LIPUS) was investigated on the repair of articular cartilage and chondrocyte migration from this study. Also, optimal conditions of LIPUS dose on chondrocyte migration were investigated for the repair of articular cartilage.

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CHAPTER 1

INTRODUCTION

Ultrasound has been widely used as diagnostic imaging tools in medical applications such as fetal images before giving a birth since it is widely known as to be safe of non-ionizing radiation. As an imaging modality, ultrasound is advantageous that is inexpensive, portable and visualizing based in real time compared to the magnetic resonance imaging (MRI) and computed tomography (CT). Over a past few decades, ultrasound has been extended its applications beyond the diagnostic tools into therapeutic, surgical and industrial applications. Especially in medical applications, it is mostly utilized that high intensity focused ultrasound (HIFU, intensity generally ranged from 5,000 to 15,000 W/cm^2 and inducing selective temperature increase causing necrosis of tumor), lithotripsy, targeted ultrasound drug delivery, accelerating bone fracture healing and physical therapy. For industrial applications, ultrasound can be used for measuring thickness of testing materials and ultrasonic welding, cleaning and blazing. Low intensity ultrasound (LIPUS, intensity generally ranged from 0.5 to 3,000 mW/ cm^2) first applied in 1970's for bone fracture healing and has been extended its application into healing of soft tissue and stimulating cells. The beneficial effect of LIPUS also has been reported on the repair of articular cartilage and is also widely applied in the field of physical therapy. Although there have been several reports about the beneficial effect of LIPUS, its mechanism has not been fully understood and still under investigation.

Osteoarthritis is a joint disorder that can be caused by a joint wear or tear and the causes are not fully understood yet, however, it is mainly due to aging, genetic inheritance, obesity, long-term overuse and fractures and its typical symptoms include pain, stiffness, cracking sound, and swelling of the joint. Articular cartilage is a firm and rubbery connective tissue found in several areas in the human body especially in a joint and intervertebral disc. It functions as cushion for the bone to bone and it allows gliding over. Since there is no blood supply in articular cartilage, it causes the arrested the repairing process when the articular cartilage is injured. Although several treatment methods are available in clinics such as direct surgery, medications and physical therapy, those methods are still in development and cannot provide full recovery of injured cartilage so far.

Low-intensity pulsed ultrasound (LIPUS) has been applied for accelerating the repair process of soft tissues and its potential benefits in the repair of injured articular cartilage have been reported frequently. However, the effect of LIPUS was inconsistent depend on the ultrasound dose such as a type of ultrasonic wave, frequency, intensity and duration are different. As such, optimal dose of ultrasound on soft tissue healing is still under investigation and is not fully understood with only appreciation of beneficial effect of LIPUS.

Therefore, specific goal of this study was to investigate how chondrocytes were affected by the ultrasound parameters, input energy instead of intensity, duration and frequency, for the repair of articular cartilage.

CHAPTER 2

BACKGROUND

2.1 Physics of Ultrasound

Ultrasound energy can be measured by the sum of potential and kinetic energy of interacting particles in the ultrasound field as follows.

$$E = E_p + E_k = \int \frac{p^2}{2 \cdot \rho \cdot c^2} dV + \int \frac{\rho \cdot v^2}{2} dV$$

Here, E is the ultrasound energy, E_p is the potential energy of particles, E_k is the kinetic energy of particles, p is the ultrasound pressure, ρ is the density of medium, and c is the speed of ultrasound in the medium.

Ultrasound power is measured by ultrasound energy per unit time, or

$$P = \frac{p^2 \cdot A}{Z}$$

Here, P is the ultrasound power ([W], [J/s]), p is the ultrasound pressure [pa], A is the surface area of transducer or area of ultrasound field, and the Z is the acoustic impedance of the medium.

Ultrasound intensity is defined as the ultrasound power per surface area of

transducer, or the area of ultrasound field as follows.

$$I = \frac{P}{A}$$

Here, I is the ultrasound intensity [W/cm^2], P is the ultrasound power, and A is the surface area of transducer.

2.1.1 Generation of Ultrasound

Ultrasound is produced by electrical signal applied to a transducer or a piezoelectric crystal, which is usually made of quartz or lead zirconate titanate (PZT). The piezoelectric crystal changes its shape as a function of the polarity of applied electrical signal by expanding and contracting as shown in Figure 2.1, in turn, molecular compression and rarefaction in front of the transducer take place and these pressure waves propagate in the medium. In other words, the ultrasonic transducer converts the electrical energy into a mechanical wave and conversely, the transducer converts the reflected echo into an electrical signal. Thickness of the piezoelectric crystal determines the frequency of ultrasound. Thicker piezoelectric crystal is used for generating lower frequency of ultrasound, while thinner piezoelectric crystal is used for generating higher frequency of ultrasound. The ultrasound frequency affects the quality of the ultrasound image. Higher frequency, which has shorter wavelength, has better image resolution but less penetration depth into the body, however, lower frequency has longer wavelength and has poorer image resolution with better penetration depth.

2.1.2 Propagation of Ultrasound

Ultrasound is cyclic high frequency of sound wave which is generally above the 20 kHz and the frequency is higher than the upper limit of human hearing. Propagation ability of ultrasound highly depends on its frequency or its wavelength and the spectrum of ultrasonic frequency is shown in Figure 2.2. One cycle of ultrasound represents the one repetitive periodic oscillation. Therefore, a number of oscillations of ultrasound is defined as its frequency [1/s] and the wavelength [m] is defined the length of one complete cycle. Speed of ultrasound is defined by the product of frequency and wavelength.

$$C = f \cdot \lambda$$

Here, C is the speed of ultrasound, f is the frequency of ultrasound, and λ is the wavelength of ultrasound. Ultrasound propagates through a medium at a certain speed which differs depend on the type of media and the velocity of ultrasound table is shown in Table 2.1. For instance, ultrasound propagates at constant speed in the medium such as 330 m/s in air, 1,540 m/s in soft-tissue and 4,080 m/s in bone. When ultrasound propagates through the medium, there are four types of ultrasonic interactions take place in the medium; reflection, transmission, refraction and attenuation.

2.1.3 Reflection of Ultrasound

Reflection of ultrasound occurs at the border of two different media when it passes through. This is due to the acoustic impedance mismatch of two media

and ideally there is no reflection of ultrasound occurred in homogeneous material. Acoustic impedance can be expressed by sound pressure per particle velocity per area and the characteristic impedance, plane waves with single-frequency, of a medium can be calculated by the density multiplied by sound wave speed as follows.

$$Z_0 = \rho \cdot c$$

Here, Z_0 is the characteristic acoustic impedance ([$M \cdot L^{-2} \cdot T^{-1}$]; $N \cdot s/m^3$ or $Pa \cdot s/m$), ρ is the density of the medium ([$M \cdot L^{-3}$]; kg/m^3), and c is the longitudinal wave speed ([$L \cdot T^{-1}$]; m/s).

As stated, reflection of ultrasound occurs when it passes through the border of two different media where acoustic impedance mismatch exists. Example of the percentage reflection and transmission is shown in the Figure 2.3. The greater acoustic impedance mismatch, the greater percentage of ultrasound can be reflected at the border. The percentage of reflection from the incident sound waves can be calculated below, called reflection coefficient.

$$R = \left(\frac{Z_2 \cdot \cos\theta_2 - Z_1 \cdot \cos\theta_1}{Z_2 \cdot \cos\theta_2 + Z_1 \cdot \cos\theta_1}\right)^2$$

Here, R is the reflection coefficient [%], Z_1 is acoustic impedance of the first medium ([$M \cdot L^{-2} \cdot T^{-1}$]; $N \cdot s/m^3$ or $Pa \cdot s/m$), Z_2 is acoustic impedance of the second

medium ([M·L^{-2}·T^{-1}]; N·s/m^3 or Pa·s/m), θ_1 is the angle of incidence, and θ_2 is the angle of refraction.

2.1.4 Transmission of Ultrasound

Transmission of ultrasound takes places when ultrasound passes through the border of two different media where acoustic impedance mismatch exists. However, if the impedance difference of two media is large enough, there will be almost no transmission of ultrasound. Since total incident energy is the sum of transmission and reflection energy, the transmission coefficient can be calculated by subtracting reflection coefficient from one hundred percent or the calculation below.

$$T = \frac{4 \cdot Z_2 \cdot Z_1 \cdot \cos^2 \theta_1}{(Z_2 \cdot \cos \theta_1 + Z_1 \cdot \cos \theta_2)^2}$$

Here, T is the Transmission coefficient [%], Z_1 is acoustic impedance of the first medium ([M·L^{-2}·T^{-1}]; N·s/m^3 or Pa·s/m), Z_2 is acoustic impedance of the second medium ([M·L^{-2}·T^{-1}]; N·s/m^3 or Pa·s/m), θ_1 is the angle of incidence, and θ_2 is the angle of refraction.

2.1.5 Refraction of Ultrasound

When ultrasound passes through two different media at an oblique angle, both reflection and refraction take place, at the same time refraction occurs due to the different media properties such as velocity. The characteristic of ultrasonic refraction is same as light and follows the Snell's law, which describes the

relationship between the refraction and incident angle, when it passes through two different media. Illustration of Snell's law is shown in Figure 2.4.

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{v_1}{v_2}$$

Here, θ_1 is the angle of incidence, θ_2 is the angle of refraction, v_1 is the longitudinal wave velocity in first medium, and v_2 is the longitudinal wave velocity in second medium.

2.1.6 Absorption or Attenuation of Ultrasound

Attenuation is the reduction of ultrasound amplitude as a function of distance in the medium when ultrasound propagates. In other words, ultrasound energy is absorbed in the medium during propagation. Within a soft tissue, the absorption of ultrasound energy results in the increase of temperature. Amount of attenuation of ultrasonic pressure can be calculated below.

$$p(x) = p_0 \cdot e^{-\alpha x}$$

Here, p(x) is the ultrasound pressure at distance of x from transducer, α is the attenuation coefficient, and p_0 is the incident ultrasonic pressure.

Attenuation coefficient [α] can be used to quantify the total attenuation in the medium in dB during ultrasound passes through.

$$\text{Attenuation} = \alpha \cdot \left[\frac{\text{dB}}{\text{MHz} \cdot \text{cm}}\right]$$

For instance, 5Mhz of ultrasound propagated total 2 cm length in fat, the total attenuation of ultrasound can be calculated. From the table 2.2, the attenuation coefficient of the fat is 0.48 [dB/(MHz ・ cm)]. Threrefore,

$$0.48[\text{dB}/(\text{MHz} \cdot \text{cm})] \times 2[\text{cm}] \times 5[\text{MHz}] = 4.8\ [\text{dB}]$$

total 4.8 dB of input intensity of 5 MHz ultrasound decreased after propagating through the 2 cm distance in the fat.

2.1.7 Measure of Ultrasound Strength

Since ultrasound is generated from a number of points at surface of transducer, ultrasound waves are interacting or interfering with each other within the ultrasound field. The interaction of two or more ultrasound waves results in the sum of each ultrasound pressure or intensity. Therefore, the interference of ultrasound waves results in the fluctuations of ultrasound pressure in the ultrasound field and this field is called a near field or Fresnel zone. Relatively uniform ultrasound waves are formed from the fluctuation at the end of the near field and this field is called far field which is shown in

the Figure 2.5. And the illustration of fluctuation of ultrasonic field is shown in the Figure 2.6. The calculation that divides the near field and far field is below.

$$N = \frac{D^2}{4 \cdot \lambda}$$

Here, N is the length of near field from the transducer, D is the diameter of transducer, and λ is the wavelength.

Since ultrasound pressure fluctuates spatially and temporally in the ultrasound field, spatial and temporal strength of ultrasound should be regarded when measuring the strength. In general, ultrasonic pressure is measured by hydrophone and radiating force balance is also common method to measure the ultrasonic power.

Spatial Peak and Temporal Peak Intensity (SPTP) is the measure of peak intensity over the ultrasound field when the intensity is peak during the entire time. Spatial Average and Temporal Average Intensity (SATA) is the measure of averaged intensity over the ultrasound field during the pulse repetition period. Spatial Average and Temporal Peak Intensity (SATP) is the measure of averaged intensity over the ultrasound field when the intensity is peak during the entire time. Spatial Peak and Temporal Average Intensity (SPTA) is the measure of peak intensity over the ultrasound field during the pulse repetition period. Spatial Peak and Pulse Average Intensity (SPPA) is the measure of peak intensity over the ultrasound field when the pulse duration is on. Spatial

Average and Pulse Average (SAPA) is the measure of averaged intensity over the ultrasonic field during the pulse duration is on.

The strength of ultrasound intensity will vary depend on the denoting methods.

$$I_{SATA} < I_{SPTA} < I_{SATP}(I_{SAPA}) < I_{SPTP}(I_{SPPA})$$

Lowest ⟷ Highest

2.2. Biophysical Characteristics of Therapeutic Ultrasound

Ultrasound intensity level can be classified into high intensity ranged from 5,000 to 15,000 W/cm^2, and low intensity ranged from 0.5 to 3,000 mW/cm^2. The high intensity focused ultrasound (HIFU) can be used for selective heating which causes tumors burned up in the body. When ultrasound propagates through biological tissues, it causes to generate mechanical micro strain by molecular interaction and collisions that results in the biochemical events at the cellular level [20, 21]. The absorption of ultrasound in the biological tissues may increase the temperature depend on ultrasonic frequency, intensity and tissue density. Conveniently, biophysical effects of ultrasound can be separated into thermal effects which typically caused by continuous ultrasound and non-thermal effects which typically caused by pulsed ultrasound [2]. However, it is hard to say that there is only single effect either thermal or non-thermal when the ultrasound propagates through tissue, because both thermal and mechanical interactions take place at the same time. For instance, pulsed ultrasound does not completely remove the heating effect, even though it reduces the increase of temperature

inversely proportional to the duty cycle. Even so, in general, a thermal effect of ultrasound is associated with the increase of temperature in tissue and a non-thermal effect of ultrasound is associated with acoustic cavitations.

2.2.1 Thermal Effects of Therapeutic Ultrasound

When ultrasound is propagating through in tissue, its energy is attenuated or absorbed by the tissue which results in the conversion of ultrasonic energy to heat [3]. Parameters of tissue that cause to absorb ultrasound are related to the compositions of tissue such as protein contents and collagen, in addition, wave form of ultrasound either pulsed or continuous, duration of exposure and frequency are also critical parameters. In general, pulsed ultrasound reduces the temperature increase in tissue proportional to the on, off pulse ratio [4]. A study on human muscle, the gastrocnemius muscle at a depth of 3cm was increased by 5 ºC in condition of 1 MHz of frequency, 1.5W/cm^2 of intensity and 10 minutes of duration [5].

2.2.2 Non-thermal Effects of Therapeutic Ultrasound

Non-thermal effects of ultrasound can be divided into generating acoustic cavitations, the formation of tiny gas bubbles as a result of ultrasound vibration, and other mechanical effects [6]. The gas bubbles can be generated and expanding and contracting in tissue when ultrasonic pressure goes to positive peak and negative peak. The cavitations can be classified into stable cavitations and unstable cavitations. The stable cavitations, also called non-inertial cavitations, are described in which the bubble is forced to oscillate its size and shape due to the change of ultrasonic pressure. Ultrasonic cleaning is the

example of use of such cavitations in industry. Unstable cavitations, also called inertial cavitations, are described in which the bubble collapses rapidly and produces a shock wave with very high local temperature increase which produces toxic free radicals in the tissue. ter Haar et al. reported that the beneficial effects of ultrasound are due to the non-thermal interaction rather than the heating effect [6]. At the intensity range of therapeutic ultrasound, the difficulty of demonstrating the cavitations in vivo has proved. Therefore, it is not accepted the formation of acoustic cavitations at the therapeutic ultrasound generally.

