Developing a Living Composite Ligament by Combining Prolotherapy and Nanoparticles as Treatment for Damaged Connective Tissue

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Keywords: prolotherapy, carbon nanohorns, healing, ligament, tendon, tissue engineering

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Abstract

Significant cost and debilitation results from connective tissue injury and disease every year. Prolotherapy is an effective medical treatment used to increase joint stability. However, most associated studies are retrospective or case studies, rather than comprehensive laboratory investigation originating with the cellular response to exposure to the proliferant solutions. As a parallel consideration, nanoparticles are being investigated for use in drug delivery and heat shock treatment of cancerous tissue due to their unique structural and thermal properties. The phenomenal strength and stiffness of carbon nanoparticles have been used for commercial purposes in composite materials, but investigation of biomedical applications is still fairly nascent. In an attempt to develop a non-surgical approach to supporting and healing damaged ligaments and tendons resulting from injury or disease by combining prolotherapy and the use of nanoparticles, the author presents studies investigating the cellular response to proliferative therapy solution as well as tendon and ligament tissue’s mechanical and cellular response to exposure to nanoparticles. In the prolotherapy solution cell studies, the results suggested that there is an optimal dosage of the proliferant for in vitro studies, different responses between cell types, and a dosage-dependent response in cell viability and collagen production to the solution P2G in preosteoblasts. In the nanoparticle studies, cell populations tolerated nanoparticles at the levels tested, tendon mechanical properties were increased (stiffness significantly so), and bright field and transmission electron microscopic histological images were taken of connective tissue and carbon nanohorn interactions.
Dedication

This thesis is dedicated to Digby, the love of my life, and to our precious daughters, Anthea and Amelia.

This work is for you.
Acknowledgements

This has been a long and challenging journey, with many who have helped along the way.

I would like to thank first of all, God, the Father, who placed me on this path and led me through its entirety, even when that meant side trips to destinations unknown. You are faithful and good, and I pray that this work glorifies you.

My greatest earthbound thanks go to my husband and best friend, Digby, without whose love, encouragement, and support I would not have even made it to step one. Digs, I am so very glad it’s you.

Our daughters, Anthea and Amelia, have also been terrific cheerleaders. Girls, thank you for encouraging me to continue, even when that meant less time at home, and for offering to make chocolate pie when I was stressed out.

I’d like to thank other members of my family: my mom and dad, who taught me I could do anything and to never give up; my wonderful and fun sisters, Mary Bea, Lisa, and Ruthie; my brother, Jerry (the best big brother I could wish for); and my adopted daughter, Hincal, who believed in me even at my most disabled. God has blessed me by having each of you in my life.

I thank Dr. Roop Mahajan for introducing me to carbon nanoparticles at an ASME dinner in December of 2007, and for connecting me to the right people to pursue testing my idea.

I have deep gratitude for my committee members, who have backed me over the long haul. Dr. Joseph Freeman, you took me on in your Musculoskeletal Tissue Engineering Lab in 2008, let me run with this concept, and have provided thoughtful and continuous guidance, even from afar. Thank you. Dr. Nichole Rylander, I appreciate your bright encouragement, also from the beginning and all the way through. Dr. Gunnar Brolinson, your enthusiasm, knowledge, and support have been great gifts.
I thank Dr. Wally Grant, in whose summer of ‘91 Biomedical Engineering class I became smitten with biomed. Dr. Grant, I am so thankful that you are still here and were part of my return to Virginia Tech.

I thank my numerous labmates, past and present, especially Emmanuel Ekwueme, who has methodically labored to further this research, and continues to do so, and Albert Kwansa, whose brilliant yet gentle intellect and sweet spirit have blessed both me and my family. I also thank Jung Ki Hong for his excellent work on the CNCs, and contributions to our shared studies.

Tess Sentelle, you took great care of me, as you do all of your students; thanks for fighting the dragons for us. I’m grateful to Hal and Bill in the Assistive Technology Lab, who set me up with the coolest desk on campus.

Thanks to Dr. John Roberston (a.k.a. “Dr. Bob”), who was my pig parts Santa Claus, and for Chip Aardema, both of whom generously provided space, knowledge, and exquisitely sharp hunting knives for dissection. I also thank Kathy Lowe who cheerfully and tenaciously hunted down elusive nanohorns among tendon fibrils with me on the TEM.

I am grateful to all of my extraordinary physicians, who put and keep this zebra together, including Drs. Amy Doolan, Logan Brooke, Rick Williams, Mark Rogers, David Harden, Daniel Osimani, and especially Mark Dean.

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Lastly, I thank my friend and mentor, John Roetling, in memoriam, who was a gift to me and taught me to follow my curiosity and continue learning as long as I live. John, I hope you can see how much you have blessed my life. Thank you.
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Attributions

Several advisors and colleagues contributed to the research and writing presented in this thesis. A brief description of the way in which they supported this work is included below.

Joseph Freeman, PhD, was the head of the Musculoskeletal Tissue Regeneration (MoTR) Laboratory in the School of Biomedical Engineering and Sciences (SBES) at Virginia Tech during the majority of my time of research, and is now a professor at Rutgers University in Biomedical Engineering and head of the MoTR Lab there. Dr. Freeman is the primary advisor and committee co-chair for this research. He guided me in the design and execution of the experiments, as well as in the interpretation of their results, and provided editorial input for each manuscript. Dr. Freeman is also the Primary Investigator for the grant that funded this research.

M. Nichole Rylander, PhD, is the head of the Tissue Engineering, Nanotechnology, and Cancer Research Lab, in SBES and the Mechanical Engineering department at Virginia Tech. Dr. Rylander served as my secondary advisor and committee co-chair. She also provided equipment and carbon nanoparticles for the nanoparticle experiments, and provided general guidance throughout my research.

P. Gunnar Brolinson, DO, is the Associate Dean for Clinical Research and Discipline Chair of Sports Medicine at the Edward Via College of Osteopathic Medicine (VCOM) in Blacksburg, Virginia. Dr. Brolinson served as my medical mentor for the tissue experiments, shared his expertise in prolotherapy mechanism, use, and technique, and provided the P2G solution for the proliferant studies. Dr. Brolinson also served as a member of my committee and provided editorial feedback on the manuscripts.

John Robertson, VMD, PhD, is a research professor in the Department of Mechanical Engineering and in SBES at Virginia Tech. Dr. Robertson aided in obtaining tissue for the
majority of the skin and tendon experiments, as well as sources for the primary tendon rat cells. Dr. Robertson also provided space and dissection equipment for the histological tissue studies.

Albert Kwansa, PhD in Biomedical Engineering, was a fellow labmate in the Musculoskeletal Tissue Regeneration Laboratory in SBES at Virginia Tech. Albert helped with mechanical testing and data analysis, taught me about collagen behavior, and generally lent a hand whenever needed using his extensive knowledge base regarding laboratory equipment and collagen. Albert also provided editorial support for the manuscripts.

Emmanuel Ekwueme, a PhD student in the Musculoskeletal Tissue Regeneration Laboratories in SBES at Virginia Tech and in the Biomedical Engineering department of Rutgers University, aided in cell and tendon experiments and statistical analyses, as well as in writing of the manuscripts. Emmanuel is also currently continuing work on combining prolotherapy with nanoparticles in vivo at Rutgers.

Danielle Paynter, BS in Chemical Engineering at Virginia Tech, served as an undergraduate research assistant. Danielle helped conduct the proliferant cell studies, and preliminary nanoparticle cell, skin, and tendon studies. Danielle also aided with mechanical testing and data analysis.

Katelyn Colacino, PhD, a member of the Biomedical Optical Devices Laboratory in SBES at Virginia Tech, taught me some of the intricacies of advanced Microsoft Word use and aided me in formatting the thesis text, figures, and tables.

Chapter 3:

Jung Ki Hong, a PhD student in the Macromolecular Science and Engineering Department at Virginia Tech, co-laborated with me on the nanoparticle cell and tendon studies. In addition, Jung Ki manufactured cellulose nanocrystals, as well as conducted AFM and DLS characterization for
the cellulose nanocrystals and carbon nanohorns, and aided in writing about the materials characterizations in this manuscript.

*Maren Roman*, PhD, is the head of the Bio AFM Lab in the Department of Sustainable Biomaterials at Virginia Tech. Dr. Roman served as Jung Ki’s advisor and provided some editorial feedback for this manuscript.

*Allison Pekkanen*, PhD student in the Tissue Engineering, Nanotechnology, and Cancer Research Lab in SBES at Virginia Tech, performed additional material characterization of the carbon nanoparticles and taught me to operate laboratory equipment in Dr. Rylander’s lab.

