LOW-INTENSITY THERAPEUTIC ULTRASOUND: ROLE OF FREQUENCY AND ITS IMPACT ON THE MECHANOSENSITIVE SIGNALING PROCESSES IN CHONDROCYTES

by

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LOW-INTENSITY THERAPEUTIC ULTRASOUND: ROLE OF FREQUENCY AND ITS IMPACT ON THE MECHANOSENSITIVE SIGNALING PROCESSES IN CHONDROCYTES

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This dissertation primarily focuses on understanding the implications of ultrasonic frequency in low-intensity ultrasound (LIUS) mediated cellular processes. Our central hypothesis is that the ultrasonic frequency influences the key signaling events in the mechanotransduction pathway. Our hypothesis is predicated on the premise that for the optimal stimulation of cells via ultrasound (US), the frequency of an US signal must be maintained close to a natural resonant frequency of the cells. Our collective results provide evidence that the nuclear processes (i.e. transcription of load-inducible genes) responds to US with frequency dependence, and it was maximized at 5.0 MHz. Our results also lend credence to the notion that LIUS promotes euchromatin (less condensed chromatin) formation to allow easier access of transcription factors to their target DNA sequences. Collectively, these findings suggest that the US signal is able to transverse a cell and directly impacts the nuclear machinery as a function of frequency.

LIUS treated bovine articular chondrocytes (BACs) exhibited increased mRNA expression of SOX9 (master transcription factor for collagen-II). The LIUS mediated stimulation of BACs also induced the phosphorylation of Erk1/2 (MAP kinases with diverse cellular functions), upregulated the mRNA expression of hsp27 (small heat shock protein with cytoprotective function), activated the RhoA (a small GTPase known for

regulating actin structure), and influenced the remodeling of actin microfilaments. Confocal images also depicted an increase in length and bending of the primary cilium (a "sensory cellular antenna" with mechanosensory function) in BACs upon US stimulation. These biochemical events are significant and represent potential signaling pathway(s) that can influence the regulation of SOX9 at the transcriptional level.

To capitalize upon the positive bioeffects of US, an US assisted bioreactor (UBR) was fabricated. 2D-DIGE analysis of protein lysates from BACs subjected to US showed a differential expression of 138 unique proteins.

In summary, this dissertation represents for the first time that the frequency content must be considered when investigating LIUS for therapeutic benefit and points towards potential signaling pathways of US mediated chondrogenesis. This may have far-reaching implications in optimizing the LIUS treatment conditions for cartilage tissue engineering.

PREFACE

This dissertation is a collection of seven chapters. Each chapter is organized into separate sections: introduction, material and methods, results and discussion. An appendix is included at the end of the thesis that catalogs all the protocols employed in this thesis and vendors and sources of key reagents are also listed. A short write-up about media selection, and other cell isolation parameters is also included. Chapter-1, provides an introduction to the thesis and presents a current perspective on the different signal transduction pathways engaged in the communication of external stimuli to the cells that results in a desired biosynthetic response. Chapter-2, titled "Mechanotransduction of ultrasound is frequency dependent below the cavitation threshold" combines both theory and experimentation to investigate the frequency dependent effects of ultrasound (US). Our work has shown that US does not merely produce on-off effects, but provides a finely tuned response at 5 MHz. In Chapter-3, titled "Low-intensity continuous ultrasound activates RhoA via the integrin mediated signaling pathway and influences the reorganization of actin microfilaments in chondrocytes", actin reorganization under varying US frequencies was explored. In Chapter-4, titled "Primary cilium: modulation of its length and shape in response to ultrasound stimulation and association with MAPK/Erk signaling in chondrocytes", the role of primary cilium in mediating signaling under US was ascertained, where ciliated and deciliated cells were exposed to US and the expression of select signal transduction markers were carried out.Chapter-5, titled "Ultrasonic bioreactor as a platform for studying cellular response" describes the characterization of an US assisted bioreactor developed at the University of Nebraska-Lincoln. Chapter-6, titled "Ultrasound stimulation upregulates the gene expression of

