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1-21-2020

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Shah, Ishani D.; David, Michael; and Lake, Spencer, "Exploration of Drug Therapies for Post-Traumatic Elbow Joint Contracture" (2020). *Mechanical Engineering and Materials Science Independent Study*. 116. <https://openscholarship.wustl.edu/mems500/116>

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Exploration of Drug Therapies for Post-Traumatic Elbow Joint Contracture

Isha Shah

December 18, 2019

Abstract

This semester I worked in the Musculoskeletal Soft Tissue Laboratory to explore the potential drug therapies for post-traumatic joint contracture (PTJC), or arthrofibrosis, in the elbow following a traumatic joint injury. To simulate the potential effects of these drugs in the elbow post-injury, this study utilized an in-vitro model using collagen gels and NIH-3T3 cells to mimick the contraction of capsule tissue in the elbow and the cells thought to contribute to disease progression in the capsule, namely fibroblasts and myofibroblasts. The first part of the study tested the effects of two drugs, losartan and simvastatin, on decreasing contraction. The standard testing procedure required that the gels be seeded with cells, then administered the appropriate drug concentration and mixture, and then observed for a period of six days. Once we narrowed down the drug formulations to a few possible candidates, we considered the issues related to timing of treatment and pharmacokinetics of these drugs if administered into the human elbow; more specifically, we wanted to understand how factors like drug clearance, or the treatment of PTJC *after* its onset, would affect the drug's effectiveness. To this end, the second part of the study looked at how exposure time of the drug to the cells affected the drug's function; more specifically, the impact of both time delay and a shorter exposure period on the performance of the drugs were explored. It quickly became apparent through these studies that simvastatin could halt contraction, but did nothing to reverse it. The third and final part of the study tested the ability of the hormone relaxin on decreasing contraction. By the end of the semester, we determined that, within the construct of our experimental set-up and parameters, simvastatin was the only promising candidate for treatment of PTJC.

Introduction

Arthrofibrosis is characterized by stiffness of the joint and decreased range of motion, and patients can also experience varying levels of pain. It can occur after injury or severe trauma, and even as the result of surgery to address an injury or trauma. Arthrofibrosis is caused by the excess formation of extracellular matrix in the affected area [6]. As a part of the normal regulatory process, spindle cells called fibroblasts regularly produce extracellular matrix for cell scaffolding and injury response (Figure 1). Upon injury and in the presence of specific signals, like transforming growth factor β 1 (TGF β -1), fibroblasts can be influenced to differentiate into myofibroblasts [3]. Myofibroblasts are essential in the wound healing process. Their antecedents, proto-myofibroblasts, utilize stress fibers to pull cells together and reduce inter-cellular gaps; once evolved to full myofibroblasts, they secrete α -smooth muscle actin (α -SMA), which organizes further and more rigorous contractile activity in the area [3]. In a healthy immune response to an injury, the appropriate amount of fibroblasts would differentiate into myofibroblasts, and once the healing process had ended, the myofibroblasts would undergo apoptosis. However, abnormalities in this typical immune response often result in an elevated number of myofibroblasts in the injured area, and therefore the excessive buildup of extracellular matrix; this causes adhesions in joint capsules and immoderate contracture of connective tissue.

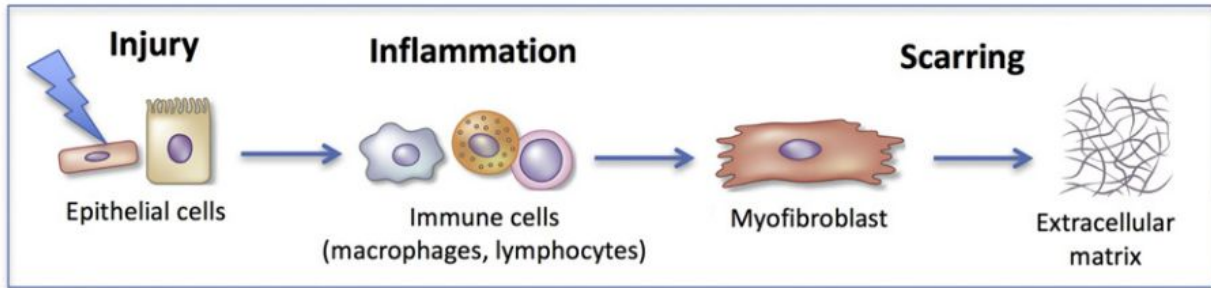


Figure 1: Progression from initial injury to immune response, triggering differentiation of fibroblasts into myofibroblasts and ultimately resulting in formation of extracellular matrix [4]

This contraction is the cause of the loss of range of motion in the elbow of many patients.

