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An in vivo improvement of range of motion in shoulder contractures with relaxin in animal models

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AN IN VIVO IMPROVEMENT OF RANGE OF MOTION IN SHOULDER CONTRACTURES WITH RELAXIN IN ANIMAL MODELS

by

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AN IN VIVO IMPROVEMENT OF RANGE OF MOTION IN SHOULDER CONTRACTURES WITH RELAXIN IN ANIMAL MODELS

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ABSTRACT

Introduction

Arthrofibrosis, which occurs in a substantial portion of the population, is a pathologic accumulation of scar tissue that presents in patients as a painful decrease in joint range of motion. Since an individual's quality of life can be significantly impacted by arthrofibrosis and because there are limitations in current treatments, this thesis focuses on examining the use of the hormone relaxin to alleviate shoulder arthrofibrosis.

Methods

A set of 20 Sprague Dawley rats were given secondary shoulder contractures and separated into groups to examine the efficacy of relaxin using intravenous delivery, intra-articular delivery, and different treatment frequencies. The differences across groups were examined through mechanical range of motion testing as well as histologic sampling.

Results

Multiple doses of intra-articular injections of relaxin showed a complete return to the normal range of motion ($P < 0.01$) when compared with the surgical control, whereas other delivery methods and frequencies failed to show meaningful improvements. This was further confirmed in histologic analysis through the lack of fibrotic adhesions within

the capsular space after multiple intra-articular relaxin treatments when compared with the surgical control.

Discussion

Although significant improvements to range of motion were seen after multiple doses of intra-articular relaxin, potential tissue degradation was also observed within the joint space after histologic examination. Further research is necessary to fully understand the proper dosing needed to avoid potential negative side effects caused by excess use of relaxin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACL..... Anterior Cruciate Ligament

ANOVA .....Analysis of Variance

BIDMC ..... Beth Israel Deaconess Medical Center

COL1A1..... Collagen Type I Alpha I

COL1A3.....Collagen Type I Alpha III

COX ..... Cyclooxygenase

ECM.....Extracellular Matrix

EDTA..... Ethylenediaminetetraacetic Acid

FGF .....Fibroblast Growth Factor

H&E .....Hematoxylin and Eosin

IA ..... Intra-articular

IACUC .....Institutional Animal Care and Use Committee

IV .....Intravenous

MMP .....Matrix Metalloproteinase

MUA ..... Manipulation Under Anesthesia

NSAID ..... Nonsteroidal Anti-Inflammatory Drug

PBS .....Phosphate-Buffered Saline

PDGF .....Platelet-Derived Growth Factor

ROM ..... Range of Motion

SSNB.....Suprascapular Nerve Block

TGF-ß.....Transforming Growth Factor Beta

TIMP .... Tissue Inhibitor of Matrix Metalloproteinase

INTRODUCTION

Background of Arthrofibrosis

Arthrofibrosis presents a significant public health issue with limited and varied outcomes given current treatment options. Arthrofibrosis is defined as a pathologic accumulation of excessive scar tissue or adhesions found within or around a joint space. The excessive scar tissue results in a painful, gradual restriction of passive and active range of motion (ROM) (1-3). Development of arthrofibrosis is still unclear and is thought to arise through synovitis, trauma or injury, or prolonged immobilization of the joint. The disease may also occur idiopathically (1, 4). Those affected by arthrofibrosis observe a direct impact on their quality of life, with their afflicted joint making daily tasks more difficult. This disease may occur in any joint and affects articular areas such as the hip, ankle, wrist, elbow, and most notably the knee and shoulder.

Within the knee, arthrofibrosis that necessitated surgical intervention after articular fractures or post-intra-articular trauma was reported at 14.5% incidence (5). After anterior cruciate ligament (ACL) reconstruction, the incidence was reported to be as high as 35% (6). Further, the incidence of arthrofibrosis after a total knee arthroplasty was close to 15% (7). With regard to arthrofibrosis within the shoulder, in a random sampling of ten thousand individuals, 8.2% of men and 10.1% of women were shown to have some degree of arthrofibrosis (8). It is estimated that arthrofibrosis of the shoulder has an incidence between 2% and 5% of the general population, with 20%-36% prevalence within those suffering from diabetes (1, 9-12).

Treatment options are similar across the different joint cases and do little to improve patient outcomes. Nonoperative treatments include physical therapy, nonsteroidal anti-inflammatory drugs (NSAIDs), intra-articular corticosteroid injections, intra-articular sodium hyaluronate injections, and nerve blocks. Although these methods may aid to provide moderate or temporary relief from the symptoms, they are only marginally effective (1, 4). In more extreme cases, surgical intervention may prove to alleviate the patient from symptoms of arthrofibrosis. These procedures include manipulation under anesthesia (MUA), arthroscopic capsulotomy, and more rarely an open capsulotomy. Whereas an open capsulotomy was shown to do little to improve ROM and pain, arthroscopic capsulotomies and MUA were shown to improve ROM and reduce pain; however, they may also cause complications that can worsen the condition (1).

Since arthrofibrosis covers a wide range of pathologies, it is helpful to limit the focus of this study to a single type of arthrofibrosis. The priority of this thesis is to examine a potential treatment for arthrofibrosis within the shoulder. Although the above treatment options are available to patients with arthrofibrosis, there remains a need for a treatment that more completely targets the symptoms or the causes of the disease.

Pathologic and Epidemiologic Significance Within the Shoulder

Arthrofibrosis of the shoulder, also known as shoulder contracture, adhesive capsulitis, or more colloquially frozen shoulder, is characterized by a progressive fibrosis and contracture of the capsule within the shoulder, leading to a reduction in both active

and passive glenohumeral motion (**Figure 1**) (13). This disease manifests typically after trauma, prolonged immobilization, or synovitis of the affected area. It may also occur idiopathically. Pathologically, this disease is established as an increased deposition of type I and type III collagen matrix from an increased recruitment of fibroblastic and myofibroblastic cells to the area of injury (14, 15). Even though the presence of important inflammatory mediators and mechanisms, such as T cells, B cells, synovial cells, fibroblasts, and transforming myofibroblasts, have been shown to exist in patients undergoing surgical capsular release, the exact manner of causation has yet to be discovered.

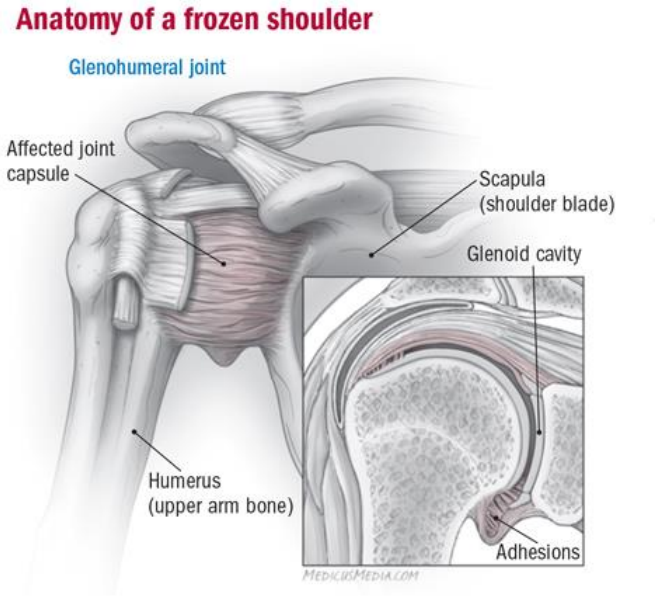

Figure 1. Anatomy of a frozen shoulder. The illustration shows the anatomy of the glenohumeral joint and the affected areas within the joint for cases of adhesive capsulitis. The intra-articular space is also known as the synovial space or the space within the joint. Adapted from Harvard Medical School, 2014 (16).

Shoulder contracture can be classified as either primary (idiopathic) or secondary (known extrinsic/intrinsic cause), and both cause a significant reduction in ROM (17, 18). In the general population, women are known to be more often affected by shoulder

contracture than men, but there is no known genetic or racial correlation (19). Macroscopically, capsular contracture is identified as a thickened joint capsule with adhesions interfering with the axillary fold. The fibrotic capsule can then adhere to itself and the anatomic neck of the humerus, causing a decrease in the intra-articular volume and thus reducing the amount of synovial fluid within the joint (20).

