NEURAL AND COLLAGEN TISSUE PROPERTIES AS THEY RELATE TO GLENOHUMERAL JOINT LAXITY AND STIFFNESS

By

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Abstract

Shoulder instability is the most frequently occurring shoulder injury. Instability is caused by repetitive stress or a traumatic event that leads to excessive joint laxity. A major concern with instability is the high recurrence rate post treatment. Although the occurrence and recurrence of instability are primarily associated with capsule and labrum injury, the tissue characteristics of the capsule and labrum are not well understood. Therefore, we sought to explore the tissue properties of the capsule and labrum. We also explored the relationship between the tissue properties and passive joint motion.

To carry out this body of work, we dissected five fresh and three frozen human cadaveric shoulder pairs, removing the muscle and subcutaneous tissue while leaving the capsule and labrum intact. The cadaveric specimens included four males and four females of ages 23, 55, 58, 62, 76, 81 (x2), and 98. We then assessed glenohumeral joint laxity and joint stiffness using a materials testing machine. Following this assessment, we further dissected the shoulder removing the capsule and labrum from the anteroinferior (most common region of injury) and posteroinferior (least common region of injury) areas of the glenohumeral joint. The majority of the tissue was used for studying mechanoreceptors. A small portion was stored and used later for determining collagen content. Mechanoreceptor distribution and neural count were determined by first staining the tissue using our improved gold chloride staining method and light microscopy. Collagen content was determined using an acid-pepsin digestion for extracting collagen and the Sircol Collagen Dye assay for quantifying collagen.

Joint laxity and joint stiffness assessment revealed a significant relationship ($r = -.824$, $P<.001$) between joint laxity and joint stiffness where joint stiffness decreased as joint laxity increased. From the neural staining, we created neuroanatomical maps for the capsule and labrum based on mechanoreceptor distribution. Not only did we discover a mechanoreceptor

distribution pattern, but we also observed a strong positive relationship between neural count and joint laxity specifically in the capsule ($r = .710$, $p = .003$). Though this relationship was not significant in the labrum, when taking the capsule and labrum neural count into consideration, the overall relationship was significant ($r = .646$, $p = .009$). Unlike neural count and joint laxity, we did not observe a significant relationship between collagen content and joint stiffness in the capsule or labrum except when injury was taken into consideration. In the presence of injury, we observed strong negative relationships in the capsule ($r = -.803$, $p = .016$), labrum ($r = -.755$, $p = .030$), and overall, when including the capsule and labrum ($r = -.814$, $p = .014$).

Based on our results, we were able to identify potential contributing factors to the unstable shoulder that may help guide future treatment thereby reducing the recurrence rate. We identified mechanoreceptor scarce regions from the neuroanatomical map that may be more apt for surgical repair that allows for mechanoreceptor preservation. We also observed a relationship between neural count and joint laxity that further supports the need to preserve neural structure integrity during treatment. The relationship between collagen content and joint stiffness, in the presence of injury, suggests that changes in collagen content should be considered during rehabilitative treatment when joint stiffness is compromised. Joint laxity, joint stiffness, mechanoreceptor distribution, neural count, and collagen content are all factors that should be studied in greater detail when developing treatments for the unstable shoulder.

Acknowledgements

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CHAPTER 1

Introduction

1.1 Overview

Stability of the glenohumeral joint is provided by dynamic (muscles) and static (ligaments, capsule, and labrum) stabilizers (Fig. 1). Injury to these structures compromises their ability to stabilize the glenohumeral joint (Dumont et al. 2011). Capsular elongation, capsular microtears, and labral tears (Bankart lesions) are the most common pathological findings (Fig. 2) of instability; however the capsule (without ligaments) and labrum are the least studied tissues (Matsen et al. 2009, Pagnani and Warren 1994, Rowe et al. 1984). Capsular laxity and labral tears have also been shown to be the main causal factors of increased joint laxity related to recurrent glenohumeral joint instability (Hara et al. 1996, Omoumi et al. 2011).

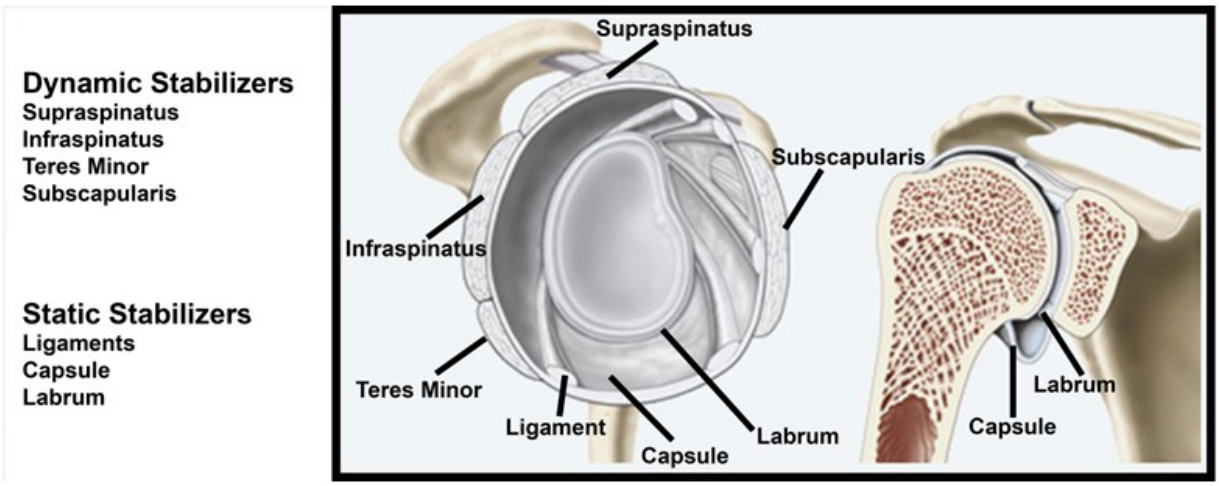

Figure 1. Illustration of dynamic and static stabilizers of the glenohumeral joint. (*Reconstructive Orthopaedic Center-Houston)*

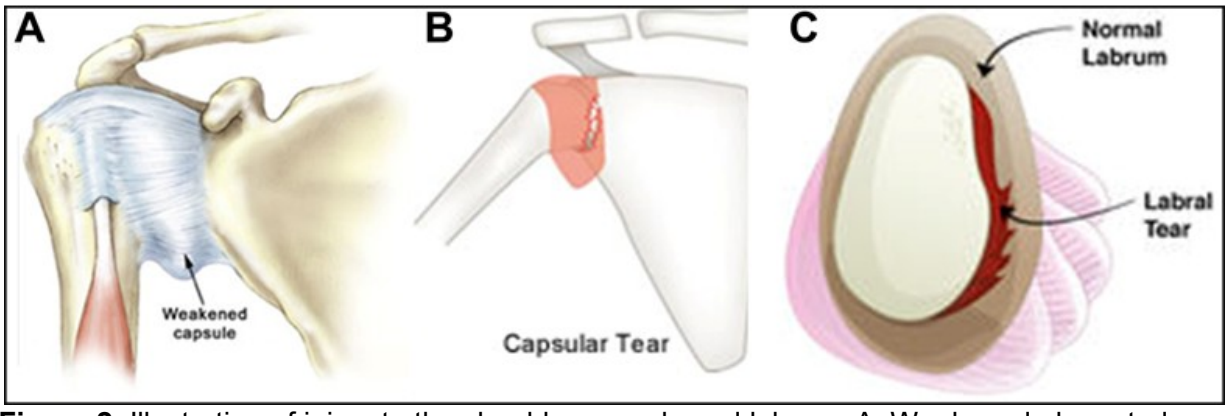

Figure 2. Illustration of injury to the shoulder capsule and labrum. A. Weakened elongated glenoid capsule. B. Microtear in the glenoid capsule. C. Microtear in the glenoid labrum. (*Injured Shoulder 2013;* Gollogly et al. 2013; *L. Mead 2013)*

The glenohumeral joint is a complex joint that sacrifices stability for mobility, and so laxity is a normal attribute of the shoulder (Taylor et al. 2009, Finnoff et al. 2004, Robinson and Dobson, 2004, Wilk et al. 1997). Consequently, the most frequently occurring shoulder injury is instability (subluxations and dislocations) which is characterized by excessive joint laxity. Figure 3 is an illustration of an unstable shoulder where the humeral head is dislocated. The unstable shoulder occurs secondary to repetitive stress or a traumatic event (Sherry 2003). In response, damage is incurred to the static stabilizers such as the capsule and labrum limiting their ability to resist glenohumeral traslation thereby contributing to excessive joint laxity. Eventually, excessive joint laxity leads to the occurrence and recurrence of subluxations and/or dislocations which can become career ending for the workforce as well as for athletes (Wang and Arciero 2008).

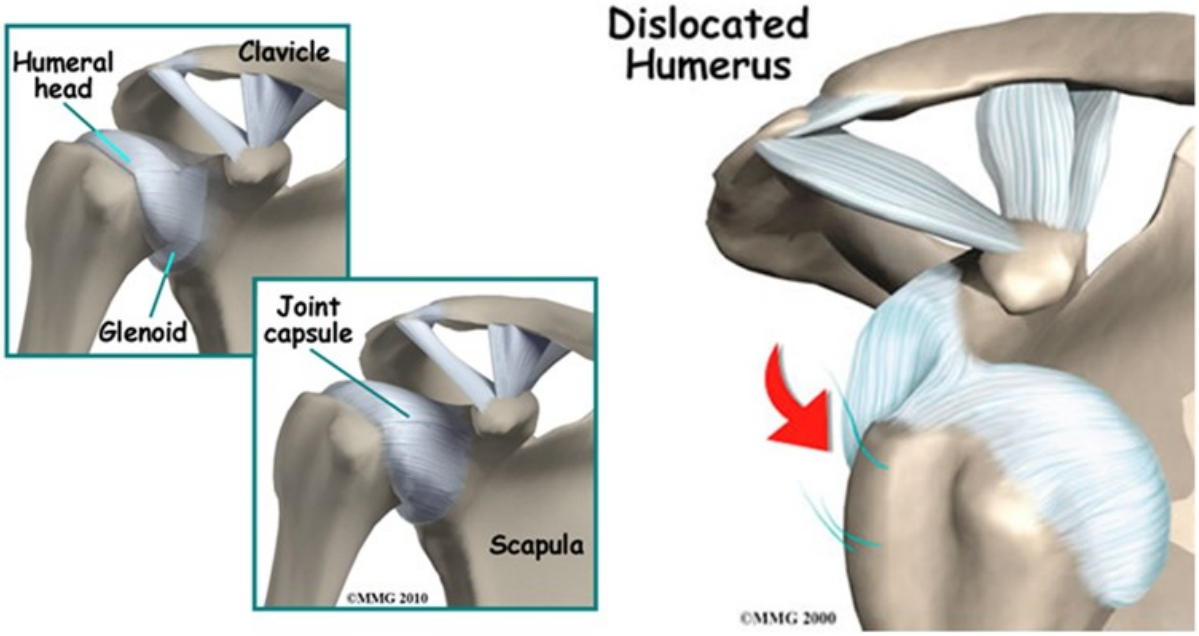

Figure 3. Illustration of a normal and a dislocated shoulder. (*A. Rumian 2000)*

Since the glenoid capsule and labrum are the primary stabilizers involved in glenohumeral joint instability, these tissues are the focus of our study. The ability of the capsule and labrum to limit glenohumeral translation is determined by their extracellular matrix with collagen being its major component (Rodeo et al. 1998), therefore we aimed to study the tissue

properties of these tissues and their relationship to joint laxity and stiffness. The tissue properites of focus in this study include the mechanoreceptors embedded in the collagen fibers, and the collagen content.