Current treatment options for pain and loss of range of motion that often result from PTJC include continuous passive motion, surgical intervention, bracing, and physical therapy, but these options can be expensive, invasive, uncomfortable, or may even exacerbate symptoms [4].

The specific drugs we were exploring were losartan and simvastatin, traditionally used to treat high blood pressure and to lower cholesterol, respectively. Both drugs were selected because of their antifibrotic properties, for which they have been used to treat other fibrotic diseases[7][8]. The first goal was to evaluate the effectiveness of the drugs under continuous application, mimicking repeated injections in the elbow; then, the issue of real-life applicability needed to be addressed. One of the factors that must be considered when working with an injectable drug is drug clearance; blood flow is constant and can wash out the majority of any substance injected into any area of the body relatively quickly. As a result, the concentration of the drug that is exposed to the cells of interest will dramatically decrease over time. In addition, in reality, the patient will not always come to the doctor at the exact moment contraction sets in. We wanted to see if the drug would still be effective in inhibiting contracture regardless of the time point at which the contracture set in. To test how both of these situations might alter the

effectiveness of the drug in the elbow, two testing parameters were implemented: shorter time exposure and time delay. The shorter time exposure parameters simulated the effects of blood flow in the body, where the gels sat in media containing the drug for only two days out of the six-day observation period. The time delay simulated a patient coming in to receive treatment *after* a contraction had set in, and the drugs were applied two days after the gels were seeded with cells.

In addition to simvastatin and losartan, we tested the hormone relaxin. Relaxin releases matrix-metalloproteinases (MMPs), which are enzymes that degrade extracellular matrix [1]. It is found in both men and women, but is most prevalent during the female reproductive cycle. It is responsible for relaxing the uterus wall during the menstrual cycle, the first trimester of pregnancy to allow for implantation, and before childbirth to allow for the opening of the cervix [5]. A collaborator's in-vitro study showed relaxin to reduce contraction in the shoulder, making it a prime candidate for investigation for use on the elbow; our study design and predictions of the outcomes was based on the fact that they used a 700 ng/injection concentration,

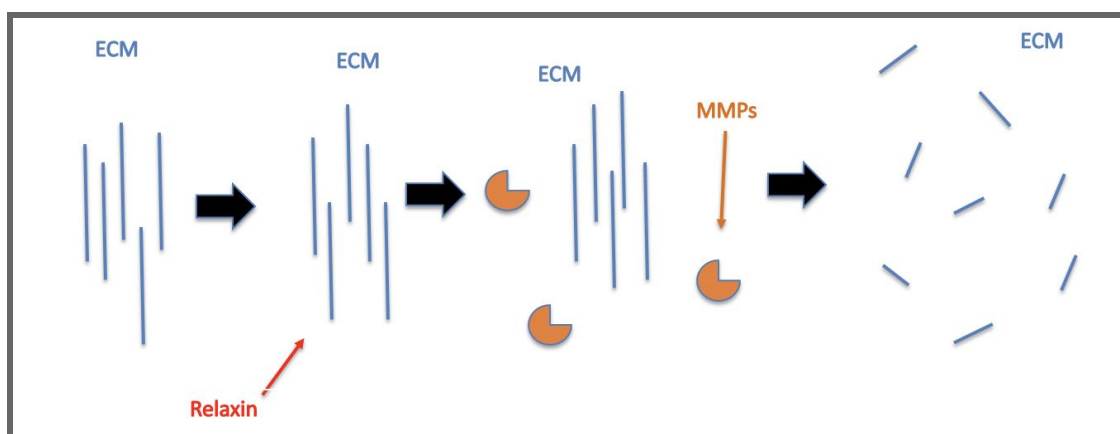


Figure 2: Relaxin activates matrix-metalloproteinases, which break down extracellular matrix

To assess the effectiveness of all drug formulations used, gel area and cell viability were analyzed for all samples. We expected that all of the drugs that were tested would have a dose-dependent effect.