hsp27 but not of hsp70 or hsp90, in bovine articular chondrocytes" describes the characterization of an US assisted bioreactor developed at the University of Nebraska-Lincoln. Chapter-6, titled "Ultrasound stimulation upregulates the gene expression of hsp27 but not of hsp70 or hsp90, in bovine articular chondrocytes" explores the heat shock response under US stimulation, where the gene and protein expression of heat-shock-factors and proteins were carried out and compared with cellular response to thermal stresses. Chapter 7 reports the summary, conclusions and recommendations based on the experimental results.

DEDICATION

I dedicate this dissertation work to my family. A special feeling of gratitude to my loving parents, Bharat Bhushan and Sudesh Kumari for their love and support over the years. My sisters Rupam has never left my side and is very special. This dissertation is also in debt to my beloved grandmother, Saraswati Devi, and my uncle, Raj Kumar, who have always been there to support me and to encourage me to succeed.

I also dedicate this work to my amazing wife, Priyanka whose sacrificial care for me and my family made it possible for me to complete this work, and to my wonderful son, Sanjam who is indeed a blessing and brings so much joy to my life.

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Many thanks to former colleagues: Dr. Sanjukta Guha Thakurta and Dr. Nicholas P. Whitney for sharing their knowledge, and providing insights on conducting experimental research. A special thanks to current group member Neety Sahu for illustrating Figure1-1 and providing assistance in executing experiments.

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GRANT INFORMATION

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CHAPTER 1.

INTRODUCTION

1.1. Ultrasound and its biological applications

Ultrasound (US) is an acoustic vibration and is similar to a sound wave, but with frequencies above the limit of human hearing range of 2-20 kHz.[1] The interaction of US with biological materials has been reported since the 1940s and its non-invasive nature has allowed its widespread usage in ultrasound assisted diagnostics for various pathologies and in the imaging of normal and diseased tissues and organs.[2] However, applications of US are not restricted to these well-defined applications; US has also found extensive usage in therapeutic applications as in physiotherapy and using high-amplitude acoustic shock wave to disintegrate kidney stones (lithotripsy).[3, 4] Emerging applications of ultrasound include the use of high intensity focused ultrasound in the non-invasive treatment of tumors, the use of US in gene and drug-delivery, thrombolysis and tumor therapy.[5-10]

Biological applications of US can be divided according to the intensity of US signal employed. For example: (a) diagnostic ultrasound, uses a low-intensity ultrasound (LIUS) signal (1 to 5 mW/cm^2) between 3 and 5 MHz; (b) disruptive ultrasound, uses high intensity US signal (8 W/cm^2) between 20 to 60 kHz, and (c) therapeutic US, used moderate intensity US signal (0.1 to 2 W/cm^2) between 1 and 3 MHz.[11-13] Our research interest is concentrated especially in exploiting the therapeutic potential of LIUS for cartilage tissue engineering.