1.2 Structure and Function

1.2.1 Capsule

Generally, joint capsules function to stabilize synovial joints by preventing the loss of synovial fluid required for joint lubrication and limiting joint movement. The capsule of the glenohumeral joint not only prevents synovial fluid loss and limits glenohumeral translation, but it also allows mobility thus providing an inherent joint laxity. (Peat 1986, Singh 2005)

The glenohumeral joint capsule is reinforced by tendons and ligaments. Together, they are known as the cuff-capsule complex consisting of five layers (Fig. 4): 1) superficial layer (closest to muscle), 2) closely packed tendon fibers, 3) tendon fibers organized differently from layer two, 4) loose connective tissue with thick bands of collagen, and 5) the deepest layer which is a complex "thin (1.5-2.0mm thick) continuous sheet of interwoven collagen fibrils", otherwise known as the "true" capsule (Clark and Harryman 1992, Peat 1986, Rainis et al. 2009, Williams and Gillespie 2012). Most researchers, when describing the capsule, focus on the glenohumeral ligaments located between the fourth and fifth layers that reinforce the capsule (Rainis et al. 2009, Peat 1986). This study focused solely on the capsule

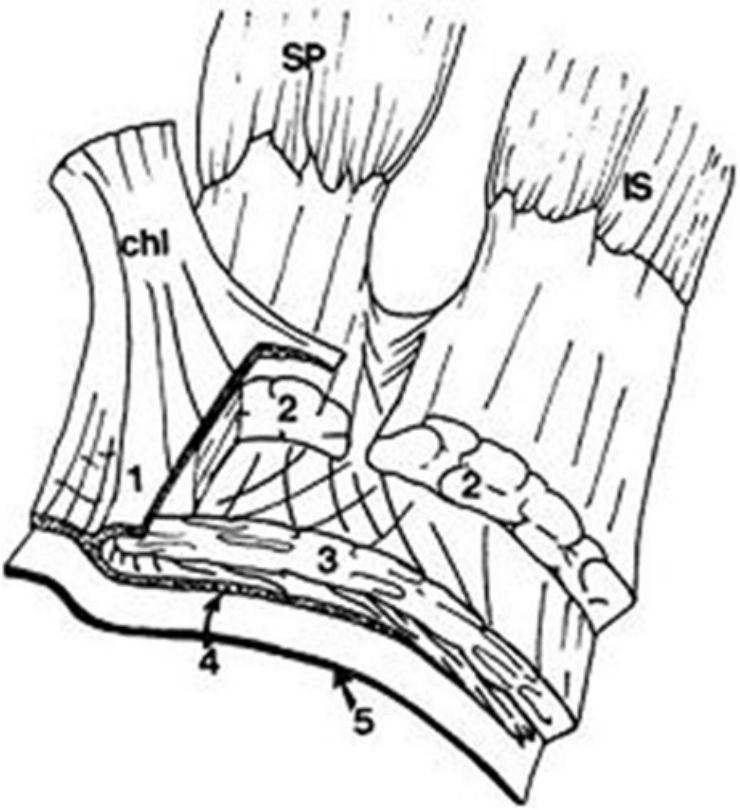

Figure 4. Illustration of the cuff-capsule complex layers: **1)** superficial layer (closest to muscle), **2)** closely packed tendon fibers, **3)** tendon fibers organized differently from layer two, **4)** loose connective tissue with thick bands of collagen, and **5)** the deepest layer which is a complex thin (1.5-2.0mm thick) continuous sheet of interwoven collagen fibrils. SP=supraspinatus, IS=infraspinatus, chl=coracohumeral ligament. *(Funk et al., 2005)*

without the reinforcements. Thus, we will refer to the capsule as the structure that has three layers only.

The glenohumeral joint capsule three layers include the synovial membrane layer, subsynovial layer and the dense fibrous layer (Morag et al. 2005, McFarland et al. 2002). Together, the layers form a flexible fibrous cuff around the glenohumeral joint by attaching to the labrum medially and the neck of the humerus laterally (Finnoff et al. 2004, Buckwalter 1999, Peat 1986). The most lateral aspect of the glenoid capsule consists of Sharpey's fibers, which are responsible for anchoring the capsule to the humerus (Clark and Harryman 1992). Since the capsule is loose fitting and flexible, the cuff allows for at least 2-3mm to 2-3cm of distraction (Peat 1986, Hammer 2005) and glenohumeral translation (Buckwalter 1999), respectively. The synovium is the deepest internal layer of the glenoid capsule. This layer is in direct contact with and lubricates the articular surface of the humeral head. (Clark and Harryman 1992)

The subsynovial layer consists of loose areolar connective tissue and adipose tissue (McFarland et al. 2002, Buckwalter 1999). Loose areolar connective tissue contains loosely packed coiled collagen fibrils (McFarland et al. 2002). Throughout the collagen fibrils are plexuses of small blood vessels and nerves that penetrate and supply the dense fibrous layer and the outer surface of the synovial layer (Buckwalter 1999).

The dense fibrous layer consists of "multilayered collagen fiber bundles of differing strength and orientation (Wilk et al. 1997)." (McFarland et al. 2002) Collagen fibers are oriented in a radial, circular, or spiral pattern. Radial fibers of the capsule are attached to each other by circular structures that allow rotational forces to build tension in these areas resulting in glenohumeral compression and the centering of the humeral head. Longitudinal stretch of the capsule in response to distractive forces also builds tension resulting in greater glenohumeral compression and intra-articular pressure. The collagen fibers with a circular orientation function to absorb stress and tension whereas the spiral shaped fibers are responsible for limiting joint

movement. (Wilk et al. 1997) More specifically, the absorption and transmission of forces by collagen fibrils allow the capsule to limit glenohumeral translation. In addition to collagens, the extracellular matrix contains proteoglycans which provide viscoelastic properties that allow for reversible transformation of the glenohumeral capsule after loading. (Silver et al. 2005) Not only does the capsule function to provide mechanical restraint, but it also aides in functional stability. Mechanoreceptors have been consistently identified in the capsule (Backenkohler et al. 1996, Guanche et al. 1999, Maass et al. 2001, Solomonow et al. 1996) and are of great importance. The presence of mechanoreceptors implies the capsule plays a role in joint proprioception influencing functional stability.

1.2.2 Labrum

The glenoid labrum provides stability to the glenohumeral joint by deepening the socket and limiting glenohumeral translation serving as a “chock block” (Matsen et al. 2009, Singh 2005). The structure of the labrum allows it to absorb forces and transmit loads from glenohumeral translation and compression during shoulder movements (Singh 2005). For this reason, it is currently suggested that the primary function of the labrum involves its ability to transfer and resist tensile forces, rather than just deepening the socket as it was previously thought (Smith et al. 2008).

The labrum more closely resembles, of all structures, the menisci of the knee joint. Each have a triangular cross-section, function in load transmission, and have three collagen fiber layers arranged to convert glenohumeral compressive and translation forces into “hoop” stresses (Singh 2005, Brindle et al. 2001, Carey et al. 2000). The three collagen fiber layers include the superficial mesh layer, circumferential braided core, and a peri-core zone (Hill et al. 2008; Cooper et al. 1992). These layers are thought to accommodate different types of loads (Hill et al., 2008).

The superficial mesh layer consists of a wrinkled fine fibril network (Hill et al. 2008) that is in direct contact with the humeral head as well as the glenoid cavity. The superficial mesh layer is suggested to be responsible for reducing surface friction with the humeral head through lubrication (Hill et al. 2008). The portion of the labrum that is in contact with the glenoid cavity consists of Sharpey's fibers. Sharpey's fibers are thick collagen fibrils that anchor soft tissue, in this case, the labrum to bone (Hill et al. 2008, Weiner 2010, Johnson and Martinez 1998). Post injury, in the periodontal ligament, Sharpey's fibers have been shown to reattach the ligament to the resorptive site of the bony surface (Johnson and Martinez 1998). This may also occur in the labrum post injury.

The innermost labral layer is the dense circumferential braided core. Collagen fibrils within this layer are radially oriented. Functionally, it is hypothesized that the braided core absorbs "hoop" stress. The ability of the core to absorb "hoop" stress would result in decreased contact stress between the labrum and chondral surfaces. (Hill et al. 2008) Tamai et al. noted the circumferential orientation was specific for accommodating "hoop" stress whereas the radial fibers therein function to resist forces that would lead to detachment of the labrum from the glenoid cavity (Tamai et al., 1986) as cited in (Hill et al. 2008, Tamai et al. 1986). The distribution of forces as a result of the "hoop" mechanism is hypothesized to prevent glenohumeral translation (Hill et al. 2008). The innermost layer, also called the central core zone, is suggested to be the most critical region of the labrum (Smith et al. 2008).