Of the 2% to 5% of the population afflicted with adhesive capsulitis (11, 12), a majority of that population is subdivided into the ages of 40-60 years (19). Adhesive capsulitis is often seen as a self-limiting disease, with patients regaining mobility within 1 to 3 years. However, a significant number of patients never regain a full normal range of mobility (21). Although there are instances in which patients achieve a spontaneous resolution and attain full ROM, up to 60% have a lasting reduction in ROM, and 50% have residual pain for several years after the onset of the disease (22). As with other arthrofibrotic joints, treatments through physical therapy are often long and painful and still fail to resolve this issue.

Current Treatments and their Effectiveness in Shoulder Arthrofibrosis

Nonsurgical Interventions

Nonsurgical interventions for capsular fibrosis vary widely in their effectiveness but are ultimately lacking in their ability to treat the disease. Physical therapy is generally used as the first line of treatment for those that have early stages of a shoulder contracture (1). However, physical therapy as an individual treatment has little support showing its efficacy (23). Although the core motivation behind physical therapy is early mobilization,

there remains to be seen a consensus in both frequency and intensity for the treatment. One study reported that 63% of their patients who underwent intense physical therapy improved when compared with 90% of those who did gentler therapy, whereas another study performed by Vermeulen et al. (24)showed no difference between therapy intensities (1, 24, 25). Despite the improvements within patients common across studies, other studies have shown no differences between those that received physiotherapy and those that did not (4).

NSAIDs can also be prescribed to treat adhesive capsulitis. Since COX-1 and COX-2 have elevated expression within the capsular and bursal tissues, anti-inflammatory drugs prove to reduce pain by targeting the inflammatory pathway (26). This is often beneficial when used in conjunction with physical therapy, as pain management encourages the pursuit of additional activity. However, when used alone, the anti-inflammatory treatment does little more than improve the pain associated with the contracture (27). An alternative pharmacological therapy, intra-articular corticosteroid injections, has shown to provide reductions in fibromatosis and myofibroblasts within shoulder contractures. In one study, it was shown that intra-articular methylprednisolone injections resulted in initial improvements in ROM and pain beyond that of physical therapy, ice therapy, and no therapy groups (28). This improvement was short-lived, however, as there was unfortunately no difference between all groups after 6 months. Another injection type, sodium hyaluronate, is used because it can be chondro-protective. This unbranched polysaccharide was shown to produce similar outcomes as intra-articular injections of corticosteroid (29).

Other nonsurgical interventions include suprascapular nerve blocks (SSNB) and hydrodilation. The SSNB works by reducing pain perceived at the shoulder, since 70% of the glenohumeral joint nerve supply originates from the suprascapular nerve (30). Of course, this improves pain in the short-term but fails to improve ROM (31). Hydrodilation, or distention arthrography, is a process in which the capsular space is dilated through injection of air or fluid under fluoroscopic guidance. This procedure aims to stretch the capsule, under anesthesia, to counteract the contracted capsule (32, 33). Hydrodilation was shown to provide similar improvement in pain and ROM with both injection of normal saline and a corticosteroid-infused saline; however, this improvement was similar to that observed in intra-articular corticosteroid injections (34).

Surgical Interventions

When patients do not respond well to conservative treatments, surgical intervention is possible. These interventions, however, do not come without downsides. MUA for the shoulder aims to tear the capsular adhesions under a controlled setting beyond the limits imposed by pain on a patient. Although MUA is considered a safe procedure, cases of hemathrosis, capsular tear, labral detachment, superior labral anterior and posterior lesions, and humeral or glenoid fracture have been known to occur (35-37). The effectiveness of this treatment is still debated, as some studies have shown moderate improvement in pain and ROM, whereas other studies have shown worse outcomes than other nonsurgical therapies (38, 39). MUA is also less effective in those with diabetes (40).

A more effective procedure is an arthroscopic capsulotomy which comes with two advantages. An initial diagnostic arthroscopy can be performed to confirm the presence and exact location of the fibrosis while simultaneously ruling out other potential causes for shoulder pain. In this way, a capsular release can be performed directly at the area of interest (41-43). Multiple studies have shown improvements in most, if not all, patients who have undergone arthroscopic capsulotomies, which are usually concluded with little complication (44-46). Although generally safe, infection of postoperative arthrofibrosis can occur. Unfortunately, while diabetic patients show slight improvements, they do not show the same improvement as seen in nondiabetic patients (47). On the other hand, open capsulotomies are rarely performed because of the increased time required for postoperative recovery, although they achieve a similar effect to the arthroscopic variant (1, 48, 49). While these surgical procedures may be effective, their invasiveness and potential for complication underline the need for a more efficient therapy for shoulder arthrofibrosis.

Biochemical Basis for Relaxin

Capsular fibrosis is highlighted by an excessive accumulation of extracellular matrix (ECM) components, including fibrillary collagens type I and type III, as a result of expression of transforming growth factor beta (TGF-β), a potent fibrogenic agent. A decreased clearance of ECM components also occurs because of decreased secretion of matrix metalloproteinases (MMPs) (13, 14, 50). These MMPs are important for their degradation of collagen and their subsequent role in increasing the endogenous inhibitors,

tissue inhibitors of metalloproteinases (TIMPs). These interactions are normal in a healing response after an injury to tissue. However, if these interactions are not stopped, a simultaneous decrease in clearance and an increased production of ECM result in excessive tissue scarring (50). Thus, the contracted capsule has a mechanical resistance to motion while causing pain from inflammation. Additionally, gene and protein expression assays have found products related to fibrosis, inflammation, and chondrogenesis, including increased collagen type I alpha 1 (COL1A1) and collagen type I alpha 3 (COL1A3) genes, interleukin-6, platelet-derived growth factor (PDGF), and fibroblast growth factors (FGF) (51). This information further suggests that inflammatory changes initiate the recruitment of fibroblasts and immune cells, encouraging the fibrotic process and an inappropriate deposition of collagen. However, an alternative idea is that fibrotic changes occur before inflammation, making fibrosis the underlying disease process. In this model, it is thought that the error lies within the defective cell signaling pathways that mediate collagen remodeling (52).

Relaxin is a 6-kDa hormone, derived from the insulin superfamily, that naturally plays a role in inhibiting uterine contractions and assists in cervical dilation during pregnancy by aiding to soften and grow the cervix (50). Similar to insulin, relaxin is processed from a prepro-form to the mature hormone-containing A and B peptide chains, which are connected by two interchain disulfide bridges and one intrachain disulfide within the A chain (53). However, relaxin is also an antifibrotic hormone that causes an increase in MMPs and fibronectin degradation while simultaneously decreasing the expression of TIMPs and TGF-β-induced fibronectin levels (54-56). With this

understanding, it may be possible that the use of relaxin may reduce or reverse the biochemical effects of a capsular contracture if a reliable delivery method can be developed. Recently, a highly purified recombinant form of H2 relaxin, or relaxin 2, has been tested in many in vitro and in vivo systems to evaluate both its ability to modify connective tissue and its potential antifibrotic properties. So far, several studies have reported that relaxin acts at multiple levels to inhibit collagen overexpression and fibrogenesis within pulmonary, renal, cardiac, and hepatic environments (50). What is yet to be seen, however, is whether relaxin can be delivered to maximize an efficacy to alleviate the symptoms related to shoulder arthrofibrosis.

Because relaxin has a half-life of only 2.5 hours when delivered intravenously (57), it is difficult to say how relaxin should be most effectively administered to affect the joint space. To investigate the most successful method of delivery, this thesis will examine multiple methods of both different temporal dosing and different administration locations. It is therefore hypothesized that the effective delivery of relaxin will increase the ROM, in addition to reducing pain, in those diagnosed with shoulder contracture. Furthermore, it is thought that an intra-articular injection of relaxin will shield the protein from normal systemic degradation, extending its half-life, and that multiple injections will be required to maintain a level necessary to reverse the fibrosis.