Acoustic streaming, localized liquid flow around the vibrating bubbles, is another major effect. It can be distinguished between bulk streaming and micro-streaming which is more mechanically powerful than bulk streaming. Bulk streaming is a movement of the fluid in a single direction in the ultrasound field in a liquid [7], however, a micro-streaming form eddies of flow. The difference between bulk streaming and micro-streaming is that bulk streaming occurs in vivo but micro-streaming do not occur in vivo, because the micro-streaming is always associated with the cavitations [8, 9]. It is the micro-streaming that can only alter cell membrane permeability and stimulate the cell activity.

2.3. Articular Cartilage

In human body, three types of cartilage can be distinguished by their composition of matrix such as structure, strength and elasticity; articular cartilage, fibro-cartilage and elastic cartilage. First, articular cartilage, or called hyaline cartilage which covers gliding surfaces of joint, is a complex living soft tissue that lines the bony surface of joints and provides a low friction surface

that enables the joint to withstand weight bearing needed to perform daily activities, such as walking, work-related activities, climbing stairs and athletic activities. Articular cartilage is also called a very thin shock absorber. Second, fibro-cartilage is generally found in knee meniscus and intervertebral discs consisting of fine collagen fibers, type I collagen and type II collagen. Unlike uniform appearance of hyaline cartilage, fibro-cartilage has spongy-like structure between lacunae and collagen fiber bundles which can provide fibro-cartilage a good shock absorbing ability. Last, elastic cartilage found in the outer ear and epiglottis which contains elastic fiber networks and collagen fibers. Elastic cartilage contains many yellow elastic fibers forming bundles which give elastic cartilage a good flexibility to be able to withstand repeated bending.

2.3.1 Composition of Articular Cartilage

Articular cartilage is composed of collagen matrices with proteoglycan (PG) and interstitial fluid with the composition of sixty to eighty percent of water, ten to twenty percent of collagen and ten to twenty percent of PGs based on the wet weight. Water content in the articular cartilage allows load-dependent deformation and provides nutrition and medium for lubrication in addition to the low-friction gliding surface. The type II collagen is primary type of collagen in the articular cartilage and provides a high tensile stiffness, in turn, contributes to the cartilage to form a network providing withstanding the tensile and shear stresses.

Aggrecan is the most abundant type of PGs in the articular cartilage and the subunits of PGs are glycosaminoglycan which are disaccharide molecules,

chondroitin and keratin sulfate, connected to the core protein. The schematic diagram of PG is shown in the Figure 2.7. The aggrecan molecules bind to the core of hyaluronic acid and from negatively charged aggregates which preserve the electrolyte balance and water bound in the articular cartilage contributing the tissue to withstand sustained compressive stresses. The illustration of the structure of aggrecan is shown in the Figure 2.8.

2.3.2 Structure of Articular Cartilage

In articular cartilage, it can be classified depend on the morphological changes of chondrocytes and matrix that four named zones from the cartilage surface to subchondral bone region; superficial zone, transitional zone, middle or deep zone and calcified zone shown in the Figure 2.9. The concentrations of water, PG and collagen in matrix are different in each zone and chondrocytes are also different in the shape, size and metabolic activity [39]. This may respond differently to mechanical loading which suggests that the development and maintenance of articular cartilage depend on the differentiation of phenotypically distinct populations of chondrocytes [40].

2.3.2.1 Superficial tangential Zone

Superficial zone is the thinnest among all zones and covered by lubricin which provides a gliding surface to the articular cartilage. The superficial zone contains about 85% of collagen contents by dry weight and the collagen fibrils are arranged parallel to the cartilage surface which can provide the high tensile strength and resistance to the shear stresses primarily so that protects the deeper layer from shear stresses. Chondrocytes in this zone are flattened in shape and

most abundant. Supposed to disrupt of the superficial zone, the mechanical properties of the articular cartilage would be rapidly changed and osteoarthritis would be developed.

2.3.2.2 Middle or Transitional Zone

Middle zone represents 40 % to 60 % of total articular cartilage volume and provides functional bridge between the superficial and deep zones. Chondrocytes in this zone are spherical in shape at low cell density. Collagen fibrils are thicker than in superficial zone and are organized obliquely. Middle zone is the first line of resistance to compressive forces from the cartilage surface.

2.3.2.3 Deep or Radial Zone

Deep zone represents about 30 % of total articular cartilage volume and is responsible for providing the greatest resistance to compressive forces and for distributing the compressive forces. Collagen fibrils are arranged perpendicular to the surface and have the largest diameter. It contains the most abundant amount of PG and the lowest concentration of water in this zone. There is a thin basophilic line, called tidemark, which separates the deep zone from calcified zone.

2.3.2.4 Calcified Zone

Calcified zone separates the deep zone from the subchondral bone and there is a small number of chondrocytes in the calcified zone. There are some regions that chondrocytes are surrounded by calcified cartilage which suggests that the chondrocytes have little metabolic activity [40]. In addition,

chondrocytes function synthesizing the type X collagen which provides structural integrity and shock absorber along with the subchondral bone [12]. The calcified zone contains the tidemark which spreads out along the boundary between the calcified and un-calcified cartilage.

2.4 Osteoarthritis

Articular cartilage injuries can occur by a variety of causes, such as traumatic accidents, previous knee injuries, immobilization for long periods or wear and tear over time. Articular cartilage cells, chondrocytes, have ability to repair the injured cartilage depend on the extent of the damage and the location, however, it is generally very hard to be repaired due to no direct blood supply in the articular cartilage. Mechanical degeneration of articular cartilage results in loss of structure, loss of functions, softening of cartilage and fragmentation of cartilage which can lead to osteoarthritis (OA). OA is the most common joint disease which can be caused by joint degeneration and the most common symptoms of OA are pain and stiffness in the joints. Although the causes of OA can be considered by increasing age, being overweighting and over-use at work or in sports, the pathophysiology of the joint degeneration leading to the OA still remains poorly understood. Current medical treatment is not able to give patients neither full treatment nor satisfactory pain relief [13]. For these reasons, the OA could be a considerable economic burden for patients.

2.4.1 Diagnosis of OA

One of the first signs of OA is the pain and the decreased activity of movement which could be due to the loss of articular cartilage. Although scanning the joint by

imaging techniques such as X-rays, CT and MRI has been applied for the diagnosis of OA, it could not be represented that the successive diagnosis of OA due to the uncertainty of the correlation between the severity of OA and the diagnostic imaging.

2.4.2 Classification of OA

OA can be classified into primary or secondary depend on whether the cause of OA is identifiable or not. The primary OA is described that the cause of joint degeneration is unknown, however, it is considered to be related to aging. Secondary OA can be caused by traumatic injury, inflammatory, obesity, diabetes, metabolic or developmental diseases.

2.4.3 Prevalence and Cost

The World Health Organization (WHO) estimates that the 10% of people in the world suffers from the OA and more than 20 million people in the US suffer from the OA [14, 15]. It is widely known that the OA increases with aging and more than one third of people in the age of over 45 years sensate the symptoms of OA. In addition, more than 75% of people in the age of over 65 years suffer from the OA. According to the research from Stony Brook University, it costs 186 billion dollars per year that medical care costs for the OA in the US.

2.4.4. Risk Factors and Restoring Degenerated Articular Cartilage

Although there is the number of risk factors of OA such as genetic predisposition, excessive mechanical loading, repetitive joint overuse, post-traumatic joint incongruity, obesity and joint laxity, the most important risk factor is related to aging in all population. There have been little progress on regeneration of degenerated articular cartilage for a long time, but surgical procedures, such as penetrating the subchondral bone, altering

joint loading and soft tissue grafts, have been applied for regeneration of articular surface in the last 50 years. Transplantation of stem cells, growth factors and artificial scaffolds has been studied and it shows the regeneration of injured joint. However, most of these approaches have limitations and have not been able to provide satisfactory solution for the regeneration of articular cartilage.

2.5. Literature Review of the Effects of Therapeutic Ultrasound on the Repair of Articular Cartilage.

2.5.1 History of Therapeutic Ultrasound

In 1930's, it was discovered that sound energy could be absorbed into skin and some people in 1940's started to believe that the ultrasound could be beneficial therapeutically for human despite the fact that there were not enough evidence to support. In 1970's, the first successful results of therapeutic ultrasound were performed by the Institute of Environmental stress in the University of California Santa Barbara. They found that the low intensity ultrasound stimulated the generation of osteoblasts on the femur fracture of rat model. Since then, the low intensity ultrasound has become widely used not only for the bone fracture healing, but also for soft tissue healing. In 1994, the Food and Drug Administration (FDA) approved to use of ultrasound for bone fracture healing. Although there are not currently clear mechanisms available, the investigations on both the application and the mechanism of the benefits are consistently undergoing.

2.5.2 Biological Responses by Therapeutic Ultrasound

There have been frequent reports about the beneficial effect of low-intensity ultrasound on both cellular and tissue level. However, the results of

those studies such as the existence of beneficial effects or optimal ultrasound dose are inconsistent and even contradictory at times. This is probably due to the fact that not only the differences of experimental samples and environments, but also differences of the parameters of ultrasound such as intensity, frequency, duration and the type of ultrasound wave either continuous or pulsed. Nevertheless, there have been few researches that reported the adverse effects of therapeutic ultrasound.

2.5.2.1 Responses of Soft Tissue by Therapeutic Ultrasound

Table for the responses of soft tissue by therapeutic ultrasound is summarized in Table 2.3. Rawool et al. in 2003 reported that enhancement of angiogenesis and blood flow around the bone fractured site by LIUS (1.5 MHz, 30 mW/cm^2, 20 min/day) was investigated [19]. Cook et al. in 2001 showed that the LIPUS (1.5 MHz, 30 mW/cm^2, 20 min/day) improved the repair of osteochondral defects on rabbits in vivo by examining morphological feature and histology [26]. Min et al. in 2006 showed that LIUS (1 MHz, 200 mW/cm^2, 10 min/day) increased type II collagen and PG synthesis of human cartilage explants donated from OA patients. However, there was no significant proliferation of chondrocytes [31]. Naito et al. in 2010 reported that LIPUS (1.5MHz, 30 mW/cm^2, 20 min/day) increased type II collagen synthesis on surgically induced OA model of rat in vivo. However, LIPUS could not attenuate the progression of type II collagen degradation after OA induced [46]. Gurkan et al. showed that LIPUS (1.5 MHz, 30 mW/cm^2, 20 min/day for 3 to 10 months) attenuated the progression of cartilage degeneration on pig joint in vivo [47]. Korstjens et al. in 2008 showed that LIPUS (1.5

MHz, 30 mW/cm^2, 20 min/day) stimulated chondrocytes proliferation and matrix production of human articular cartilage explants donated from OA patients in vitro [48].

2.5.2.2 Cellular Responses by Therapeutic Ultrasound

Table for the cellular responses by therapeutic ultrasound is shown in Table 2.4. Lee et al. in 2006 showed that LIUS (1 MHz, 200 mW/cm^2, 20 min/day) treated rabbit mesenchymal stem cells increased cartilage matrix formation, type II collagen synthesis, aggrecan synthesis and inhibition of matrix metalloproteinase-2 (MMP-2) expression indicating that LIUS increased the cartilage matrix integrity [10]. Zhang et al. in 2003 studied two different LIPUS (1.5 MHz, 20 min/day) intensity levels, 2 and 30 mW/cm^2, on chondrocytes, which were isolated from the chick embryos and cultured in 3D alginate bead, viability, proliferation, type II collagen and aggrecan synthesis. They found that both intensities did not affect the chondrocytes viability, however, proliferation, type II collagen, type X collagen and aggrecan synthesis were affected intensity-dependently. 2 mW/cm^2 of ultrasonic intensity had more significant effect on type II collagen synthesis, chondrocyte proliferation and inhibition of type X collagen synthesis rather than 30 mW/cm^2 [23]. Tien et al. in 2008 showed that LIPUS (1 MHz, 48 mW/cm^2, 20 min/day) increased the aggrecan and type II collagen synthesis on the human chondrocytes cultured in 3D agarose gel, however, there was no significant difference in chondrocytes proliferation [27]. Schumann et al. in 2006 showed that LIPUS (1.5 MHz, 30 mW/cm^2, 40 min/day) increased chondrogenic differentiation of mesenchymal stem cells cultured in 3D scaffold containing 70% estered hyaluronan and 30% gelatin. However, there was no significant effect with 20 minutes of LIPUS stimulation [49]. Ebisawa et el in 2004 showed that LIPUS (1 MHz, 30 mW/cm^2, 20

min/day) increased TGF-b mediated chondrocytes differentiation of Mesenchymal stem cells and cartilage matrix formation without cell proliferations [50].

2.5.2.3 Molecular Responses by Therapeutic Ultrasound

Table for the molecular responses by therapeutic ultrasound is shown in Table 2.5. Chapman et al. in 1980 reported that there was decrease in potassium level of rat thymocytes in vitro by ultrasound exposure (3 MHz, 2 W/cm^2, 40 min/day). However, the potassium level increased back to be normal after ultrasound exposure [17]. Li et al. in 2003 investigated the effect of LIPUS (1 MHz, 30 mW/cm^2, 20 min/day) on cytokine release and showed increased in the transforming growth factor-β1 (TGF-β1) secretion and decreased in the IL-6 and tumor necrosis factor- α (TNF- α) in vitro culture of rat osteoblasts [18]. Zhou et al. in 2004 showed that LIPUS (1.5 MHz, 30 mW/cm^2) activated the Ras homolog gene family, member A (RhoA) which induced the F-actin stress fiber formation and LIPUS increased the extracellular signal-regulated kinases ½ (ERK ½) activation and DNA synthesis which resulted in the cell proliferation [22]. Guzman et al. in 2001 showed that calcein molecules, which cannot cross intact cells, were delivered into cells when focused ultrasound was induced on cancer cells with contrast agent required for the phenomenon called cavitations effect [37].

2.5.3 Optimal Dose of Therapeutic Ultrasound

Table for the study of optimal dose of therapeutic ultrasound is shown in Table 2.6. Although there have been appreciation of the beneficial effect of therapeutic ultrasound, the optimal dose of ultrasonic stimulation has not been in agreement by a number of studies. Zhang in 2003 reported that 2 mW/cm^2 of LIPUS (1 MHz, 20 min/day)

was more effective rather than 30 mW/cm^2 on chondrocytes of the sternum of chick embryos proliferation and type II collagen synthesis [23]. Cook et al. in 2001 showed that the 40 minutes of duration was the most effective on the repair of osteochondral defects on rabbits [26]. Tien in 2008 showed that 48 mW/cm^2 of LIPUS (1 MHz, 20 min/day) was the most effective on aggrecan synthesis among the various intensities, 18, 48, 72 and 98 mW/cm^2 from the human articular chondrocytes cultured in 3D agarose gel [27]. Parvizi et al. in 1999 showed that both 50 and 120 mW/cm^2 of LIPUS (1 MHz, 10 min/day) increased the aggrecan mRNA expression from rat chondrocytes, while the rate of PG synthesis was much higher in the 50 mW/cm^2 of intensity [28]. Choi et al. in 2006 showed that 200 mW/cm^2 of LIUS (1 MHz, 10 min/day) was the most effective among 100, 200 and 300 mW/cm^2 on increasing the chondrocytes viability isolated from OA patients, PG synthesis and type II collagen synthesis [30]. Schumann et al. in 2006 showed that 40 minutes of LIPUS (1.5 MHZ, 30 mW/cm^2) had significant effect on type II collagen and PG synthesis, while 20 minutes of LIPUS had no significant effect [49]. Min et al. in 2006 showed that 200 mW/cm^2 of LIUS (1 MHz, 10 min/day) was the most effective for type II collagen and PG synthesis among the 40, 200, 500 and 700 mW/cm^2. However, there was significant decrease in type II collagen synthesis above 500 mW/cm^2 and significant decrease in PG synthesis above the 700 mW/cm^2. Thus they suggested that 500 mW/cm^2 could be a threshold for LIUS stimulation [31]. As such, the optimal dose has been hard to be in general agreement, albeit there have been sufficient benefits and appreciations of LIPUS.