*Chalmers Brown* served as an undergraduate research assistant in the Musculoskeletal Tissue Regeneration Lab at Rutgers University. Chalmers aided in mechanical testing.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>alpha-minimum essential medium</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CNC</td>
<td>cellulose nanocrystal</td>
</tr>
<tr>
<td>CNH</td>
<td>carbon nanohorn</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos Syndrome</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GIMP</td>
<td>GNU Image Manipulation Program</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>MC3T3</td>
<td>mouse preosteoblast cell line</td>
</tr>
<tr>
<td>MCL</td>
<td>medial collateral ligament</td>
</tr>
<tr>
<td>MTS</td>
<td>cell viability assay composed of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate</td>
</tr>
<tr>
<td>MTS</td>
<td>mechanical testing machines from MTS Systems Incorporated</td>
</tr>
<tr>
<td>NP</td>
<td>nanoparticle</td>
</tr>
<tr>
<td>P2G</td>
<td>proliferant solution composed of phenol, glycerin, and glucose</td>
</tr>
<tr>
<td>PAA/PVA</td>
<td>poly (acrylic acid)/poly (vinyl alcohol)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(-caprolactone)</td>
</tr>
<tr>
<td>PDI</td>
<td>polydispersity index</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>penicillin-streptomycin</td>
</tr>
<tr>
<td>PT</td>
<td>patella tendon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RIT</td>
<td>regenerative injection therapy</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TCPS</td>
<td>tissue culture treated polystyrene</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>WST</td>
<td>cell viability assay composed of water soluble tetrazolium salts</td>
</tr>
</tbody>
</table>
Chapter 1. INTRODUCTION

1.1. Rationale for Investigating Connective Tissue Repair

A majority of connective tissue injuries are represented by damage to tendons, ligaments, cartilage, and skin. More specifically, ligament and tendon injuries are a significant problem in the United States where there are over 32 million traumatic and overuse injuries annually (Vunjak-Novakovic, Altman, Horan, & Kaplan, 2004). Sprains and strains are the most common types of injury to ligaments and tendons, having an estimated cost of 30 billion dollars and affecting 5.7 million people per year (Butler et al., 2008; McCaig & Nawar, 2006). Sprain injuries typically occur as a result of damage to a ligament after an excessive load to the joint resulting in non-physiologic motion, while strain injuries occur as a result of damage to a tendon or muscle due to overuse, trauma, or tensile overload. In addition, there can be damage to tendons and ligaments due to connective tissue disease, such as Ehlers-Danlos Syndrome (EDS), a disorder that causes the body to produce faulty and weak collagen (“What is EDS?,” 2014).

Both ligaments and tendons are dense collagenous tissues composed primarily of fibroblasts embedded in a complex network of extracellular matrix (ECM). The ECM components, including collagen, elastin, and proteoglycans, give these tissues unique viscoelastic biomechanical properties.

Biological soft tissue stress-strain behavior can be separated into three distinct regions (i.e., a triphasic response) (Figure 1.1). The first region, the toe region of the curve, corresponds to the straightening of the collagen fibrils and reorganization of the ECM components. The second or linear region corresponds to the translation of strain to the matrix molecules. Lastly, the yield region corresponds to matrix defibrillation and tissue failure. The degree and types of injuries to connective tissue vary, but symptoms often include joint pain, stiffness, swelling, and bruising.
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(Beynnon, Renström, Haugh, Uh, & Barker, 2006; Brown, Padua, Marshall, & Guskiewicz, 2008). These injuries are biomechanically characterized by disruptions in the collagen fibrils resulting in lax connective tissues that have diminished mechanical properties (Freeman, Woods, & Laurencin, 2007; Kwansa, Empson, Ekwueme, Walters, Freeman, & Laurencin, 2010).

![Triphasic mechanical behavior of ligaments and tendons in tension](image)

*Figure 1.1. Triphasic mechanical behavior of ligaments and tendons in tension (adapted from Freeman et al., 2007).*

The conventional treatment for these types of injuries include rest, ice, compression, and elevation (Lynch, & Renström, 1999). Due to the lack of sufficient vascularization, ligament and tendon injuries are often characterized by incomplete healing, leaving patients with reduced mobility and prone to re-injury. In the presented research, two approaches to tissue reinforcement were investigated, with the goal of combining them in future studies in order to develop a “living composite ligament.” These treatments are proliferative therapy and nanoparticle (NP) reinforcement of damaged tissue.
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1.2. Cellular Response to Prolotherapy

Proliferative therapy, also called prolotherapy or regenerative injection therapy (RIT), is a currently available alternative treatment for damaged tissues. Most often prolotherapy is employed for ligaments and tendons (Hackett, 1958; Linetsky et al., 2001; Reeves, & Hassanein, 2000a), but it has also been investigated for articular cartilage (Reeves & Hassanein, 2000a). It involves the injection of an irritant solution into the tissue, such as a damaged tendon or bone-ligament junction, which is hypothesized to stimulate the body’s wound healing cascade. The damaged tissue is repaired, and new collagen is thought to be laid down which gradually contracts, forming a denser, stronger, and tighter ligament or tendon (Hackett, 1958; Banks, 1991). In persons with normal collagen production, only 4 - 6 treatments are typically needed and the repair may be permanent (Gearhardt, 1987; Hackett, 1958; Klein, Dorman, & Johnson, 1989; Leedy, 1987).

Common proliferant solution choices include dextrose, phenol, glucose, glycerin, sodium morrhuate (a cod liver oil derivative), or a combination of these. An example of a popular combination in use is “P2G,” which is composed of phenol, glycerin, and glucose. While the mechanism of action of prolotherapy is not yet clearly understood and is still being investigated, it is believed that the different types of irritant solution yield potentially different pathways to the same end of healing damaged tissue. It is hypothesized that dextrose causes local cell necrosis due to osmotic shock, P2G causes cell irritation on a local level, and sodium morrhuate brings about inflammatory mediators through chemotactic attraction (Rabago, Slattengren, & Zgierska, 2010). These methods of intentional small-scale cell trauma at the injection site, or “therapeutic trauma,” hypothetically initiate the body’s wound healing cascade of inflammation, granulation tissue formation, and matrix formation and remodeling (Banks, 1991). According to the wound
healing cascade model, tissue trauma brings an influx of granulocytes which then attract monocytes and macrophages. Macrophages secrete polypeptide growth factors which in turn attract and activate fibroblasts. Fibroblasts deposit new collagen at the wound site which eventually contracts, causing the tissue to tighten. As stresses are placed on the new tissue, the new collagen fibers align in the direction of the stress (Banks, 1991). While there is clinical evidence for success with prolotherapy (Reeves, & Hassanein, 2003; Scarpone, Rabago, Zgierska, Arbogest, & Snell, 2008; Topol & Reeves, 2008; Reeves & Hassanein, 2000b), other studies have yielded inconclusive results (Kim, Stitik, Foye, Greenwald, & Campagnolo, 2004; Yelland, Glasziou, Bogduk, Schluter, & McKernon, 2004; Jensen, Rabago, Best, Patterson, & Vanderby, 2008). Some physicians are using prolotherapy on a variety of tissues, such as tendons, ligaments, and even cartilage. It is also used as treatment for a variety of ailments, including strains, sprains, osteoarthritis, and hypermobility due to Ehlers-Danlos Syndrome. Unfortunately there is still a lack of research focusing on the effects of prolotherapy on a cellular level and many unanswered questions. Different physicians use various treatment regimens including different dosages of the same proliferant; optimal dosing is not known. Although it is believed that the healing cascade is responsible for the new tissue growth, the role of the local cell population in healing is not well understood. The potential for use of prolotherapy in bone and other tissues, as well as whether or not there is a difference in cellular response between the cells of different tissues, has not been widely investigated.

In this study (Chapter 2), we explored the effects of different proliferant dosages on cellular response. We examined the effects of exposure to an irritant solution on cell proliferation and type I collagen production of two different cell types, mouse preosteoblast cells (MC3T3-E1) and patella tendon (PT) fibroblasts, in vitro. It was hypothesized that there would be increased
cell death with increased dosage of the proliferant solution, and that there would be an optimal dosage for increasing collagen production. The results presented may elucidate the role the response of the local cell population has in the tissue healing process after exposure to proliferant solution.

1.3. Nanoparticle Reinforcement of Damaged Tissue

Recent work has shown the ability of NPs to modulate cellular responses (Jiang, Kim, Rutka, & Chan, 2008; Fisher, et al., 2010; Kwan, Liu, To, Yeung, Ho, & Wong, 2011) and matrix mechanical properties (Cadek, 2004; Bhattacharyya, Salvetat, & Saboungi, 2006; Li, Zhou, & Zhang, 2009; Deeken, Cozad, Bachman, Ramshaw, & Grant, 2011). One prolotherapy technique employs the injection of irritant particulates such as ground pumice to activate inflammatory cells which, in turn, attract fibroblasts to deposit new collagen and other ECM components in order to strengthen damaged tissue (Banks, 1991). The cellular and molecular mechanisms involved have not been thoroughly investigated in the literature and thus warrant further rigorous scientific inquiry. Also, recent work has shown the ability of silver NPs to modify collagen deposition and alignment in a mouse skin wound-healing model. Although the exact signaling pathway has yet to be determined, the enhanced collagen alignment resembled that of normal skin tissue and led to improved mechanical properties (Kwan et al., 2011). In addition to triggering a cellular response, NPs have also been shown to enhance the mechanical properties of natural polymer matrices; examples include cellulose whiskers added to both chitosan and regenerated cellulose matrices, and single walled carbon nanotubes crosslinked to decellularized porcine diaphragm tendon (Deeken et al., 2011; Li et al., 2009; Qi, Cai, Zhang, & Kuga, 2009). However, few studies have shed light on the influence of NP shape and size on the mechanical properties of natural matrices or the utilization of NPs as biocompatible nanofillers to locally
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reinforce injured and lax connective tissues in vivo. The diameters of NPs are similar to ECM components, allowing for enhanced NP interaction with matrix components. Additionally, modulating NP dispersion within the matrix influences the interfacial stress transfer between matrix components and the NPs, allowing for control of composite mechanical properties (Albanese, Tang, & Chan, 2012; Bhattacharyya et al., 2006; Cadek et al., 2004). Here (in Chapter 3), we explore the in vitro biomechanical and cell response of this therapy for damaged connective tissues.