Animal Model and Hypothesis Testing

This hypothesis will be tested in a rat model based on an induction of frozen shoulder through a prolonged extra-articular internal fixation of the shoulder (see **Figure**

2) (58), which mimics the clinical observations with shoulder contracture. This model has demonstrated the same long-lasting effects to the joint mechanics within the shoulder that are of interest for this study. Additionally, the synovial hyperplasia related to an increased content of type III collagen is also observed (59). This model also reliably reproduces a decrease in ROM in both internal and external rotational directions, a characteristic of the clinical manifestation of shoulder contracture in humans.

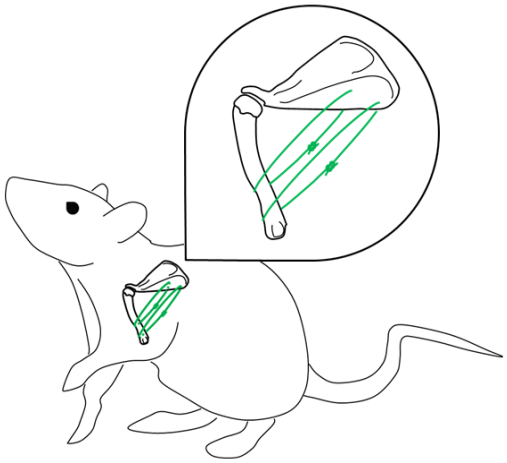

Figure 2. Representation of the animal model for extra-articular, internal fixation of the glenohumeral joint. Braided polyester sutures are tied around the humerus and through the inferior border of the scapula and tightened to restrict movement. This method avoids any major vessels and nerves while providing maximal restriction. Adapted from Villa-Camacho et al., 2015 (58).

With a reliable animal model and the same robotic testing apparatus (58), the change in ROM will be observed over time following a similar 8-week immobilization period under different conditions for relaxin exposure. Furthermore, immunohistology will be performed on samples taken from the rats to observe the effects of relaxin on collagen I, collagen III, and MMP expressions within the joint. These samples will also be evaluated for anatomic changes due to excessive fibrosis, or lack thereof. Because it is possible for symptoms of frozen shoulder to spontaneously resolve, the rate of this occurrence, as well as the degree to which the rats return to normalcy, will be examined.

Goals and Specific Aims

To reliably determine whether relaxin affects the transient outcome of those diagnosed with frozen shoulder, an animal model will be used to address the following aims:

Aim 1: Determine whether the location of injection of relaxin, either intra-articular or systemic, significantly affects the prognosis of ROM in the rat model.

Aim 2: Determine whether a single injection or prolonged exposure to relaxin has any appreciable effect on prognosis.

Aim 3: Examine the effects of relaxin on the glenohumeral joint capsule and histologically compare those results to controls.

METHODS

Specimen Preparation

On the approval of the Institutional Animal Care and Use Committee (IACUC), 20 Sprague Dawley rats (250-300 g, Charles River Laboratories, Inc., Wilmington, MA, USA) were chosen for this study. Before the rats were immobilized to create adhesive capsulitis, baseline ROM measurements were taken for both forelimbs. Torque measurements were recorded at 100^{o} of internal rotation (τ_{INT}) and 60^{o} of external rotation (τ_{OUT}) (**Figure 3**). These measurements were required as they indicate a baseline for normal torque necessary to achieve both rotations. These angles were chosen to ensure minimal scapular recruitment while simultaneously providing maximum humeral rotation within the joint space. Scapular deviation was monitored through fluoroscopy. Each ROM measurement was repeated three times to ensure consistency. These measurements were also performed under anesthesia to prevent any active muscle activation from interfering with the passive capsular resistance. Induction of the rats was performed at 5% isoflurane inhalation, and maintenance was managed at a 2% isoflurane dose.

After the baseline measurements and while still anesthetized, the 20 rats were subjected to the immobilization procedure as outlined in Villa-Camacho et al. (58) to induce fibrosis. On the left limb, an incision was created longitudinally, perpendicular to the scapular spine to expose both the scapula and humerus. A No. 2-0 Ethibond polyester suture (Ethicon, San Lorenzo, PR, USA) was used to immobilize the humerus to the scapula by passing two loops through the medial border of the scapula and against the

humeral shaft (**Figure 2**). Care was taken to ensure the sutures avoided critical vasculature, musculature, and nerves. Each rat was maintained under fixation for 8 weeks. After the eighth week, the suture fixations were removed, and the rats were randomly placed in four groups: (1) intra-articular relaxin, single dose (n = 5); (2) intra-articular relaxin, multiple doses (n = 5); (3) intravenous relaxin, multiple doses (n = 5); and (4) untreated controls (n = 5).

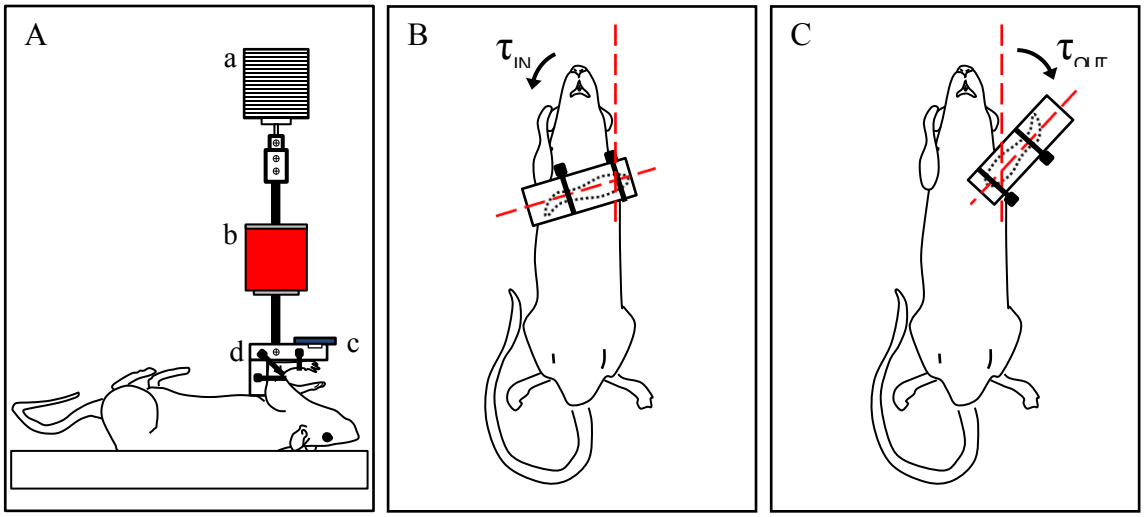

Figure 3. Range of motion measurement apparatus and method. (**A**) The core components of the range of motion measurement apparatus and measurement method are: (a) stepper motor, (b) torque sensor, (c) inclinometer, and (d) arm clamp. Rotation of the rat forelimb is shown in internal (**B**) or external (**C**) rotation of 100° and 60°, respectively. Adapted from Villa-Camacho et al., 2015 (58).

Mechanical Testing Apparatus

The mechanical testing apparatus was assembled with four core components and controlled with a computer through custom-built software written on MATLAB 7.13.0.564 (The MathWorks, Inc., Natick, MA, USA). Movement of the forelimb was

mediated by a stepper motor controlled by a microcontroller (UNO R3; Arduino, Torino, Italy). The motor was then positioned axially with the reaction torque sensor (TFF400; FUTEK Advanced Sensor Technology, Inc., Irvine, CA, USA), which measured the experienced torques and provided an input torque feedback for the system. Along the same axis, the arm clamp and the 3-axis inclinometer (3DM-GX3-15; MicroStrain, Inc., Williston, VT, USA) were attached on the sensing side of the torque sensor. The inclinometer also provided positional feedback as well as measurements for the system. The entire assembly was positioned above the rat with the sensing plane parallel to the ground to ensure that gravity had little impact on the torque measurements (**Figure 3**). The apparatus was programmed to move to a specified torque or angle for internal and external rotation for each rat. Plastic zip ties were used to secure the rat forelimb in the apparatus. Care was taken to prevent any injury, and the apparatus was programmed with an internal and external kill switch.