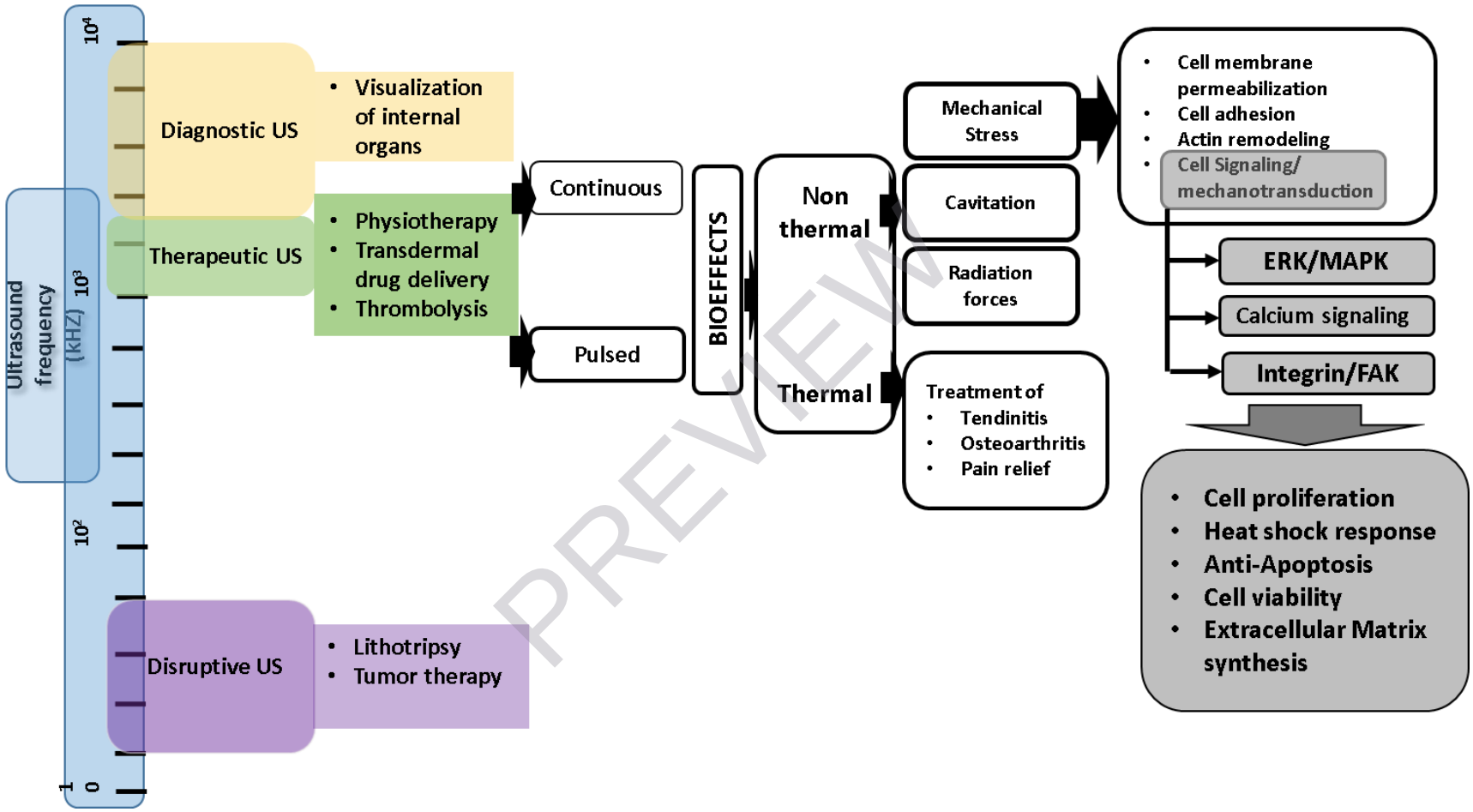

Figure 1-1 Biological applications of ultrasound corresponding to its frequency range.

1.2. Cartilage Tissue Engineering

Tissue engineering, is a promising therapeutic approach that aims to replace, repair, or enhance the biophysical functions of a diseased or an injured tissue.[14] It is an interdisciplinary field that utilizes the applications of engineering and life sciences, in harvesting tissue specific cells, growing them *in vitro* in the presence of various biochemical and physiological factors, mimicking their natural environment and ultimately developing a tissue construct that can be transplanted into the patient at the specific site so it can restore or improve the function or a whole tissue.[15-17]

Optimal performance of an articular cartilage is determined by its biomechanical properties attributed to the composition of its extracellular matrix (ECM). Even small changes in the structural integrity of the cartilage matrix due to mechanical injury or aging, can permanently alter its biomechanical properties and compromise its functionality which may further predispose the joint to degenerative changes such as osteoarthritis.[18-20] Moreover, lack of blood vessels in cartilage coupled with a low replicative ability of the chondrocytes results in a limited capacity for natural regeneration of articular cartilage.[21] Therefore, articular cartilage repair is the prime target for tissue engineers. The aim of articular cartilage tissue engineering is to either repair these defects or to develop a viable replacement before they give rise to a long-term disability.[21, 22]