The peri-core zone is a loosely packed region of the labrum that surrounds the dense braided circumferential core. The fibril orientation of the peri-core zone implies this region also contains fluid and is able to respond to compressive loads in a viscoelastic manner (Hill et al. 2008). The extracellular matrix of any cartilaginous tissue is considered to have two phases, a solid phase and a fluid phase. The solid phase primarily consists of collagen and proteoglycans whereas the fluid phase consists of water and ions. It is important to note that the solid phase

has low permeability and thus a high frictional resistance to the flow of fluid. In turn, this creates high interstitial fluid pressure, which contributes to load transmission. Together, the increased frictional resistance due to low permeability and the resulting high pressure may be attributed to the stiffness and viscoelastic properties of cartilaginous tissue. (Pearle et al. 2005) In support of the viscoelastic properties, the peri-core zone has been proposed to absorb and disperse different forces while the innermost layer is the primary stabilizer (Nishida et al. 1996).

To summarize, the labrum has always been thought to provide limited stability, and has therefore been suggested to play a small role in joint stability (Lippitt et al. 1993). More recently, researchers have taken a greater interest in the structural organization of the labrum and its contribution to glenohumeral joint stabilization (Hill et al. 2008, Nishida et al. 1996, Smith et al. 2008). Within the last few years, a greater appreciation for the labrum and its role in instability has been established. Now, the labrum is believed to be a major contributor to glenohumeral joint stability (Karahan et al. 2012). The collagen fibrillar orientation, the distinct layers that make up the labrum, the load transmission throughout the labrum, and the labrum serving as an attachment site for other glenohumeral passive stabilizers (Karahan et al. 2012, Johnson 2007) are an indication that the labrum likely plays a greater role in instability beyond deepening the socket. In addition to its collagenous structure, the labrum has been proposed to possibly play role in joint proprioception. There have been three studies, to our knowledge, that sought to identify mechanoreceptors in the labrum and the findings of these studies are controversial (Guanche et al. 1999, Bresch and Nuber 1995, Vangsness et al. 1995). The controversies may be due to the staining techniques used. Together, the structural organization of the labrum and suggested presence of the mechanoreceptors in the labrum imply the labrum plays a critical role in passive and functional glenohumeral joint stability.

1.2.3 Summary

The glenoid capsule and labrum are fibrous connective tissues that rely on their collagenous structural organization to provide mechanical restraint, thus joint stability. In addition, the presence of mechanoreceptors identified within the collagens of these tissues allows the capsule and labrum to influence joint proprioception. For these reasons, this study focused on the tissue properties of the capsule and labrum including their collagen composition and the mechanoreceptors therein.

1.3 Capsule and Labrum: Tissue Properties

Tissue material properties influence the mechanical behavior (Hill et al. 2008). An understanding of the capsule and labrum tissue properties would provide further insight regarding the role of the capsule and labrum in glenohumeral stabilization.

1.3.1 Fibrous Connective Tissues

The glenoid capsule and labrum are comprised of collagen fibrils structurally organized to withstand forces with different collagen types providing the strength and elasticity of the tissue. “The strength of fibrous connective tissues, such as the joint capsule, is determined by the extracellular matrix” (Rodeo et al. 1998). Dense fibrous connective tissues are composed of an extracellular matrix (ECM) responsible for tissue mechanical and biochemical properties. (Silver et al. 2005, Peat 1986, Gelse et al. 2003) Two primary components of the ECM (Fig. 5) include collagen and ground substance (proteoglycans, glycosaminoglycans, and glycoproteins) with collagen being the most abundant component (Silver et al. 2005, Bach and Hasan 1997). Not only is collagen the primary protein of the ECM, but it is also the building block determining mechanical tissue properties. Several types of collagen exist. Collagen types I, II, III, V, and XI are the fibril forming collagens and are thus responsible for the structural framework of dense fibrous tissues. It has been reported that the joint capsule primarily consists of type I collagen and contains small amounts of type III collagen. (Silver et al. 2005) The other collagen types are often located at the soft tissue junction with bone (Silver et al. 2005) and are not of interest in this study and will not be discussed further.

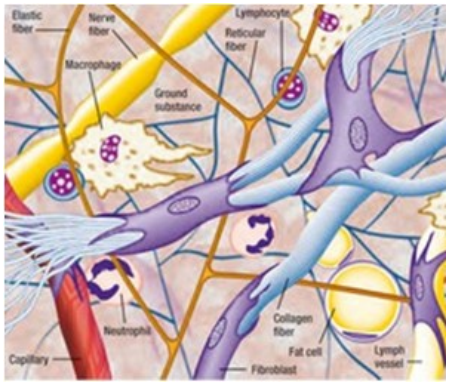

Figure 5. Illustration of connective tissue extracellular matrix. *(B. Cummings 2001)*

Type I and type III collagens are of clinical importance in the presence of tissue injury (Pataridis et al. 2009). Both collagen types are extractable from soft tissues, allowing for the analysis of

the collagenous tissue properties. Collagen concentration and its structural organization strongly impact the tissue mechanical behavior. (Holzapfel 2001)

1.3.2 Collagen

Collagen is considered to be the main load carrying molecule of soft tissues (Holzapfel 2001). Several factors influence the collagen composition, thus tissue integrity, including collagen fibril diameter, cross-linking, molecular organization, type, and concentration (Holzapfel 2001).

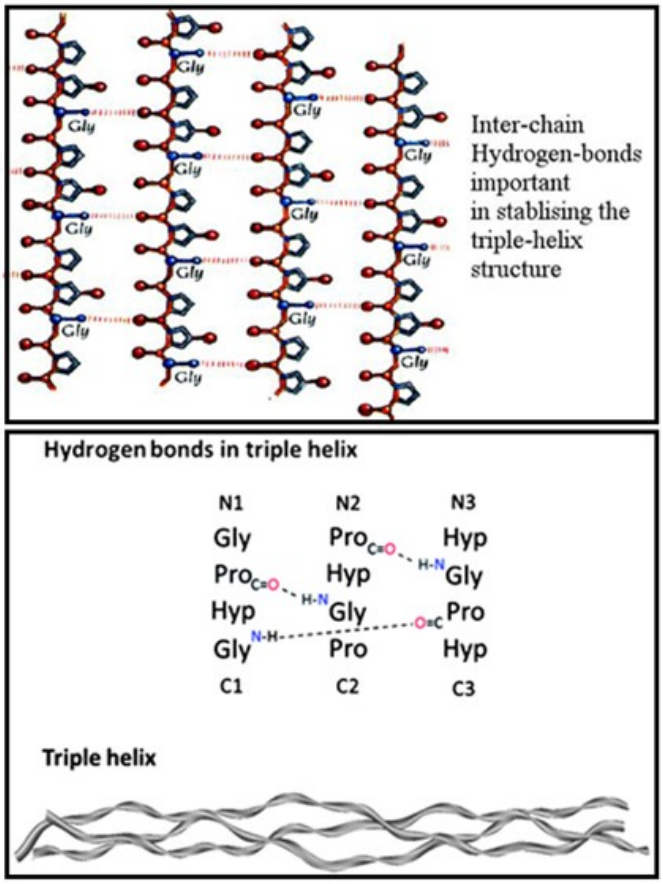

Figure 6. Illustrations of hydrogen bonds formed between glycine residues of the collagen alpha chains to hold the chains together in a triple helical formation. *(D. Jamieson, 2013; Maeda and Matsui, 2012)*

In articular tissues, type I collagen is the most abundant collagen, with type III collagen being the next in abundance of all collagens (Boudko et al. 2008). Type I and type III collagens are fibrillar forming collagens composed of different polypeptide chains. A type I collagen molecule is composed of three chains: two α_1 and one α_2, making it a heterotrimeric protein (not all three chains are the same). A type III collagen molecule is composed of three α_1 chains, making it a homotrimeric protein (all three chains are the same). The collagen polypeptide chains wind into a triple helix (Kadler et al. 1996). These polypeptide chains include two focal amino acids, proline and hydroxyproline, that form interchain hydrogen bonds responsible for

folding the chains together into a helix and maintaining their helical conformation (Brodsky et al. 2003, Freeman 2000).

The enhancement of the helical stabilization is achieved due to the fact that most collagen molecules consist of glycine (Gly) residues (33% of all amino acid residues) in addition to the proline (Pro; 15%) and hydroxyproline (Hyp; 15%) residues (Holzapfel 2001) making them the three most abundant amino acids that make up the triple helical structure of collagens. Together, they make up the repeating motif Gly-Pro-X where X represents any amino acid (Fig. 6). (Freeman 2000) Glycine is responsible for holding the three alpha chains together. More specifically, the side chain of glycine is a hydrogen atom. The hydrogen atom fits into the center of the triple helix forming a hydrogen bond with a polypeptide from an adjacent alpha chain, and these hydrogen bonds hold the alpha chains together. (Freeman 2000) Located at the ends (N- and C-termini) of the collagen helices are telopeptides. Cleavage and removal of the telopeptides allows the collagen molecules to form cross links with the triple helical structure of adjacent collagen molecules forming collagen fibrils (Canty and Kadler 2002, Kadler et al. 1996, Eyre et al. 1984). These collagen fibrils aggregate forming collagen fibers. Collagen fiber

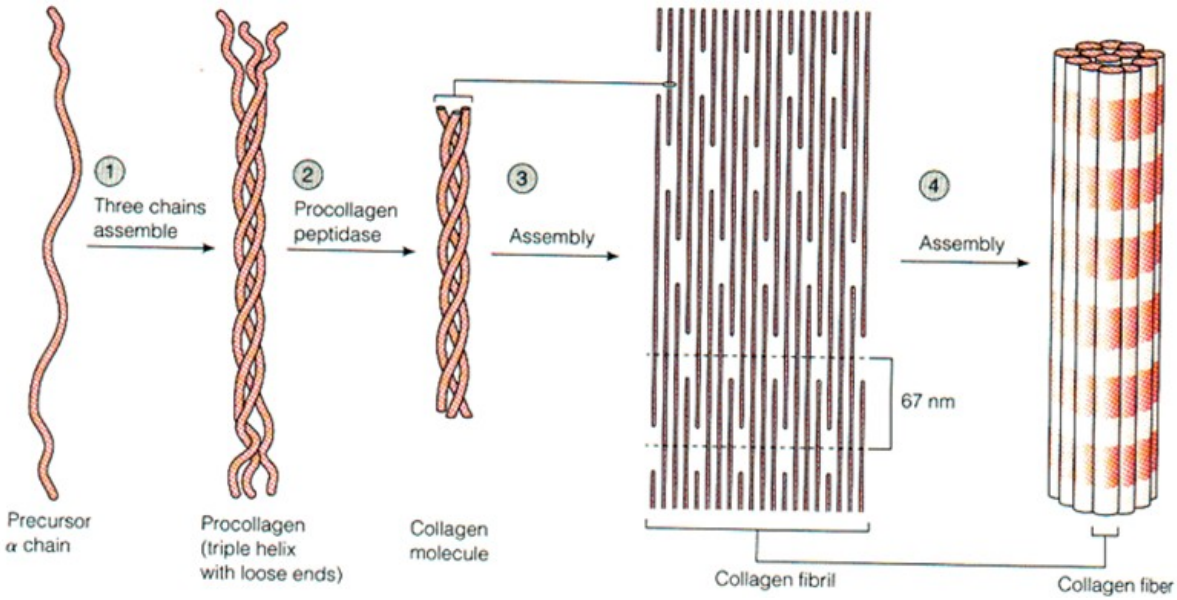

Figure 7. Illustration of collagen fiber formation. *(Pearson education, 2012)*

formation is illustrated in Figure 7. Intra and intermolecular crosslinking is not only critical for collagen fibril formation, but it also contributes to the tensile strength of tissues (Canty and Kadler 2002).