Table 2.1. The table of ultrasound velocity [25]

Acoustical Properties Of Common Materials					
Material	Ultrasonic Velocity				
	Longitudinal		Transverse (Shear)		Impedance
	in / us	mm / us	in / us	mm / us	Z
METALS					
Aluminum 1100-0	0.248	6.229	0.121	3.073	17.1
Aluminum 2024-T4	0.251	6.375	0.124	3.150	17.6
Aluminum 6061-T6	0.248	6.299	0.124	3.150	17.0
Beryllium	0.507	12.878	0.350	8.890	23.5
Brass (70% Cu - 30% Zn	0.172	4.369	0.083	2.108	37.1
Bronze (Phosphor 5%)	0.139	3.531	0.088	2.235	31.3
Copper (CP)	0.187	4.750	0.092	2.337	42.5
Gold	0.128	3.251	0.047	1.194	62.6
Hastelloy C	0.230	5.842	0.114	2.896	52.2
Hastelloy X	0.228	5.791	0.108	2.743	47.7
Inconel (Wrought)	0.308	7.823	0.119	3.023	64.5
Iron (Cast), Various Alloys	0.138-0.220	3.505-5.588	0.087-0.126	2.210-3.200	24.3-41.2
Lead (94Pb-6Sb)	0.085	2.159	0.032	0.813	23.5
Magnesium, Various Alloys	0.215-0.228	5.461-5.791	0.119-0.122	3.023-3.099	9.24-10.6
Monel	0.211	5.359	0.107	2.718	47.2
Nickel (CP)	0.222	5.639	0.117	2.972	50.0
Silver (0.99 Fine)	0.142	3.607	0.063	1.600	37.8
Steel 1020	0.232	5.893	0.128	3.251	45.4
Steel 4340	0.230	5.842	0.128	3.251	45.6
Steel , CRES 300 Series	0.221-0.226	5.613-5.740	0.120-.0123	3.048-3.124	44.6-45.4
Steel , CRES 400 Series	0.212-0.237	5.385-6.020	0.118-0.132	2.997-3.353	41.3-46.3
Titanium, 6Al-4V	0.243	6.172	0.130	3.302	27.3
Zircaloy	0.186	4.724	0.093	2.362	44.2
Zirconium	0.183	4.648	0.089	2.261	30.1
POLYMERS					
Acrylics	0.105-0.109	2.667-2.769	0.044-0.057	1.118-1.448	3.15-3.51
Cellulose Acetate	0.096	2.438	No Shear Component		3.19
Nylon	0.016	2.692	No Shear Component		--------
Phenolic	0.056	1.422	No Shear Component		1.90
Polycarbonate	0.090	2.286	No Shear Component		2.71
Polyethylene	0.105	2.667	No Shear Component		2.94
Polystyrene	0.094	2.388	0.045	1.143	2.52
Rubber (Natural)	0.061	1.549	No Shear Component		1.74
Rubber (Carbon Filter)	0.066	1.676	No Shear Component		--------
Rubber (Silicone)	0.037	0.94	No Shear Component		1.40
Teflon	0.054	1.372	0.250	6.35	3.00
MISCELLANEOUS SOLIDS					
Alumina (Al2O3)	0.427	10.846	No Shear Component		43.1
Concrete	0.167-0.207	4.242-5.258	0.135	3.429	12.4
Glass (Plate)	0.227	5.766	No Shear Component		14.5
Granite	0.156	3.962	0.076	1.93	10.9
Ice (-16C)	0.150	3.81	No Shear Component		3.60
Quartz, Natural	0.226	5.74	0.139	3.531	15.2
Quartz, Fused	0.219	5.563	0.302	7.671	14.5
Sapphire	0.469	11.913	0.157	3.988	47.2
Tungsten Carbide	0.262	6.655	No Shear Component		67.6
COMPOSITE MATERIALS					
Fiberglass (50 v/o)	0.124	3.15	0.068	1.727	6.04
Graphite/Epoxy (60 v/o)	0.117	2.972	0.077	1.956	4.65
Boron/Epoxy (50v/o)	0.131	3.327	0.072	1.829	6.38
LIQUIDS					
Ethylene Glycol	0.064	1.626	No Shear Component		1.80
Glycerin	0.076	1.93	No Shear Component		2.42
Oil (SAE 20)	0.069	1.753	No Shear Component		1.51
Water (20C)	0.058	1.473	No Shear Component		1.48
Gases					
Air (20°C)	0.014	0.356	No Shear Component		0.00041
Nitrogen (20°C)	0.014	0.356	No Shear Component		0.00041
Oxygen (20°C)	0.013	0.33	No Shear Component		0.00043

Table 2.2. The table of ultrasonic attenuation coefficient [24].

Material	$\alpha(\mathrm{dB}/(\mathrm{MHz}\cdot\mathrm{cm}))$
Blood	0.2
Bone, cortical	6.9
Bone, trabecular	9.94
Brain	0.6
Breast	0.75
Cardiac	0.52
Connective tissue	1.57
Dentin	80
Enamel	120
Fat	0.48
Liver	0.5
Marrow	0.5
Muscle	1.09
Tendon	4.7
Soft tissue (average)	0.54
Water	0.0022

Table 2.3. The effect of therapeutic ultrasound on soft tissue healing.

Reference	Author & year	Sample type	US dosage	Results
19	Rawool et al. (2003)	fractured midshaft of the ulna on dog	LIUS, 1.5 MHz, 30mW/cm^2, 20min/d	LIUS enhanced angiogenesis and blood flow around the bone fractured site.
26	Cook et al. (2001)	osteochondral defects in vivo on rabbits	LIPUS, 1.5 MHz, 30mW/cm^2, 20min/d	Enhanced the repair of osteochondral defects on rabbits in vivo by examining morphological feature and histology.
31	Min et al. (2006)	Human cartilage explants from OA patients	LIUS, 1 MHz, 200mW/cm^2, 10min/d	There were increased type II collagen and PG synthesis, but no significant proliferation of chondrocytes
46	Naito et al. (2010)	Rat OA model in vivo	LIPUS, 1.5 MHz, 30mW/cm^2, 20min/d	LIPUS increased type II collagen synthesis on surgically induced OA model of rat in vivo. However, LIPUS could not attenuate the progression of type II collagen degradation after OA induced
47	Gurkan et al. (2010)	Guinea pig OA model in vivo	LIPUS, 1.5 MHz, 30mW/cm^2, 20min/d	LIPUS attenuated the progression of cartilage degeneration on pig joint in vivo
48	Korstjens et al. (2008)	Human cartilage explants from OA patients	LIPUS, 1.5 MHz, 30mW/cm^2, 20min/d	LIPUS stimulated chondrocytes proliferation and matrix production of human articular cartilage explants donated from OA patients in vitro

Table 2.4. Cellular responses by the effect of therapeutic ultrasound.

Reference	Author & year	Sample type	US dosage	Results
10	Lee et al. (2006)	rabbit mesenchymal stem cells	LIUS, 1 MHz, 200mW/cm^2, 20min/d	LIUS treated rabbit MSCs increased type II collagen synthesis, aggrecan synthesis and inhibition of MMP-2 expression indicating that LIUS increased the cartilage matrix integrity
23	Zhang et al. (2003)	chondrocytes isolated from chick embryos	LIPUS, 1.5 MHz, 2 & 30mW/cm^2, 20min/d	Both intensities did not affect the chondrocytes viability. However, 2 mW/cm^2 was more effective on type II collagen synthesis, chondrocyte proliferation and inhibition of type X collagen synthesis rather than 30 mW/cm^2
27	Tien et al. (2008)	human child chondrocytes	LIPUS, 1 MHz, 48mW/cm^2, 20min/d	LIPUS increased the aggrecan and type II collagen synthesis on the human chondrocytes cultured in 3D agarose gel, however, there was no significant difference in chondrocytes proliferation.
49	Schumann et al. (2008)	human mesenchymal stem cells	LIPUS, 1.5 MHz, 30mW/cm^2, 40min/d	LIPUS increased chondrogenic differentiation of mesenchymal stem cells cultured in 3D scaffold. However, there was no significant effect with 20 minutes of LIPUS stimulation
50	Ebisawa et al. (2004)	human mesenchymal stem cells	LIPUS, 1 MHz, 30mW/cm^2, 20min/d	LIPUS increased TGF-b mediated chondrocytes differentiation of MSCs and cartilage matrix formation without cell proliferations.

Table 2.5. Molecular responses by the effect of therapeutic ultrasound.

Reference	Author & year	Sample type	US dosage	Results
17	Chapman et al. (1980)	rat thymocytes	3 MHz, 2 W/cm^2, 40min/d	There was decrease in potassium level of rat thymocytes in vitro by ultrasound exposure. However, the potassium level increased back to be normal after ultrasound exposure
18	Li et al. (2003)	osteoblasts in vi	LIPUS, 1 MHz, 30mW/cm^2, 20 min/d	LIPUS increased TGF-β1 secretion and decreased in the IL-6 and TNF- α in vitro culture of rat osteoblasts
22	Zhou et al. (2004)	human fibroblasts	LIPUS, 1.5 MHz, 30mW/cm^2	LIPUS activated the RhoA which induced the F-actin stress fiber formation, LIPUS increased the ERK ½ activation and DNA synthesis which resulted in the cell proliferation.
37	Guzman et al. (2001)	human prostate cancer cells	Focused ultrasound	Calcein molecules, which cannot cross intact cells, were delivered into cells when focused ultrasound was induced on cancer cells with contrast agent required for the phenomenon called cavitations effect

Table 2.6. Study of optimal dose of therapeutic ultrasound.

Reference	Author & year	Sample type	US dosage	Results
23	Zhang et al. (2003)	chondrocytes isolated from chick embryos	LIPUS, 1.5 MHz, 2 & 30mW/cm^2, 20min/d	2 mW/cm^2 of LIPUS was more effective rather than 30 mW/cm^2 on chondrocytes of the sternum of chick embryos proliferation and type II collagen synthesis
26	Cook et al. (2001)	osteochondral defects in vivo on rabbits	LIPUS, 1.5 MHz, 30mW/cm^2, 20min/d	40 minutes of duration was the most effective on the repair of osteochondral defects on rabbits
27	Tien et al. (2008)	human articular chondrocytes	LIPUS, 1 MHz, 18, 48, 72 & 98mW/cm^2, 20min/d	48 mW/cm^2 of LIPUS was the most effective on aggrecan synthesis among the various intensities, 18, 48, 72 and 98 mW/cm^2 from the human articular chondrocytes cultured in 3D agarose gel
28	Parvizi et al. (1999)	rat chondrocytes	LIPUS, 1 MHz, 50 & 120mW/cm^2, 10min/d	Both 50 and 120 mW/cm^2 of LIPUS increased the aggrecan mRNA expression from rat chondrocytes, while the rate of PG synthesis was much higher in the 50 mW/cm^2 of intensity
30	Choi et al. (2006)	Human OA chondrocytes	LIUS, 1 MHz, 100, 200 & 300mW/cm^2, 10min/d	200 mW/cm^2 of LIUS was the most effective among 100, 200 and 300 mW/cm^2 on increasing the chondrocytes viability isolated from OA patients, PG synthesis and type II collagen synthesis
49	Schumann et al. (2008)	human mesenchymal stem cells	LIPUS, 1.5 MHz, 30mW/cm^2, 40min/d	40 minutes of LIPUS had significant effect on type II collagen and PG synthesis, while 20 minutes of LIPUS had no significant effect
31	Min et al. (2006)	Human cartilage explants from OA patients	LIUS, 1 MHz, 200mW/cm^2, 10min/d	200 mW/cm^2 of LIUS was the most effective for type II collagen and PG synthesis among the 40, 200, 500 and 700 mW/cm^2. However, there was significant decrease in type II collagen synthesis above 500 mW/cm^2 and significant decrease in PG synthesis above the 700 mW/cm^2. Thus they suggested that 500 mW/cm^2 could be a threshold for LIUS stimulation

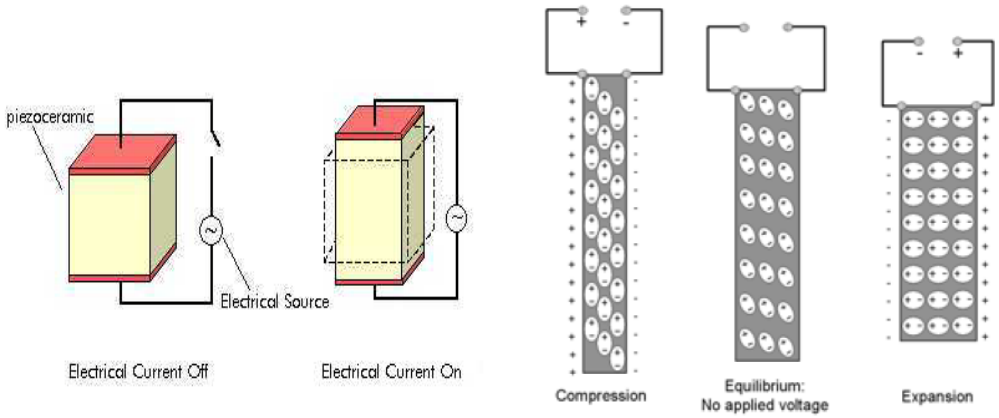

Figure 2.1. Illustration of piezoelectricity. Electrical pulses cause the mechanical strain of piezoelectric crystal which generates ultrasound. Conversely, mechanical strain causes the piezoelectric crystal to convert electrical pulses [42].

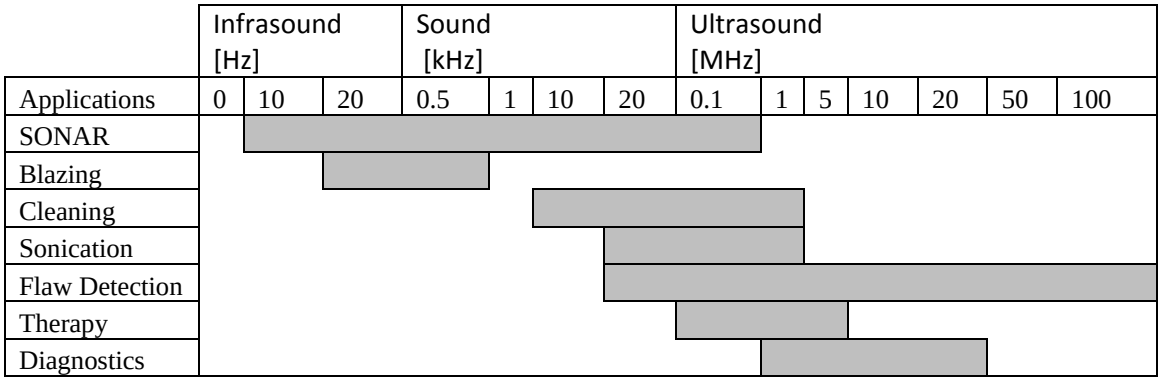

Figure 2.2. Spectrum of sound frequency and its applications. Sound below the frequency range of human hearing is called infrasound and the sound above the frequency range of human hearing is called ultrasound.