Methods

To model the tissues that the drugs would be applied to, we used collagen gels and NIH-3T3 cells. The collagen that was used was extracted from rat tail tendons. The gels were seeded with NIH3T3 cells at 5×10^5 cells/ml, released from the well, and then observed for a six-day period to capture gel contraction. In the first part of the study, losartan and simvastatin were each continuously applied, i.e. the cells were constantly exposed to the drugs for six days in 1, 10, and 100 μM concentrations. Initial trials also experimented using these concentrations of drugs with and without the presence of TGF β -1. Throughout the experiment period, drugs, media, and TGF β -1 were replenished every two days.

The outcomes that we used to assess the effects of the drugs and hormone were gel area and cell viability. To monitor the contraction of the gels over this six-day observation period, a set-up was used where an iPhone was placed under a plastic stand holding the culture dish/gels and used to take photos daily from beneath each individual gel. These photos were analyzed using a Matlab program to assess gel area. After the observation period, the cells in the gels were stained with calcein AM, ethidium homodimer, and hoescht to assess the number of live cells, dead cells, and total cells, respectively. After staining, the gels were cut in half, and the gel's top surface ($\sim 100 \mu\text{m}$) was imaged using an epifluorescence microscope at 10x. Cell viability (% of

cells alive relative to total cells) were quantified from fluorescent images using a Matlab algorithm.

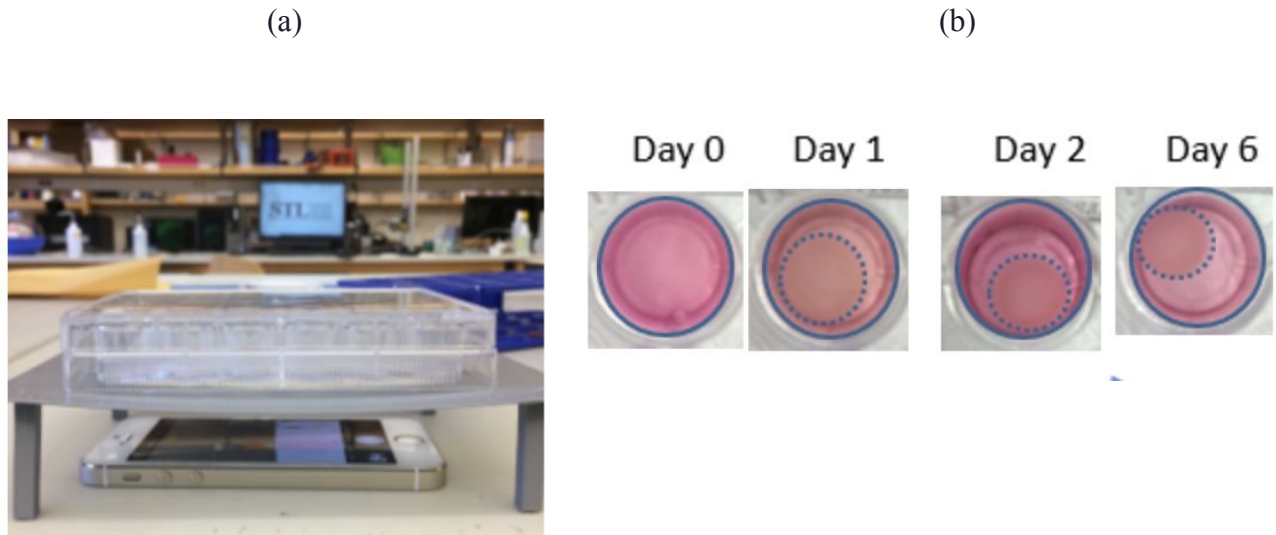


Figure 3: (a) Set-up to take photos used to analyze gel area (b) Example of progression of gel contraction through a six-day period. TGF β -1 was not added in this sample, so the gel contracted much less than one with the addition of TGF β -1 would.

