Published in final edited form as:

Proc SPIE Int Soc Opt Eng. 2017 ; 10066: . doi:10.1117/12.2255761.

Low intensity pulsed ultrasound (LIPUS) for the treatment of intervertebral disc degeneration

Devante Horne^{a,b}, Peter Jones^b, Vasant Salgaonkar^b, Matt Adams^{b,c}, B. Arda Ozilgen^{a,b}, Peter Zahos^b, Xinyan Tang^c, Ellen Liebenburg^c, Dezba Coughlin^c, Jeffrey Lotz^{b,c}, and Chris Diederich^{a,b}

^aUC Berkeley – UC San Francisco Graduate Program in Bioengineering, CA, USA

^bDepartment of Radiation Oncology, UC San Francisco, CA USA

^cDepartment of Orthopaedic Surgery, UC San Francisco, CA, USA

Abstract

Discogenic back pain presents a major public health issue, with current therapeutic interventions limited to short-term symptom relief without providing regenerative remedies for diseased intervertebral discs (IVD). Many of these interventions are invasive and can diminish the biomechanical integrity of the IVDs. Low intensity pulsed ultrasound (LIPUS) is a potential treatment option that is both non-invasive and regenerative. LIPUS has been shown to be a clinically effective method for the enhancement of wound and fracture healing. Recent *in vitro* studies have shown that LIPUS stimulation induces an upregulation functional matrix proteins and downregulation of inflammatory factors in cultured IVD cells. However, we do not know the effects of LIPUS on an *in vivo* model for intervertebral disc degeneration. The objective of this study was to show technical feasibility of building a LIPUS system that can target the rat tail IVD and apply this setup to a model for acute IVD degeneration. A LIPUS exposimetry system was built using a 1.0 MHz planar transducer and custom housing. *Ex vivo* intensity measurements demonstrated LIPUS delivery to the center of the rat tail IVD. Using an established stab-incision model for disc degeneration, LIPUS was applied for 20 minutes daily for five days. For rats that displayed a significant injury response, LIPUS treatment caused significant upregulation of Collagen II and downregulation of Tumor Necrosis Factor – α gene expression. Our preliminary studies indicate technical feasibility of targeted delivery of ultrasound to a rat tail IVD for studies of LIPUS biological effects.

Keywords

Low intensity pulsed ultrasound; intervertebral disc; disc degeneration; rat tail; TNF; Aggrecan; Collagen; Interleukin

1. INTRODUCTION

Discogenic back pain (DBP) is one of the most significant non-lethal medical ailments in society. The American Academy of Orthopaedic Surgeons estimates that more than 6M people are diagnosed with DBP annually, with 80% of the US population experiencing DBP sometime in their lives. The treatment costs of lower back pain exceed \$27 billion annually,

but the societal cost of the disease exceeds \$86 billion¹⁻⁴. The intervertebral disc (IVD) is an articulating, load-bearing structure between the vertebral bodies of the spine. In the center of the disc is the nucleus pulposus (NP); this proteoglycan-rich structure imbibes water and provides compressive resistance. Surrounding the NP are lamellae of collagen fibers called the annulus fibrosis (AF) which accounts for tensile strength. Between the IVD and each vertebral body is the cartilage end-plate which serves as a barrier for nutrient and waste exchange between the IVD and vertebra. Intervertebral disc degeneration is associated with the loss of extracellular matrix (ECM) molecules, resulting in modification in biochemical and biomechanical tissue properties. While the etiology of IVD degeneration is unclear, it is suspected that it is the result of metabolic imbalance within the tissue⁵. This imbalance may cause a gradual loss of important matrix proteins such as aggrecan and collagen II, which may lead to depressurization of NP and breakdown of the AF. Another factor likely related to disc degeneration is cartilage end-plate damage which may impair proper nutrient and waste exchange, leading to a loss of cells due to apoptosis. Current therapeutic options for treating discogenic back pain include procedures such as discectomy (removing part of most of the disc nucleus) and laminectomy (removing part of the bone anatomy), which are largely focused on addressing manifested symptoms (i.e. pain) rather than functional causes. The major limitations of these procedures are that they are invasive and may actually lead to biomechanical failure and further degeneration. Incidence rates and costs associated with biomechanical damage from these interventions are significant, creating a significant need for noninvasive alternatives. Recently emerging alternative strategies for treating IVD degeneration including cell transplantation, gene therapy, and growth factor injection are under investigation. While these options have advantages over invasive surgeries, their primary limitations include concerns about sustainability and biological safety. Mechanical stimulation of the IVD cells may be a nontoxic alternative that could be used alone or in conjunction with other forms of biochemical treatment.