Type I collagen is characterized by its “rod-like structure” thus determining the strength of the tissue. It contributes to mechanical stability of the tissue and largely determines the functional properties of dense fibrous connective tissue (Silver et al. 2005, Berisio et al. 2008). Type III collagen is responsible for elastic properties of tissue thus contributing to tissue flexibility. In fibrous tissues, type III collagen is mixed within type I collagen fibrils at low levels (Boudko et al. 2008, Gelse et al. 2003).

Within this study, we analyzed the collagen content in the glenoid labrum and capsule from areas that correspond to the most common (anterior) and least common (posterior) sites of injury. To further explore the role of both the glenoid capsule and labrum tissue properties in shoulder stability, we determined whether their total soluble fibrillar collagen correlated with glenohumeral joint laxity and stiffness.

1.3.3 Mechanoreceptors

The capsule and labrum not only contribute to glenohumeral joint mechanical stability by providing passive restraint given their collagen content and composition, but these tissues also contribute to glenohumeral joint functional stability. Embedded within the collagens of the capsule (with ligaments) (Bresch and Nuber 1995, Backenkohler et al. 1996, Maass et al. 2001, Solomonow et al., 1996) and in the labrum, although this is currently controversial (Bresch and Nuber 1995, Guanche et al 1999, Vangsness et al. 1995), are mechanoreceptors which function in joint proprioception. (Myers and Lephart 2002, Wilk et al. 1997)

Mechanoreceptors are sensory receptors characterized by their specialized nerve endings, surrounding a nerve terminal of articular nerve fibers, that are sensitive to mechanical displacement (Freeman et al. 1965, Kandel et al. 2000, Warner et al. 1996). In articular tissues,

these receptors are also called sensory nerve endings, proprioceptive mechanoreceptors, or proprioceptors (Warner et al. 1996, Purves et al. 2001). Freeman and Wyke developed a numerical classification system for the nerve endings to avoid the confusion and varying terminology used to describe them (Freeman and Wyke 1967, Hogervorst and Brand 1998). There are four types of nerve endings (Table 2) grouped based on their primary morphological features, types 1 through 4 (Freeman and Wyke 1967). Each type has different functional characteristics and responds to different mechanical stimuli. In this study, we are interested in type 1 (Fig. 8) and type 2 (Fig. 9) sensory nerve endings as these are the only mechanoreceptors previously identified in these tissues.

Table 2. Classification of mechanoreceptors according to Freeman and Wyke (1967).

Kind	Morphology	Diameter (µm)	Functional Characteristic	Eponyms
1	Globular or ovoid	5-8 (myelinized)	Mechanoreceptor; slow-adapting, low threshold	- Ruffini's - Golgi-Mazzoni endings - Meissner's Corpuscle
2	Cylindrical or tapered	8-12 (myelinized)	Mechanoreceptor; fast-adapting, low threshold	- Krause's vater - Vater-Pacini Corpuslce
3	Spindle-like	13-17 (myelinized)	Mechanoreceptor; very slow-adapting, high threshold	- Golgi endings - Golgi-Mazzoni corpuslce
4	-Non-myelinized plexus -- Non myelinized free endings	-2-5 (myelinized) -<2 (non-myelinized)	- Pain receptor - Pain receptor	N/A

Type 1 and type 2 nerve endings have varying morphology that with time resulted in different descriptive names. In articular tissues, type 1 nerve endings are generally known as Ruffini Corpuscles or Ruffini endings whereas type 2 nerve endings are generally known as Pacinian Corpuscles. (Johansson et al. 2000) Both types of mechanoreceptors have been

identified in articular tissues (Borsa et al. 1994). Mechanoreceptors with Ruffini endings (type 1) are slow adapting, and so they respond to prolonged and constant stimuli such as stretch, compression, and rotation (Kandel et al. 2000). Type 2 nerve endings (Pacinian), on the other hand, are rapid adapting receptors that respond to the beginning and end of stimuli (Kandel et al. 2000).

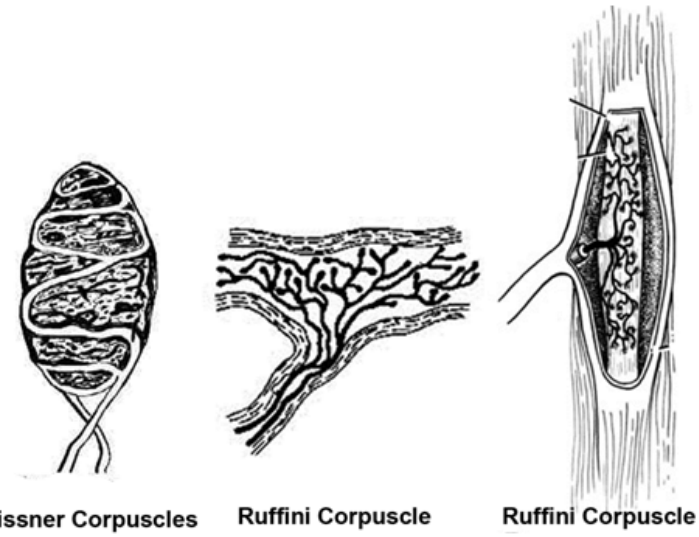

Figure 8. Drawings of type 1 sensory nerve endings. *(H. Gray, 1918; library.thinkquest.org; myhumanbody.ca)*

1.3.3.1 Mechanoreceptors and Joint function

Mechanoreceptors, in articular tissues, function to transduce mechanical deformation into neural signals that transmit proprioceptive information along the afferent pathways (Riemann and Lephart 2002, Myers et al. 2009). Proprioception is a neurologic feedback mechanism that provides information about joint position and joint motion (Lephart et al. 1997), which is critical for neuromuscular control (Riemann and Lephart 2002). Ruffini Corpuscles and Golgi Tendon Organ (GTO)-like Ruffini endings have low threshold slow adapting

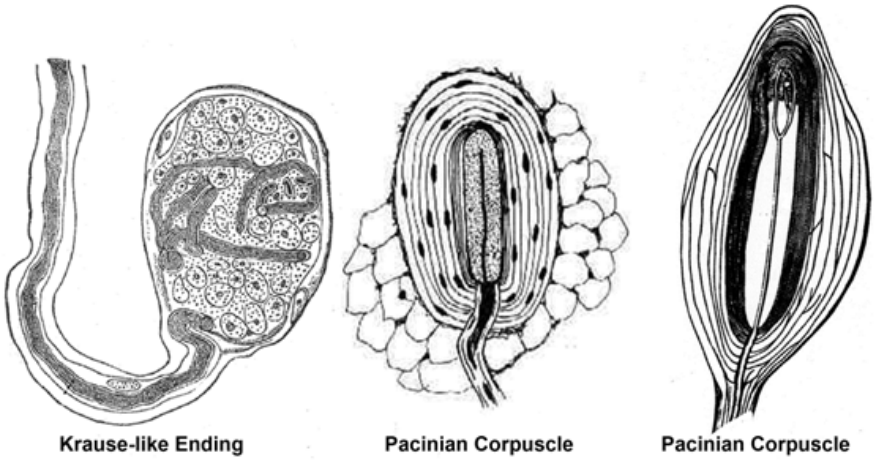

Figure 9. Drawings of type 2 sensory nerve endings. *(H. Gray, 1918; K. Hertzog, 2002; intropsych.com)*

characteristics allowing these nerve endings to register joint position (Borsa et al. 1994). “Ruffini corpuscles and Golgi Tendon Organ-like endings of the capsuloligamentous structures are stimulated most during motion and changes in joint position” (Pagnani and Warren 1994). Ruffini endings, identified by histochemistry in the cat knee capsule, elicited neural signals after exposure to axial and compressive loads. The length of time the stimuli was applied and the continued neural signals during that time confirms these nerve endings are slow adapting. (Grigg and Hoffman 1982) Pacinian Corpuscles, on the other hand, are low threshold rapid adapting sensory nerve endings that function to register joint motion. More specifically, these structures register the acceleration and deceleration of joint motion. (Borsa et al. 1994) Pacinian Corpuscles have been shown to demonstrate a neural discharge rate that ends within milliseconds in response to a continuous stimuli (Frontera et al. 2006, Lephart et al. 1992) supporting their rapid adapting characteristics.

Proprioceptive information signaled by these mechanoreceptors provides efferent control over neuromuscular activity thereby permitting reflexive contractions of the muscles surrounding the joint. The resulting joint stiffness provides dynamic (functional) stabilization during joint motion (Warner et al. 1996, Nyland et al. 1998, Frontera et al. 2006). Dynamic joint stability is considered “the product of the proprioceptive system” (Frontera et al. 2006). It refers to the ability of the muscular and mechanical stabilizers of a joint to work together (Frontera et al. 2006) thereby creating a balance between inherent joint laxity and joint stiffness.