Treatment and Measurement of Study Groups

Immediately after removal of the restraining sutures, relaxin was administered to the noncontrol group rats. These groups were randomly selected for relaxin treatment. Relaxin was administered by intra-articular (IA) injection into the anesthetized rats under fluoroscopic guidance. Each dose was comprised of 0.0005 mg relaxin diluted in 100 μL of phosphate-buffered saline (PBS; 0.0015 mg/kg). Relaxin that was dispensed by intravenous (IV) injection through the tail was dosed at 0.17 mg relaxin diluted in 100 μL PBS (0.5 mg/kg). In the group that required multiple doses of relaxin, intra-articular and

intravenous injections were provided every 2 days over the first 10 days of the post-immobilization period (5 doses; total relaxin: IA 0.0025 mg, IV 0.85 mg). Injection of each intra-articular aliquot of relaxin was performed with a 27-gauge needle (PrecisionGlide; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Subsequent kinematic measurements were performed randomly and in a blinded manner after treatment. Each measurement was longitudinally spaced in the follow-up period of 8 weeks as determined by a previous study (58). These measurements examined the change in ROM, both internal and external angles, given the τ_{INT} and τ_{OUT} recorded at baseline. The apparatus was programmed so that each rat was measured on the basis of its own individual torque values. This was done to measure any resulting kinematic differences by keeping the force required to reach a full ROM consistent for each rat, since variation across rats existed (58). Each of these measurements occurred biweekly within the first two weeks and then weekly throughout the follow-up period. This schedule was done to limit specimen exposure to isoflurane, mainly because during the prior experience establishing this model, kinematic changes had been found to occur rapidly within the first two weeks and become generally steady for the remainder of the period (58). During each measurement period, measurements were taken under anesthesia and were repeated three times for both forelimbs to ensure accuracy.

Histological and Radiographic Analysis

On the conclusion of the follow-up period, the rats were euthanized according to IACUC guidelines. The rats were weighed and then subjected to CO_2 exposure for

euthanasia, which was subsequently confirmed through a bilateral thoracotomy. The shoulders were bilaterally harvested by disarticulating the humerus from the ulna and the scapula from the clavicle and thoracic cavity. Any excess muscle tissue not immediately surrounding the glenohumeral joint capsule was removed. The excised shoulders were decalcified for two months in a solution of ethylenediaminetetraacetic acid (EDTA), which was changed every two to three days. Once decalcified, the shoulders were affixed in a solution of 10% formalin and then mounted in paraffin stacks for histological sectioning at the Beth Israel Deaconess Medical Center (BIDMC) Histology Core. These stacks were mounted so that anterior-posterior slicing could be performed on the specimens. The slices were stained with hematoxylin and eosin (H&E) and examined for any morphological changes.

The specimens chosen to undergo histological analysis were the surgical control group and the multiple-dose intra-articular relaxin group. The multiple-dose intra-articular relaxin group was chosen because this group received the highest and most frequent amount of relaxin dosing and would therefore be the best group to showcase any changes to the joint space due to relaxin. The contralateral shoulders from the surgical control group were used to model a healthy control shoulder for histologic comparisons.

Data and Statistical Analysis

Comparisons in kinematic changes were done by comparing the change in ROM between the baseline measurement and the measurements that followed immobilization and treatment. The change in ROM was calculated using MATLAB script to ensure

randomization and blinded data processing for the comparisons. ROM measurements were shown as total ROM averages along with standard errors. All variances were described by standard errors. Standard error was chosen to represent the precision of the population mean as it can be more useful for interpretation in this case rather than examining the variation within each group (60). Changes in ROM were examined across groups at each measurement time point. Statistical differences across groups and over time were performed by repeated measures analysis of variance (ANOVA). Significance was determined using an alpha level of 0.01 ($P < 0.01$). A significance level of 0.01 was chosen because it better represented meaningful trends that were observed in this study. Histologic images were processed and analyzed using the Fiji distribution of ImageJ (61) to measure intra-articular intima thicknesses of the glenohumeral capsule.

RESULTS

Mechanical Testing

At the baseline measurement, all rats were measured and attained a full ROM of 159.17° (0.21°) and a standard deviation of 1.14°, with external measures of 59.58° (0.17°) and an internal range of 99.58° (0.14°) with standard deviations of 0.77° and 0.63°, respectively. Immediately after suture removal from the immobilization period, all rats attained a total ROM of 91.17° (4.52°), correlating to a 43.22% (2.82%) total reduction from the baseline. Externally and internally, the rats exhibited ranges of 35.96° (4.97°) and 55.20° (5.10°), respectively. The rats together had a significant decrease in ROM ($P < 0.0001$), with the control group alone holding a similar significance ($P < 0.0001$). At day 0 immediately after immobilization, the multiple-dose intra-articular group ($P = 0.2721$), the single-dose intra-articular group ($P = 0.4347$), and the multiple-dose intravenous group ($P = 0.6074$) were shown to be statistically as restricted as the control group.

At the final time point, the control group attained a total of 130.93° (5.38°) of rotation, the multiple-dose intra-articular group measured at 162.27° (3.60°), the single-dose intra-articular group measured at 138.67° (7.08°), and the multiple-dose intra-venous group measured at 130.80° (5.02°). Interestingly, the multiple-dose intra-articular group ended with a 3.47°, or a 2.17%, increase in ROM from baseline and was found to be significantly improved when compared with the control group at $P = 0.0013$. All other groups were not significantly different from the control group (**Table 1**). The control group had a -28.14°, or a -17.59% ($P = 0.0048$), reduction from baseline measurements.

Similarly, the intravenous group displayed a -27.87°, or a -17.42% (P = 0.0056), reduction when compared with baseline. The single-dose intra-articular group showed more of a slight improvement from baseline with a difference in ROM of -21.46°, or -13.41% (P = 0.0385). However, this increase was not found to be a significant improvement when compared with the control.

Table 1. Final Total Ranges of Motion for Each Group[a]

	Baseline		Final		Difference		
Group	ROM (°)	SEM (°)	ROM (°)	SEM (°)	ROM (Δ°)	Percent	P
Control	159.07	0.61	130.93	5.38	-28.14	-17.59	1.0000
IA Multiple	158.80	0.48	162.27	3.60	3.47	2.17	0.0013
IA Single	160.13	0.40	138.67	7.08	-21.46	-13.41	0.4090
IV Multiple	158.67	0.55	130.80	5.02	-27.87	-17.42	0.9860

[a]A complete range of motion is expected to be near 160°. A negative change in ROM describes a difference in final ROM that is lower than a full ROM. A positive change indicates a final ROM that is greater than the baseline measurement. Significance is determined at $\alpha = 0.01$. The P-value is the result of a comparison between the final control ROM and the ROM of each of the different groups.

On examination of the full ROM data across different time points (**Figure 4**), the multiple-dose intra-articular group appeared to be a significant improvement over the control group. The multiple-dose intra-articular group was significantly greater than the control group at days 7, 49, and 56 with $\alpha = 0.01$, and at all days except day 0 and day 21 with $\alpha = 0.05$. All other groups showed no significance throughout the time points. For the multiple-dose intra-articular group, its variance increased as it approached day 21 and decreased thereafter. All other groups did not exhibit any pattern with variance. Another important trend to note was the sharp improvement in ROM in the single-dose intra-articular group immediately after administering relaxin, similar to that of the multiple-dose intra-articular group. Improvements showed a 29.20° increase in ROM as opposed

to only 11.33° seen in the control group. This increase was not significant (P = 0.4213). After the second measurement, however, the single-dose intra-articular group began to drop and trend below all the other groups for some time. This trend was not found to be significantly lower than the control group at any point in time.

Additionally, at the final measurement, the multiple-dose intra-articular group was statistically similar (P = 0.4304) to its healthy baseline measurement. The single-dose intra-articular group showed an improvement toward baseline that was ultimately not statistically significant (P = 0.0385). Both the intravenous and control groups remained significantly worse than the healthy condition at P = 0.0056 and at P = 0.0048, respectively.