Low intensity pulsed ultrasound (LIPUS) is a mechanical treatment option that is potentially both noninvasive and regenerative. LIPUS is a low energy, pulsed waveform, removing the thermal component found at higher intensity, continuous wave systems. LIPUS technology typically operates at low acoustic intensity values ($30\text{mW}/\text{cm}^2$) and transmits from low-frequency acoustic sources (1.5 MHz) using short duty cycles (20%) and a pulse repetition rate around 1 kHz. These sonication parameters are significantly different from those applied for heat-induced interventions, which rely on acoustic intensities that are 1 – 4 orders of magnitude larger than LIPUS. LIPUS has been shown to accelerate healing of cortical and cancellous bone fractures⁶, including non-union fractures, possibly due to enhanced callus formation and angiogenesis near the fracture site⁷. LIPUS is widely used in physiotherapy for the treatment of soft tissue injuries, likely stimulating increased perfusion to the injury site⁸.

IVD degeneration is known to result from alteration in biosynthesis of proteoglycan (PG) and pro-inflammatory cytokines in the disc⁵. It has been established that LIPUS stimulates PG and collagen synthesis, and induces an anti-inflammatory and anti-catabolic responses in human IVD cells⁹. These LIPUS effects have been shown to be mechanically regulated through the FAK/PI3K/Akt pathway⁹. Promoting the synthesis of major ECM proteins and reducing inflammatory factors may stall or even reverse the degenerative cascade. While

findings support the application of LIPUS for repair of IVD damage and degeneration, it is unclear whether these LIPUS effects are translatable to an *in vivo* IVD degeneration model. We hypothesize that LIPUS can be applied noninvasively to stimulate favorable biological activity within the acutely damaged rat tail IVD. The objective of this research project was to demonstrate the technical feasibility of building a LIPUS system that can target the rat tail IVD and apply this setup to a model for acute IVD degeneration.

2. METHODOLOGY

2.1 Ultrasound exposimetry system

Ultrasound exposimetry systems were devised and fabricated, following our generalized schema (Figure 1a), specific to delivering calibrated and characterized LIPUS to rat tail intervertebral discs. Three configurations considered consisted of 2.5 cm diameter PZT4 transducers (1.0 MHz, $f=1$; 1.6 MHz, $f=3.8$; 1.0 MHz planar), mounted water-tight with air-backing on a 3D-printed assembly, and integrated within a degassed water-filled plastic housing with a thin Mylar window marked with central cross-hairs (Figure 1b). The housing is designed as an acoustic standoff to place the distal focus, or uniform far-field zone, within the targeted rat tail disc and to prevent the influence of transducer heating. The acoustic energy is isolated to a single targeted disc and portions of the adjacent vertebral body by selection of a standoff distance which gives an appropriate beam cross-section. Additional isolation was achieved by use of peripheral brass reflectors and a custom acoustic absorber placed beyond the target (Figure 1a).

2.2 Acoustic characterizations

LIPUS applicators were evaluated with comparative beam plot measurements to assess whether the systems can deliver LIPUS to the narrow ($\sim 1.5 \text{ mm} \times 5 \text{ mm}$) rat tail disc. The planar device with a 5 cm standoff provided a more uniform beam of appropriate width ($\sim 8 \text{ mm}$) for easier alignment and full exposure of the disc (Figure 2a). Partial disc exposure may be achievable with focused devices ($< 3 \text{ mm}$) (Figure 2b, c). Radiation force observations and intensity measurements, with and without insertion of sectioned rat tails *ex vivo*, were made to evaluate whether sufficient intensity could reach the center of the rat tail disc (Figure 3). It was confirmed that approximately 45% incident energy was delivered to central disc while reducing exposure to significant portions of the adjacent bone and with negligible ($< 1^\circ\text{C}$) temperature elevation measured.

2.3 Rat tail stab model

A well-characterized and established protocol demonstrates that a single stab incision through the rat tail intervertebral disc induces inflammation that mirrors the early stages of IVD degeneration¹⁰. Five three-month-old female Sprague-Dawley rats were used in this study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Rats were anesthetized and an analgesic (Buprenorphine, 0.01 mg/kg) was delivered before stab-incisions were administered. Rat tails were sanitized with alcohol wipes and betadine topical antiseptic solution. The 3 most proximal discs were selected; two to be stabbed and the remaining disc as a control. Under fluoroscopic guidance, a 20-gauge, short-bevel needle (Fisher Scientific, Cat. No. 305179)

was laterally inserted through the disc and stopped just before penetrating the outer annulus on the opposite side. The needle was turned 180° before fully retracting the needle. Injury to multiple discs does not result in systemic inflammation in rats nor does it affect healthy adjacent discs. Female rats were chosen over male rats since previous studies have suggested that male rats are more aggressive and may bite the injury site of other rats in the cage, potentially affecting results. Relative disc location was marked with a tissue pen and verified daily with fluoroscopy prior to LIPUS treatment.