The third part of the study, which tested relaxin, had four different initial conditions, and two additional conditions added later: 10 ng/mL, 100 ng/mL, 100 ng/mL after a two-day delay, and 100 ng/mL + 10 μ M simvastatin after a two-day delay, and then 500 ng/mL and 1000 ng/mL. All of the conditions also included TGF β -1.

Results

Of the two drugs tested, only simvastatin, not losartan, inhibited gel contraction. Of the three concentrations of simvastatin tested, 100 μ M was the most effective in preventing gel contraction, as the gel area remained very close to 100% of its initial value. Similarly, 10 μ M simvastatin was also relatively effective, resulting in gel contraction of about 25%. On the other

hand, 1 μM did not prevent gel contraction. Similar observations were seen with (Figure 4) and without (data not shown) $\text{TGF}\beta\text{-1}$

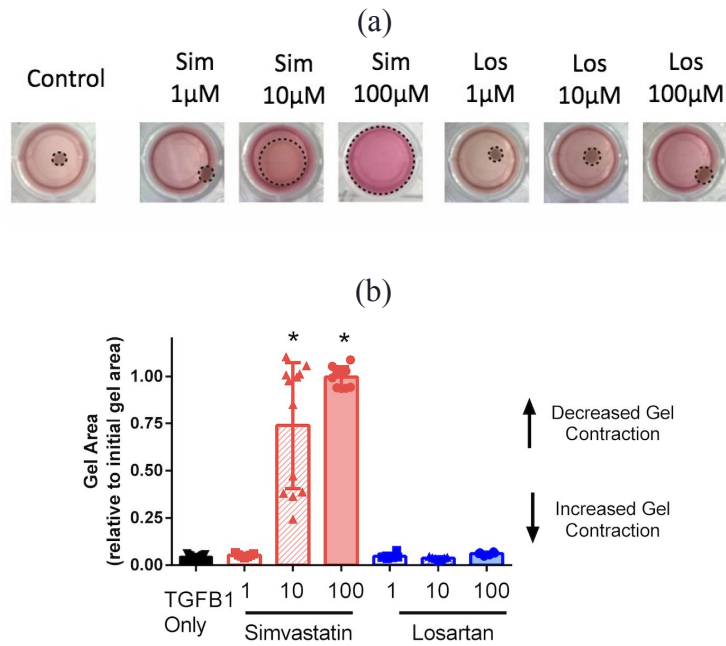


Figure 4: (a) Gels after 6 days under each of the applied-drug conditions. Only $\text{TGF}\beta\text{-1}$ was added to the control sample. The losartan samples, even the 100 μM concentration, ended up looking very similar to the control sample by the end of the observation period. (b) Shows normalized gel area relative to the initial gel area for all applied-drug conditions.

It was also found that at the end of the 6-day observation period, simvastatin, but not losartan, reduced cell number and viability; more specifically, the 100 μM concentration of simvastatin significantly reduced cell number and viability.