1.4 Glenohumeral Joint Laxity and Stiffness

Glenohumeral joint laxity is a normal attribute of shoulder motion. Inherent joint laxity refers to passive translation of the humeral head on the glenoid fossa (Wilk et al. 1997). Passive stabilizers of the glenohumeral joint, function to allow a limited amount of glenohumeral translation and to resist excessive translation (Sein et al. 2010). Theoretically, injury to these stabilizers due to microtrauma results in increased glenohumeral translation presented as excessive joint laxity with decreased joint stiffness (Crawford and Sauers 2006). Joint stiffness is considered to be an important clinical variable for assessing glenohumeral joint instability (Wright 1970; Woo 1990). It is characterized by the magnitude of force required to resist glenohumeral translation thereby limiting the amount of laxity allowed in the joint (Borsa et al. 2002). Essentially, stiffness is considered to be a better indicator of tissue mechanical behavior since it encompasses displacement as a function of applied forces (McQuade et al. 1999).

From a clinical perspective, anterior, posterior, and inferior are the most important directions of glenohumeral translation (Borsa et al. 2002). Since anteroinferior is the most common direction of instability and posterior is the least common, we focused on anterior and posterior laxity and stiffness. Figure 10 demonstrates glenohumeral translation in the anterior and posterior directions. Between studies, there have been a broad range of values for anterior-posterior laxity in the "normal shoulder" ranging from 10 to 32 mm (Borsa et al.

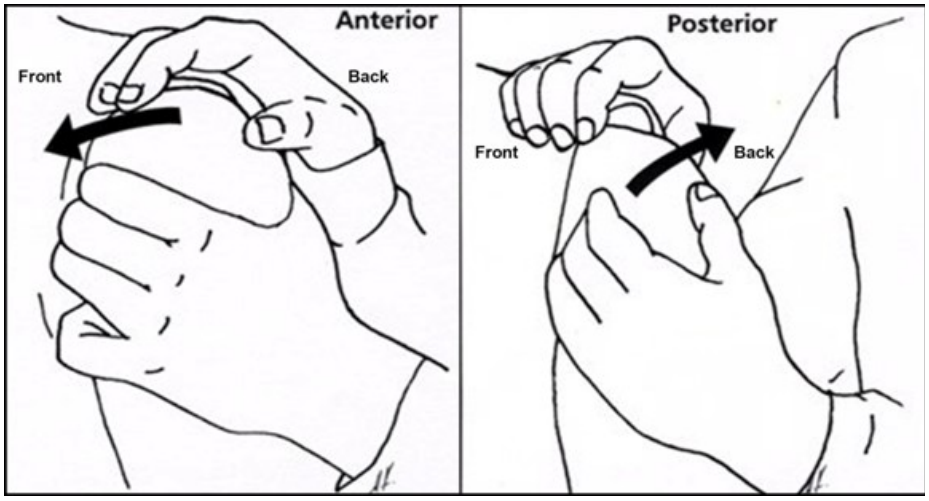

Figure 10. Illustration showing the direction of anterior and posterior glenohumeral translation. *(Worldortho.com)*

2000, Pizzari et al. 1999, Marquardt et al. 2006, Borsa et al. 2001, Sauers et al. 2001). In the presence of injury, the amount of glenohumeral joint laxity increases (Marquardt et al. 2006). Controversy is involved in differentiating between normal joint laxity and excessive joint laxity since judgement may vary from clinician to clinician and the amount of laxity in the joint may vary from person to person (Carpenter et al. 1998). For these reasons, shoulders are compared bilaterally. More importantly, joint stiffness seems to be as clinically relevant as joint laxity for assessing joint stability (Wright 1973; Woo et al. 1990). The amount of stiffness within the joint is considered to be characteristic of the joint structural and mechanical properties (Borsa et al. 2002). During loading, a stiffer joint has been shown to be able to absorb more forces compared to less stiff joints. It is therefore suggested that a stiffer joint has a reduced risk of instability. The amount of stiffness in the normal shoulder ranged from 9.2 to 28.4 N/mm depending on the direction of translation (Anterior: 9.6-26.6; Posterior: 10.4-23.4). (Borsa et al. 2002)

Occupation and repetitive activities influence glenohumeral translation as does gender. Women, compared to men, presented with significantly greater anterior joint laxity and concomitant decreased joint stiffness than posterior whereas men exhibited greater posterior joint laxity than anterior (Borsa et al. 2000). However, no significant difference was found in glenohumeral joint stiffness between genders and the direction of translation (Borsa et al. 2002). In summary, inherent joint laxity varies depending on occupation, frequency in extracurricular overhead activity participation, and gender. Joint stiffness varies with varying amounts of glenohumeral joint laxity. Although the amount of joint laxity varies, there is excessive joint laxity with reduced stiffness in comparison to the norm when an injury is incurred by the static stabilizers of the glenohumeral joint.

1.5 Glenohumeral Joint Stability: The Alliance

Stability within the glenohumeral joint refers to the "state of the humeral head remaining or promptly returning to proper alignment within the glenoid fossa through an equalization of forces" (Myers et al. 2006). In theory, the capsule and labrum have two lines of defense that work together to contribute to stability of the glenohumeral joint: mechanical restraint and proprioception (Buckwalter 1999, Nyland et al. 1998). Both mechanical restraint and proprioception limit glenohumeral translation thereby regulating the amount of laxity in the joint.

Mechanical restraint provided by the glenoid capsule and labrum contributes to glenohumeral stability by absorbing the tensile forces (Smith et al. 2008, Wilk et al. 1997) resulting from joint movement and physically limiting translation functioning as barriers (Snyder 2003, Wilk et al. 1997). It is believed that proprioception is dependent on the tissue structural properties and the ability of the tissue to generate tension (Nyland et al. 1998). Fukami and Wilkinson (1977) studied mechanoreceptor potentials in response to varied static tension in the muscles of cats. They found the magnitude of receptor potentials to be proportional to the applied static tension. (Fukami and Wilkinson 1977) Essentially, this study showed that the neural response of mechanoreceptors becomes greater as the tension builds in tissues. Activation and signaling of the mechanoreceptors in response to joint motion (mechanical stimuli) and tension in the connective tissue result in a protective neuromuscular response around the joint further contributing to joint stiffness (Nyland et al. 1998). During the midrange of glenohumeral movements, the capsule is lax. However, it is suggested that tension builds, during midrange, increasing the stiffness of the capsule in response to rotator cuff muscular contractions. For this reason, the capsule is considered to be a part of the dynamic stabilizing system. (Finnoff et al. 2004).

In summary, the alliance between the mechanical and proprioceptive properties of the connective tissues provides two means of limiting and controlling glenohumeral translation. These tissue properties contribute to both mechanical and functional stability.

1.5.1. Mechanical Stability

Glenohumeral compression and translation inflict direct stress on the capsule and labrum. Gross shoulder movements are accompanied by compression and the coupling of humeral head translation and rotation on the glenoid fossa.

During active shoulder movements while the passive tissues are lax, the dynamic stabilizers compress the humeral head into the glenoid fossa consequently increasing the load required to translate the humeral head (Hammer 2005, Lephart and Jari 2002). It is believed that joint compression combined with an intact labrum together are effective in stabilizing the joint during midrange movements (Pagnani and Warren 1994). Halder et al. (2001) assessed the stability of the glenohumeral joint with and without an intact labrum using a stability ratio (peak translational force/peak compressive force) where the compressive forces were controlled. Overall, the results from this study showed the stability of the glenohumeral joint varied with position. The glenoid with an intact labrum, showed a higher stability ratio in the inferior, posteroinferior, and anteroinferior directions in comparison to the glenoid without a labrum. (Halder et al. 2001) Based on the stability ratio, these results suggest more force is required to translate the humeral head with an intact labrum versus the absence of a labrum supporting the effectiveness of combined compressive forces and intactness of the labrum. Negative intra articular pressure within the joint, maintained by the capsule, also provides stability to the glenohumeral joint and is most effective in resisting translation when the muscles, capsule, and capsular ligaments are fairly lax (Cole et al. 2007, Taylor et al. 2009). Negative intra articular pressure has a vacuum stabilizing effect that prevents instability more so in the inferior direction (Finnoff et al. 2004).

The joint dynamic stabilizers are not only responsible for creating compressive forces, but they also initiate movements that lead to the tightening of the capsule and capsular ligaments (Taylor et al. 2009). As mentioned previously, the capsule and capsular ligaments are more lax during midrange movements. However, at end range movements, the capsule and capsular ligaments passively tighten and are responsible for limiting translation and centering the humeral head in the glenoid fossa (Halder et al. 2001, Pagnani and Warren 1994).

1.5.2 Functional Stability

Interaction between mechanical (capsule and labrum) and dynamic (musculature) restraints yields functional stability mediated by the sensorimotor system (Myers et al. 2006). The sensorimotor system allows for integration of proprioceptive information with other areas of the nervous system resulting in an efferent motor response, hence neuromuscular control (Myers et al. 2004). In response to joint movement, the articular tissues are placed under tension resulting in mechanical deformation of the mechanoreceptors within the tissues. Subsequently, electrical signals from the mechanoreceptors are sent to the central nervous system. (Pagnani and Warren 1994) Previous research has already shown that glenohumeral joint mechanoreceptors stimulate a protective muscular response for proper limb stiffness in relation to the perceived articular surface and load (Nyland et al. 1998).

The shoulder relies on proprioceptive input from several structures including the dynamic tension of the glenoid capsule (Myers et al. 2004). Stimulation of the mechanoreceptors of the glenoid capsule, including the axillary nerve branches, elicits electromyographic activity from the rotator cuff muscles and biceps brachii (Guanche et al. 1995). The loss in a synergistic muscular response of the unstable shoulder, results in repetitive microtrauma (Bicos et al. 2006, Frontera et al. 2006, Lephart et al. 1997). Repetitive microtrauma, as mentioned previously, results in instability.

1.6 Instability: General

Ninety-five percent of glenohumeral joint instability cases occur in the anterior and anteroinferior directions (Sherry 2003). Since anterior instability is the most prevalent, ***this was the focal point of our study*** with secondary analysis of posterior instability. Instability only exists when opposing sides of the capsule are damaged (Halder et al. 2001). Clinically, glenohumeral joint instability is determined by several tests. These tests include the load and shift, apprehensive, relocation, anterior release and sulcus tests. The gold standard for assessing anterior and posterior instability is the load and shift test when used with the Hawkins grading scale to create consistency. (Tzannes and Murrell 2002) It is believed by many clinicians that shoulder laxity is difficult to assess, in particular, to distinguish between normal or pathologic laxity (Carpenter et al. 1998).

To appreciate joint stability, clinicians must be able to recognize instability in relation to not only laxity, but also stiffness (Huxel et al. 2008). Repetitive stress elongates the glenoid capsule as well as introduces microtears to the glenoid capsule and labrum compromising their ability to provide passive restraint (McCluskey and Getz 2000, Myers et al. 2004, Wilk et al. 2009).