2.4 LIPUS delivery

LIPUS was delivered shortly following the completion of stab incisions. Five daily LIPUS exposures (I_{SPTA} 60 mW/cm², 200 μ s burst, 1 kHz repetition frequency, 20 min duration) were applied to one disc and compared to stab only and normal controls. Acoustic coupling gel was applied between the applicator and tail, and a silicone absorber coupled with gel was mounted on tail (Figure 4) to best reduce standing waves and reflections off of the dorsal surface. Day 1 exposure was 30 min after injury, and Day 5 sacrifice was 2–3 hours post-LIPUS.

2.5 Histology

After day 5 treatment, a cohort of rats was euthanized and motion segments were harvested. Discs and adjacent vertebrae were harvested, fixed in formalin, decalcified in formic acid, dehydrated, and embedded in paraffin. Sagittal sections were cut through the motion segments, perpendicular to the direction of the stab. Sections were stained with Heidenhain's stain. Disc and vertebral morphology was examined at 2X magnification.

2.6 Measurement of gene expression

The remaining cohorts of rats were euthanized following day 5 treatment and complete discs were harvested and snap frozen in liquid nitrogen. Disc tissue was homogenized and total RNA was extracted using the using Trizol reagent (Life Technologies, Cat. No. 15596026) and the RNeasy mini kit (Qiagen, Cat. No. 74104); cDNA was synthesized using iScript reverse transcriptase (BioRad, Cat. No. 1708840). The mRNA expression of matrix related (ACAN, Col2a1, Sox9) and pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) were measured by RT-qPCR with TaqMan Fast Advance Master Mix (Life Technologies, Cat. No. 4444557) using the BioRad CFX96 RealTime Thermal Cycler. Relative gene expression was calculated using the $\Delta\Delta$ Ct method and normalized to B2M and the control group. Results were compared using *t*-tests. *P* values < 0.05 were considered significant.

3. RESULTS

3.1 Stab induced morphological changes

All control discs appeared to have a rounded nucleus and well-organized collagen lamellae in the annulus (Figure 5). Stab incisions resulted in depressurization of the disc and reduced disc height (Figure 5). Stabbed discs displayed an irregularly shaped nucleus and denatured collagen fibers that appear lax in the inner annulus. These are all positive signs of acute disc injury and precursors to further degeneration.

3.2 LIPUS effect on gene expression

Real-time quantitative PCR analysis was performed at 5 days after injury to assess the impact of LIPUS stimulation on extracellular matrix and inflammatory gene expression. Previous *in vitro* studies suggest that LIPUS treatment stimulates proteoglycan and collagen synthesis in IVD cells while inhibiting pro-inflammatory cytokine expression^{9,11–13}. Results indicated favorable trends, but exhibited large variation when gene expression was averaged over all animals (n=5), showing no statistical significance between stab only and stab plus LIPUS groups. However, as typically applied with the stab-injury model, when selecting the subset (n=2) of animals that had successful stabs which produced a significant injury response, LIPUS treatment had an anti-inflammatory effect and anabolic response (Table 1): Col2a1 expression was significantly upregulated ($p = 0.03$) and TNF- α expression was significantly downregulated ($p = 0.05$). Expression of proinflammatory cytokines IL-1 β and IL-6 was not significantly affected, but results suggest a trend towards downregulation. Unexpectedly, LIPUS appears to downregulate Aggrecan and Sox9 gene expression.

4. CONCLUSIONS

This preliminary study demonstrates technical feasibility of delivering LIPUS isolated to a targeted rat tail IVD for the study of LIPUS exposure and biological effects. Our analyses indicate that previously observed *in vitro* effects seem to carry over to our *in vivo* model. While several favorable trends were observed, statistical significance was only achieved in two genes of interest, likely due to large animal to animal variation. One of the major limitations of the rat tail stab model is that minute variations between individual's stabbing technique may significantly affect the animal's injury response. Future studies will include additional animals to improve statistical power as well as implementation of a refined stab technique to ensure more a consistent injury response. Subsequent studies will evaluate a wider variety of genes to determine the healing mechanism by which LIPUS most significantly acts (e.g. anti-inflammatory, anti-catabolic, anabolic). Additional end points (14 and 28 days) will be included to assess the healing response over time. Successful completion of these studies can lead to similar evaluations in larger IVD degeneration models (e.g. ovine, bovine), to more closely replicate the dimensions of the human IVD and lead to development of delivery strategies for treating discogenic back pain in humans.

Acknowledgments

This project was supported by an award from the Focused Ultrasound Foundation and NIH R21EB024347. Histology services were provided by Ellen Liebenburg from the Department of Orthopaedic Surgery at the University of California, San Francisco.