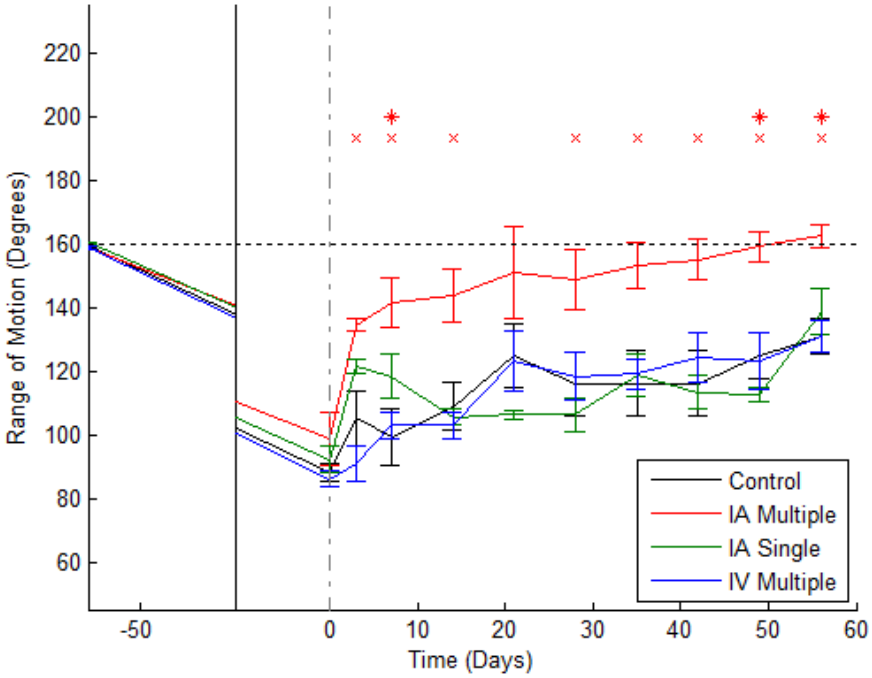

Figure 4. Change in total ROM post-surgery. Results are presented as the means with standard errors. The dotted line indicates a healthy ROM, and the vertical dashed line highlights the day of suture removal. Significance at $\alpha = 0.01$ is denoted by an asterisk, and significance at $\alpha = 0.05$ is shown as a cross.

Breaking up the total ROM into external and internal ROMs highlighted the stark differences between the two. A closer look at external ROMs noted no major differences between the baseline and final measurements (**Table 2**). The control group had a change in ROM of 2.87°, or 1.79%. The control group also showed no significant difference when comparing baseline with the final measurement (P = 0.6199) or when comparing day 0 with the final measurement (P = 0.0418). These results contrasted with the significance found within internal rotation, with a significant difference in both day 0 (P = 0.0002) and final day measurements (P = 0.0078) when compared with baseline, which

suggests to a successful contracture model internally. The absence of a significant decrease in ROM for the external rotation case was unexpected from the previous stiffness model (58). In comparison, the other groups—multiple-dose intra-articular (P = 0.7316), single-dose intra-articular (P = 0.6329), and multiple-dose intravenous (P = 0.2271)—also showed a similar lack of difference from baseline to their final measurements. Based on these findings, external ROM by itself showed negligible change in ROM. This was further observed in the temporal data plotted in **Figure 5**. There was, however, one single point at day 7 of measurement that showed a strong significant difference when comparing the single-dose intra-articular group and the control group (P = 0.0018). Although once the data were aggregated, this significance was lost.

Table 2. Final External Ranges of Motion for Each Group[b]

	Baseline		Final		Difference		
Group	ROM (°)	SEM (°)	ROM (°)	SEM (°)	Range (°)	Percent	P
Control	59.33	0.43	62.20	5.27	2.87	1.79	1.0000
IA Multiple	59.67	0.26	57.13	7.12	-2.53	-1.58	0.5831
IA Single	60.00	0.32	63.47	6.78	3.47	2.17	0.8863
IV Multiple	59.33	0.38	50.07	6.74	-9.27	-5.79	0.1940

[b] A complete external range of motion is expected to be near 60°. A negative change in ROM describes a difference in final ROM that is lower than a full ROM. A positive change indicates a final ROM that is greater than the baseline measurement. Significance is determined at $\alpha = 0.01$. The P-value is the result of a comparison between the final control ROM and the ROM of each of the different groups.

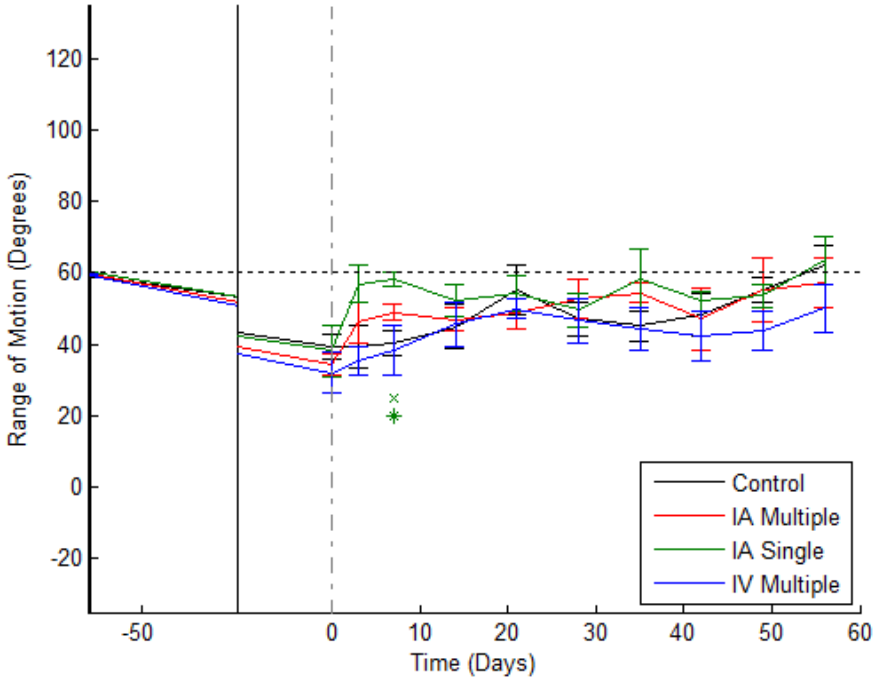

Figure 5. Change in external ROM post-surgery. Results are presented as means with standard errors. The dotted line indicates a healthy ROM, and the vertical dashed line highlights the day of suture removal. Significance at $\alpha = 0.01$ is denoted by an asterisk, and significance at $\alpha = 0.05$ is shown as a cross.

Contrary to external rotation results, the control group for internal rotation displayed a significant total decrease in ROM of 31.00°, or 19.38% (P = 0.0078), after the final measurement. Additionally, immediately after immobilization, the internal rotation of the control group exhibited a decrease of 51.73°, or 51.67% (P = 0.0002) on day 0. Each of the other groups was similarly restricted when compared with the control group, as indicated by the multiple-dose intra-articular group with P = 0.0875, the single-dose intra-articular group with P = 0.4621, and the multiple-dose intravenous group with P = 0.3627. At the final measurement, only the multiple-dose intra-articular group was shown to have a significant improvement from the control contracture group, with a total

range of 105.13°, or a 6.05% improvement (P = 0.0073) (**Table 3**). This improvement, however, was statistically similar to the healthy baseline measurement (P = 0.5142).

Table 3. Final Internal Ranges of Motion for Each Group[c]

Group	Baseline		Final		Difference		
	ROM (°)	SEM (°)	ROM (°)	SEM (°)	Range (°)	Percent	P
Control	99.73	0.32	68.73	6.15	-31.00	-31.08	1.0000
IA Multiple	99.13	0.27	105.13	8.13	6.00	6.05	0.0073
IA Single	100.13	0.31	75.20	7.42	-24.93	-24.90	0.5213
IV Multiple	99.33	0.24	80.73	7.90	-18.60	-18.73	0.2651

[c] A complete internal range of motion is expected to be near 100°. A negative change in ROM describes a difference in final ROM that is lower than a full ROM. A positive change indicates a final ROM that is greater than the baseline measurement. Significance is determined at $\alpha = 0.01$. The P-value is the result of a comparison between the final control ROM and the ROM of each of the different groups.