Due to its availability and biocompatibility, cellulose may be considered a suitable natural material for the reinforcement of biopolymer matrices. Cellulose is a polysaccharide that occurs in the form of highly crystalline microfibrils in all plant cell walls and some sea animals and is also produced by certain bacteria. Cellulose microfibrils can be broken down into nanoscale crystals by acid hydrolysis (Azizi Samir, Alloin, Sanchez, & Dufresne, 2004; Eichhorn et al., 2010; Habibi, Lucia, & Rojas, 2010). Nanocarbons including carbon nanotubes, carbon nanohorns, and graphenes have also been shown to enhance matrix mechanical properties; their small size, superior strength, and functional capabilities make them attractive options for nanofillers for injured ligaments and tendons (Bhattacharyya et al., 2006; Cadek et al., 2004).

Recently, using several in vitro assays, Deeken and colleagues (2011) demonstrated that amine-functionalized single-walled carbon nanotubes could be crosslinked to an acellular porcine diaphragm tendon at concentrations up to 1 mg/ml. Results from those studies showed that the carbon nanotubes and the processing (functionalization) had no detrimental effects on the tissue matrix. None of the conditions or concentrations studied significantly improved scaffold physiochemical or mechanical properties; however, it is possible that the concentrations studied were below the range necessary to elicit a significant mechanical response (Deeken et al., 2011).
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In the presented nanoparticle study (Chapter 3) we hypothesized that the controlled and localized injection of biocompatible NPs into damaged connective tissues would enhance matrix mechanical properties, evidenced by increased stiffness and yield strength. This technique would be a particulate reinforcement-based alternative modality for the treatment of damaged connective tissues. The effects of NPs, namely, single walled carbon nanohorns (CNHs) and cellulose nanocrystals (CNCs), on damaged connective tissue mechanical properties were examined. The CNHs and CNCs differ in overall size, architecture, and chemical composition thus enabling the comparison of their effects on matrix mechanical properties. Two different types of porcine connective tissues were employed in this study, specifically, skin and tendon. Porcine skin was employed with the injury model because of its similarity to ligaments and tendons both in matrix composition (fibrous with a high type I collagen content) and in tensile mechanical behavior (similar trends in stress-strain curves, seen in Figure 1.1). Porcine digitorum tendons were employed as a model for the treatment of target tissues using the optimal NP. A strain injury model was utilized to disrupt the tissue matrix and mimic sprain and strain injuries. In addition, a preliminary biocompatibility test was performed to assess the biological response of tendon fibroblasts to the NPs in vitro. NP dispersion was explored through a histological study of porcine tendon injected with CNHs. The results from these preliminary studies will be used to understand the feasibility of mechanically reinforcing damaged connective tissues by injecting biocompatible NPs in vivo.
Chapter 2. PROLOTHERAPY STUDIES

This chapter and portions of Chapter 1 are based on the article, “Effect of prolotherapy on cellular proliferation and collagen deposition in MC3T3-E1 and patellar tendon fibroblast populations,” by Freeman JW, Empson YM, Ekwueme EC, Paynter DM, and Brolinson PG, published in 2011 in the journal Translational Research.

2.1. Introduction

In this prolotherapy study, we explored the effects of different proliferant dosages on cellular response. We examined the effects of exposure to an irritant solution on cell proliferation and type I collagen production of two different cell types, mouse preosteoblast cells (MC3T3-E1) and patella tendon (PT) fibroblasts, in vitro. It was hypothesized that there would be increased cell death with increased dosage of the proliferant solution, and that there would be an optimal dosage for increasing collagen production. The results presented may elucidate the role that the response of the local cell population has in the tissue healing process after exposure to proliferant solution.

2.2. Methods

2.2.1. Proliferant

A commonly used proliferant, P2G (Buderer Drug Company, Perrysburg, OH, USA), was administered to MC3T3-E1 preosteoblasts (ATCC, Manassas, VA, USA) in various dosages. P2G is a solution composed of 2% phenol, 25% dextrose, and 25% glycerin in a sufficient quantity of sterile water for injection. It was chosen because of its popularity in clinical use and its availability to the authors. Optimal in vitro dosing was determined in a preliminary study for use in the secondary studies.
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2.2.2. Cell Culture

MC3T3-E1 preosteoblast cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM, Cellgro, Mediatech, Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS, Cellgro, Mediatech, Inc., Manassas, VA, USA) and 1% penicillin-streptomycin (PS, Cellgro, Mediatech, Inc., Manassas, VA, USA). These cells were chosen because of their use in determining biocompatibility of biomaterials (Andric, Sampson, & Freeman, 2010; Khan & Laurencin, 2008; Laurencin et al., 1993; Wright, Andric, & Freeman, 2011), as well as to explore the response of other cell types and tissues, specifically bone, in addition to fibroblasts. Approximately 30,000 cells per well were seeded onto the bottom surfaces of 24-well tissue culture treated polystyrene (TCPS) plates. Each well plate had four designated groups of three wells each with twelve of the wells left empty so that each well plate could be independently tested at specific weekly time periods. The cells were permitted to attach for 30 minutes before adding culture medium to attain a final volume of 1 ml per well.

Primary PT fibroblasts from male Sprague-Dawley rats were harvested and cultured as reported earlier (Lu et al., 2005). Rat tendon tissue harvest was conducted in accordance with the ethical standards established by Virginia Tech’s Institutional Animal Care and Use Committee (IACUC). Briefly, patella tendons from Sprague-Dawley rats were dissected, minced, and rinsed with Hank’s Buffered Salt Solution (HBSS). Sequential digestion of the tendon explants was performed with 0.1% collagenase and trypsin. The cells were collected from the fourth digestion, grown to confluency, and counted. The fibroblasts were cultured in alpha-minimum essential medium (α-MEM, Fisher) at pH 7.6 supplemented with 1% PS, and 10% FBS. Culture medium was replaced 3 times a week. Cells were seeded with the same protocol used for seeding MC3T3 preosteoblasts.


2.2.3. Preliminary Preosteoblast Cell Study

MC3T3 cell populations were permitted to grow for six days prior to treatment, and were treated on day 7 of the study. Three groups were treated with the P2G proliferant solution, and one group was used as a positive control. The solution was delivered in 25 µl, 50 µl, or 75 µl volumes, added to the 1 ml volume of cell medium in each cell well. The cells were exposed to the treatment for 24 hours after which the medium was removed and replaced with fresh, proliferant-free media. The medium was changed 2-3 times a week. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells were cultured for a total period of 36 days, and cell viability data were collected as described below on days 1, 7, 14, 21, 28, and 35 post-treatment. Dosages that caused a degree of cell death from which the population could not recover were eliminated from consideration for use in the secondary studies.

2.2.4. Secondary Preosteoblast and Fibroblast Studies

Based on the results of the preliminary study, the cell study was repeated with 3 optimized dosages of P2G using preosteoblasts (15 µl, 25 µl, and 35µl) and 2 dosages for fibroblasts (15 µl and 25 µl). Cells were permitted to grow for four days prior to treatment, and were treated on day 5 of the study. Along with groups treated with the P2G proliferant, an additional group was used as a positive control for each cell type. As in the preliminary cell study, the proliferant was added to the 1 ml volume of cell medium in each cell well. The cells were exposed to the treatment for 24 hours after which the medium was removed and replaced with fresh, proliferant-free media. The medium was changed 2-3 times per week. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Preosteoblasts were cultured for a total period of 34 days, and data were collected on days 1, 7, 14, 21, and 28 post-treatment. Fibroblasts were
cultured and data were collected on days 7, 14, and 21 post-treatment. Cellular viability and amount of collagen production were determined as described below.

### 2.2.5. Cellular Viability

Cellular proliferation was evaluated using the colorimetric CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Each well was emptied by removing the cell culture medium. Then, 60 µl of the CellTiter 96 AQueous One Solution Reagent, containing the MTS compound, and 300 µl of fresh cell culture medium were added to each well. The plates were incubated for three hours. The volume in each well was diluted (1:4) with deionized water, and the absorbance was read at 490 nm with a SpectraMax M2 spectrophotometer (Sunnyvale, CA, USA).

### 2.2.6. Histological Staining

Each well was emptied and stained for cytoplasm content and total type I collagen production using Masson’s Trichrome staining protocol (Sigma-Aldrich Inc., St. Louis, MO, USA). After staining, photomicrographs of each well were taken. For analysis of percent coverage, GNU Image Manipulation Program (GIMP 2.6.8) was employed to isolate the color of the stain for collagen (blue) and cytoplasm (red). Cytoplasm staining was only performed on the preosteoblasts. Saturation modifications were made to isolate the targeted colors. Afterward, the threshold option was used to convert the images to black and white pixels. With the resulting image, the percent coverage was calculated. This technique is similar to that used by Panneton (2002).
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2.2.7. Statistical Analysis

A one way ANOVA with a Tukey test (SAS JMP 8) was employed for comparing the varying proliferant solution dosages for each time point. Differences were considered statistically significant for $p$-values less than 0.05.