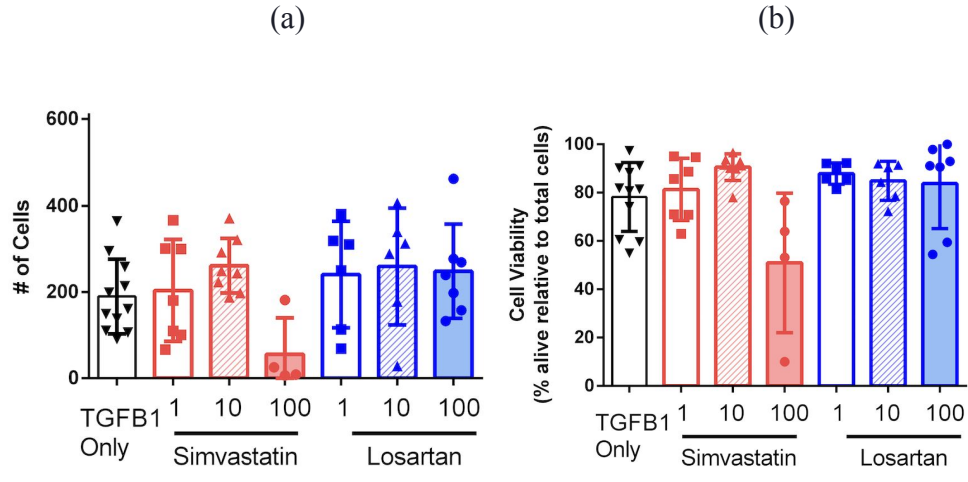


Figure 5: (a.) Shows the number of cells counted at the end of the 6-day observation period for each of the applied-drug conditions. The 100 μM concentration of simvastatin caused the most drastic drop in the number of cells alive. (b.) Shows the percentage of cells found to be alive at the end of the 6-day observation period for each of the applied-drug conditions. The 100 μM concentration of simvastatin resulted in nearly 50% drop in viability.

Because only simvastatin affected gel contraction, it was used to study the effect of time delay and shortened exposure period on the effectiveness of a drug. Additionally, given that the effects seen with simvastatin were similar with and without TGFβ-1, simvastatin with TGFβ-1 only was used in this study; these samples were deemed to be more representative of the in vivo environment, as TGFβ-1 will almost always be present in the elbow post-injury. Of all of the conditions, the 100 μM concentration of simvastatin applied for only the first 2 days of the 6-day observation period was the most effective in inhibiting contraction; it maintained the gel area relative to the initial very near 100%. Both the 1 and 10 μM concentrations of simvastatin did nothing to prevent gel contraction. It was observed that when simvastatin in 10 and 100 μM concentrations was added 2 days after the observation period began, it stopped the contraction from getting worse, but the gel area did not increase back to initial size.

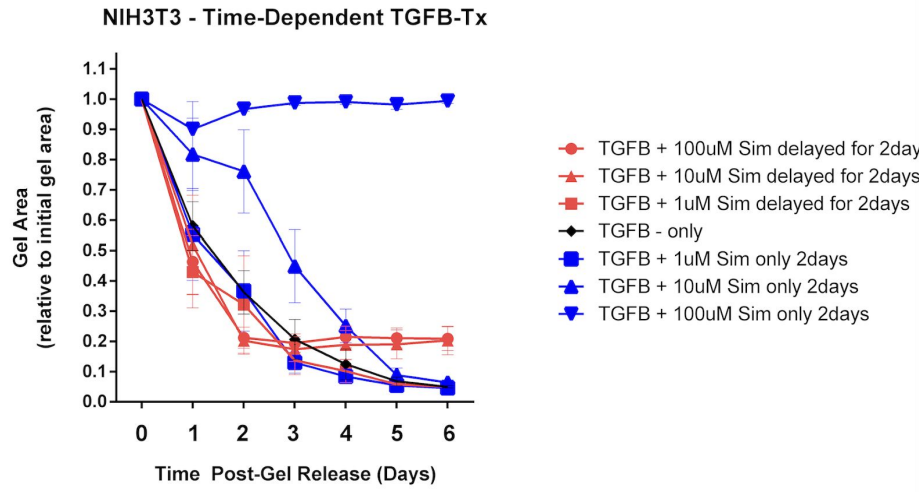


Figure 6: Shows the gel area relative to the initial for cells under each drug-application condition for the entirety of the 6-day observation period. The red points indicate trials where simvastatin was added after two days, and the blue points indicate trials where simvastatin was added for only two days.