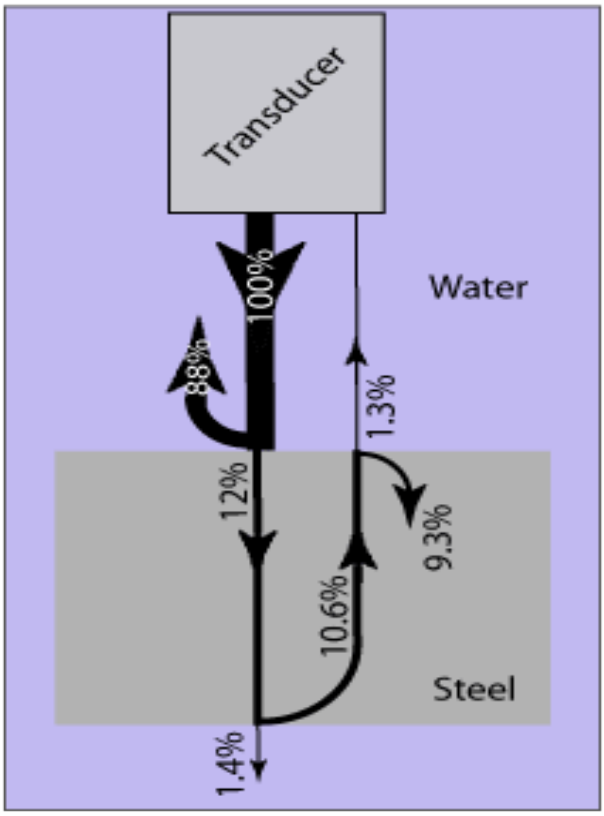

Figure 2.3. Example illustration of the reflection and transmission of ultrasound. Due to acoustic impedance mismatch, ultrasound can be reflected at the border of two media.

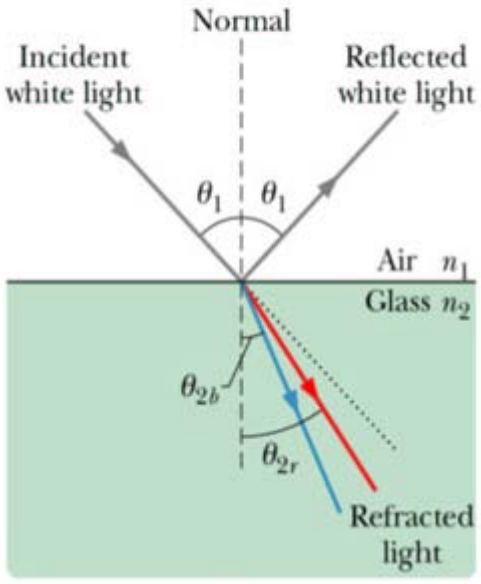

Figure 2.4. Illustration of Snell's Law. Ultrasound propagation follows the Snell's law. θ_1 represents incident angle and $\theta_2$represents refracted angle [41].

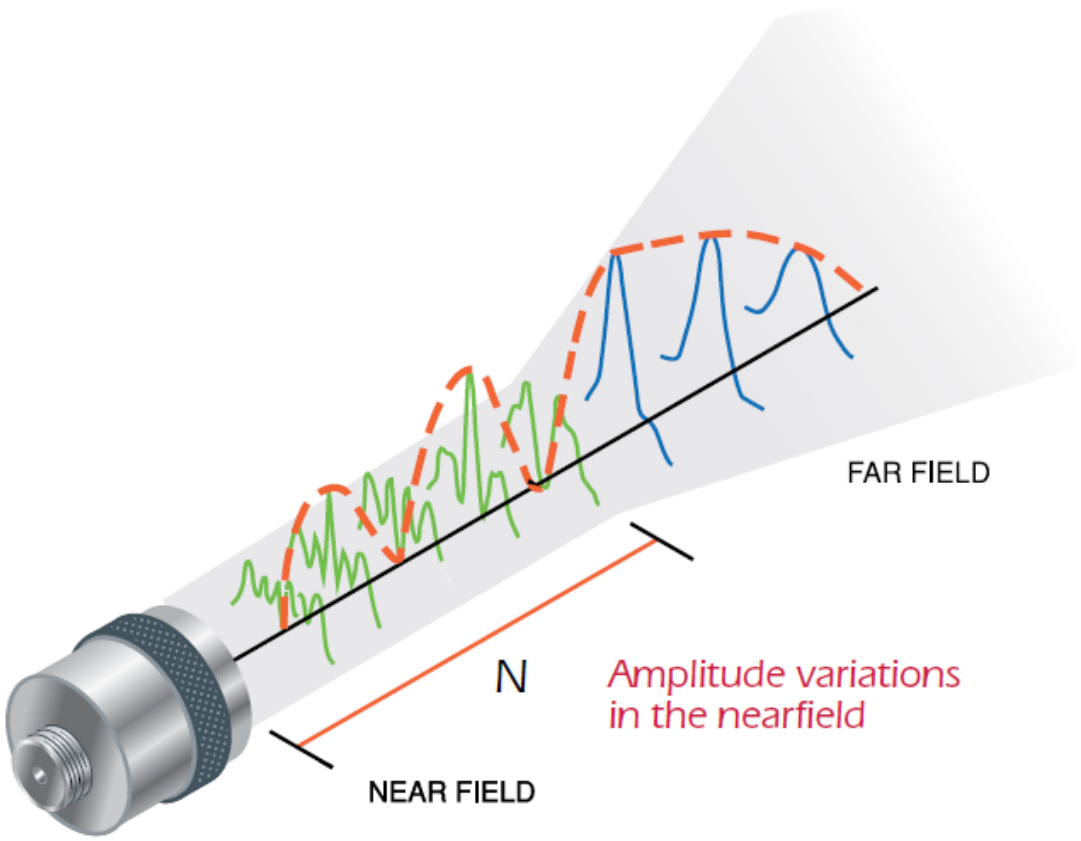

Figure 2.5. Illustration of near field and far field in the ultrasound beam. Two fields can be classified in the ultrasound beam depend on the fluctuation of sound pressure. N represents the length of near field [29].

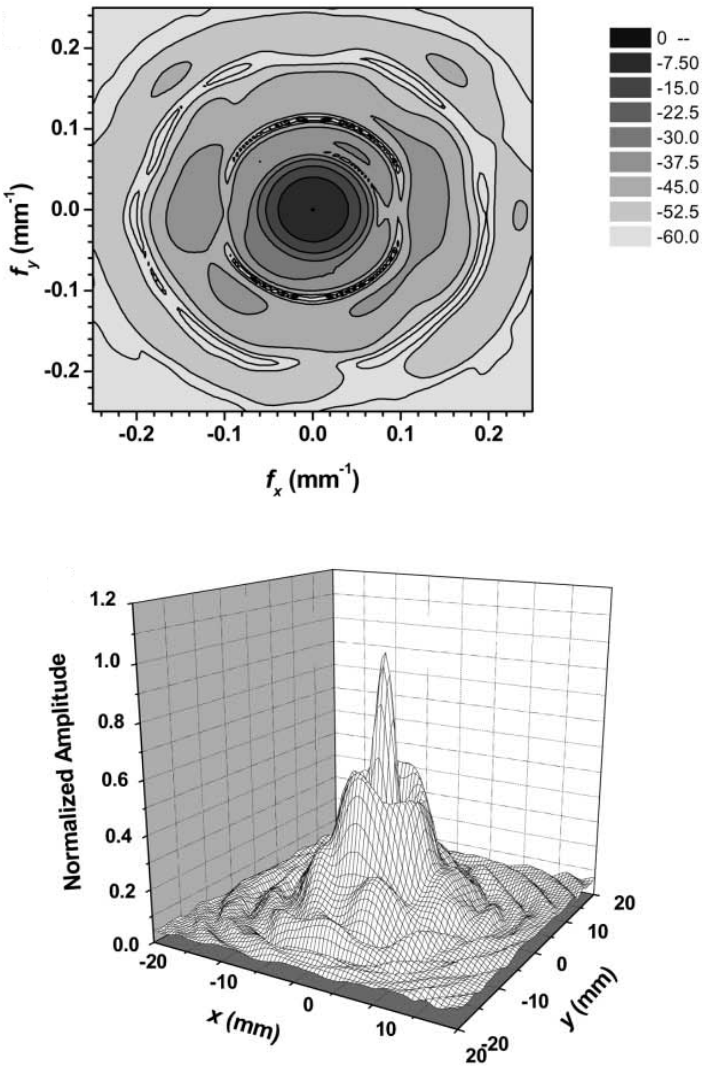

Figure 2.6. Profile of the normalized sound pressure amplitude of a transducer and the reconstructed pressure amplitude of the ultrasound field [38].

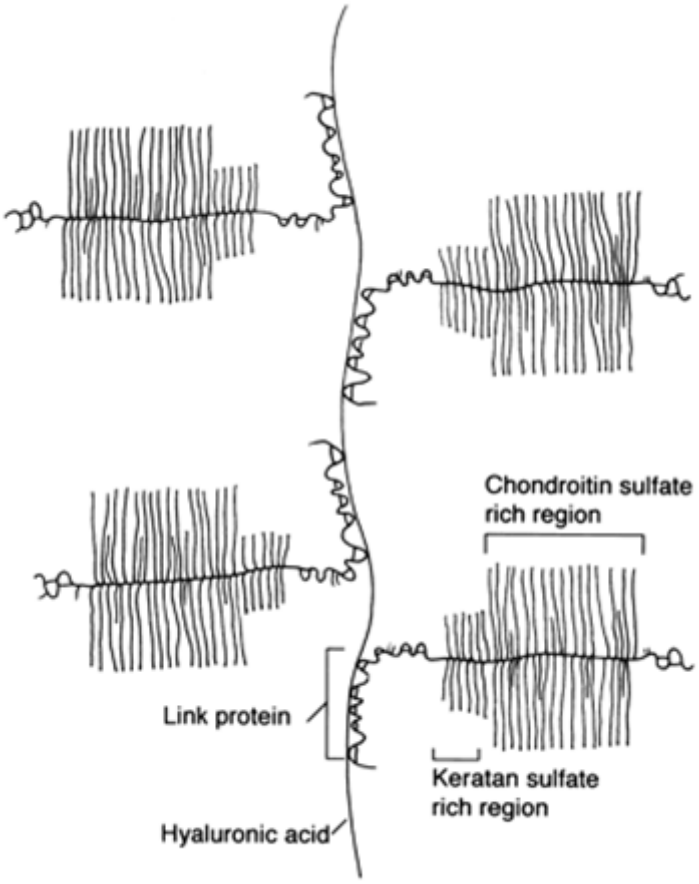

Figure 2.7. Illustration of proteoglycan which shows PG molecule and structural relationships between two main moieties, keratan sulfate and chondroitin sulfate [44].

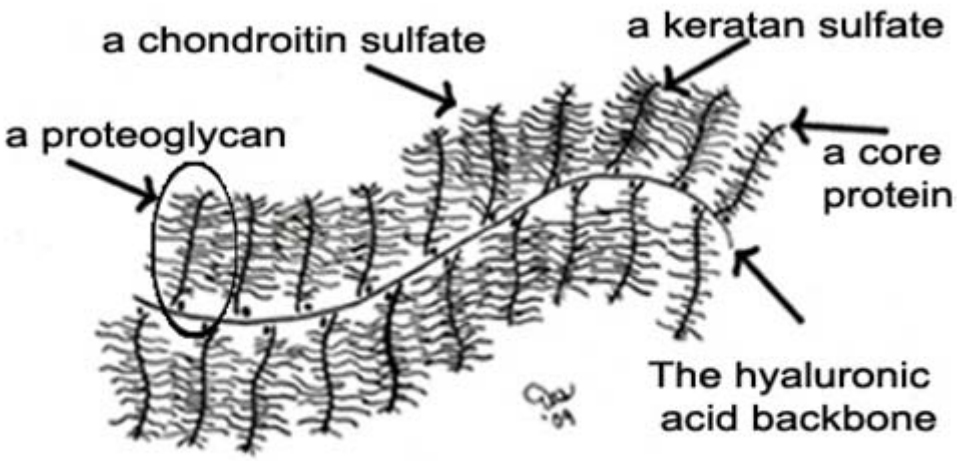

Figure 2.8. Structural illustration of aggrecan [43].

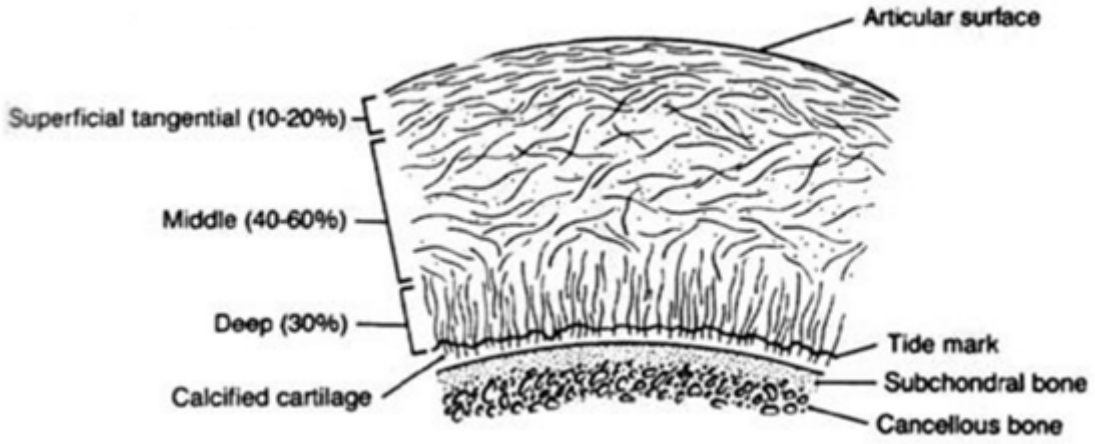

Figure 2.9. Illustration of zonal structure of articular cartilage [45].

CHAPTER 3

INVESTIGATION OF OPTIMAL ULTRASOUND DOSE ON HONDROCYTE MIGRATION FOR THE REPAIR OF ARTICULAR CARTILAGE

3.1 Purpose of Study

The phenomenon of cell migration is critical phase during embryonic development or gastrulation because they migrate to target locations and differentiate to specialized cells that make different tissues or organs. Chondrocytes are responsible in the articular cartilage for maintenance of extracellular matrix (ECM) by producing a variety of proteins such as PG, type II collagen and fibronectin. Since injured cartilage is hard to be repaired due to no blood vessels, chondrocyte migration is indispensable for the repair of cartilage and regeneration. Although there are several approaches to repair of articular cartilage, ultrasound has been frequently reported as one of the promising approaches for the cartilage repair [26, 31].

Recently, there have been frequent reports that therapeutic ultrasound has beneficial effect on the repair of articular cartilage. To confirm this, it was tested that low-intensity pulsed ultrasound (LIPUS) could repair the osteochondral defect model both simple scratches on the surface of cartilage and 2 mm full thickness of biopsy punch.

It was investigated how LIPUS affected to the activity of chondrocyte migration, simple scratched lines were made on monolayer culture plate and examined how LIPUS treated chondrocytes migrated toward the scratched area. Ultrasound safety test was first

conducted to confirm that LIPUS did not have any adverse effect on chondrocyte viability.

There have been several reports that compared two or more ultrasound doses to find better therapeutic conditions such as ultrasound intensity, duration and frequency, however, the optimal ultrasound dose is still unknown. This is probably due to the fact that different researches use different type of ultrasound wave such as continuous or pulsed, intensity, duration and frequency, and the different source of test samples. Therefore, the purpose of this study is to investigate optimal dose of LIPUS on the chondrocyte migration from the various combination of ultrasound dose; frequencies, duration and input energy (tested frequencies were 1, 3.5 and 5 MHz, tested durations were 10, 20, 40 and 60 minutes and tested input energy was ranged from 0.04 to 6.4 W). By adjusting three parameters of those ultrasound variables, the optimal dose of ultrasound on chondrocyte migration activity was investigated.

Finally, chondrocyte proliferation test was conducted to confirm that covered area from the chondrocyte migration assay was due to the chondrocyte migration, not by proliferation.

3.2 Material and Methods

3.2.1 Osteochondral Explants Harvest

Osteochondral explants were harvested from the bovine tibia plateau of the stifle joints and cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (1:1 mixture) supplemented with 10% fetal bovine serum (FBS), 50 ㎍/㎕ L-ascorbate, 100 U/㎕ penicillin, 100 ㎍/㎖ streptomycin, and 2.5 ㎍/㎕ Fungi zone after washed by Hank's Balanced Salt Solution (invitrogen, California, USA).