2.3. Results

2.3.1. Preliminary Preosteoblast Cellular Viability Study

In the preliminary cell study preosteoblasts were exposed to various volumes of proliferant (25 µl, 50 µl, and 75 µl per ml of media). These volumes were chosen to assess cell behavior over a wide range of dosages. The results of this preliminary study were used to select more “optimized” dosages for the next stage of the study. After exposure to the P2G, all of the groups exhibited a decrease in viability at day 7 (Figure 2.1). This was followed by an upward trend of increased viability over the following weeks in the cells exposed to 25µl of P2G (Figure 2.1). The cell proliferation rate was highest in the cells exposed to 25µl of P2G between weeks 2 and 3 (Figure 2.1B). Cells exposed to 50µl displayed an increase followed by a decrease by week 5; weeks 3 and 4 are not significantly different from each other (Figure 2.1A). The cells exposed to 75µl of P2G did not recover after initial cell death (Figure 2.1A).
2.3.2. Secondary Preosteoblast Viability Study

Based on the results of the preliminary study, a secondary cell study measured the viability of preosteoblasts treated with 15μl, 25μl, and 35μl of the P2G per ml of media solution over a period of 5 weeks, n=3. All groups showed a decrease in cellular viability followed by an
increase (Figure 2.2). The cells exposed to 15µl displayed the largest increase after 14 days, followed by the 25µl group and the 35µl group. Moreover there was an initial significant difference in viability between the control group and the three treatment groups (p < 0.0001). By week 5 of the study, the control group was only significantly greater (p < 0.0061) than the 35µl group.

2.3.3. Fibroblast Viability Study

Based on the results of the secondary preosteoblast study, the fibroblast cell study measured the viability of fibroblasts treated with 15µl and 25µl of the P2G per ml of media solution over a period of three weeks, n=3 (Figure 2.3). There were no differences between the treated groups and the control groups at any time point. There was an increase in cell number in the 25µl from
day 7 to day 21 (p<0.05). No statistically significant increase in cell number was seen in any group.

### 2.3.4. Preosteoblast Histological Staining

The secondary preosteoblast cell study wells were stained with Trichrome for cells (red for cytoplasm) and collagen (blue) while fibroblast wells were stained for just collagen (Figure 2.4). In order to analyze the wells for cell proliferation and collagen deposition separately, images were transformed by removing all blue (to view the cells alone) or removing the red (to view the collagen alone) (Figure 2.4B). Purple was kept in both analyses because it represented areas where both cells and collagen are present. After removal, the remaining color was enhanced and the picture was converted into black and white pixels (Figure 2.4D). Post-conversion, the total area covered by the black was calculated at each time point.
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The cytoplasm amount and collagen protein production of the cells were assessed during the course of the study using Trichrome staining, (n=3). Figure 2.5 shows a significant decrease in the amount of cytoplasm in the treatment groups when compared to the control group (p<0.05).

Figure 2.4. Images of a well during transformation for the percent coverage image analysis: (a) The unaltered image of the well. (b) The image modified to enhance the red color (cytoplasm). (c) The image of the well modified to enhance the blue color (collagen). (d) The image after employing the threshold technique.

Figure 2.5. Percent coverage of cytoplasm of wells seeded with preosteoblast MC3T3 cells treated with varying dosages of P2G proliferant solution and a control group in the secondary study.
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Preosteoblast cell collagen production from the secondary study is presented in Figure 2.6A. During the first week, the control group’s protein production was significantly greater than that of the treatment group (p<0.05) (Figure 2.6A).

![Graph showing collagen production over weeks with control and treatment groups comparison.]

Figure 2.6. Percent coverage of collagen of wells seeded with (a) preosteoblast MC3T3 cells and (b) patellar tendon fibroblasts. Wells were treated with varying dosages of P2G proliferant solution except for the control group.
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2.3.5. Fibroblast Histological Staining

Fibroblast wells were stained with Trichrome for collagen (Figure 2.6B). Wells were analyzed as described earlier. Collagen production for the fibroblasts increased in the control and 15μl groups between 7 and 21 days (p<0.05). The amount of collagen for the 15μl group at 21 days was larger than the 15μl group at 14 days (p<0.05). There was no statistical difference between the amount of collagen in the control group and 25μl group at 14 days and all the groups at 21 days.

2.4. Discussion

According to Kim et al., the use of prolotherapy as a common treatment option for hypermobile and painful joints has been limited in part by the lack of heterogeneous variables and methodologies in treatment and research (Kim et al., 2004). The primary goal of the present study was to measure the effects of dosage on cell behavior, particularly cell proliferation and collagen production. The use of multiple cell studies allowed us to find a maximum dosage for the proliferant P2G that still permitted the remaining cells to recover and produce collagen. The data also show a dosage-dependent response to P2G in preosteoblasts; as the P2G dosage decreased in the preliminary study, the cellular response at week 2 increased, with the highest proliferation rate occurring between weeks 2 and 3 for that dosage (Figure 2.1). The absorbance values for the secondary preosteoblast viability assay (MTS) and the percentage of the well covered by cells after Trichrome staining were significantly larger for the 15μl dosage than the other two dosages at week 2 (Figure 2.2 and Figure 2.5). The 15μl group nearly attained the population of the control group toward the end of the study (Figure 2.2). The other two treatment groups also showed an increase in proliferation, but it was not as pronounced as the
response of the 15μl group (Figure 2.2). The rate of proliferation was inversely proportional to proliferant dosage in both studies (Figure 2.1 and Figure 2.2). Two of the dosages from the second preosteoblast study (15μl and 25μl) were used in the fibroblast study. The P2G had no effect on fibroblast viability at any time point, but did hinder collagen production after 7 days (similar to preosteoblasts) (Figure 2.3 and Figure 2.6B). Collagen production increased from day 7 onward for each group (Figure 2.6B).

It is believed by some health practitioners who use prolotherapy that the treatment works by causing a small amount of cell irritation or death, which activates the healing cascade. The healing cascade is thought to bring in growth factors that signal cells to make more extracellular matrix (ECM) (Banks, 1991). The presence of this new ECM strengthens the tissue as it heals. The data from this study support aspects of this theory. In the preosteoblast studies, significant cell death occurred immediately after treatment with P2G. There was no significant difference between any of the treatment groups in cell viability or proliferation (Figure 2.1, Figure 2.2, and Figure 2.5) and collagen deposition (Figure 2.6A) at week 1. The amount of collagen production by the preosteoblasts did not reach that of the control during this study. For the fibroblast population, however, there were no changes in cell viability when treated with P2G (Figure 2.3). There was a decrease in the amount of collagen laid down by the cells after week 1, but the cells displayed collagen production comparable to the control group by week 2 for 25μl and week 3 for 15μl (Figure 2.6B). It is possible that increased dosages of P2G would cause an initial decrease in fibroblast viability and collagen production followed by an increase as seen in the preosteoblast studies. Based on the trends for both cell types, collagen production of the treated populations could surpass that of the controls, but further studies with longer time points are necessary to determine if this is true.
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The effects on different cell types cannot be determined at this point, but future studies will be conducted in an attempt to better understand what appears to be a tissue-specific response. In another study (data not shown), similar results to the fibroblast study presented were observed with another population of fibroblasts, which supports the possibility of tissue-specific response to proliferative solutions. If this is the case, the important factor to consider is the end goal of increased collagen production for tissue healing. As long as the exposure to proliferant leads to tissue healing, the potential tissue-specific response may not indicate a need to increase the dosage, but perhaps instead suggest that cell death may not be a necessary step in the proliferative process for tissue healing in some tissue types.

The results of this study display aspects of the perceived healing process induced by prolotherapy, initial cell trauma followed by collagen production. They also suggest that the response of the cells over a 3-4 week period near the injection site may not be robust enough to heal the tissue alone; the cells did not produce the same amount of collagen as the control. There was also a dosage-dependent cell response to P2G. According to this data, healing may not only be a result of factors and cells being brought to the injury site from other areas of the body. There may also be a local response, although small, to the cell irritation or death that leads to increased proliferation of the remaining cells. Unlike tests in animal models or clinical settings, our experiments took place in a closed system with one cell source and a controlled media source. The increases in cell number and collagen content were not due to new cell sources or factors released from other parts of the body. These are local responses to the proliferant and cell trauma by the remaining cells. It is possible that upon trauma or death, cells release certain factors that enhance proliferation by the remaining cells.
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While aspects of this study support the previously proposed mechanism for healing by prolotherapy, they also raise potential new mechanisms and new questions. Although intuitively an increase in cellular proliferation makes sense as a source for new collagen it is not cited as a mechanism for tissue healing with prolotherapy. Therefore the increase in collagen content may not be due to an upregulation in collagen production by the remaining cells alone. It may also be caused by additional cells producing collagen and adding to the overall collagen content of the damaged area. Future cell studies will focus on longer time periods, additional proliferants, the use of reverse transcription polymerase chain reaction (RT-PCR) to measure the upregulation and down-regulation of genes in response to prolotherapy, and the use of enzyme linked immunosorbent assay (ELISA) to better measure protein production. Animal studies will also be conducted to compare the response of isolated cell populations versus that of complete biological systems to prolotherapy.