References

1. Martin BI, et al. Expenditures and Health Status Among Adults With Back and Neck Problems. JAMA. 2008; 299:656–664. [PubMed: 18270354]
2. Ricci JA, et al. Back Pain Exacerbations and Lost Productive Time Costs in United States Workers. SPINE. 2006; 31:3052–3060. [PubMed: 17173003]
3. Stewart WF. Lost Productive Time and Cost Due to Common Pain Conditions in the US Workforce. JAMA. 2003; 290:2443–2454. [PubMed: 14612481]

4. Peng B-G. Pathophysiology, diagnosis, and treatment of discogenic low back pain. *World J Orthop.* 2013; 4:42. [PubMed: 23610750]
5. Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol.* 2013; 10:44–56. [PubMed: 24166242]
6. Lerner A, Stein H, Soudry M. Compound high-energy limb fractures with delayed union: our experience with adjuvant ultrasound stimulation (exogen). *Ultrasonics.* 2004; 42:915–917. [PubMed: 15047406]
7. Harrison A, Lin S, Pounder N, Mikuni-Takagaki Y. Mode & mechanism of low intensity pulsed ultrasound (LIPUS) in fracture repair. *Ultrasonics.* 2016; 70:45–52. [PubMed: 27130989]
8. Haar, Ter G., Dyson, M., Oakley, EM. The use of ultrasound by physiotherapists in Britain, 1985. *Ultrasound in Medicine & Biology.* 1987; 13:659–663. [PubMed: 3686730]
9. Zhang XJ, Hu ZM, Hao J, Shen JL. Low Intensity Pulsed Ultrasound Promotes the Extracellular Matrix Synthesis of Degenerative Human Nucleus Pulposus Cells Through FAK/PI3K/Akt Pathway. *SPINE.* 2016; 41:E248–E254. [PubMed: 26571160]
10. Rousseau M, Ulrich JA, Bass EC, Rodriguez AG. Stab incision for inducing intervertebral disc degeneration in the rat. *SPINE.* 2007; 32:17–24. [PubMed: 17202887]
11. Iwashina T, et al. Low-intensity pulsed ultrasound stimulates cell proliferation and proteoglycan production in rabbit intervertebral disc cells cultured in alginate. *Biomaterials.* 2006; 27:354–361. [PubMed: 16099036]
12. Kobayashi Y, Sakai D, Iwashina T, Iwabuchi S. Low-intensity pulsed ultrasound stimulates cell proliferation, proteoglycan synthesis and expression of growth factor-related genes in human nucleus pulposus. *Eur Cell.* 2009
13. Miyamoto K, An HS, Sah RL, Akeda K, Okuma M. Exposure to pulsed low intensity ultrasound stimulates extracellular matrix metabolism of bovine intervertebral disc cells cultured in alginate beads. *SPINE.* 2005; 30:2398–2405. [PubMed: 16261116]

Figure 1.

Generalized schematic of the LIPUS exposimetry system designed for LIPUS delivery at calibrated intensity exposures to rat-tail IVDs *in vivo* (a). LIPUS applicators – various transducer assemblies were constructed with various frequencies and focusing (b).

Figure 2.

Axial beam plots with selected standoff distance marked by vertical line. Plots are representative of a planar transducer (a) and transducers of different focusing (b and c).

Figure 3. Contour plots of intensity measurements using 1.6 MHz, focused transducer; without sectioned tail (a) and with sectioned tail in beam path (b).

Figure 4. Rodent positioning and LIPUS delivery system during treatment. Caudal vertebral segment of anesthetized rat positioned over center of aperture (a). Gel and apical absorber secured prior to treatment (b). Assembly uses a 1.0 MHz planar transducer with a 5-cm standoff (c), delivering an 8-mm diameter beam (i.e. FWHM = 8 mm) to the surface of the tail (c).

Figure 5.

Heidenhain stained, mid-sagittal sections of rat-tail motion segments. Control discs (a) display a rounded nucleus and lamellae of collagen in the annulus, while stabbed discs (b) are depressurized and have lax collagen fibers in the annulus. Note that stabs were into the page.

Genes investigated after 5 days of LIPUS treatment. Data have been normalized by their respective control group. Fold change is reported as an average among animals with an injury response (n=2).

Table 1

Fold Change (Normalized to Control)				
Gene	Gene Name	Description	Stab Only	Stab + LIPUS
IL-1 β	Interleukin - 1 β	Pro-inflammatory Cytokine	5.54	1.00
IL-6	Interleukin - 6	Pro-inflammatory Cytokine	3.57	0.20
TNF α	Tumor Necrosis Factor - α	Pro-inflammatory Cytokine	1.29	0.02
ACAN	Aggrecan	ECM Protein	2.48	1.61
Col2a1	Collagen type 2a1	ECM Protein	1.56	16.31
Sox9	Sox9	Transcription factor for Collagen II synthesis	2.61	1.65
				0.62