3.2.2 Chondrocyte Isolation

After 3-4 days of explants culture, chondrocytes were isolated from the cartilage tissue by 0.25 mg/ml collagenase (Sigma-Aldrich, Missouri, USA). The chondrocytes were seeded in 6-well plates with 8 x 10^4 cells and cultured in 5% O_2 and 5% CO_2 at 37°C until cells approximately 80% confluent.

3.2.3 Cartilage Injury Model

Osteochondral explants were harvested and scratched for mimicking injury model with 22 G needle causing local chondrocyte death, and LIPUS stimulation was induced for 20 minutes per day and consecutively 14 days. To see the amount of chondrocyte migration affected by LIPUS, human fibrin hydrogel using Tisseel^{TM} kit (Baxter AG, Vienna, Austria) was injected into 2mm biopsy punched osteochondral defects. Injured sites were imaged by confocal laser scanning microscope images (Bio-Rad, MRC-1024) as shown in the Figure 3.1.

3.2.4 Devices for Ultrasound Generation

Manually controlled Pulser-Receiver (5077PR, Olympus Panametrics-NDT), was used to apply electrical pulses to the transducers and to generate pulsed ultrasound waves (Figure 3.3). Briefly, 5077PR generated negative square pulse waves and was advantageous to adjust optimized pulse energy which resulted in superior signal to noise characteristics and maximizes the response in scattering materials. In addition, 5077PR automatically tunes the pulse width of the square pulse wave to half of the transducer's center frequency, the pulse energy to the transducer increases and it provides fast pulse rise and fall time, typically less than 10 nano-seconds, compared to spike pulse. The 5077PR has four selectable pulse voltages, -100, -200, -300 and -400 [V] with various

pulse repetition frequencies (PRF), 0.1, 0.2, 0.5, 2 and 5 KHz, in turn, various input energy could be applied to transducer by combination of pulse voltages and PRF.

Three different frequencies, 1, 3.5 and 5 MHz, of transducers (ICMF016, ICMF036, ICMF056, NDT System, California, USA) were used for LIPUS stimulation and those transducers have same shape with 0.75 inches of diameter and are manufactured to immerse into water (Figure 3.4). Since the transducer was immersible, it did not have to remove the culture media from the test samples, chondrocytes and cartilage explants, and could prevent the samples from contamination.

3.2.5 Ultrasound Safety Test

After approximately 80% of chondrocytes were confluent in a 6-well culture plate, various ultrasound parameters such as frequencies, input energy and duration, were combined and induced to the chondrocytes for one time. After 24 hours, chondrocytes viability was assessed using LIVE/DEAD fluorescent assay (ethidium-homodimer and calcein-AM) and the percentage of viability was calculated as a ratio of live cells out of total cells. Live or dead chondrocytes were stained either green or red as shown in the Figure 3.5.

3.2.6 Chondrocyte Migration Assay

After approximately 80% of chondrocytes were confluent in a 6-well culture plate, two scratched lines, one vertically and one horizontally, were created on the culture plate with cell scrapper (2mm). Then, four areas on the scratched lines were monitored to measure the area covered by migrated chondrocytes during two consecutive days of LIPUS stimulation as shown in the Figure 3.9. Using software, ImageJ, the percentage of areas covered by chondrocyte migration was measured [32, 33]. Chondrocyte migration

assay was used for input energy, frequency and duration dependent test. The images of area covered by migrated chondrocytes are shown in the Figure 3.10.

3.2.7 Confirmation Test of Chondrocyte Migration

Chondrocytes were seeded in the 6-well culture plates with 1 x 10^5 of cell density with the seeding area controlled to be less than the surface area of transducer which resulted in all chondrocytes seeded were stimulated by LIPUS. After 24 hours of chondrocyte attachment, LIPUS stimulation for two consecutive days was conducted with optimal dose of LIPUS found as 3.6 W of input energy, 5 MHz of frequency and 40 minutes of duration. After 2 days of LIPUS stimulation, the density of chondrocytes was calculated and compared to the control group.

3.2.8 Statistical Analysis

Statistical analysis was conducted by One-way ANOVA with tukey pair-wise comparison. Statistical significance was set at $p<0.05$.

3.3 Result

3.3.1 Effect of LIPUS on Osteochondral Explants Injury

In order to confirm that the low-intensity pulsed ultrasound (LIPUS) has beneficial effect on the repair of articular cartilage, LIPUS was induced on osteochondral explants defect injury models. The results showed that LIPUS significantly increased chondrocyte migration into the fibrin hydrogel and scratched area compared to the control group as shown in the Figure 3.1 and Figure 3.2.

From the experiments of osteochondral defects, LIPUS stimulates chondrocyte migration into injured site. However, it was hard to quantify the effect of LIPUS on osteochondral explants since the effect of LIPUS varied depend on the conditions of

explants which is supposed by the chondrocyte density and integrity of the explants. Therefore, chondrocytes were isolated and migration test was conducted in monolayer culture to see the effect of LIPUS on the activity of chondrocytes migration.

3.3.2 Ultrasound Safety Test on Chondrocytes

Ultrasound safety test was conducted to confirm if ultrasound is safe on chondrocytes. Ultrasound uses high frequency of sound waves that propagate through in the body by molecular interaction and has been considered to be safe as its non-radiating characteristic, thus most of infants in the US were imaged by ultrasound before birth in those days. Although the adverse biological effects of ultrasound have not been reported, the National Council on Radiation Protection and Measurements (NCRP) advocates that the safety of ultrasound needs to keep studied. In order to confirm if ultrasound is safe on articular cartilage, chondrocytes viability test was conducted in monolayer culture.

Ultrasound safety test was conducted by combination of parameter, input energy, frequency and duration. First, 1 MHz of ultrasound combined with 0.32, 3.6 and 6.4 W of input energy and either 20 or 60 minutes of duration was first tested. Second, 3.5 MHz of ultrasound combined with 0.18, 0.57 and 3.6 W of input energy and either 20 or 60 minutes of duration was tested. Lastly, 5 MHz of ultrasound combined with 0.32, 1.28 and 6.4 W of input energy and either 20 or 60 minutes of duration was tested. The results show that all combinations of ultrasound parameters had over 95 % of chondrocyte viability and there were no significant difference compared to the control group which did not receive the ultrasonic stimulation. The results of each frequency test were shown in the Figure 3.6, 3.7 and 3.8.

3.3.3 Chondrocyte Migration Test by Input Energy Dependent

Although ultrasound intensity is one of the most important parameters in therapeutic ultrasound, total amount of ultrasound energy could be also important factor for the therapeutic effect. Therefore, it was focused that the amount of ultrasound energy chondrocytes received rather than the strength of single ultrasound wave energy from this study. In order to see the migration activity of chondrocytes by input energy dependent, pulse repetition frequency (PRF) and pulse voltage of 5077PR were adjusted for the various input energy combination. 1 MHz of frequency and 20 minute of duration were randomly pre-set.

It was investigated that chondrocytes migration were affected by input energy dependent. Percentage of areas covered by migrating chondrocytes was normalized by control group as shown in the Figure 3.11. Out of five different input energy levels, only 0.4 W and 3.6 W of input energy were significantly different from control group and 3.6 W of input energy was more significant effect on the chondrocytes migration activity than 0.4 W. The result indicated that the chondrocyte migration was not proportionally affected by the increase of input energy, but affected at a certain input energy level.

3.3.4 Chondrocyte Migration Test by Frequency Dependent

Ultrasound frequency is very important parameter in therapeutic ultrasound, because it determines the penetration depth, however, the effect of frequency itself has not been well studied. Therefore, the effect of ultrasound frequency was investigated on chondrocyte migration in order to see if ultrasound frequency is only related to the penetration depth. Since previous experiment resulted in 3.6 W of input energy was most

effective on chondrocytes migration, the input energy was set to 3.6 W. Then, 1, 3.5 and 5 MHz of ultrasonic frequency with 20 minutes of duration were tested.

It was investigated that chondrocyte migration was affected by ultrasonic frequency dependent. Percentage of areas covered by chondrocyte migration was normalized by control group. Although all of three ultrasound stimulated groups showed increased the activity of chondrocyte migration compared to the control group, only 5 MHz ultrasound has statistically significant difference as shown in the Figure 3.12. The result indicates that the activity of chondrocyte migration was more affected by higher frequency, or shorter wavelength, of ultrasound among the tested frequencies.

3.3.5 Chondrocyte Migration Test by Stimulating Duration Dependent

Since previous experiments resulted in 3.6 W of input energy and 5 MHz of frequency were the most effective on the activity of chondrocyte migration, they were pre-set and various durations, 10, 20, 40 and 60 minutes of LIPUS were tested.

Percentage of areas covered by migrating chondrocytes was normalized by control group and the result showed that 40 minutes of LIPUS was the most effective on the activity of chondrocyte migration as shown in the Figure 3.13. Out of four different stimulating durations, only 40 minutes of stimulation has statistically significant difference compared to the control group. The result indicates that the activity of chondrocyte migration was not proportional to the duration, but was significantly affected at certain duration.

3.3.6 Confirmation of Chondrocyte Migration Test

It was investigated that the activity of chondrocyte migration was the most effective at 3.6 W of input energy, 5 MHz of frequency and 40 minutes of duration.

However, chondrocyte proliferation could be primary to cover the area on scratched lines rather than migratory activity of chondrocytes. Therefore, chondrocyte proliferation was investigated if the area of scratched lines was covered by chondrocyte migration.

The result shows that chondrocytes were not proliferated by LIPUS stimulation and chondrocytes in control group proliferated slightly more than LIPUS treated group as shown in the Figure 3.14. However, there was no statistically significant difference between control group and LIPUS treated group. The result indicates that the areas on scratched lines were primarily covered by chondrocyte migration, but proliferation.

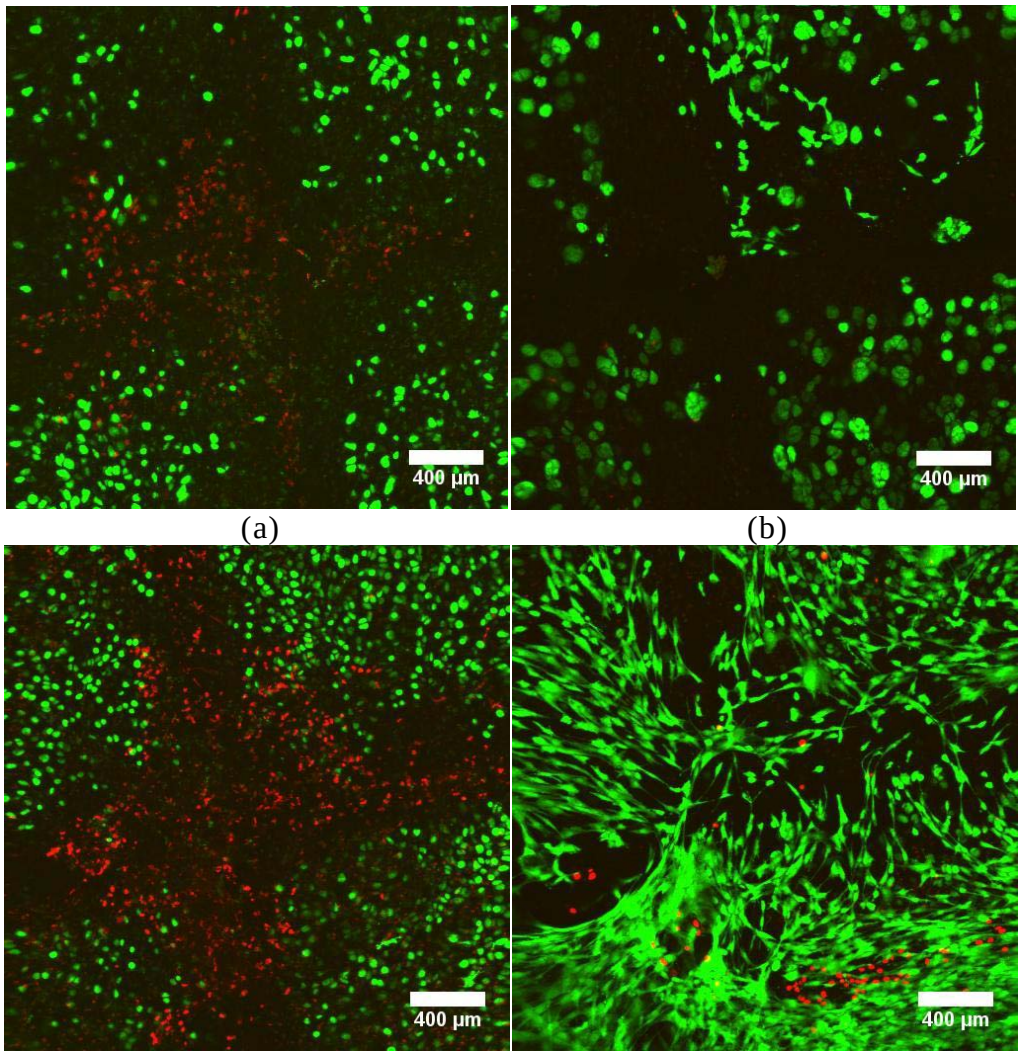

Figure 3.1. Confocal laser scanning microscope (MRC-1024, Bio-Rad) images of green (living cells) and red (dead cells) in the injured cartilage explants model. After 14 days of LIPUS stimulation, abundant elongated chondrocytes around injured sites were observed whereas the control group showed few chondrocytes migration. (a) and (b) is control group at day 0 and day 14. (c) and (d) is LIPUS induced group at day 0 and day 14.

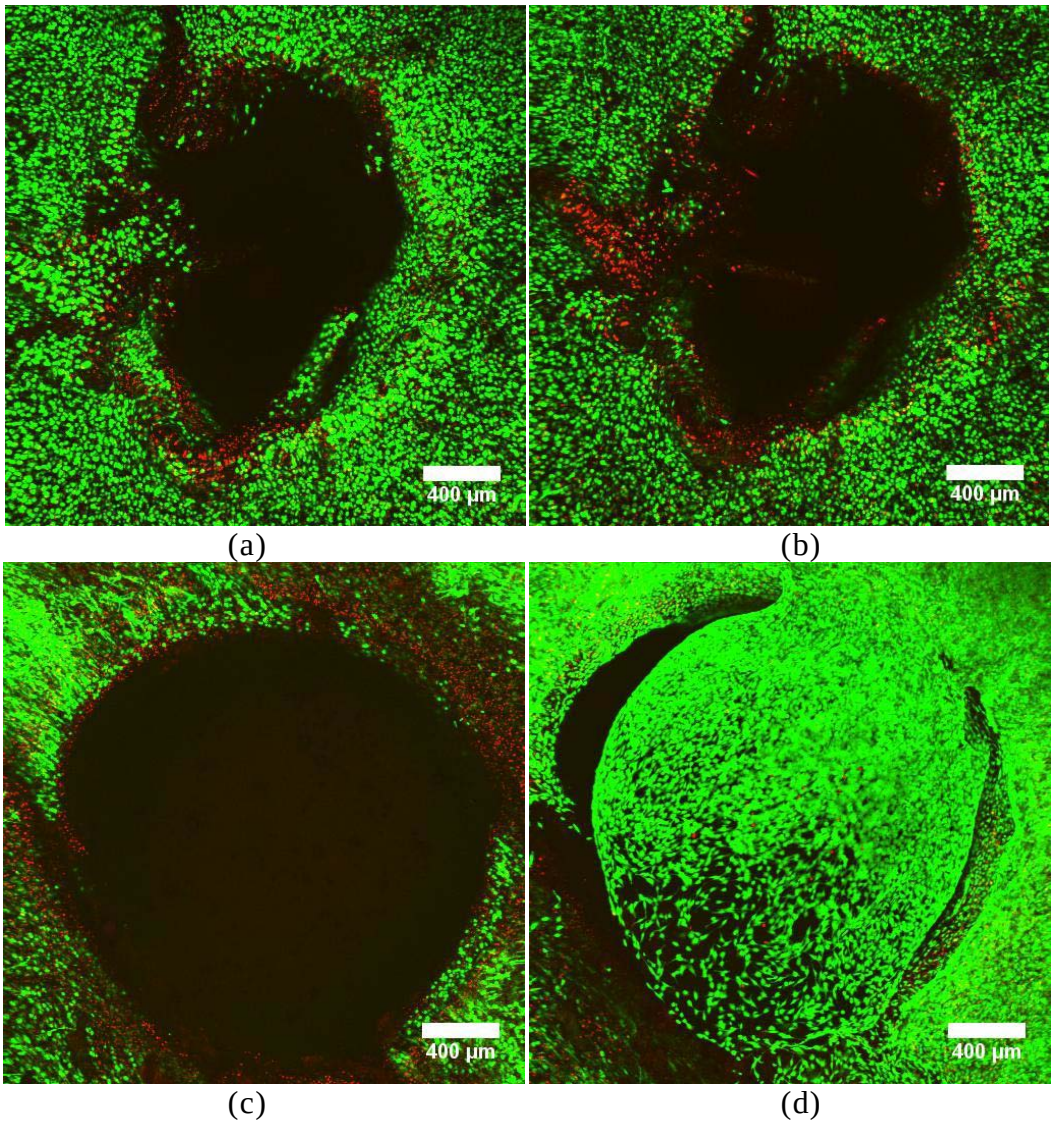

Figure 3.2. Confocal laser scanning microscope (MRC-1024, Bio-Rad) images of green (living cells) and red (dead cells) in 2mm biopsy punched cartilage explants defect model. After 7 days of LIPUS stimulation, abundant chondrocytes migrated into the human fibrin hydrogel. (a) and (b) is control group at day 0 and day 7. (c) and (d) is LIPUS treated group at day 0 and day 7.