The results of the relaxin trials contradicted most of our hypotheses; it was obvious that the relaxin did not prevent gel contraction as it was predicted to. The first of the four conditions, 10 ng/mL relaxin, looked very similar to the control as predicted. The second, 100 ng/mL relaxin, was predicted to be less contracted than the control; in reality, it looked very similar to the control. The third, 100 ng/mL after a two-day delay, also ended up looking much like the control, though it was predicted to be less contracted than the control, but more so than the second condition. Finally, the fourth condition, 100 ng/mL + 10 μ M simvastatin after a two-day delay was predicted to be the least contracted; this actually ended up being true, with extremely little loss in gel area. This was attributed to the addition of simvastatin to the gel, rather than to the relaxin. These results led us to try and increase the concentration of relaxin used, so two additional trials with 500 ng/mL and 1000 ng/mL were added. However, these higher concentrations yielded results similar to those using the 10 ng/mL and 100 ng/mL concentrations.

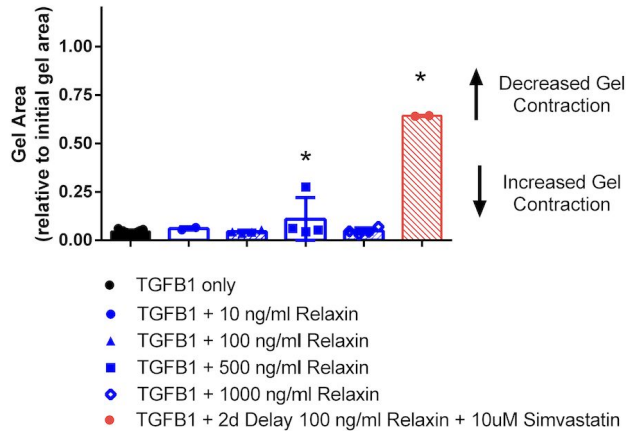


Figure 7: Quantitative results of relaxin study. The only conditions under which contraction was prevented were when 100 ng/mL + 10 μ M simvastatin were combined, leading to about a 25% decrease in gel area relative to the initial

Discussion

The broader purpose of this study was to find an effective drug therapy for post-traumatic joint contracture (PTJC) in the elbow. Given the parameters in this experiment, simvastatin was the only candidate for a drug therapy targeting PTJC. At 100 μ M concentration, simvastatin preserved nearly all of the gel area in the trials conducted.

There was a relatively large deviation in the data gathered for the 10 μ M concentration simvastatin samples; the gel areas relative to the initial varied from about 25% to 100%. This variability could be attributed to cell variability or drug concentration variability, or a combination of both. Another possible explanation is that the 10 μ M concentration of simvastatin was a “critical point” in its ability to function, causing several mixed results.

Though simvastatin was the most effective in preserving gel area, the cell viability data from the simvastatin caught our attention; if the simvastatin was causing cells to die out it could end up doing more harm than good, as cells will always be required to heal any injury in the first place. We are unsure if killing the cells off will have a truly negative effect in different systems, but in the system that we designed, it was successful in stopping contraction.

The exposure time experiments provided us with valuable information on how factors like drug clearance and delayed treatment would affect the effectiveness of the drug formulation. From the time delay experiments, it was concluded that simvastatin at both 10 and 100 μM concentrations were able to completely halt the contraction upon administration; that is, the gel did not contract any further *after* day 2 of observation than it did *on* day 2 of observation. This indicates that if a patient were to seek treatment after PTJC onset in their injury, the simvastatin treatment as it is would be able to prevent further progression of PTJC, but would not be able to reverse it. From the shortened exposure experiments, it was determined that contraction was almost completely prevented by the 100 μM concentration of simvastatin; the 1 and 10 μM concentrations, on the other hand, proved almost completely ineffective in preventing gel contraction. In real life, these results would indicate that an administration of the 100 μM concentration of simvastatin could be somewhat effective even if simvastatin is cleared rapidly from the elbow in some manner.

We originally looked to relaxin for two outcomes: the first was to inhibit contraction, and the second was to reverse contraction that had already set in. The results of the relaxin trial as designed indicated it was unsuccessful; the gels to which relaxin was applied, at all concentrations, looked similar to the control. The first variable changed immediately was the concentration; our design of the study used an administration of relaxin at a concentration much lower than that used by our collaborators. In addition to the 100 ng/mL previously tested, we also tested 500 ng/mL and 1000 ng/mL to match the work of our collaborators. Unfortunately, these trials were not successful either; they also ended up looking very similar to the 10 ng/mL and

100 ng/mL samples. Contrary to the results of our collaborators, we saw absolutely no change in the contractile patterns of the gels under any condition with relaxin tested.