At the final time point, the other two groups shared no statistical difference with the control group or with the multiple-dose intra-articular group. The differences from the control for single-dose intra-articular and multiple-dose intravenous were very similar (**Table 3**). When compared with the final result of the multiple-dose intra-articular group, the single-dose intra-articular group had P = 0.0263, which could be considered significant if $\alpha = 0.05$ was used. The intravenous group, however, was not significant at either alpha level with P = 0.0636 when compared with the multiple-dose intra-articular group.

The results for internal ROM (**Figure 6**) differed from total ROM (**Figure 4)** in that internal ROM did not share as prominent of an increase in ROM immediately after day 0. In fact, the increase in the total ROM data was a manifestation of two less obvious increases at day 3 in both external and internal ROM data sets. Also, for internal ROM,

significance from the control group was only found in the multiple-dose intra-articular group on days 42 and 56.

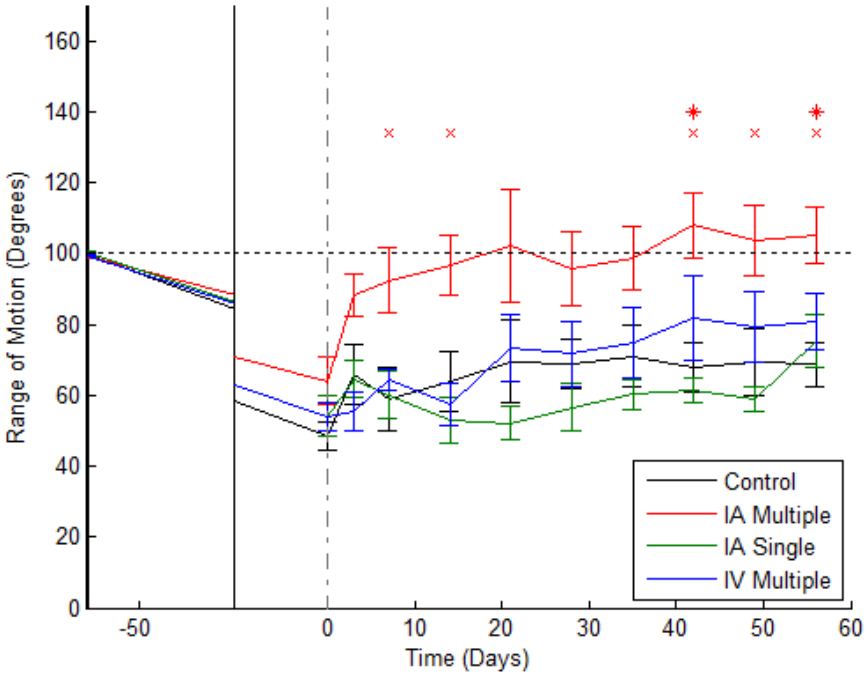

Figure 6. Change in internal ROM post-surgery. Results are presented as means with standard errors. The dotted line indicates a healthy ROM, and the vertical dashed line highlights the day of suture removal. Significance at $\alpha = 0.01$ is denoted by an asterisk, and significance at $\alpha = 0.05$ is shown as a cross.

Histology

The H&E stained sections for the surgical control group showed morphological changes to the surrounding capsular tissue when compared with the healthy control. As seen in **Figure 7,** the healthy control displayed proper separation between capsule and articular surface on the humeral head. The synovial membrane and articular cartilages

showed normal cellular organization. However, the surgical control group in **Figure 8** lacked this separation.

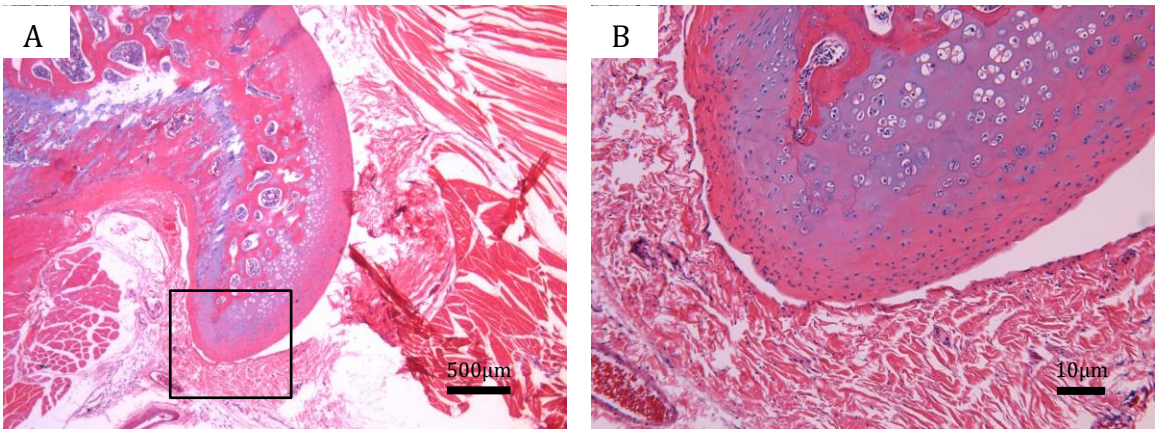

Figure 7. Coronal H&E stains of the humeral head in a healthy control. Medial and lateral directions correspond to the right and the left of the image, respectively. The top of the image is the superior direction. (**A)** Image taken at 2.5x magnification. The scale bar is set to 500 μm. (**B)** Magnified image of the inferior aspect of the humeral head taken from within the outlined box on image A. Image taken at 10x magnification. The scale bar is set to 10 μm.

In the surgical control group, fibrotic adhesions were apparent. The region outlined by the box in **Figure 8A** was the most affected area in the joint. In **Figure 8B**, two arrows delineated the differentiated synovial membrane and articular cartilage. These tissues showed the same organization as found in the healthy control. This differentiation between the tissues gradually disappeared when approaching the inferior aspect of the humeral head. Most inferiorly, the articular surface of the humeral head and the synovial membrane lost all differentiation. The membrane and cartilage nuclei failed to maintain a tangential orientation to the humeral head within the superficial zone (tangential zone) and instead showed directionality that was orthogonal to the expected surface. The cellular bodies appeared to be stretched between the capsular membrane and the

cartilaginous surface of the humerus. Evidence of these adhesions was a confirmation of the success of the contracture model.

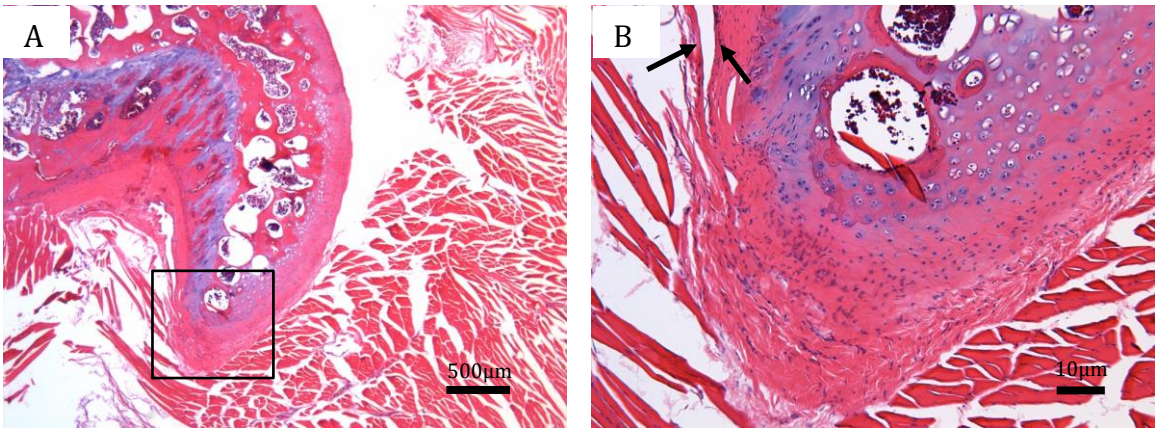

Figure 8. Coronal H&E stains of the humeral head in a surgical control. Medial and lateral directions correspond to the right and the left of the image, respectively. The top of the image is the superior direction. (**A)** Image taken at 2.5x magnification. The scale bar is set to 500 µm. (**B)** Magnified image of the inferior aspect of the humeral head taken from within the outlined box on image A. Image taken at 10x magnification. The scale bar is set to 10 µm.