Figure 3.3. Picture of manually controlled square wave Pulser-Receiver (5077PR, Olympus Panametrics-ndt). Various combinations of input energy can be adjusted by controlling pulse repetition rate, pulse voltage and pulse duration.

Figure 3.4. Picture of ultrasonic immersible transducer (ICMF016, ICMF036, ICMF056, NDT System). Three different frequencies of transducers have same shape and 0.75 inches of diameter. Since it is immersible in culture media, contamination of experimental sample can be prevented from contamination.

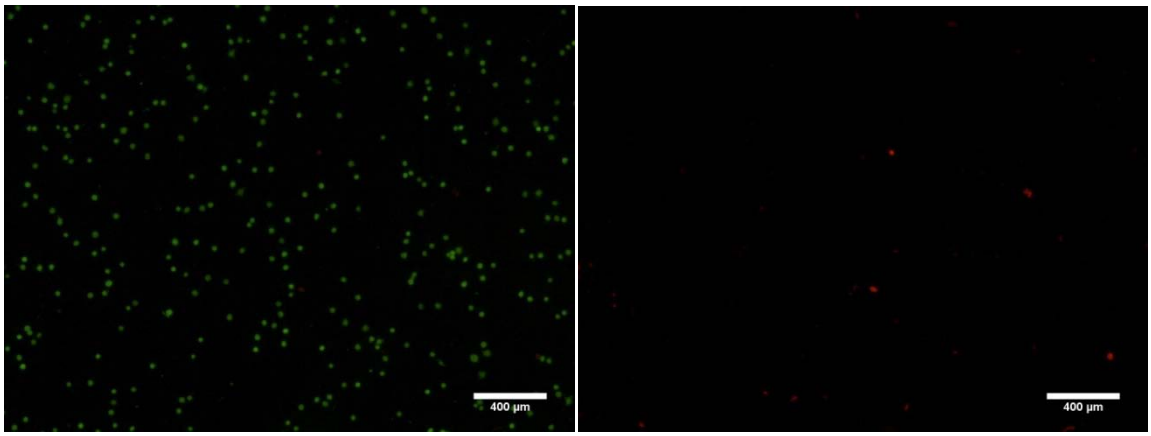

Figure 3.5. LIVE(green)/DEAD(red) fluorescent stained chondrocytes for ultrasound safety test. Using fluorescent assay, ethidium-homodimer for dead cell and calcein-AM for living cell, percentage of chondrocyte viability was calculated.

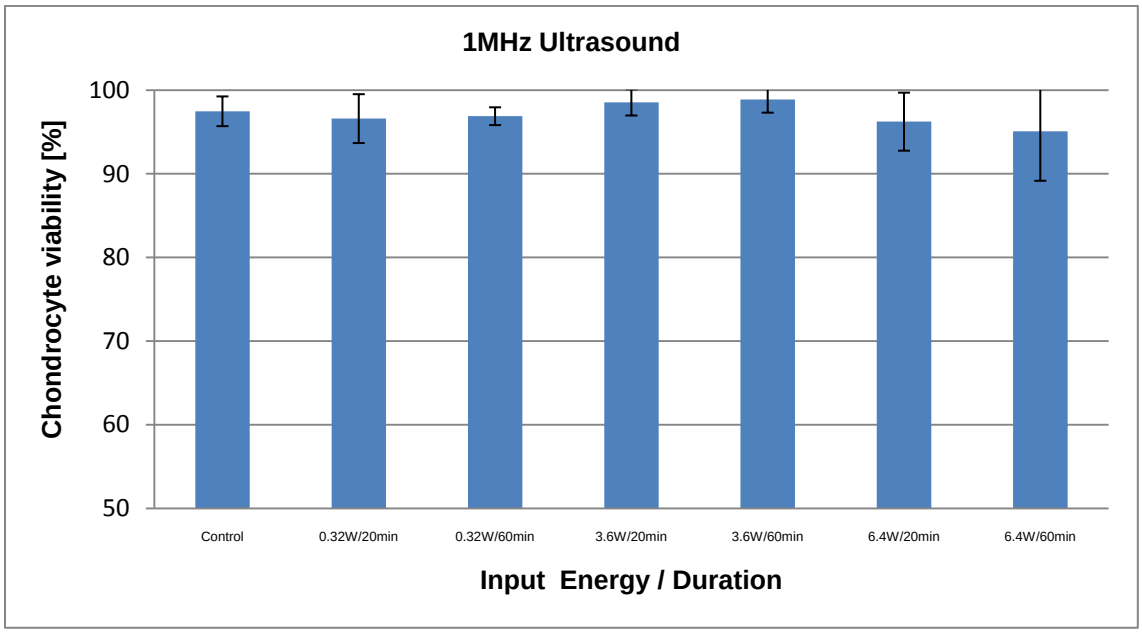

Figure 3.6. Effect of 1 MHz ultrasound on chondrocyte viability. Various input energy with either 20 or 60 minutes duration were tested. All experimental groups had over 90 % of viability and there was no statistically significant difference compared to the control group. n=2.

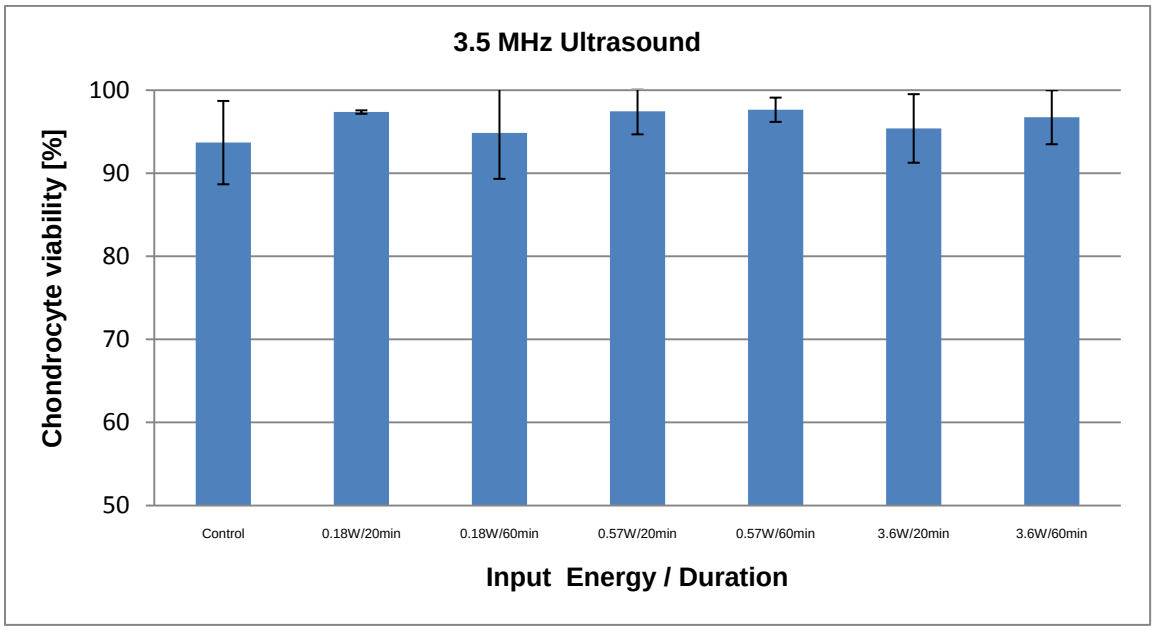

Figure 3.7. Effect of 3.5 MHz ultrasound on chondrocytes viability. Various input energy with either 20 or 60 minutes duration were tested. All experimental groups had over 90 % of viability and there was no statistically significant difference compared to the control group. n=2.

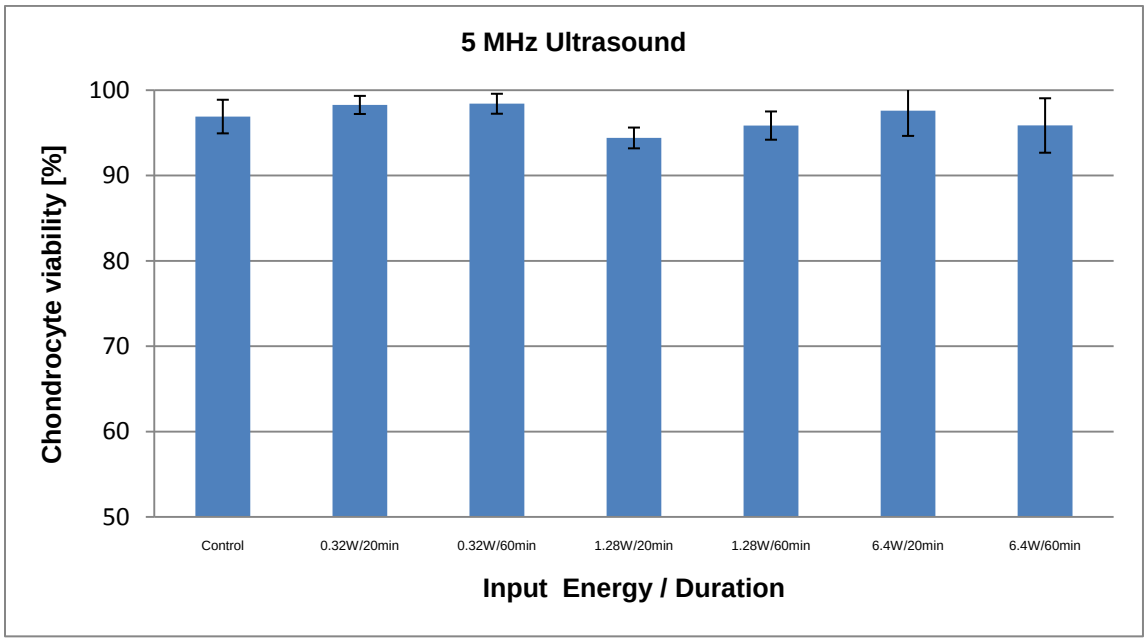

Figure 3.8. Effect of 5 MHz ultrasound on chondrocytes viability. Various input energy with either 20 or 60 minutes duration were tested. All experimental groups had over 90 % of viability and there was no statistically significant difference compared to the control group. n=3.

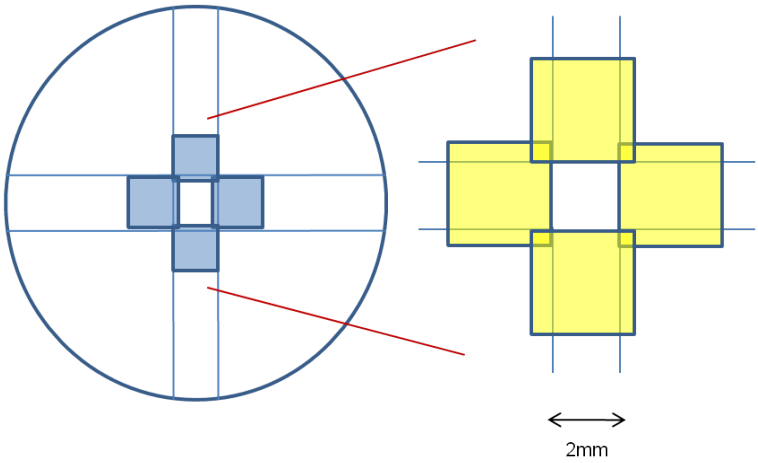

Figure 3.9. Illustration of areas monitored for chondrocyte migration in monolayer culture. Four areas on two scratched lines were monitored and measured to see the rate of coverage by chondrocyte migration compared to control group.

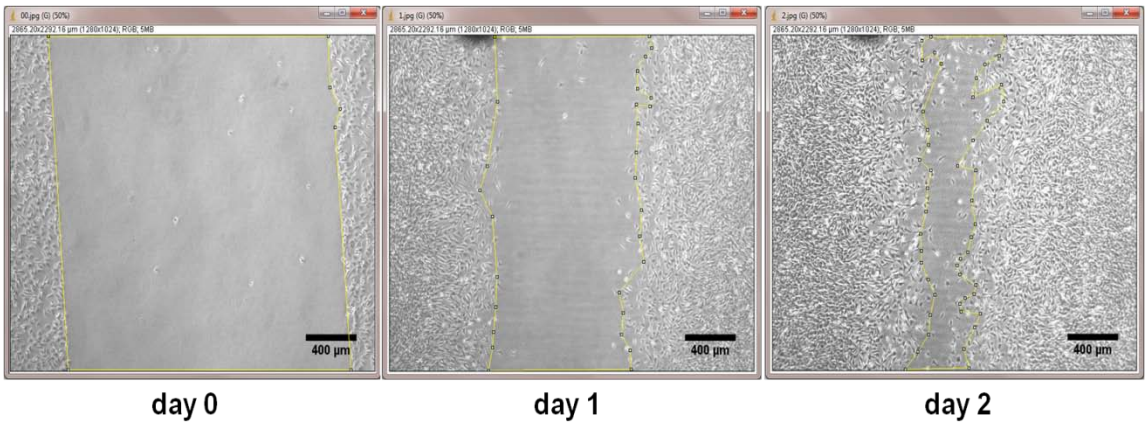

Figure 3.10. Images of area covered by chondrocyte migration in a monolayer culture plate. With 2 mm cell scrapper, the scratched area was almost fully covered by chondrocyte migration for 2 days. LIPUS was induced at day 0 and at day 1 only.