The results of this study suggest that there is an optimal dosage for the proliferant P2G in vitro. A dosage of 15μl led to the highest collagen deposition and rate of proliferation after week 1 for the preosteoblastic MC3T3 cells. Although collagen production never surpassed the control levels for the fibroblasts, it is comparable. The data presented in this study support the fundamental theory behind prolotherapy, that after local proliferant-induced trauma, the cell population recovered with an increase in collagen protein production beneficial for the healing and repair of damaged tissue.
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2.5. Acknowledgements

The authors would like to thank Albert Kwansa for his assistance in the collagen staining analysis and the Virginia Tech PREP program for support. There are no conflicts of interest associated with this study.
Chapter 3. Nanoparticle Studies

Chapter 3. NANOPARTICLE STUDIES


3.1. Introduction

In the presented nanoparticle (NP) study it was hypothesized that the controlled and localized injection of biocompatible NPs into damaged connective tissues would enhance matrix mechanical properties, evidenced by increased stiffness and yield strength. This technique would be a particulate reinforcement-based alternative modality for the treatment of damaged connective tissues. The effects of NPs, namely, single walled carbon nanohorns (CNHs) and cellulose nanocrystals (CNCs), on damaged connective tissue mechanical properties were examined. The CNHs and CNCs differ in overall size, architecture, and chemical composition thus enabling the comparison of their effects on matrix mechanical properties. Two different types of porcine connective tissues were employed in this study, specifically, skin and tendon. Porcine skin was employed with the injury model because of its similarity to ligaments and tendons both in matrix composition (fibrous with a high type I collagen content) and in tensile mechanical behavior (similar trends in stress-strain curves, seen in Figure 1.1). Porcine digitorum tendons were employed as a model for the treatment of target tissues using the optimal NP. A strain injury model was utilized to disrupt the tissue matrix and mimic sprain and strain injuries.

In addition, a preliminary biocompatibility test was performed to assess the biological response of tendon fibroblasts to the NPs in vitro. NP dispersion was explored through a histological study of porcine tendon injected with CNHs. The results from these preliminary
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studies will be used to understand the feasibility of mechanically reinforcing damaged connective tissues by injecting biocompatible NPs \textit{in vivo}.

3.2. Materials and Methods

3.2.1. Materials

CNHs, synthesized as described by Geohegan and colleagues, were kindly provided by Oak Ridge National Laboratory (Geohegan et al., 2006). Wood pulp (Temalfa 95 A) was kindly provided by Tembec, Inc. Sulfuric acid (95.9%, certified ACS plus) was purchased from Fisher Scientific. Pluronic F-127 (solid) was purchased from Biotium, Inc. Porcine skin tissue was isolated from fresh belly sections purchased from Oasis World Market (Blacksburg, VA). Porcine tendons were harvested from 14 – 16 week old female pigs. Primary patella tendon (PT) fibroblasts were harvested from 10 - 14 week old male Sprague Dawley rats (Harlan Laboratories).

3.2.2. NP Synthesis and Characterization

3.2.2.1. CNC Synthesis

CNCs were prepared from dissolving-grade softwood sulfite pulp as described previously (Dong & Roman, 2007). Briefly, milled pulp was hydrolyzed with 64 wt% sulfuric acid at 45.5 °C and an acid-to-pulp ratio of 10 ml/g. After 60 min, the acid was diluted 10-fold by addition of deionized water (Millipore Direct-Q 5 Ultrapure Water System) and removed by centrifugation and dialysis. The final product was a 10 mg/ml suspension of CNCs in deionized water.
3.2.2.2. Atomic Force Microscopy (AFM)

AFM samples of CNHs were prepared from a 0.05 mg/ml suspension in a 1mg/ml aqueous Pluronic solution after 30 min of indirect sonication in an ice bath with a 500-Watt ultrasonic processor (Sonics Vibra-Cell VC-505, probe model: CV 33) at 40% output. AFM samples of CNCs were prepared from a 0.02 mg/ml suspension of CNCs in deionized water after 10 min of sonication. The NPs were deposited onto freshly cleaved mica discs (diameter: 0.5 in., Ted Pella) by spin coating of 15 µL at 4000 rpm for 1 min. AFM images were recorded under ambient conditions in tapping (AC) mode with an Asylum Research MFP3D-Bio atomic force microscope using Olympus OMCL-AC160TS probes.

3.2.2.3. Dynamic Light Scattering (DLS)

For size analysis by DLS, a 0.05 mg/ml CNH suspension in a 1 mg/ml aqueous Pluronic solution and a 1 mg/ml CNC suspension in deionized water were sonicated for 10 min and transferred to Malvern Instruments DTS0012 disposable polystyrene cuvettes. Measurements were carried out in triplicate at 25 and 37 °C after a 10-min equilibration period with a Malvern Instruments Zetasizer Nano ZS particle analyzer equipped with a He-Ne laser (633 nm, 4.0 mW) and a photodiode detector located at 173°.

3.2.3. Mechanical Testing

3.2.3.1. Porcine Skin Injury Model

Specimen preparation: A tensile strain injury model was developed to test the hypothesis that the injection of NPs into connective tissues would result in increased strength due to mechanical reinforcement. The skin from the underbelly of female pigs was used for preliminary validation of the strain injury model (Figure 3.1). The fat layer was carefully scraped away from the skin using a surgical scalpel. Full-thickness (0.25 cm) 8 cm x 1 cm
sections were isolated and dissected from the skin, wrapped in phosphate buffered solution (PBS)-soaked gauze, and stored in a -20°C freezer for approximately four weeks. Tissue was stored and handled with the intention of thawing gently as well as minimizing the time it was kept thawed in order to maintain its properties. Tissue was thawed prior to damage, measurement, injection, and/or testing by placing the packaged tissue in a 4°C refrigerator overnight, after which it was exposed to room temperature for one hour. The tissue was unwrapped and the appropriate procedure conducted. Tissue was kept in the refrigerator between procedures (damage, measurement, injection, or testing), with a one hour exposure to room temperature prior to the next. If there were more than 12 hours between procedures, the tissue was refrozen until the subsequent procedure using the same gradual thawing protocol. Just prior to testing, specimens were soaked in PBS for ten minutes and measured for hydrated dimensions.

Mechanical damage: Pig skin sections were damaged using an MTS Tytron 250 mechanical testing machine (MTS Systems Corporation, Eden Prairie, MN, USA). A specialized set-up was used to apply damage to the skin sections. Specifically, a custom-designed grip was attached to the actuator, and a custom-designed U-shaped bracket was attached to the load cell. For each damage procedure, the skin section was placed into the grip, looped around an iron pin of the U-shaped bracket, and placed back into the grip prior to closure of the grip (i.e., both ends of the specimen were clamped within the same grip) (Figure 3.1B). This looped configuration was used to elicit damage near the middle of each skin section. Starting from a gauge length of 20 mm, each skin section was preloaded to 1 N at a rate of 10%/min. Then each skin section was damaged to 70% strain at a strain rate of 1,000%/min. After damage, each skin section was divided into smaller skin specimens for different injection treatments (Figure 3.1B).
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*Nanoparticle treatment:* A total of 0.25 ml of the NP suspension was injected from one side of the skin specimens at nine injection sites using a 25-gauge 1.5 inch needle and 1.0 ml syringe (Figure 3.1C). The skin specimen was placed between two small sheets of Plexiglass to provide a stable and consistent injection platform. A new needle was used after every two specimens. A new syringe was used with each change in concentration. The vial containing the NP suspension was agitated prior to each injection. The needle was inserted through each specimen, and the NP suspension was delivered as the needle was slowly withdrawn. A minimum concentration of 2 mg/ml was initially used for both NPs in exploratory studies to determine the effects of NPs on the tensile elastic modulus and yield strength of injured porcine skin. For the present work, it was found that pig skin samples injected with CNC suspensions did not experience substantial changes in mechanical properties at concentrations less than 10 mg/ml (data not shown). After completion of the exploratory experiments, three different concentrations of CNH and CNC solutions were studied (Table 1). CNHs were solubilized with 1mg/ml Pluronic in deionized water to enhance dispersion. Pluronic is a commonly used surfactant to enhance biomaterial solubility and has been shown to be biocompatible (Whitney, 2011). The CNC suspensions did not contain Pluronic. The skin samples were randomly placed into the following eight groups: (1) uninjured control skin samples, (2) injured untreated skin samples, (3-8) injured skin samples treated with injections of NP suspensions at the concentrations listed in Table 3.1. As the skin testing was preliminary and used in part to determine appropriate concentration levels of the NP solutions to be selected for the tendon testing, no sham was deemed necessary.
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Mechanical characterization: Tensile testing was performed using an Instron 5869 (Instron, Norwood, MA, USA) mechanical testing machine with a BioPuls bath containing PBS at 37°C (Figure 3.1D). Beginning with a gauge length of 30mm, the pig skin specimens were pulled at a displacement rate of 100 mm/min until failure. For data analysis, the elastic modulus and yield stress values were calculated from the linear and yield regions of the stress-strain curves with a

Table 3.1. Nanoparticle Treatment Concentrations for Skin Testing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CNH (mg/mL)</th>
<th>CNC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment 1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Treatment 3</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 3.1. Porcine skin injury mechanical testing workflow. (a) Full thickness tissue sections isolated from porcine skin. (b) Skin tissue sections stretch-injured using MTS mechanical testing machine. Red arrow within photo indicates tissue location. (c) Skin test specimen isolated from sections and treated with NP suspensions via injections. Dashed red arrows indicate injection pattern. Small red circles on right side of specimen indicate where needle was inserted. (d) Specimen subjected to uniaxial tensile tests using an Instron mechanical testing machine. Red arrow within photo indicates tissue location. For tendon testing, test specimens were cut along the length of tendon, with remaining test workflow similar to that of the skin. Tendon specimens were stretch-injured with the method shown in (b), injected with a single X-pattern in the center of the tendon similar to (c), and tested as shown in (d).
0.2% strain offset from the end of the linear region to determine the yield point. The sample size for each of the eight groups was n=4.