1.6.1 Mechanical Instability

Compromised stabilizing structures result in a disruption of capsular integrity and increased capsular volume (Myers et al. 2004). Most repetitive overhead motions induce persistent stress to the anterior capsule increasing joint laxity (Krishnan et al. 2004). Magnetic resonance arthrography demonstrated that capsular elongation was prominent in shoulders with anterior dislocation in human subjects. The anteroinferior capsule was significantly elongated on the involved side compared to the uninvolved side. (Urayama et al. 2003)

Excessive lengthening of the anterior capsule may result in labral damage posteriorly affecting movement not only anteriorly, but also anteroinferiorly and posteriorly with overhead

activities. Overhead movements, such as the cocking phase of throwing (Fig. 11), include abduction and horizontal abduction accompanied with external rotation (Krishnan et al. 2004; Scolaro and Kelly, 2010). Abduction involves an inferior translation of the glenohumeral head whereas the humeral head glides anteriorly with horizontal abduction and external rotation. Increased external rotation, in the throwing shoulder, results in decreased internal rotation leading to the development of a posteroinferior capsular contracture (Crawford and Sauers 2006, Whiteley 2007). In the absence of normal posterior laxity, the amount of contact between the humeral head and posterosuperior glenoid cavity is increased making the labrum susceptible to damage.

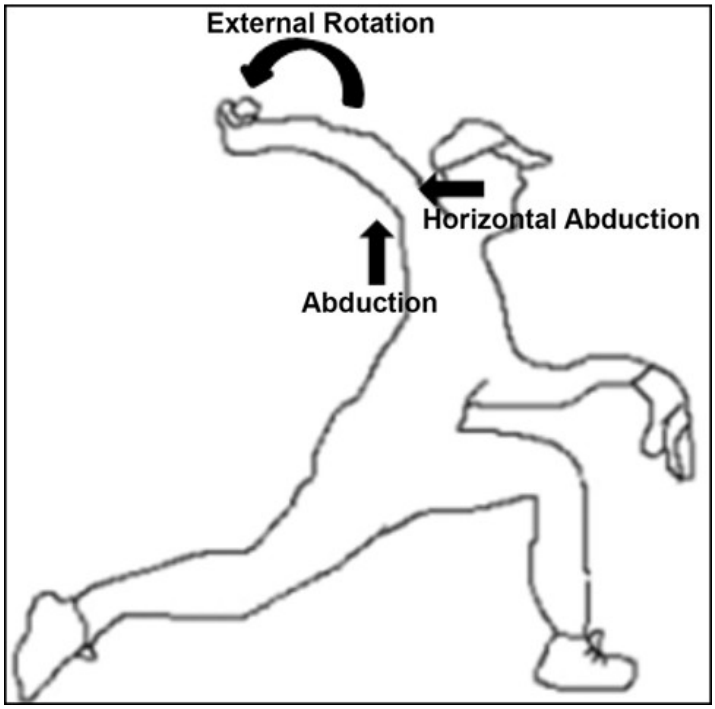

Figure 11. Illustration of the late cocking phase of the throwing shoulder demonstrating external rotation, abduction, and horizontal abduction. *(safethrow.com)*

Labral tears resulted in a 20% reduction in passive restraint (Lippitt et al. 1993, Pagnani and Warren 1994). “Excision of the glenoid labrum decreases the depth of the socket by 50% and reduces the resistance to instability by 20% (Johnson 2007)”.The greatest reduction in resistance provided by the labrum occurred inferiorly and posteroinferiorly thereby increasing the amount of translation in these directions (Pagnani and Warren 1994). Efficient compression of the humeral head in the glenoid fossa is compromised with labral injury reducing the amount of force required for translation. In addition, a tear in the labrum disrupts the intraarticular negative pressure and vacuum effect of the glenohumeral joint. (Hammer 2005) As a result, the

shoulder becomes unstable (Hammer 2005), especially in the inferior direction (Finnoff et al. 2004).

In essence, the capsule and labrum play an important role allowing joint laxity and preventing excessive joint laxity by resisting glenohumeral translation.

1.6.1.1 Collagen and Injury

Prior to disruption of the capsule and/or labrum, both structures are able to provide stability, thereby restricting glenohumeral translation, defined in large by their collagen composition (Rodeo et al. 1998). Shoulder laxity may result from repetitive microtrauma, which may be symptomatic or asymptomatic. As a result, injury to the glenohumeral joint stabilizers may persist for months or years before diagnosis and treatment thereby disrupting proper tissue healing. (Wang and Flatow 2005, Grigg and Hoffman 1982, Yuushi and Anri 2001, Cole et al. 2007, Iannotti and Williams 2006) In turn, abnormal collagen composition may persist for many months post injury which may be contributory to the development of shoulder instability (Riley 2008).

To date, a few pieces of evidence are available about collagen changes as it relates to the tissue/organ function or dysfunction; however, the glenohumeral joint has not been studied in this regard. In the presence of injury, the type III collagen levels increase relative to the amount of type I collagen in tendons (Lui et al. 2010, Eriksen et al. 2002). Dysplastic hip joint capsules, compared to normal hips, in greyhounds, contained a higher ratio of TIIIC to TIC and increased soluble collagen fraction (Todhunter and Lust 2003). Collagen degeneration, in addition to increased amounts of ground substance and decreased TIC to TIIIC ratio, was detected in overuse tendon injuries including tendonitis and tendinosis (Wilder and Sethi 2004). In this study, we evaluated the relationship between collagen content in the capsule and labrum and glenohumeral joint laxity. **We expected** the collagen content to vary as joint stiffness varied.

1.6.2 Functional Instability

"The combination of mechanical deficits and sensorimotor alterations contribute to deficits in functional stability" (Myers et al. 2006). Ligaments have been shown to provide mechanical restraint as well as contribute to neurologic feedback, due to presence of mechanoreceptors, by regulating muscle reflexes for joint stabilization. Partial deafferentation in the ligament following injury results in abnormal neuromuscular joint stabilization. (Borsa et al. 1994) Furthermore, partial deafferentation leads to repetitive injuries and a progressive decline in the joint. (Borsa et al. 1994) Finally, partial deafferentation occurs in response to trauma to mechanoreceptor containing tissues ultimately resulting in proprioceptive deficits making the joint more susceptible to reinjury (Lephart et al. 1997). Articular deafferentation includes mechanoreceptor damage or degeneration of the nerve fibers that terminally end in mechanoreceptors resulting in disruptive neural signaling thereby altering proprioceptive information (Freeman et al. 1965, Delforge 2002). Similar to ligaments, previous studies have identified mechanoreceptors in the glenoid capsule and labrum as previously mentioned. The presence of mechanoreceptors in these tissues suggests they also register glenohumeral joint position and motion (Borsa et al. 1994).

Lephart et al. (1994) analyzed the threshold for detecting passive range of motion in the stable shoulder, the unstable shoulder after pre-surgical rehabilitation, and the post surgical shoulder. The unstable shoulder pre surgery presented with a higher threshold for detection of passive movement compared to the "normal" and "post-surgical" shoulders. These results suggest that damage to capsuloligamentous tissue is accompanied by partial deafferentation, which leads to proprioceptive deficits and possible reinjury. (Lephart et al. 1994)

In the absence of partial deafferentation, proprioceptive deficits may still exist in the unstable shoulder. Increased tissue (capsular) laxity reduces the amount of tension in the capsule, which limits the ability of the mechanoreceptors to detect movement and change in

tissue tension. (Myers and Lephart 2000) Restored proprioception post surgery implies that reestablished capsular tension allows mechanoreceptors within the capsuloligamentous tissue to be properly activated in response to mechanical stimuli. (Tibone et al. 1997) However, surgical intervention for restoring mechanical restraint of the capsuloligamentous structures to the unstable shoulder provides only partial restoration of proprioception. (Frontera et al. 2006, Lephart et al. 1997, Bicos et al. 2006)

Warner et al. showed greater passive shoulder motion and decreased proprioceptive ability in shoulders with anterior instability compared to normal shoulders (Warner et al. 1996). Subjects with anterior instability demonstrated increased peak activation of the rotator cuff muscles; however, they presented with a significant reduction in the biceps brachii reflex. In addition, co-activation of the supraspinatus and subscapularis was significantly suppressed (Myers et al. 2004). Previous studies have shown that shoulder joint dysfunction, including muscular incoordination, may be associated with joint proprioception deficiency. Significantly reduced proprioceptive abilities were demonstrated in subjects with recurrent unilateral anterior dislocations (Carpenter et al. 1998, Smith and Brunolli 1989). These data suggest there is a disruption in the proprioceptive pathway between the dynamic and static stabilizers resulting in muscular incoordination. “Fine motor coordination requires accurate afferent input from the joints and muscles involved. These signals come from mechanical receptors in the periarticular tissues.” (Carpenter et al. 1998)

1.6.2.1. Mechanoreceptors and Injury

Theoretically, proprioceptive deficits result from injured neural structures, a decreased number of mechanoreceptors, or abnormal activation of uncompromised neural structures (Tibone et al. 1997). ”Mechanoreceptor type, density, distribution, and development reveal contrasts related to muscle function, but the significance of these findings and their location within muscles acting at the glenohumeral joint are presently unknown (Nyland et al. 1998).” To

the best of our knowledge, mechanoreceptor count and anatomical distribution has not been described for the capsule or labrum. Increased joint laxity from capsular elongation, damage to mechanoreceptors, decrease in proprioceptive feedback due to mechanoreceptor sensitivity, and repetitive microtrauma are proposed to be the most prominent causes of diminished proprioception (Hammer 2005).

Proprioceptive deficits in the unstable shoulder not only result from mechanoreceptor damage but also from increased capsular laxity. Increased tissue laxity reduces the amount of tension in the capsule, which leads to abnormal mechanoreceptor activation limiting the ability of mechanoreceptors to detect movement and a change in tension. (Myers and Lephart 2000, Hammer 2005) Excessive joint laxity in the female knee seems to contribute to diminished joint proprioception. Knee joint proprioception and anterior tibial translation were measured in male and female athletes. The females had a significantly greater amount of anterior tibial translation thus a higher degree of joint laxity compared to males. In addition, the threshold for detecting anterior tibial translation during knee flexion was significantly higher in females than males, thus taking longer to detect joint movement. (Rozzi et al. 1999) In essence, proprioceptive deficits result from mechanoreceptor damage due to joint injury and capsular laxity/elongation in response to joint injury.