1.3. Mechanobiology of articular cartilage and chondrocytes

1.3.1. Articular cartilage

Articular cartilage is a load bearing connective tissue of diarthrodial joints that primarily serves two important functions. First, it provides a smooth and lubricated

cushion for joint articulation with low friction coefficient and second, it helps in reducing stress in the joint by the uniform distribution of loads during joint movement and weight bearing activities.[23] Articular cartilage frequently experiences a wide variety of mechanical stresses during static and dynamic joint loading. Compression is one of the primary mechanical stresses experienced by articular cartilage. Even a normal joint movement, during a day can cause total compressive strain of 15-20%.[24] In addition, rotational and translational movement of joints can also contribute to shear stress and tensile stress within the cartilage tissue.[25]

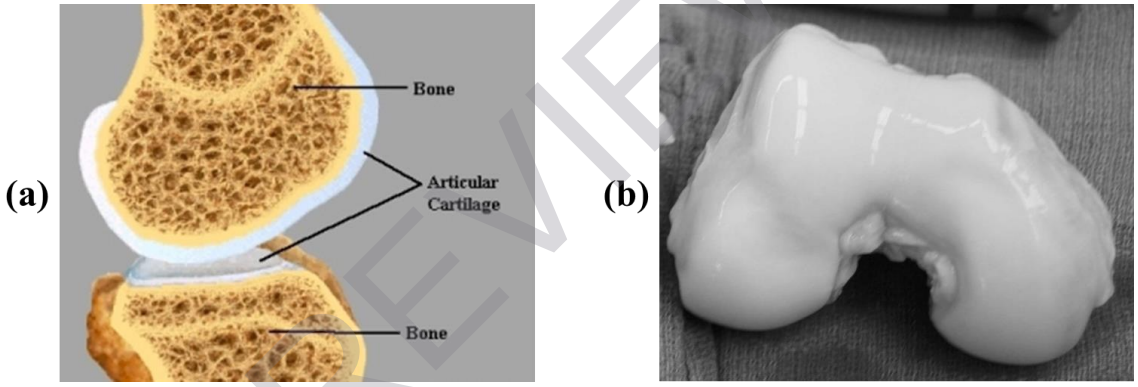

Figure 1-2 Articular cartilage (a) Pictorial representation of articular cartilage (retrieved from http://www.thaimedicalnews.com/health-care-medical/about-knee-problems-damaged-articular-cartilage-treatments/); (b) Photograph of a healthy articular cartilage removed from a human knee (retrieved from http://www.jointpreservationinstitute.com/cartilage-surgery.html)

Articular cartilage is composed of a dense ECM and sparsely distributed specialized cells called chondrocytes that occupy only 1-10% of the cartilage volume.[23, 26] It is an avascular, aneural and alymphatic tissue that meets its nutritional demand through diffusion of synovial fluid.[23] The ECM of cartilage contains 65-80% of fluid by weight with the remaining solid components made up of proteoglycans and collagen fibers.[23] Proteoglycans can take up to 30% dry weight of the cartilage.[27] It is comprised of a

protein core attached to glycosaminoglycans (chondroitin sulfate and keratan sulfate) that is further linked to the hyaluronic acid backbone to form a large macromolecule.[28] These highly negatively-charged proteoglycans play critical role in retaining water in the cartilage creating an osmotic swelling pressure that coupled with negative charge repulsion between carboxyl and sulfate groups allow the tissue to withstand high compressive loading.[29] Tensile and shear strength of the cartilage is determined by the collagen network of the matrix. Collagen-II is the major protein of the ECM that makes up 60-70% of the dry weight of the tissue. Other collagen such as type VI, XI, X and XI are also present in the matrix, but to lesser extent.[23, 26, 29]