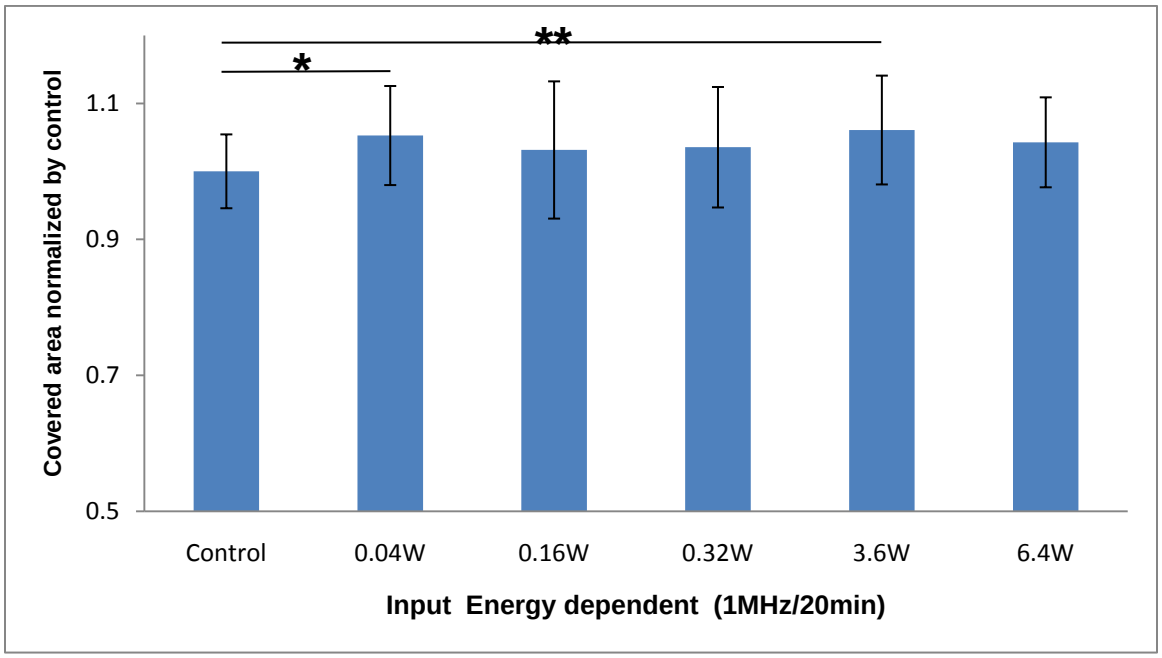

Figure 3.11. Effect of LIPUS on chondrocyte migration by input energy dependent. Percentage of covered area was normalized by control group. One-way ANOVA with tukey pair-wide comparison was used for statistical analysis at significant level, p=0.05. (*p<0.05, **p<0.01) n=10.

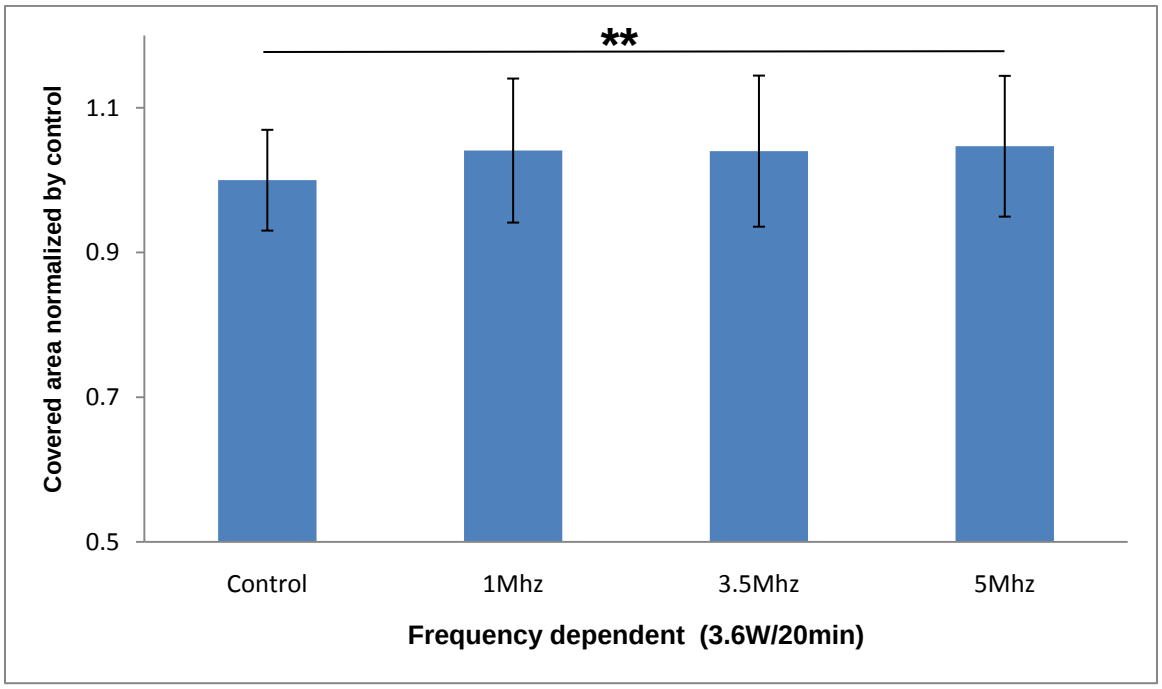

Figure 3.12. Effect of LIPUS on chondrocyte migration by frequency dependent. Percentage of covered area was normalized by control group. One-way ANOVA with tukey pair-wide comparison was used for statistical analysis at significant level, p=0.05. (**p<0.01) n=16.

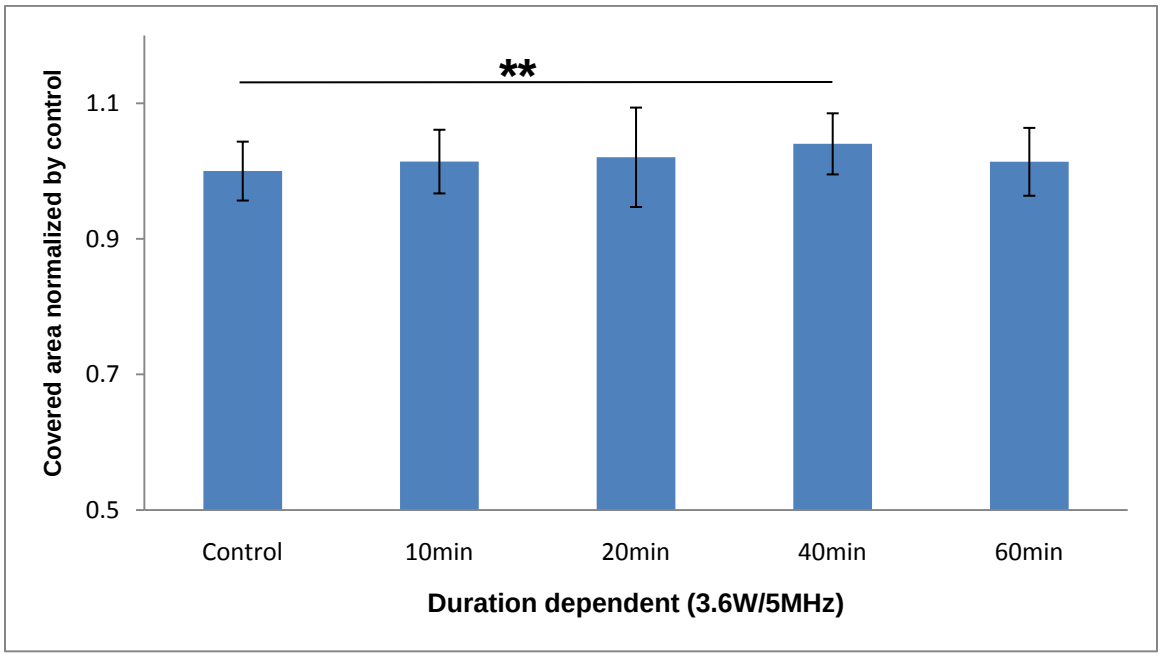

Figure 3.13. Effect of LIPUS on chondrocyte migration by duration dependent. Percentage of covered area was normalized by control group. One-way ANOVA with tukey pair-wide comparison was used for statistical analysis at significant level, p=0.05. (**p<0.01) n=6.

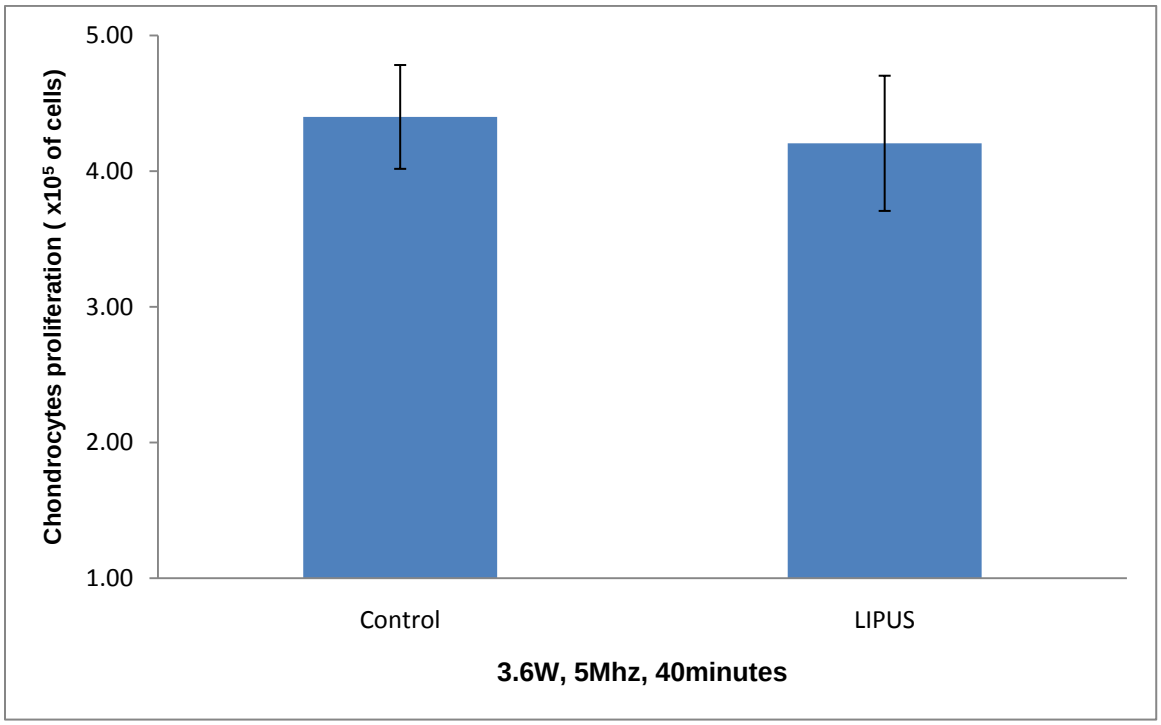

Figure 3.14. Effect of optimized dose of LIPUS on chondrocyte proliferation in a monolayer culture. There was no statistical significant difference between two groups. n=6.

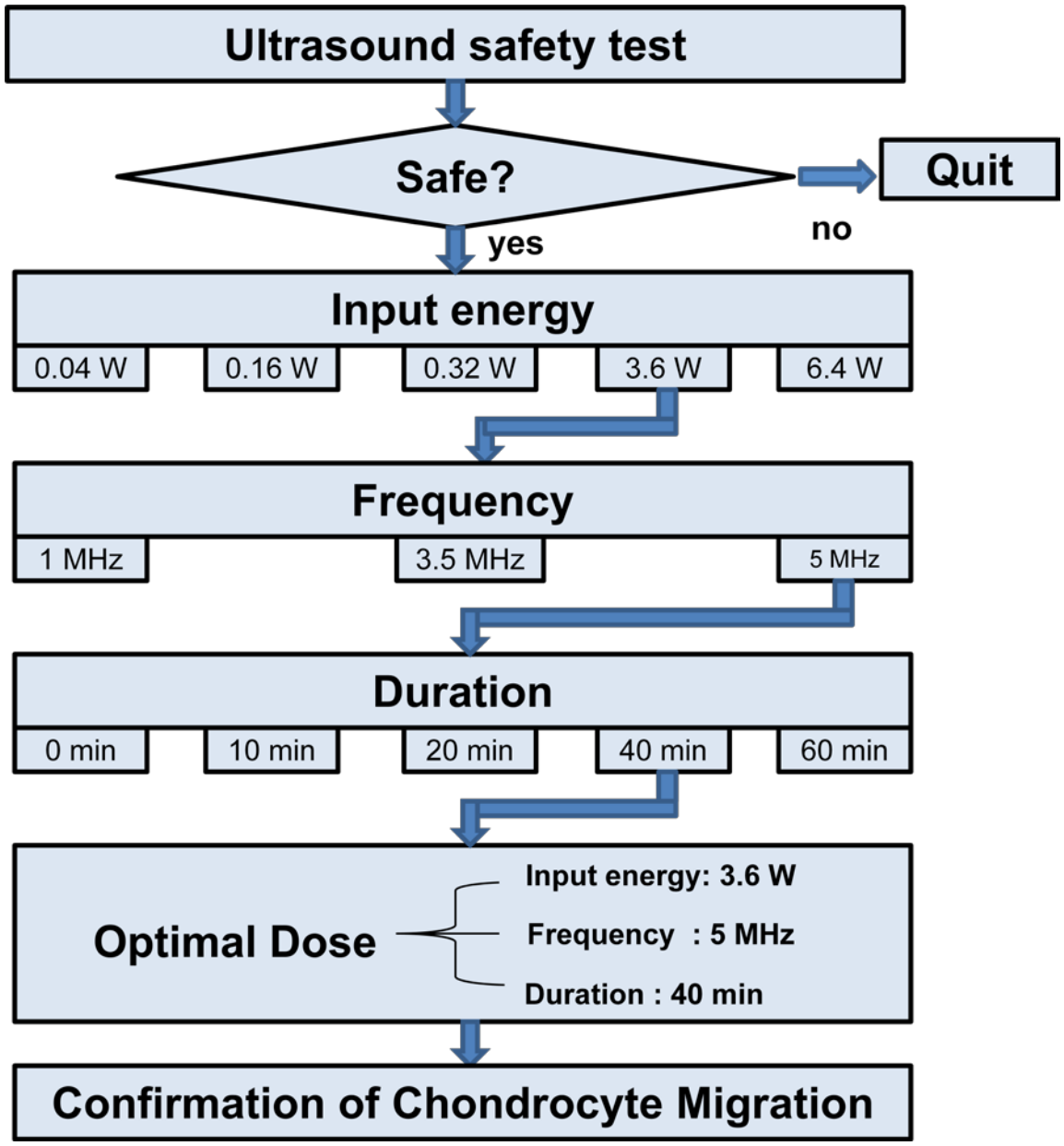

Figure 3.15. Flow chart of investigating the optimal dose of LIPUS on the activity of chondrocyte migration.

CHAPTER 4

DISCUSSION AND CONCLUSION

Ultrasound has been widely used as medical diagnostic tools such as imaging a fetus before giving birth since ultrasound is considered safe and it is supported that there have been few reports its adverse effects. Recently, ultrasound has been extended its application into physical therapy and frequently reported its benefits for the repair of fractured bone and damaged cartilage without its adverse effect. Even so, it cannot emphasize the importance much enough to make sure confirming safety of ultrasound diagnostically as well as therapeutically. In order to confirm that low-intensity pulsed ultrasound (LIPUS) is safe and has therapeutic benefits on damaged articular cartilage, chondrocytes viability test first conducted. By controlling three different ultrasonic variables; frequencies, input energy and duration, various combinations of ultrasound dose were examined to confirm its safety on chondrocytes in monolayer culture. LIPUS stimulation was then induced only one time when the chondrocytes in a 6-well culture plate were to confluent. After 24 hours, Live/Dead fluorescent assay (Ethidium-homodimer and Calcein-AM) was used to calculate the percentage of live cells out of total cells. For statistical analysis, One-way ANOVA with tuckey pair-wire comparison was used with significance level at $p<0.05$. The result showed that the chondrocytes were not affected its viability by various combinations of ultrasound dose and all experiments had over 95% chondrocyte viability and there were no statistical significant differences compared to the

control group. Therefore, it could conclude that the range of LIPUS dose applied to the experiments is safe and could be applied to the repair of injured articular cartilage.