3.2.3.2. Tendon Mechanical Testing

*Specimen preparation:* After observing the effects of the NP injections on skin mechanics, we investigated the effects of the NP injections on tendon mechanics. The stretch injury model was applied to the digitorum longus tendons of female pigs. Tendons were isolated and dissected from hindlimbs, wrapped in PBS-soaked gauze, placed in polystyrene tubes, and stored in a -20°C freezer. Each tendon was cut into two equal lengths of 8 cm with one section serving as the control and the other section serving as the treated portion. The tendon sections were stored in the freezer for approximately four weeks. Tissue was thawed using the protocol described above.

*Mechanical damage:* Pig tendon specimens were damaged using an MTS Tytron 250 mechanical testing machine. A specialized set-up identical to that used to damage the pig skin sections was utilized to damage the pig tendon specimens. Starting from a gauge length of 30 mm, each tendon specimen was preloaded to 100 mN at a rate of 10%/min. Then each tendon specimen was damaged to 13% strain at a strain rate of 1,000%/min.

*Nanoparticle treatment:* The tendons were randomly placed into the following four groups: (1) uninjured control tendons, (2) injured untreated tendons, (3) injured tendons injected with deionized H₂O as a sham treatment, (4) injured tendons injected with 4 mg/ml of CNHs in a 1mg/ml Pluronic F-127 deionized water solution. The deionized water sham was employed to examine the effect of the needle and the base solution being inserted into the tissue. Pluronic F-127 is a nonionic surfactant polyol used to facilitate the solubilization of carbon NPs. As CNHs have a much larger elastic modulus, around 1 TPa (Kumar, Verma, Bhatti, & Dharamvir, 2011)
than a Pluronic solution, the potential effect of the Pluronic on the mechanical properties of the
tendon was deemed negligible compared to the CNHs, making a separate Pluronic sham
unnecessary. Tendon specimens were injected in a similar fashion to the skin, described in
Section 3.2.3.1 (Figure 3.2). The volume of the injected NP solution was calculated by
multiplying the tendon specimen volume (cm$^3$), measured by calipers in the hydrated state, by a
factor of 0.4 to yield a dosage in ml. Two injections were needed for the tendons and were
applied in an X-configuration in the center of the tendon (Figure 3.2B). The deionized water
solution was mixed with one drop of liquid blue food dye in 30 ml deionized water so that it was
easily detected when injected.

Figure 3.2. Injection of tendons. (a) Tendon specimen with needle inserted prior to injection of CNH solution.
(b) Tendon specimen after injection of CNH solution.

**Mechanical characterization:** Tensile testing was conducted using an Instron 5869. Tendons
were thawed in PBS and soaked for an additional 10 minutes prior to testing. Beginning with a
gauge length of 20 mm, the tendons were preloaded to 0.5 N and pulled at a displacement rate of
100 mm/min in air at room temperature until failure. Due to the short duration of the mechanical
tests, the tendons were expected to remain hydrated due to soaking in PBS. For data analysis,
the elastic modulus and yield strength values were calculated from the linear and yield regions of
stress-strain curves. The sample size for each of the four groups was n=4.
3.2.4. Cell Culture

Primary PT fibroblasts from male Sprague-Dawley rats were harvested and cultured as reported earlier (Fu, Cheuk, Chan, Hung, & Wong, 2008). Briefly, patella tendons from the rats were dissected, minced, and rinsed with Hank’s Buffered Salt Solution (HBSS). Sequential digestion of the tendon explants was performed with trypsin. The cells were collected from the fourth digestion and cultured in α-MEM at pH 7.6 supplemented with 1% (v/v) penicillin streptomycin and 10% (v/v) fetal bovine serum (FBS, Fisher Scientific Co. Suwanee, GA, USA). The cells were seeded onto 24-well plates at a density of $3 \times 10^4$ cells per well and incubated for 24 hours before being dosed with 50 µL of 10mg/ml CNC aqueous solution or of 2mg/ml CNH with 1mg/ml Pluronic aqueous solution added to 950 µL media per well once, and the cells were cultured for up to 28 days. The differences in NP concentrations seen in mechanical tests were replicated in the NP concentrations to evaluate their effects on metabolic activity. Half of the culture medium was replaced with fresh medium 3 times a week.

3.2.5. NP Biocompatibility

Cell activity was measured as previously described (Holthaus, Treccani, & Rezwan, 2013). At 7, 14, and 21-day time points the medium was removed and the cells washed twice with PBS to eliminate excess NPs not taken up by the cells. Cellular mitochondrial activity was evaluated using the colorimetric WST-1 assay via the absorbance of the reactive formazan product with live cells. After each well was emptied, 300 µL of fresh cell culture medium and 30 µL of the WST-1 assay were added to each well. The plates were incubated for three hours. Afterward, the volume in each well was diluted (1:4) with deionized water and the absorbance read at 450 nm with a SpectroMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).
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A second cell study was conducted using the same methods of harvest, culture, seeding, and treatment as described above in order to observe and image potential cell uptake of the carbon NPs used in the tendon injection study. The treated cells were dosed with 50 µL of 2mg/ml CNH with 1mg/ml Pluronic solution per well once and were cultured for seven days. Half of the culture medium was replaced with fresh medium 3 times a week. Bright field (BF) microscopic images were taken of the cell populations on days 1, 3, 5 and 7 using a Leica DM IL microscope (Leica Microsystems, Inc., Buffalo Grove, IL).

3.2.6. Data Analysis

A one-way ANOVA with a Bonferroni’s multiple comparison post-hoc analysis was employed for comparing the varying NP suspension concentrations in the mechanical testing experiments. A two-way ANOVA with a Bonferroni’s multiple comparison post-hoc analysis was employed for comparing groups for the cell study experiments (GraphPad Prism Software 5.0, La Jolla, CA, USA). Differences were considered statistically significant for p-values less than 0.05, unless otherwise stated.

3.2.7. CNH Distribution in Porcine Tendon

In order to investigate CNH distribution within treated tissue, additional porcine digitorum tendons were isolated and dissected from forelimbs, and injected as described above. Specimens were sectioned to 3 cm lengths and prepared for BF microscopy and transmission electron microscopy (TEM). The BF specimens were taken as longitudinal sections. They were fixed in 10% buffered formalin and processed using a hematoxylin and eosin (H&E) stain. The TEM specimens were taken in cross-section and fixed in 2.5% glutaraldehyde in PBS solution. They were either stained with uranyl acetate and lead citrate or left unstained. All specimens were
placed in fixative within 30 minutes of injection. BF images were taken on a Leica DMI 6000B microscope (Leica Microsystems, Inc., Buffalo Grove, IL) and TEM images were taken using a JEOL JEM 1400 (JEOL USA, Peabody, MA).

3.3. Results

3.3.1. NP Characterization

Figure 3.3 shows AFM height images of the NPs. Dried on a flat substrate, the CNHs had a disk-like morphology with a mean diameter of 39.6 ± 10.9 nm and a mean thickness of 1.3 ± 0.5 nm whereas the CNCs had an oblong morphology with a mean length of 119.3 ± 27.7 nm and a mean thickness of 3.7 ± 0.7 nm.

![AFM height images of (a) CNHs and (b) CNCs.](image)

Both NPs had monomodal DLS intensity size distributions (Figure 3.4). The z-average hydrodynamic diameters and polydispersity indexes (PDIs) of the NPs in aqueous suspensions at 25 and 37°C are listed in Table 3.2.
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Table 3.2. z-Average hydrodynamic diameters and PDIs of CNHs and CNCs at 25 and 37 °C measured by dynamic light scattering.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>CNH</th>
<th>CNC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diameter* (nm)</td>
<td>PDI‡</td>
</tr>
<tr>
<td>25</td>
<td>188.8 ± 0.7</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>37</td>
<td>245.9 ± 0.8</td>
<td>0.26 ± 0.01</td>
</tr>
</tbody>
</table>

* z-average hydrodynamic diameter, ‡ Polydispersity index

As seen in Table 3.2, in the hydrated state, the CNHs had larger dimensions than the CNCs. It should be noted, however, that the CNH suspension contained 1 mg/ml Pluronic F-127. This concentration is below the critical micelle concentration of Pluronic F-127 at 25 °C but exceeds it at 37 °C (Alexandridis, Holzwarth, & Hatton, 1994). Pluronic micelles are known to adsorb onto carbon NPs and form surface layers of about 20 nm thickness (Lin & Alexandridis, 2002). Thus, the actual diameter of the CNHs suspended in water is likely much smaller than that measured at 37 °C. The presence of Pluronic micelles in the CNH suspension at 37 °C might
also explain the higher PDI, compared to that measured at 25 °C. In contrast, the length of the CNCs suspended in water is likely larger than the measured hydrodynamic diameter because DLS size analysis assumes a spherical particle shape and the equivalent spherical diameter of a prolate spheroid is smaller than its major axis (length) (Jennings & Parslow, 1988). The dimensions of CNCs suspended in water are likely to be similar to their dry dimensions because crystalline cellulose does not swell significantly in water. In summary, in aqueous suspensions the CNHs likely have an oblate spheroidal or spherical shape with a mean diameter above ~40 and below ~190 nm and the CNCs likely have an ellipsoidal or rod-like shape with a mean length of around 120 nm and a mean thickness of around 4 nm.