To extend proprioceptive research and to contribute to the shoulder instability field, ***our study*** focused on determining whether the number of mechanoreceptors related to glenohumeral joint laxity. The amount of mechanoreceptors may be a key component in shoulder instability occurrence as well as recurrences. Proprioceptive research in the glenohumeral joint extends to the capsule with less attention given to labral injury. However, we believed the contribution of the labrum to proprioception was warranted studying.

Labral lesion size affects glenohumeral translation such that the greater the lesion, the more glenohumeral translation is present (Tauber et al. 2004). We believe the labral lesion size

may also be contributory to a reduction in mechanoreceptor number such that the location of the labral lesion (loss of labral tissue or a labral tear) may affect the amount or integrity of mechanoreceptors. In addition, different types of surgery, including plications, shifts and overtightening of the capsule, contribute to disrupting mechanoreceptor orientation and concentration thereby interrupting communication between the muscle and joint (Nyland et al. 1998). A reduction in neural number secondary to surgery and/or injury may result in decreased spatial details (characteristics joint position and orientation in space) thus resolution related to joint position sense, a delay in muscle contraction at the beginning and the end of a stimulus and/or a shortened prolonged stimulus (Kandel et al. 2000). The above discrepancies are all possible causal factors for functional instability.

1.6.3 Summary

"It is proposed that one mechanism for gradual development of shoulder instability may be cumulative injury to capsuligamentous structures with loss of the proprioceptive feedback mechanism and thus reflexive muscular protection against excessive glenohumeral translation and rotation" (Warner et al. 1996). Essentially, repetitive microtrauma to the glenohumeral joint leads to cumulative injury to the static stabilizers resulting in mechanical and functional instability. Injury incurred by the static stabilizers includes capsular elongations, tears in the capsule, and/or tears in the labrum. These injuries result in mechanical instability including excessive glenohumeral translation during shoulder movements. Subsequent damage, in response to tissue injury, includes the deafferentation or loss of mechanoreceptors within the injured articular tissues. Mechanoreceptor damage, on the other hand, leads to proprioceptive deficits resulting in functional instability. Functional instability, in turn, repeats the cycle of repetitive microtrauma to the stabilizers of the glenohumeral joint and the resultant injury/damage contributing to recurrent instability.

1.7 Clinical Relevance

"Laxity testing of the shoulder in an anterior and posterior direction has been recommended as a tool for determining nonoperative or operative treatment" (Bahk et al. 2007). Incorrect diagnosis of instability may result in an inappropriate rehabilitation program (conservative treatment) leading to a failure to improve symptoms and performance (Bahk et al. 2007, Marquardt et al. 2007, Randelli et al. 2008). On the other hand, even with the correct diagnosis along with an inappropriate rehabilitative program or inefficient surgical repair, a patient may still be at risk for recurrent instability (Marquardt et al. 2007). Early diagnosis combined with an injury specific rehabilitation program is essential for the administration of an effective treatment within a timeframe that will reduce the risk for surgical intervention, inappropriate surgery and/or recurrent instability (Satterwhite 2000).

According to the American Academy of Orthopedic Surgeons (AAOS), the earlier shoulder conditions are diagnosed, the more beneficial it is for the patient long-term. A study performed by Yuushi and Anri indicated that 77% of their subjects did not report shoulder symptoms until a year post onset of an unstable shoulder making it difficult for early diagnosis (Yuushi and Anri 2001). Delaying a musculoskeletal examination may make it difficult to identify the original pathology and therefore increasing the difficulty in determining the best treatment type. The correct diagnosis and etiology of instability is very important, especially since failure to respond to conservative treatment results in surgery, which may induce drastic changes in the tissues of the shoulder joint (Nyland et al. 1998). These changes may encompass the collagen and the mechanoreceptors therein.

1.7.1 Conservative Treatment

Conservative (nonoperative) treatment usually consists of strengthening of the rotator cuff muscles, range of motion activities and scapular stabilization (Buss et al. 2004). Buss et al. studied the effects of conservative treatment on athletes with atraumatic anterior instability.

Following treatment, 37% of the subjects had a recurrent episode whereas 59% improved and did not have a recurrent episode. However, by the end of the season, 53% of the total subjects underwent surgery. A rehabilitation program that focuses solely on strengthening usually results in recurrent instability (Cuccurullo 2004). According to Burkhead and Rockwood, a strengthening program for the muscles surrounding the glenohumeral joint is the optimal conservative and pre-surgical treatment (Burkhead and Rockwood 1992). Combined neuromuscular and proprioceptive rehabilitative programs have also been established to restore proprioceptive deficits during glenohumeral movements by retraining the proprioceptive neuromuscular afferent pathways (Lephart et al. 1997, Lephart et al. 1996).More research is required to determine the effectiveness of the different rehabilitative programs (Hayes et al. 2002). Additionally, it is important to identify the etiology of shoulder instability to administer the appropriate rehabilitation protocol (Burkhead and Rockwood 1992)

We strongly believe the treatment should consider the tissue properties of the glenoid capsule and labrum. Rehabilitative programs (strengthening, neuromuscular, proprioception) only provide partial recovery of proprioceptive neuromuscular control (Lephart et al. 1997). This may be because proprioceptive neuromuscular rehabilitative programs would not restore tissue length to an elongated capsule to allow the capsular mechanoreceptors to have detection sensitivity to mechanical stimuli. For this reason, this would not be considered an appropriate treatment. Collagen, on the other hand, possesses neither contractile nor signal conducting properties and thus cannot be strengthened using a random strengthening program. However, collagenous tissue may be strengthened through progressive strengthening exercises as it increases collagen synthesis and improves collagen fiber alignment. The use of a progressive exercise program may be beneficial or detrimental depending on the phase of healing. (Potach and Grindstaff 2008) Restoration of collagen content for regaining strength within a joint may require treatment strategies that improve collagen turnover rate and/or crosslink formation.

During the repair phase of healing, the damaged tissue is replaced with new collagen, but this process may be delayed if too little or too much stress is applied to the tissues during treatment resulting in more tissue damage (Potach and Grindstaff 2008). “The ability of collagen molecules to assemble into crosslinked fibrils is an important requirement for the development of tissue strength” (Walsh 2005). The development of tissue strength is critical during the remodeling phase of healing. Proper collagen fibril alignment is important for strengthening the tissue during the remodeling phase and is determined by the type and magnitude of applied stress to the newly synthesized collagen fibers (Potach and Grindstaff 2008). Conservative treatment may be good as the first line of defense in some instability cases with surgery as the second, and vice versa in other instances.

“Normal” practice reflects treatment options that cater to diagnostic theories (Randelli et al. 2008). Since treatment is controversial, this may be a contributing factor to increased instability recurrence rates with conservative care (Wang and Arciero 2008). As mentioned previously, failure of conservative treatment results in surgery that may or may not be necessary and/or beneficial, but rather detrimental (Mallon and Speer 1995).

1.7.2 Surgical Treatment

Surgical repair of the glenoid capsule and labrum has been shown to be the most effective treatment in preventing recurrent instability compared to no surgery, yet the recurrence rate post surgery is close to 20%, especially in the active population (Robinson and Dobson 2004). Surgical techniques have been established to tighten the elongated capsule and repair the microtears within the capsule and labrum. In general, these techniques were developed to solely restore mechanical restraint provided by the involved tissues. However, there is an increasing interest regarding the effect of surgery on tissues containing mechanoreceptors (Delforge 2002).

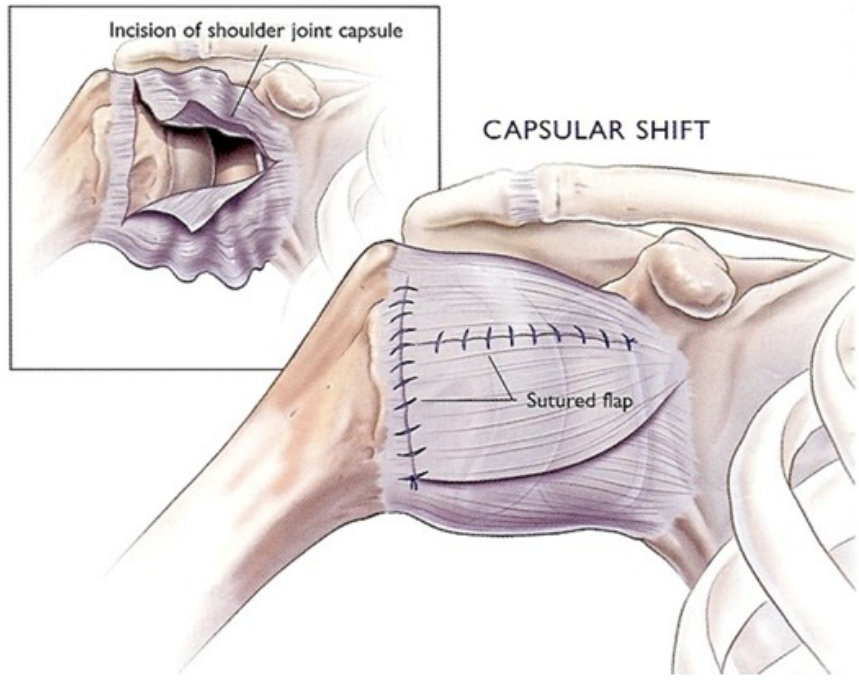

Figure 12. Illustration of capsular shift surgery. During a capsular shift procedure, the surgeon makes an incision in the shoulder joint capsule forming a flap and then sutures the flap to tighten the joint. *(orthosurgeon.com)*

Capsular shift, capsular plication, and thermal capsulorraphy are surgical techniques used to tighten an elongated capsule (Nyland et al. 1998, Levine et al. 2004, Sullivan et al. 2008). Capsular shift (Fig. 12) is an open surgery that requires cutting the capsule for complete exposure of the joint and capsule (Ticker and Warner 2000). This procedure has the lowest rate of recurrent instability (Sullivan et al. 2008). Open surgeries allow surgeons to have greater knowledge of the pathoetiology that led to instability, and this is suggested to be the reasoning for lower recurrence rates compared to arthroscopic surgeries (Dumont et al. 2011).