There have been several reports that two or three different ultrasound intensities were compared on the repair of articular cartilage in vivo or in vitro and it was found more effective intensity from each study. However, different studies had different optimal intensity; there was not consistent optimal intensity level from the studies. It was probably due to several factors such as variations from experimental sources, different type of ultrasound waves and type of transducers. Therefore, it was investigated that optimal ultrasound dose on chondrocyte migration by combinations of input energy, frequency and duration of ultrasound. Total amount of ultrasound energy induced to chondrocytes were considered as the total input energy to the transducer, because it could be regarded that the amount ultrasound energy is proportional to the amount of input energy. That is, the more input energy density to the transducer, the more ultrasonic energy density is induced to the chondrocytes.

The chondrocyte migration assay in a monolayer culture allowed to calculating the rate of chondrocyte migration. Since it was possible that the area on scratched lines was covered by chondrocyte proliferation, real time imaging microscope (Olympus IX81 Inverted Light Microscope) was used to confirm that the area was covered by chondrocyte migration. By examining the 24 hours real time imaging, the area was covered primarily by the chondrocyte migration, however, it was hard to control the chondrocytes culturing environment such as O2, CO2 and humidity

during 24 hours of imaging. Therefore, the chondrocytes proliferation test was conducted to confirm and quantify the scratched area was covered by chondrocyte migration, not by proliferation. The results shown in the Figure 3.12 indicate that the proliferation of chondrocytes was not affected by LIPUS stimulation. Compared to the control group, LIPUS treated group showed less proliferative chondrocytes activity, however, there was no statistically significant different between two groups. Therefore, it could conclude that the area on scratched lines was covered by primarily by the activity of chondrocyte migration rather than proliferation.

From the input energy dependent test, the result showed that 0.04 W and 3.6 W of input energy significantly increased the activity of chondrocyte migration compared to the control group. The chondrocytes were not affected proportional to the amount of ultrasound energy and 3.6 W of input energy was more significant on the activity of chondrocyte migration. There have not been clear answers for the mechanism of therapeutic ultrasound, thus it is hard to say that there is optimal ultrasound intensity or amount of ultrasound energy for the experimental sample to be received. However, the results from previous studies showed that experimental samples such as tissues or cells were responded at most with certain ultrasound intensity differently. This might be related to the condition of experimental sample such as freshness of samples, not for the intensity. Although further investigations would be needed to clarify this point, total ultrasound energy to the experimental sample could be one of clues for mechanism of therapeutic ultrasound as well as intensity.

The ability of ultrasonic propagation is inversely proportional to its frequency, because lower ultrasound frequency has longer wavelength which

allows ultrasound to penetrating into tissue deeper than higher ultrasound frequency. The reason why most of LIPUS studies use 1 or 1.5 MHz of ultrasonic frequency is probably due to its better penetration ability and originated from bone fracture stimulation [10, 23, 26, 27, 28, 30, 31]. However, the propagation of ultrasound into articular cartilage from the skin of knee could be affected by not only components of medium and difference of acoustic impedance at the border of two different media, but also frequency, intensity and the position of the transducer [34, 35]. 1, 3.5 and 5 MHz of ultrasound frequencies were tested on the activity of chondrocyte migration with fixed 3.6 W of input energy. By setting of input energy as 3.6 W for all three frequencies, chondrocytes could be received same amount of ultrasonic energy. Same chondrocyte migration assay in a monolayer culture plate was used for comparing the activity of chondrocyte migration by frequency dependent. From the experiment, all three different ultrasound frequencies, propagating to chondrocytes in culture media, could be regarded as there was little ultrasonic attenuation due to the fact that attenuation does not usually take place in water. The result showed that 5 MHz ultrasound was the most effective among the tested frequencies and was statistically significant different compared to the control group. Since ultrasound is not usually attenuated in the water, it could be regarded that the activity of chondrocyte migration is positively affected by shorter wavelength rather than longer wavelength of ultrasound. Therefore, it should be considered as optimal frequency for chondrocyte stimulation, even if 5 MHz ultrasound has poorer penetration ability than 1 MHz ultrasound.

Although there have been frequent reports investigating the optimal ultrasound intensity for the repair of soft tissue or cells, there have been a very few reports investigating the optimal duration of LIPUS stimulation. Most of studies just induce 10 or 20 minutes of duration without sufficient explanation. [23, 27, 28, 30, 31]. Nevertheless, there have been a few studies investigating the duration of ultrasound stimulation. Rodrigo Oyonarte et al. in 2009 showed that 20 minutes of LIPUS significantly increases the growth of mandibular condylar cartilage than 10 minutes of LIPUS [36]. And Stephen D. Cook et al. in 2001 showed that 40 minutes of LIPUS duration was the most effective on the repair of osteochondral defects model among the 5, 10, 20 and 40 minutes [26]. Therefore, the optimal duration was investigated on the activity of chondrocyte migration among 10, 20, 40 and 60 minutes. Same chondrocyte migration assay in a monolayer culture was used and the input energy and frequency were preset to 3.6 W and 5 MHz because they were found as the most effective input energy and frequency on the chondrocytes migration. The result showed that 40 minutes of LIPUS stimulation was the most effective among the 0 minute as control, 10, 20, 40 and 60 minutes and this result is consistent to the study from Stephen D. Cook et al. in 2001 [26]. Even so, it is hard to conclude that 40 minutes of duration is optimal due to the fact that there are a number of variables to make different responses of the repairing process such as type of input, continuous or pulsed, type of transducers and different testing samples.

In order to confirm that the areas in a monolayer culture plate were covered by chondrocyte migration or by proliferation, chondrocyte proliferation test was conducted. Since the optimal ultrasonic dose on chondrocytes was found as 3.6 W of input energy, 5

MHz of frequency and 40 minutes of duration, the dose was applied to chondrocytes proliferation test. The result showed that there were not statistically significant different between control group and LIPUS treated group.

In conclusion, 3.6 W of input energy was the most effective on the activity of chondrocyte migration with prefixed frequency and duration as 1 MHz and 20 minutes. And 5 MHz ultrasound was the most effective on chondrocytes among the 1, 3.5 and 5 MHz frequencies with prefixed duration with 20 minutes and 3.6 W of input energy. For the duration test, 3.6 W of input energy and 5 MHz of frequency were fixed and 0, 10, 20, 40 and 60 minutes of duration was tested. Among those durations, 40 minutes was the most effective on the activity of chondrocyte migration. As a result, the optimal dose of LIPUS on the activity of chondrocyte migration from this study was found at 3.6 W of input energy, 5 MHz of frequency and 40 minutes of duration. Finally, it was confirmed that the areas on the scratched lines was covered by chondrocyte migration, not by the proliferation.

The whole process of flow chart to find the optimal does of LIPUS is shown in the Figure 3.15.

CHAPTER 5

FUTURE WORKS

There have been appreciation of beneficial effect of LIPUS and the activity of chondrocyte migration was the most effective at 3.6 W of input energy, 5 MHz of frequency and 40 minutes of duration from this study. However, it is not still unknown why chondrocytes were responded at most at the LIPUS dose. Therefore, it needs to be investigated how ultrasound affects to intracellular environments in order to figure out why the LIPUS dose was the most effective on chondrocyte migration. Cytoskeleton studies, such as molecular changes and stress fibers formation, could provide how chondrocytes are affected to its migration activity by ultrasound. As well, intracellular signaling pathway such as focal adhesion kinase (FAK) also needs to be studied to see how ultrasound energy acts on proteins and gene expression in the chondrocytes.

REFERENCES

1. O'Brien, W.D., Jr., *Ultrasound-biophysics mechanisms.* Prog Biophys Mol Biol, 2007. **93**(1-3): p. 212-55.

2. Kitchen SS, P.C., *A review of therapeutic ultrasound, part 1: background and physiological effects.* Physiotherapy, 1990. **76**.

3. Wu J, D.G., *Temperature elevation in tissues generated by finite-amplitude tone bursts of ultrasound.* J Acoust Soc Am, 1990. **88**: p. 1562-1577.

4. ter Haar, G., *Basic physics of therapeutic ultrasound.* Physiotherapy, 1978. **64**(4): p. 100-3.

5. Draper, D.O., et al., *A comparison of temperature rise in human calf muscles following applications of underwater and topical gel ultrasound.* J Orthop Sports Phys Ther, 1993. **17**(5): p. 247-51.

6. G., t.H., *Biological effects of ultrasound in clinical applications.* In: Suslick KS, ed. Ultrasound: Its Chemical, Physical, and Biological Effects. New York, NY: VCH Publishers Inc, 1988: p. 305-320.

7. Wu, J., A.J. Winkler, and T.P. O'Neill, *Effect of acoustic streaming on ultrasonic heating.* Ultrasound Med Biol, 1994. **20**(2): p. 195-201.

8. FA., D., *Acoustic streaming and radiation pressure in diagnostic applications: what are the implications?* In: Barnett SB, Kossoff G, eds. Safety of Diagnostic Ultrasound. New York, NY: Parthenon, 1998.

9. Matre, K. and S.H. Eik-Nes, *[Biological effects of ultrasound and potential hazards in diagnosis. Review of the literature].* Tidsskr Nor Laegeforen, 1983. **103**(22): p. 1497-501.

10. Lee, H.J., et al., *Low-intensity ultrasound stimulation enhances chondrogenic differentiation in alginate culture of mesenchymal stem cells.* Artif Organs, 2006. **30**(9): p. 707-15.

11. Pietrzak, W.S., *Musculoskeletal tissue regeneration: biological materials and methods.*

12. Bhosale, A.M. and J.B. Richardson, *Articular cartilage: structure, injuries and review of management.* Br Med Bull, 2008. **87**: p. 77-95.

13. Buckwalter, J.A. and J.A. Martin, *Osteoarthritis.* Adv Drug Deliv Rev, 2006. **58**(2): p. 150-67.

14. *See, World Health Organization (WHO), http://www.who.int/en/.*

15. Lawrence, R.C., et al., *Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States.* Arthritis Rheum, 1998. **41**(5): p. 778-99.

16. Kotlarz, H., et al., *Insurer and out-of-pocket costs of osteoarthritis in the US: evidence from national survey data.* Arthritis Rheum, 2009. **60**(12): p. 3546-53.

17. Chapman, I.V., N.A. MacNally, and S. Tucker, *Ultrasound-induced changes in rates of influx and efflux of potassium ions in rat thymocytes in vitro.* Ultrasound Med Biol, 1980. **6**(1): p. 47-58.

18. Li, J.K., et al., *Cytokine release from osteoblasts in response to ultrasound stimulation.* Biomaterials, 2003. **24**(13): p. 2379-85.

19. Rawool, N.M., et al., *Power Doppler assessment of vascular changes during fracture treatment with low-intensity ultrasound.* J Ultrasound Med, 2003. **22**(2): p. 145-53.

20. Speed, C.A., *Therapeutic ultrasound in soft tissue lesions.* Rheumatology (Oxford), 2001. **40**(12): p. 1331-6.

21. Baker, K.G., V.J. Robertson, and F.A. Duck, *A review of therapeutic ultrasound: biophysical effects.* Phys Ther, 2001. **81**(7): p. 1351-8.

22. Zhou, S., et al., *Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts.* J Biol Chem, 2004. **279**(52): p. 54463-9.

23. Zhang, Z.J., et al., *The effects of pulsed low-intensity ultrasound on chondrocyte viability, proliferation, gene expression and matrix production.* Ultrasound Med Biol, 2003. **29**(11): p. 1645-51.

24. *See Wikipedia,The Table of Attenuation, http://en.wikipedia.org/wiki/Attenuation.*

25. *See Advanced NDT LTD, www.advanced-ndt.co.uk.*

26. Cook, S.D., et al., *Improved cartilage repair after treatment with low-intensity pulsed ultrasound.* Clinical Orthopaedics and Related Research, 2001(391): p. S231-S243.

27. Tien, Y.C., et al., *Effects of pulsed low-intensity ultrasound on human child chondrocytes.* Ultrasound Med Biol, 2008. **34**(7): p. 1174-81.

28. Parvizi, J., et al., *Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression.* J Orthop Res, 1999. **17**(4): p. 488-94.

29. *See Olympus Panametrics-NDT, http://www.olympus-ims.com/en/panametrics-ndt-ultrasonic/*

30. Choi, B.H., et al., *Low-intensity ultrasound stimulates the viability and matrix gene expression of human articular chondrocytes in alginate bead culture.* J Biomed Mater Res A, 2006. **79**(4): p. 858-64.

31. Min, B.H., et al., *Effects of low-intensity ultrasound (LIUS) stimulation on human cartilage explants.* Scand J Rheumatol, 2006. **35**(4): p. 305-11.

32. *Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.*

33. *Abramoff, M.D., Magelhaes, P.J., Ram, S.J. "Image Processing with ImageJ". Biophotonics International, volume 11, issue 7, pp. 36-42, 2004.*

34. White, D., et al., *Can ultrasound propagate in the joint space of a human knee?* Ultrasound in Medicine and Biology, 2007. **33**(7): p. 1104-1111.

35. White, D., et al., *Modelling the Propagation of Ultrasound in the Joint Space of a Human Knee.* Ultrasound in Medicine and Biology, 2010. **36**(10): p. 1736-1745.

36. Oyonarte, R., M. Zarate, and F. Rodriguez, *Low-intensity Pulsed Ultrasound Stimulation of Condylar Growth in Rats.* Angle Orthodontist, 2009. **79**(5): p. 964-970.

37. Guzman, H.R., et al., *Ultrasound-mediated disruption of cell membranes. I. Quantification of molecular uptake and cell viability.* J Acoust Soc Am, 2001. **110**(1): p. 588-96.38. Boutkedjirt, T. and R. Reibold, *Reconstruction of ultrasonic fields by deconvolving the hydrophone aperture effects II. Experiment.* Ultrasonics, 2002. **39**(9): p. 641-648.

39. Aydelotte, M.B.S., B. L.; and Kuettner, K. E, *Heterogeneity of articular chondrocytes. In Articular Cartilage and Osteoarthritis.* Edited by K. E. Kuettner, R. Schleyerbach, J. G. Peyron, and V. C. Hascall. New York, Raven Press, 1992: p. 237-249.

40. MANKIN, J.A.B.a.H.J., *Instructional Course Lectures, The American Academy of Orthopaedic Surgeons - Articular Cartilage. Part I: Tissue Design and Chondrocyte-Matrix Interactions**. J Bone Joint Surg Am, 1997. **79**: p. 600-611.

41. *See Wikipedia, Illustration of Snell's Law, http://en.wikipedia.org/wiki/Snell's_law*

42. *See the NDT Resource Center, Piezoelectric Transducers, http://www.ndt-ed.org/EducationResources/CommunityCollege/Ultrasonics/EquipmentTrans/piezotransducers.htm.*

43. *See Hendersonville Orthopedic Associates, structure of aggrecan, http://hendersonvilleorthopaedics.com/*

44. *See the Journal of Nuclear Medicine, http://jnm.snmjournals.org/.*

45. *See Orthoteers, http://www.orthoteers.org/.*

46. Naito, K., et al., *Low-intensity pulsed ultrasound (LIPUS) increases the articular cartilage type II collagen in a rat osteoarthritis model.* J Orthop Res, 2010. **28**(3): p. 361-9.

47. Gurkan, I., et al., *Modification of osteoarthritis in the guinea pig with pulsed low-intensity ultrasound treatment.* Osteoarthritis Cartilage, 2010.

48. Korstjens, C.M., et al., *Low-intensity pulsed ultrasound affects human articular chondrocytes in vitro.* Med Biol Eng Comput, 2008. **46**(12): p. 1263-70.

49. Schumann, D., et al., *Treatment of human mesenchymal stem cells with pulsed low intensity ultrasound enhances the chondrogenic phenotype in vitro.* Biorheology, 2006. **43**(3-4): p. 431-43.

50. Ebisawa, K., et al., *Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells.* Tissue Eng, 2004. **10**(5-6): p. 921-9.