3.3.2. Porcine Skin Mechanical Testing

The injured porcine skin samples were treated with three different concentrations of CNH solutions (2 mg/ml, 5 mg/ml, 10 mg/ml) and CNC solutions (10 mg/ml, 20 mg/ml, 50 mg/ml) (Table 1). With the injury model, there appears to be a trend of increasing tissue stiffness with increasing NP concentration (Figure 3.5). All three concentrations for both NPs had similar effects on skin mechanics.

Figure 3.5. (a) Mean percent change of the elastic moduli and mean yield strength (b) of injured porcine skin samples injected with varying concentrations of CNHs (shaded box) and CNCs (unshaded box) normalized to the uninjured control group at 100% (dashed line). Asterisk (*) designates statistical significance (p<0.01) compared to other groups.
3.3.3. Porcine Tendon Mechanical Testing

After confirming the injury model with the porcine skin, the effects of NPs on the mechanical properties of porcine tendon samples were investigated. In the skin testing, it was found that the higher concentration solutions were difficult to inject. Since the CNH particles are far larger than those of the CNCs, the CNH concentrations were lower in comparison. CNHs were selected as the NP to investigate in the tendon study. A concentration was chosen for the tendon testing that was expected to affect the tendon mechanical properties, based on the skin study, without being too difficult to inject using previously developed injection tools and protocol; the lowest previously used concentration for the CNHs of 2 mg/ml was doubled for this study (4 mg/ml solution).

In the tendon injury model, damaged digitorum tendons of pigs were injected with the 4 mg/ml CNH solution. Tendons injected with sham deionized water were also used as a control. There was no statistical difference between the control and injured groups. After injections of CNH suspensions, there was an increase in the elastic modulus of the injured tendons, with statistical significance found between the CNH treated tendons and the control and injured untreated groups (Figure 3.6A). The values for elastic modulus were highest for the tendons treated with CNHs. There were no statistically significant differences between the yield strengths of the control, injured, and treated tendons (Figure 3.6B).
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3.3.4. NP Biocompatibility

Primary rat PT fibroblasts were exposed to CNH or CNC solutions in a similar range of concentrations (50 µL of 10mg/ml CNC aqueous solution or of 2mg/ml CNH with 1mg/ml Pluronic aqueous solution) to those used in the tissue studies to ensure that cell survival at those concentrations was examined. Cellular activity was measured using a WST-1 assay up to 21 days after exposure. Throughout the course of the experiments, all groups showed a general trend of increasing cell activity over time (Figure 3.7). Additionally, after 21 days of NP exposure, the fibroblasts in the CNC group showed significantly higher cell activity than the control group (p<0.01) (Figure 3.7B).

Figure 3.6. (a) Mean elastic moduli and (b) mean yield strength of damaged porcine digitorum tendons injected with 4 mg/ml solution of CNHs. Asterisk (*) designates statistical significance (p<0.05) compared to other groups.

Figure 3.7. (a) WST-1 absorbance of PT fibroblasts exposed to CNH solution and (b) CNC solution. Asterisks designate statistical significance compared to the control group: * (p<0.01); ** (p<0.001).
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The CNH cellular uptake revealed that the treated fibroblasts engulfed and incorporated clusters of the CNHs into the cell body as well as within the extracellular matrix (ECM) they produced (Figure 3.8B and D). The control and treated cell populations displayed similar cell growth and ECM production throughout the seven days (Figure 3.8).

3.3.5. CNH Distribution in Porcine Tendon

Porcine digitorum tendon specimens were isolated and injected with CNH solution using the same method and concentration (4 mg/ml CNHs with 1mg/ml Pluronic in aqueous solution) as in the tendon testing in order to evaluate CNH distribution and infiltration within the tissue after
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treatment (Figure 3.9). Immediate dispersion of the CNH solution upon injection was observed. Wicking of the solution by capillary effect from the injection site in the center to the ends of the specimen was visible to the naked eye and occurred immediately. The entire tendon specimen changed in appearance from pristine white (Figure 3.9A) to light grey, with patches of black where the tendon sheath was disrupted from dissection or injection, as well as in the longitudinal regions between tendon fiber bundles (Figure 3.9B).

![Figure 3.9. Tendon specimen (3cm length) prepared for BF microscopy (a) in injection fixture prior to injection (b) after injection in specimen cartridge.](image)

The BF microscopic images confirmed dispersion along the pathways between tendon fiber bundles from the injection site (Figure 3.10). The needle track was evident from the swath of disrupted fibers in the image of the injected tendon (Figure 3.10A). CNH solution was discernable leading away from the injection site in pathways between the fiber bundles (Figure 3.10A), through the midway point between the injection site and the end of the specimen (Figure 3.10B), to the end of the specimen (Figure 3.10C). A higher magnification image more clearly revealed native structures of the tissue specimen, including fibroblast nuclei and blood vessel walls, as well as CNH solution in trails aligned with the structures (Figure 3.10D). The CNH solution appeared well dispersed along the length of the tendon specimen, with the regions between fiber bundles serving as effective conduits.
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TEM images further unveiled the extent of the CNH dispersion (Figure 3.11). Only a small fraction of the cross-section was found to have nanohorns, indicating that their distribution was limited to those pathways between fiber bundles that were exposed to the solution from the injection. In the nanohorn-populated portion, the nanohorns were plentiful and appeared as dark or glowing metallic spiny spheres in single or small cluster form in the midst of the biological tissue. At low magnification, individual collagen fibrils are visible in cross-section, with borders of the fibril bundles clearly evident (Figure 3.11A and B). Between the fibril bundle borders are channel-like areas in which the CNHs are more concentrated (Figure 3.11A and C), but some of

Figure 3.10. BF microphotographs of CNH solution injected porcine tendon. (a) Needle tracks and CNH solution trails at injection site (b) Treated tissue midway between injection site and end of specimen (c) Treated tissue at end of specimen (d) Detail of native tissue structures and CNH trail.
the nanohorns are visible amongst the fibrils. The nanohorns are approximately the same size as
the fibril diameter and in contact with the fibrils. At higher magnification (Figure 3.11C and D),
the dahlia-like construction of the nanohorns is clearly exhibited.

All of the images in Figure 3.11 are unstained, as the stained specimens resulted in increased
definition of cellular and tissue architecture but more difficulty in detecting the nanohorns.

### 3.4. Discussion

To study the effects of injected NPs on soft tissue mechanics, we induced stretch injuries in
porcine tissue. Porcine tissue is often utilized as a suitable tool to study human soft tissue due to
similarities in matrix composition and mechanical behavior (Deeken et al., 2011; Lim, Hong,
Chen, & Weerasooriya, 2011; Shergold & Fleck, 2005). The strain injury model was employed to mimic sprains and strains of ligaments and tendons. Previous studies have shown that mechanical and cellular damage to the medial collateral ligament (MCL) is not substantially different than that with pure axial stretch of an excised ligament (Provenzano, Hayashi, Kunz, & Markel, 2002; Provenzano, Heisey, Hayashi, Lakes, & Vanderby, 2002). It is likely that after the damage, the collagen fibrils and other matrix components undergo plastic deformation and reorganization (Freeman et al., 2007; Kwansa et al., 2010). We hypothesize that upon introduction and dispersion of both NPs, there is stress transfer between the embedded NPs and the matrix components. The observed increase in modulus indicates that the hypothesis is correct and inspires investigation into the effect of NP dispersion on tissue matrix mechanical response. To validate our injury model, the first set of mechanical experiments aimed to investigate the concentration-dependent effects of the NPs on skin mechanical properties; the injury model was utilized to disrupt the tissue matrix. After treating the skin with increasing concentrations of NPs, there is a trend of increasing percent change in the elastic modulus of injured skin (Figure 3.6A). All three concentrations for both CNHs and CNCs had larger changes in modulus compared to the injured and untreated groups. This data confirms that after damage to the matrix, the addition of NPs has a concentration-dependent effect on tissue mechanical properties. Although the yield strength results showed less of a change after NP treatments in injured skin (Figure 3.6B) the data still suggests that introducing the NPs into connective soft tissue immediately alters tissue elastic modulus creating a stiffer nanocomposite tissue.