The structural organization of articular cartilage is highly anisotropic. Based on its depth, it is divided into a superficial zone, a middle zone, and a deep zone. Water content, proteoglycans concentration, organization of collagen fibers and shape, size and number of chondrocytes vary depending on the depth of the articular cartilage.[30] For example, in the superficial zone, the orientation of the collagen network is parallel to the joint surface whereas perpendicular in the deep zones.[31] Also, superficial layer has a relatively high concentration of the lubricating protein, lubricin also known as proteoglycan-4 whereas in the deep zone, a higher concentration of large aggregating proteoglycans, aggrecan predominates.[23] In addition, chondrocytes in the superficial zone are flat, small and highly dense compared to cells in the deeper region.[23]

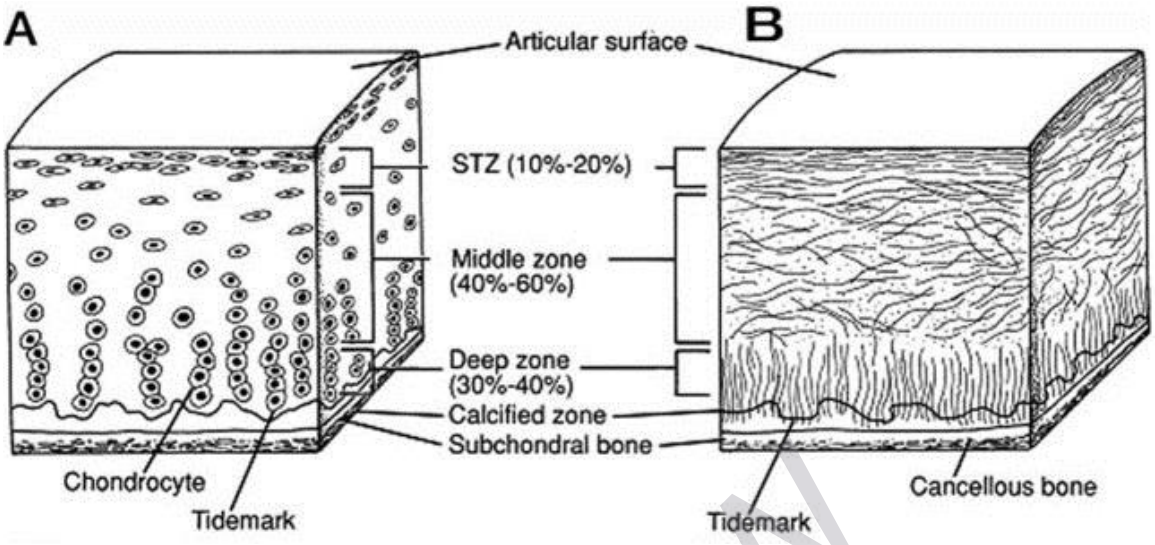

Figure 1-3 (A) Cross sectional diagram of the cellular organization in the zones of articular cartilage; (B) diagram of the collagen fiber architecture.
(Retrieved from the Journal of the American Academy of Orthopaedic Surgeons, 1994;2:192-201)[32]

1.3.2. Chondrocytes

Articular chondrocytes are the only cells present within the articular surface of the cartilage and appear to play an important role in maintaining homeostasis in a healthy cartilage. It also helps the articular cartilage in restoring proper functional activity after an injury.[33] Chondrocytes are responsible for the development, maintenance, and repair of the ECM. Chondrocytes synthesize matrix components, including proteins and glycosaminoglycan side chains.[33] Studies have also shown that within the cartilage, each chondrocyte is separately surrounded by its own pericellular matrix which provides a specialized microenvironment to chondrocytes.[34, 35] The pericellular matrix plays a critical role in mediating the communication between chondrocytes and the ECM. Mechanical stress created during loading and deformation of cartilage can be transmitted though ECM and influences the stress-strain environment within the cell's