In contrast to the surgical control, the multiple-dose intra-articular relaxin group lacked any apparent adhesions (**Figure 9**). The synovial membrane and articular cartilage surfaces remained independent from one another, maintaining proper orientation. Cellular organization of these membranes also showed the similar tangential squamous shape that was apparent in the healthy control image. Interestingly, the tissue density of the fibrous capsular tissue appeared to be less than the density found in the healthy control group. Additionally, some discontinuities of the shoulder capsule from the region just below the humeral head growth plate were seen, perhaps indicating a tear. An example of this was marked by an arrow in **Figure 9A**. The degradation of the articular cartilage was also highlighted by arrows in **Figure 9B**. This destruction was manifested as a thinning of

articular cartilage thickness in the superficial zone and delamination of the articular cartilaginous layers.

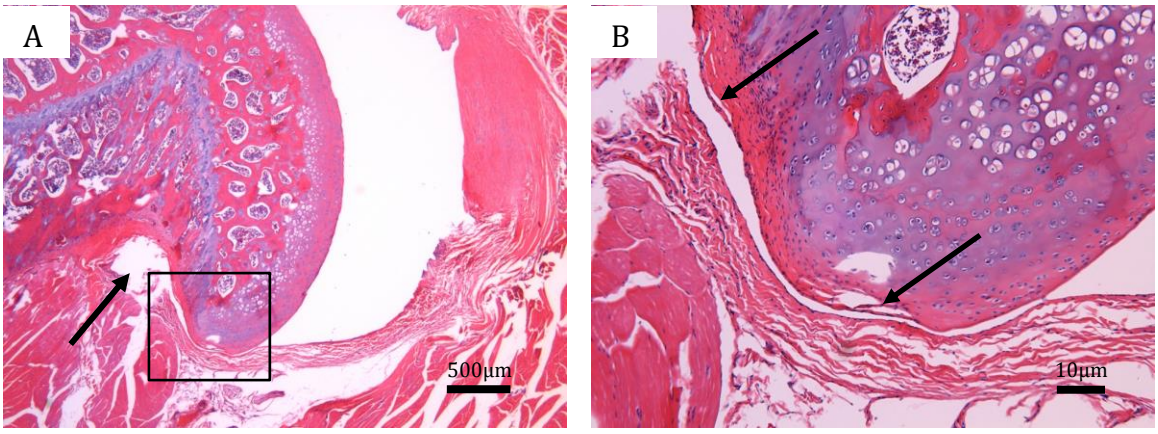

Figure 9. Coronal H&E stains of the humeral head in a relaxin-treated shoulder. Medial and lateral directions correspond to the right and the left of the image, respectively. The top of the image is the superior direction. (**A)** Image taken at 2.5x magnification. The scale bar is set to 500 µm. (**B)** Magnified image of the inferior aspect of the humeral head taken from within the outlined box on image A. Image taken at 10x magnification. The scale bar is set to 10 µm.

DISCUSSION

Support for Specific Aims

Mechanical Testing

For the aims of this thesis to be satisfactorily addressed, it is crucial to ensure that the animal model being used was properly established and that the mechanical testing apparatus achieved the precision (<1% variance) and accuracy (<1% difference from baseline) necessary for confidence in the experimental measurements. The model achieved both shoulder contracture and prolonged reduction in ROM. The total ROM of the rats after immobilization showed a 43.22% reduction from their baseline measurements. Additionally, the control group remained 17.59% more restricted than at their baseline measurements at the end of 8 weeks, suggesting that the immobilization method was a success. This amount of restriction remains consistent with previously run models (58, 59).

Given the three experimental groups for examining the effects of relaxin, the multiple-dose intra-articular relaxin group showed significant improvements above all other groups. This group finished with a 2.17% increase in ROM that was still similar to its baseline measurement. This finding suggests that the efficacy of relaxin for arthrofibrosis does exist, and that there is a potential for its use to fully resolve the ROM symptoms of shoulder arthrofibrosis. Although an increase in ROM beyond the baseline measurement may be concerning, this evidence further supports the ability of relaxin to modify existing collagen structures (50). However, since ROM greater than baseline was

achieved in some cases, proper dosing for relaxin must be carefully considered to achieve the desired effect.

Dosing effects were further observed when examining the single-dose intra-articular relaxin group. After the first intra-articular injection, although not significant from the control group, this group showed a sharp increase in ROM of 29.20° three days after injection. Because the injections occurred once every two days in the multiple-dose groups, an additional injection in the single-dose intra-articular group would have continued this trend for improvement. This result suggests that a prolonged exposure to relaxin is necessary for proper recovery from a secondary shoulder contracture. Also of importance was the observed decline in ROM after the third day of measurement in the single-dose intra-articular group. Not only did this group fail to continue improving, as seen in the multiple-dose intra-articular group, but it returned to ROM measures similar to that of the control group. This worsening of the ROM after intra-articular injection suggests that additional fibrosis may have been caused by either articular trauma induced by intra-articular injection or other reasons such as an immune response to exogenous relaxin (17, 18). This decline in ROM continued to decrease up to around 10 days after the single-dose intra-articular injection.

Further evidence supporting the contribution of articular trauma in worsening of ROM can be seen in the multiple-dose intra-articular group. Looking at the variance of each measure in this group, the values increased to a maximum on the measurement that was approximately 10 days after the final intra-articular injection for the group. This timeline fits well with other observations of the development of fibrosis after trauma in

rats, in which fibrosis was noticeable after 7-10 days (62, 63). The increase in variance may have been due in part to the variability in performing intra-articular injections with a 27-gauge needle for a small joint space. Based on how well the procedure was done, differing amounts of trauma may have been introduced into the rats' shoulders. Each of the rats may additionally have differing responses to this trauma, further compounding the variability during this time. However, despite this variability, the group trended toward a statistically full recovery and improved well beyond the controls.

In addition, the final ROM of the single-dose intra-articular group exhibited differences from all the other groups. Although the group did not show a statistically improved ROM result when compared with the control for the final measurement, neither did the group show any difference from the original baseline measurement (P = 0.0385). Though this result was not significantly different at $a = 0.01$, it was significantly different at $a = 0.05$. The fact that the final ROM measurement for this group remains a nonsignificant improvement from the control group and that it fails to show a strong difference from its own baseline measurement, suggests that there may have been an improvement in ROM from a single dose of relaxin. Because this improvement cannot be said with certainty, this result encourages a further exploration into the proper dosing and exposure of relaxin when treating shoulder contractures.

The intravenous effects of relaxin showed virtually no significant difference from the control group at any time point. Additionally, the intravenous relaxin group maintained a similar difference from baseline measurements when compared with the control. These results suggest that intravenous relaxin provides no benefit to the

betterment of a shoulder contracture. This finding makes sense, particularly because relaxin has a short half-life within the systemic circulation. As a result of its rapid degradation, relaxin may have too minimal of exposure to the joint area, especially since articular capsules are often not well perfused. Considering that intra-articular relaxin displayed more of an effect on ROM, it is possible that the joint space provides the necessary isolation from the systemic circulation to extend the time of degradation for relaxin. Furthermore, because relaxin was injected directly to the area of interest, it can also be certain that an appreciable amount reached its target.

All of these effects on total ROM were also observed when considering internal rotation only. In fact, the effects of relaxin seem to be further emphasized within internal rotation. However, this was not the case for external rotation, as all of the results remained statistically similar. This result is most likely a response to the limits placed on the ROM measurements. For internal rotation, 100° of rotation is not close to the physiologic limits of rotation, but 60° of external rotation at forward elevation is near physiologic limits (64-66). Earlier studies found that external rotation should display an increase in the torque required to reach 60° after immobilization (58), yet an increase in torque does not necessarily correlate to a decrease in ROM. In this study, because 60° is near the limits of rotation, the application of additional force would result in a minimal increase in external ROM. Therefore, although torque might increase for external rotation during a shoulder contracture, the torque required to reach 60° was overestimated as a result of the baseline measurement already nearing physiologic limits. Thus, this overestimation likely caused external ROMs of similar range to be measured despite

increases in torque. The opposite is true for internal rotation, and this may be the reason why internal rotation showed a better contracture than external rotation.