While the inability of losartan and relaxin to alter gel contraction was surprising, our experimental conditions may have influenced our results and could be worthwhile to change before completely ruling out losartan and relaxin as therapeutic potentials for PTJC in the elbow. For example, losartan and relaxin may very well affect different types of cells under different conditions. Perhaps losartan and relaxin could influence other cells that then mediate fibroblasts/myofibroblasts' role in capsule contraction *in vivo*. To mimic this multi-cellular interaction, one of the possible future directions of this study would be to attempt co-culturing the NIH-3T3 cells we used in this study with other types of cells such as inflammatory cells. Additionally, we recommend a more thorough study of relaxin's biological and functional pathways; a very specific factor could be necessary for its successful function that is not naturally present in the environment we set up for our study. In addition, any and all drug combinations could be tested in a similar manner to see if the drug's abilities can withstand the challenges presented *in vivo*. Finally, an immediate next step would be to test the drugs on cells from actual rat and human elbow capsules; this testing, though more difficult and time-consuming than the testing we carried out, would provide a more accurate look at how effective the drugs truly are in preventing post-traumatic elbow contracture following an injury to the elbow. It is entirely possible that losartan and relaxin, as well as simvastatin, might have a different effect on rat and human elbow capsule cells.

Conclusion

In conclusion, our study demonstrated that out of the 3 drug candidates tested (losartan, simvastatin, and relaxin), simvastatin seems to be a promising candidate in the treatment of PTJC in the elbow because of its ability to halt gel contraction in a dose- and exposure-dependent. Prevention of gel contraction seems to be rooted in simvastatin's ability to alter cell number and viability. Our time delay and shortened exposure experiments provided us with valuable information regarding how simvastatin would behave under conditions certain to arise in the elbow joint in vivo. Although losartan and relaxin did not prevent gel contraction in this study, additional work is warranted before dismissing losartan and relaxin as therapeutic potentials for post-traumatic elbow contracture. Overall, results from our study warrant further investigation into simvastatin therapeutic efficacy, both preclinically and clinically, for the treatment of post-traumatic joint contracture of the elbow.

Based on the very specific parameters of our study, simvastatin proved to be effective in inhibiting gel contraction, while losartan did not. Prevention of gel contraction seems to be rooted in simvastatin's ability to alter cell number and viability. However, losartan may very well work on different types of cells under different conditions. One of the possible future directions of this study would be to attempt co-culturing the NIH-3T3 cells we used in this study with other types of cells. An immediate next step would be to test the drugs on cells from actual rat and human elbow capsules; this testing, though more difficult and time-consuming than the testing we carried out, would provide a more accurate look at how effective the drugs truly are in preventing post-traumatic elbow contracture following an injury to the elbow.

The time delay and shortened exposure experiments provided us with some valuable information regarding how simvastatin would behave under conditions certain to arise in the elbow joint. In the future, any and all drug combinations can be tested in a similar manner to see if the drug's abilities can withstand the challenges presented by reality.

Finally, the results of the relaxin trial in the environment we created were unsuccessful. Contrary to the results of our collaborators, we saw absolutely no change in the contractile patterns of the gels. This could be attributed to several parameters of the study as we have set it up, but most likely can be attributed to the environment in which relaxin normally functions. In the future, we recommend a more thorough study of relaxin's biological and functional pathways; a very specific factor could be necessary for its successful function that is not naturally present in the environment we set up for our study. It is also entirely possible that relaxin might have a different effect on rat and human elbow capsule cells, as simvastatin and losartan may.

Overall, out of the 3 candidates tested (losartan, simvastatin, and relaxin), simvastatin seems to be a promising candidate in the treatment of PTJC in the elbow because of its ability to halt contracture. Simvastatin proved effective in halting contracture even in delayed application. Results from our study warrant further investigation into its abilities, both preclinically and clinically, for the treatment of post-traumatic joint contracture of the elbow.

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