The CNC suspension concentrations were considerably higher than the CNH suspension concentrations. As the CNHs are much larger in structure than the CNCs, the CNH solutions
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were less concentrated than the CNC solutions. The higher concentration solutions were found to be more difficult to inject into the skin tissue. As lower concentrations of CNHs were necessary to increase skin mechanical properties compared to CNCs, only the CNHs were utilized for the porcine tendon model. An investigation into methods of better delivery of higher concentration solutions may enhance future studies. In the porcine tendon model of this study, there was a trending increase in the elastic moduli of injured tendons treated with CNHs compared to untreated injured tendons with statistical significance found between the CNH treated tendons and the injured untreated group (Figure 3.6A). We theorize that the observed difference in NP effectiveness could be attributed to the different morphologies and mechanical properties of the NPs. The CNCs are oblong NPs, about 120 nm in average length and 4 nm in average thickness. In contrast, CNHs consist of thousands of single-walled carbon tubules with closed conical ends arranged in a dahlia flower-resembling agglomerate structure (Nakamura et al., 2011; Whitney et al., 2011) with an average diameter between ~40 and ~190 nm. It is possible that the more complex carbon nanostructures are able to more effectively interact with the matrix components than the rod-like CNCs, thus allowing for better stress transfer between the NP and matrix components. In addition, the elastic modulus of CNHs is higher than that of CNCs. Specifically, the elastic modulus of CNHs has been reported to be approximately 1 TPa (Kumar et al., 2011) whereas that of CNCs has been reported to be around 155 GPa (Wu, Wagner, Raman, Moon, Martini, & Center, 2010). This apparent difference in natural stiffness has a significant effect on composite mechanical response.

The statistical significance found between the injured tendons treated with CNH solution and those that were injured but not treated is an important finding. The untreated injured specimens represent the injuries in humans that are being studied for solutions involving improved healing
response and decreased injury recurrence. Since the yield strength of biological tissues is not approached during normal movement, the lack of statistical significance found in the yield strengths between groups is not alarming; the significant increase in elastic modulus affects normal ligament behavior and is a positive step for research involving repair of biological connective tissue and prevention of re-injury.

In the presented studies, biocompatibility tests using dosage of 50 µL of 2mg/ml CNH solution and 10mg/ml CNC solution per 1 ml well showed no adverse effects of the NPs on the cell activity of PT fibroblasts (Figure 3.7 and Figure 3.8). These results show promise for the biocompatibility of these NPs with cells from one of the target tissues in vitro. The two NPs studied, CNHs and CNCs, have been used previously for the development of reinforced nanocomposites (Thostenson & Chou, 2002; Azizi Samir et al., 2004; Lahiri et al., 2009; Li et al., 2009; Qi et al., 2009). Previous studies with CNCs have shown them to have low cytotoxicity and low non-specific cellular uptake when assessed with several different mammalian cell lines at concentrations up to 50 µg/ml and 48 hours of exposure (Dong, Hirani, Colacino, Lee, & Roman, 2012). In addition, ecotoxicological studies on the characterization of nanocrystalline cellulose found them to have low toxicity potential when exposed to rainbow trout hepatocytes (Kovacs et al., 2010). In agreement with reports of nanocarbon biocompatibility, previous work in our lab has shown the feasibility of incorporating multi-walled carbon nanotubes into coaxially electrospun poly(-caprolactone) (PCL) and poly(acrylic acid)/poly (vinyl alcohol) (PAA/PVA) nanofibers for an actuating scaffold for functional skeletal muscle tissue regeneration. Biocompatibility studies performed on the nanocomposite scaffolds showed that the carbon nanotubes had no harmful effects on rat skeletal muscle cells (McKeon-Fischer, Flagg, & Freeman, 2011). Although the CNHs are not
nanotubes, they have the same composition and are composed of many carbon nanocones arranged in a sphere-like shape.

In the histological exploration of the CNH solution treated tendon, considerable information was discovered regarding the distribution and dispersion of the NPs among the tissue structures. The NPs flow in solution by capillary action from the injection site through the natural pathways along the fiber bundles within the tendon, and migrate into the fiber bundles among the collagen fibrils. Electron microscopy revealed that the nanohorns do not appear to aggregate in large clusters, rather they predominantly separate into single units or small groups. The nanohorns are similar in diameter to the collagen fibrils, and infiltrate the local tissue from those channels exposed to the solution by injection. Furthermore, the unique structure of nanohorns was displayed and recorded.

The long-term effects of the NP reinforcement on connective tissue biomechanics will need to be assessed by cyclic loading and fatigue tests. Future mechanical tests will also include larger sample sizes. Although these preliminary biocompatibility results showed that cellular activity remained unaffected by the NPs after exposure, the assay used in the study only measures cellular activity and not cell number directly. In addition, NP cell uptake kinetics and cell phenotype were explored only by microscopic observation, and not by specific metrics. Further investigation into the NP effect on uptake and cell function will provide more knowledge regarding the biological response of in vivo NP reinforcement. Additional studies will encompass further advanced microscopy to image cell and NP interactions, assessment of ECM production, and gene expression analysis of connective tissue related markers including elastin and collagen types I and III.
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3.5. Conclusions

The purpose of these studies was to investigate the possibility of mechanically reinforcing damaged porcine connective tissues with two different NPs, determine their biocompatibility using rat PT fibroblasts, and examine the distribution of injected NPs in porcine tendons. The results presented in this study show the feasibility of using CNHs and CNCs to locally reinforce damaged connective tissues. Several mechanical testing models (injured and non-injured porcine skin and tendon), a cytotoxicity test, and histological explorations demonstrate the feasibility of the concept in preliminary studies. Thus, further studies are needed to begin to understand the effect of the NPs on the cellular response of representative cell types such as tendon/ligament fibroblasts and adult stem cells. Future studies will focus on the cellular uptake kinetics and translocation of the NPs in vitro. In addition, the effect of the NPs on cell phenotype and function will be assessed using reverse transcription polymerase chain reaction (RT-PCR) to look at the expression of connective tissue-related genes. Lastly, an investigation into the effect of the presence of the NPs on the long-term mechanical response of connective tissue will be conducted. This will be achieved by a series of tensile and fatigue tests. Furthermore, the effect of the injection of NPs into damaged connective tissues will be assessed in complete in vivo systems.
Chapter 4. SUMMARY AND FUTURE WORK

In the presented studies, foundational and essential aspects of creating a living composite ligament or tendon using prolotherapy and nanoparticles (NPs) were investigated. Multiple cell culture studies yielded an appropriate proliferant solution dosing protocol and concentration necessary to cause effect in the preosteoblast cell populations, showed a level of collagen production response from exposure to the proliferant in fibroblast cells, and provided insight into cell response to NP exposure. Tissue studies determined effective concentration levels and injection methods of NPs to bring about an increase in mechanical properties in damaged connective tissue. NP characterization of cellulose nanocrystals (CNCs) and carbon nanohorns (CNHs) was conducted using AFM and DLS analyses. Of the two types of nanoaparticles, CNHs were found to better increase the stiffness of the injured tendons at the concentration levels tested. The unique structure of CNHs was captured among the native tendon tissue architecture with transmission electron microscopy (TEM) imaging, revealing a hedgehog-like appearance (Figure 4.1). In addition, significant insight into dispersion and distribution behavior of injected NPs was gained through an extensive histological study of the treated tendon. The studies presented in this thesis yielded profitable results, not only in regard to their individual foci, but also as solid foundational work when combined to move further into the investigation of creating a living composite ligament.

Future proliferant cell studies should include longer time periods to examine the response in collagen production of treated cells in comparison with controls. Any studies with longer cell culture periods should consider use of larger well plates in order to accommodate greater cell growth and collagen production. Multiple proliferants should also be investigated. Since there was a different response in cell viability to the proliferant P2G between the preosteoblast and
fibroblast cell populations, further investigation into potential cell type-specific response should be undertaken, and the demonstrated similar collagen production response between the two cell types should be more thoroughly explored. To measure the collagen production response of the cell populations to the proliferant treatment, reverse transcription polymerase chain reaction (RT-PCR) should be employed to measure the upregulation and downregulation of specific collagen genes and enzyme linked immunosorbent assay (ELISA) should be used to measure collagen production. *In vivo* studies should be conducted to compare the responses of isolated cell populations with those of complete biological systems to proliferant solutions. Delivery of growth factors to the injury site, such as through Platelet Rich Plasma (PRP) treatments, or the addition of mesenchymal stem cells, may be investigated in the laboratory with *in vivo* studies, as well.
Chapter 4. Summary and Future Work

Regarding NP treatment, additional cell studies should be conducted in order to more closely examine the cellular response to treatment with CNHs. The presented work indicates biocompatibility to a certain degree by cellular activity and microscopic observation; future work should further analyze the effect of NP presence on cell populations by assessment of extracellular matrix production and gene expression analysis using RT-PCR and ELISA. In addition, advanced microscopy should be employed to image more detail of cell and NP interactions.

The injection of CNHs into connective tissue should also be further investigated. Methods of better delivery of higher concentration solutions may be explored. Long term effects of NP reinforcement of connective tissue biomechanics should be assessed with cyclic loading and fatigue tests, and pre- and post-loading CNH distribution patterns observed through histological studies.

After the individual prolotherapy and NP treatments have been optimized through the above cell and tissue studies, animal studies should be conducted using the combination of these two effective treatments to further develop this novel, non-surgical treatment for hypermobile and damaged joints by creating a living composite ligament.
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