Histologic Evidence

The histologic data mirror the results found during the mechanical testing. Confirming the successful creation of the in vivo shoulder contracture model was the presence of fibrotic adhesions in the inferior portion of the capsular space in the surgical controls. Additional confirmation for this model came from the expected lack of adhesions in the contralateral healthy shoulder.

The relaxin histologic images, although much more similar to the healthy control images, showed a number of changes between the other two conditions. The fact that the relaxin images showed proper differentiation between the synovial membrane and articular cartilage suggests that intra-articular relaxin aided a return to a normal condition. The surfaces seen in the superficial zone showed the characteristically parallel articular cartilage cells against the contour of the bone. In addition, a similarly squamous characteristic in the synovial membrane suggests a morphologic return to a normal condition. These findings suggest that relaxin may be able to play a role in alleviating arthrofibrosis within the shoulder.

However, differences from the normal condition did exist. The finding that the fibrous tissue comprising the capsular lining seemed to have lost some density may actually be a cause for the capsular tear observed in **Figure 9**. Because relaxin can encourage the remodeling and reuptake of collagens I and III, this lack of density may be a result of the action of relaxin on the surrounding collagen. This lessening of collagen

would result in a weakening of the tensile properties found in highly collagenous tissues and would result in tears if excessively stressed. The mechanical testing apparatus causes the rats to undergo rotational movement that is not dissimilar to physical therapy. Perhaps this force induced through measurement was enough to manifest as a tear within these histologic images.

This possible collagen degradation was also seen on the articular surface of the humerus, where there was a simultaneous thinning of the superficial zone along with cellular delamination. This degradation may simply be characteristic of some alterations of the articular surface as a result of immobilization (67). However, although it is not generally thought that relaxin affects collagen II, altering the level of MMPs within the collagen synthesis pathway has been shown to affect articular cartilage (68). If relaxin holds the ability to alter the articular surface and surrounding capsular structure within joints beyond that of reversing fibrosis, additional care must be taken to evaluate proper exposure to relaxin. The evidence, as found by these histological images, suggests that excess relaxin may not only reverse the effects of arthrofibrosis but also further degrade existing collagen structures.

Limitations

As noted in the preceding section, the ROM chosen for external rotation may have been too large. It is often expected that in frozen shoulder, external rotation is most affected (65). Because the limit of external rotation with forward elevation was overestimated in this rat model, it is possible that any effect on the contracture that

relaxin may have had would be obfuscated. Despite this problem, relaxin had a great effect on internal rotation in a manner that it dominated the results when observing a total ROM of 160°. If a smaller external rotation angle were to be chosen, perhaps a similar result would be seen externally. As a result, there is the possibility that the effect of relaxin in this study was understated.

Furthermore, the method of intra-articular injections used in this work included a comparatively large gauge needle for navigating the intra-articular space. Because of the size, the chance for additional trauma and further fibrosis may have diluted the effects of relaxin even more than necessary. Although an intra-articular injection may cause a moderate amount of trauma to the joint area, in its typical use, a more appropriately sized needle is utilized for humans. This would result in much less trauma than the rats experienced in these procedures.

Additionally, because the limitations found with the methods in this study could work to downplay the effectiveness of relaxin, the dosing duration and concentrations would not be representative values for potential treatment. Although it was successfully determined that relaxin has a positive effect on shoulder arthrofibrosis, the specifics behind delivery and dosing remain uncertain. This study is limited in its ability to rule out the ineffectiveness of any of the other drug delivery methods and instead can only propose which will work with the given concentrations and frequencies.

Although this study holds promising results, another limitation lies in the fact that these procedures were conducted in rat shoulders. Rat shoulders differ from human shoulders in that they are load-bearing appendages. Therefore, it may be difficult to say

how well these results translate to a human, as humans do not rely on their upper extremities for locomotion in normal conditions. Also, this study aimed to show the efficacy of relaxin in cases of arthrofibrosis, but only the shoulder was examined. Though the pathophysiology is similar across cases of arthrofibrosis, there is no guarantee that relaxin can be applied directly to another joint case of arthrofibrosis outside the shoulder.

Future Studies

Since there were limitations to this study, future work should aim to explore these areas further. Although relaxin was shown to alleviate a shoulder contracture in this study, further experiments should consider its efficacy in remediating cases of arthrofibrosis in other joints. Additionally, these studies may choose to examine relaxin in differing species to ensure a similar effect.

Also, proper dosing should be established, and further studies may choose to explore more efficient concentrations and frequencies for relaxin delivery. This may include a low-level, slow-release drug delivery system that reduces the amount of trauma introduced into the joint space. Additional evaluations of relaxin should also closely examine potential side effects of using relaxin as a treatment for arthrofibrosis, as excessive dosing may result in damage to articular joints.

Conclusion

A repetitive, local intra-articular injection of relaxin into the glenohumeral space was found to be effective in resolving the range of motion symptoms that occur in a

shoulder contracture and in returning the range of rotation back to a normal condition. This result was further supported through histologic evidence which showed no pathological fibrosis within the joint space. A single intra-articular injection of relaxin and an intravenous injection of relaxin were both ineffective for returning the range of motion back to baseline. Although an improvement in shoulder arthrofibrosis was shown in this study, further research is necessary to fine-tune the delivery of relaxin for use in patients suffering from arthrofibrosis.

LIST OF JOURNAL ABBREVIATIONS

Acta Orthop. ..... Acta Orthopaedica

Adv Exp Med Biol. ..... Advances in Experimental Medicine and Biology

Am J Sports Med. ..... American Journal of Sports Medicine

Am J Renal Physiol. ..... American Journal of Physiology – Renal Physiology

Ann R Coll Surg Engl. .....Annals of The Royal College of Surgeons of England

Ann Rheum Dis. .....Annals of Rheumatic Diseases

Arch Orthop Trauma Surg. ..... Archives of Orthopaedic and Trauma Surgery

Arthritis Rheum ..... Arthritis & Rheumatology

Bone Joint J. ..... The Bone & Joint Journal

Br J Anaesth. ..... British Journal of Anaesthesia

Br J Sports Med. ..... British Journal of Sports Medicine

Br Med J. .....British Medical Journal

Clin Biomech. ..... Clinical Biomechanics

Clin Orthop Relat Res. .....Clinical Orthopaedics and Related Research

Cochrane Database Syst Rev. .....The Cochrane Database of Systematic Reviews

Exp Ther Med. ..... Experimental and Therapeutic Medicine

Int Orthop.....International Orthopaedics

J Bone Joint Surg Br. .....The Journal of Bone and Joint Surgery British

J Brachial Plex Peripher Nerve Inj. Journal of Brachial Plexus and Peripheral Nerve Injury

J Rheumatol. .....The Journal of Rheumatology

J Shoulder Elbow Surg. ..... Journal of Shoulder and Elbow Surgery

Knee Surg Sports Traumatol Arthrosc. ..Knee Surgery, Sports Traumatology, Arthoscopy

Muscles Ligaments Tendons J.....Muscles, Ligaments, and Tendons Journal

N Am J Sports Phys Ther. .....North American Journal of Sports Physical Therapy

Nat Methods. ..... Nature Methods

Open Orthop J. .....The Open Orthopaedics Journal

Orthop Clin North Am.....Orthopedic Clinics of North America

Osteoarthritis Cartilage ..... Osteoarthritis and Cartilage

Pharm Res. ..... Pharmaceutical Research

Phys Ther. .....The Journal of Orthopaedic and Sports Physical Therapy

Protein Pept Lett. .....Protein & Peptide Letters

Rheumatol Rehabil. .....Rheumatology and Rehabilitation

Saudi J Anaesth.....Saudi Journal of Anaesthesia

Shoulder & Elbow.....Journal of Shoulder & Elbow Surgery

Transl Res. .....Translational Research

Traumatol Rehabil. .....Journal of Orthopaedics, Traumatology and Rehabilitation

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