Arthroscopic capsular plication (Fig. 13) requires two portals (~5mm each) introduced into the capsular regions of interest to address not only capsular laxity and/or tear, but also a labral lesion if present. Prior to beginning the surgery, the synovial layer is gently abraded to increase blood flow to the region of interest for healing. If the abrasion is too aggressive, the capsule will be completely obliterated. After abrasion, the capsule is folded on itself and sutured to the selected region of the labrum to complete the shift. If the labrum is detached, then the suture is passed through the capsule, then the labrum, and finally through the edge of the glenoid rim. (Wolf and Eakin 1998) The capsular shift and plication are the most invasive

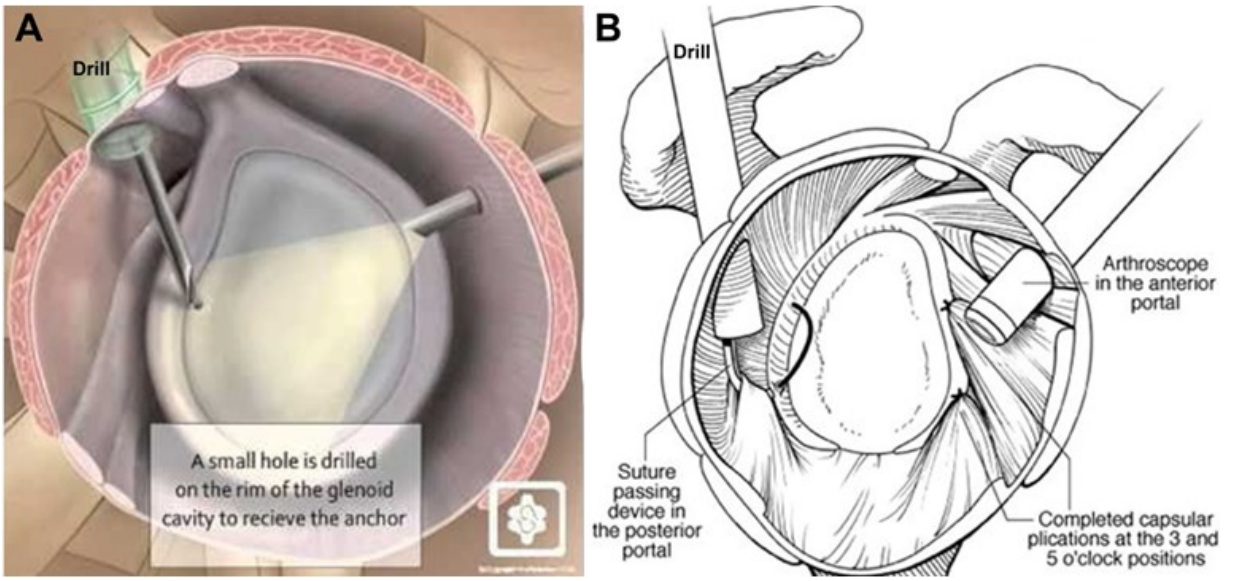

Figure 13. Illustration of capsular plication surgery of the shoulder. A hole is drilled into the glenoid rim (A) or labrum (B) for suturing the capsule to the rim or labrum thereby tightening the capsule. *(shoulderdoc.co.uk; orthopedic research of virginia)*

techniques. Figure 14 illustrates labral repair independent of capsular repair. Following invasive surgeries, the regions of the tissues with incisions, portals, and/or abrasion are susceptible to mechanoreceptor damage occuring in other regions (pre-surgical uninjured tissue) of the capsule and/or labrum thereby contributing to additional proprioceptive deficits. On the other hand, tightening the capsule is considered a mechanism for restoring shoulder proprioception. It has been shown that as the capsule tightens, detection of glenohumeral tranlsation by unharmed mechanoreceptors becomes more sensitive (Blasier et al. 1994). Warner et al. noted surgical stabilization as a technique for normalizing shoulder proprioception (Warner et al. 1996).

Capsular folding during plication may also result in misalignment of the fascicles (bundles of axons) from which axons project terminally ending as mechanoreceptors. Fascicular damage has been observed during surgery and is thought to be the cause of “restrictive” clinical deficits such as muscle weakness specific to the damaged or misaligned fascicle (Stewart

2003). Although the clinical relevance of fascicles has not been well studied, the position and distribution of the nerve bundles should also be considered during surgery.

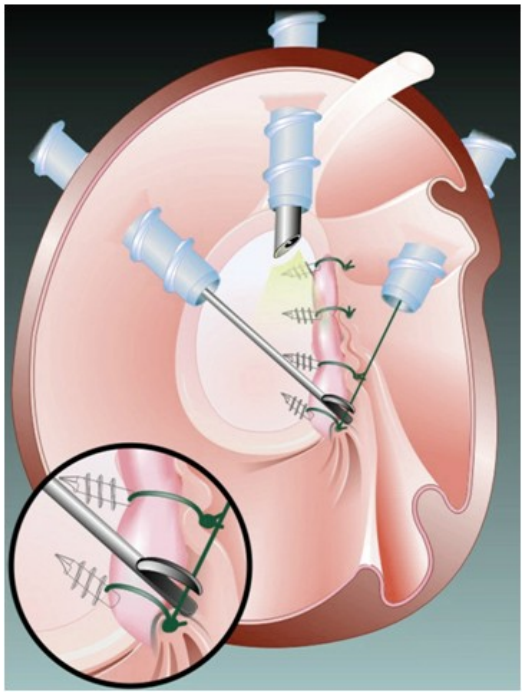

Figure 14. Illustration of labral repair surgery. *(orthopaedicdoctor.com)*

Thermal capsulorraphy is a technique that uses heat at 65°C to shrink the capsule. This technique is less invasive and is initially successful at shortening the length of the capsule. Consequently, the application of heat alters the capsular viscoelastic properties increasing the risk of restretching the capsule and recurrent instability (Levine et al. 2004). It has been shown that the collagens that make up the articular tissues significantly weaken and become denatured in response to the heat (Sekiya et al. 2003) ultimately leading to increased tissue extensibility (Sullivan et al. 2008). Unlike in the capsular shift and plication techniques, in capsulorrhaphy mechanoreceptors are left unharmed as long as the temperature does not exceed 65°C during this procedure (Levine et al. 2004). In support of this conclusion, the ability of mechanoreceptors to provide information regarding joint position was restored post surgery in the involved shoulder compared to the uninvolved shoulder (Sullivan et al. 2008).

The optimal surgical treatment for combatting functional instability and managing recurrent instability is still in the process of being determined (Sullivan et al. 2008). Each surgical type has both pros and cons. Greater knowledge and understanding regarding the pathoetiology of the primary structures involved in shoulder instability may further lessen the rate of recurrent instability post treatment and ultimately reduce the number of disability cases. The above mentioned surgical techniques may affect the collagen content and

mechanoreceptor orientation and concentration (Nyland et al. 1998, Sekiya et al. 2003). Consequently, instead of surgery being an option for return to work and/or sport, surgery may become another causal factor for why individuals cannot return to work and/or sports. On the other hand, it is the most effective treatment available today.

1.8 Significance

The following project was developed over several meetings with the University of Kansas Medical Center orthopedic surgeons, who include Drs. Kimberly Templeton, Vincent Key and Federico Adler (now deceased). The meeting discussions identified areas of research related to disabilities that the surgeons felt were lacking. They agreed that there was a lack in shoulder research, and instability was the most common injury presented.

Glenohumeral joint instability is one of the most common types of shoulder injury, but the complexity of this condition is not well understood. It is often misdiagnosed and mistreated thereby resulting in the recurrence of instability followed by disability. Since anterior instability is the most prevalent, ***this was the focal point of our study*** with secondary analysis of posterior instability.

Clinical studies have shown that treatments that address decreased muscle strength and proprioceptive deficits associated with glenohumeral joint instability, ***temporarily*** improve the overall functional mobility of the shoulder. Given the high recurrence rate, shoulder instability research should further explore the tissue integrity and mechanoreceptor characteristics of the glenohumeral joint for more effective treatment. ***Within this study***, we generated data that may progress research related to joint laxity and ultimately instability.

Tissue integrity of the static stabilizers is determined by their collagen composition. The change in collagen concentration within the injured tissues contributing to excessive translation in the unstable shoulder would imply the need for treatment and/or surgical protocols that stimulate and accelerate collagen turnover rate after injury.

Physiologically, mechanoreceptors have been identified in all the stabilizers of the glenohumeral joint including the musculature (rotator cuff muscles), glenohumeral ligaments, capsule and labrum, although controversial in the latter. The presence of mechanoreceptors in the glenoid capsule and labrum suggest these structures play a role in proprioception. An

alteration in the number of mechanoreceptors may disrupt the precision of neuromuscular signaling. Thus, techniques are needed that stimulate mechanoreceptor conductivity and/or increase the number of mechanoreceptors for improved glenohumeral joint stabilization.

This study has built upon the tissue integrity of the glenohumeral stabilizers and the potential role they may play in joint laxity and, ultimately, instability. We believe that focusing on the glenoid capsule and labrum will assist healthcare professionals in improving their ability to treat instability as well as reduce the risk of instability recurrences. It has been established that these two structures have a finite responsibility for maintaining stability during specific glenohumeral translations. ***Our study examined*** tissue resistance and proprioceptive properties of each structure, and further determined their ability to limit glenohumeral translation.

CHAPTER 2

Improved gold chloride staining method for anatomical analysis of sensory nerve endings in the shoulder capsule and labrum as examples of loose and dense fibrous tissues

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Preface: Without a successful staining protocol for sensory nerve endings in our tissues of interest, we would not have been able to carry out a major portion of our body of work. Therefore, chapter 2 marks the beginning of our body work which is the methodology for our improved Gold Chloride Staining method for sensory nerve endings. This chapter is currently a manuscript in press for publishing in the *Biotechnic and Histochemistry*, a peer-reviewed journal published by the Biological Stain Commission.

2.1 Abstract

Consistency in gold chloride staining is essential for anatomical analysis of sensory nerve endings. The gold chloride stain for this purpose has been modified by many investigators, but often yields inconsistent staining, which makes it difficult to differentiate structures and to determine nerve ending distribution in large tissue samples. We introduce additional steps and major changes to the modified Gairns' protocol. We controlled the temperature and mixing rate during tissue staining to achieve consistent staining and complete solution penetration. We subjected samples to sucrose dehydration to improve cutting efficiency. We then exposed samples to a solution containing lemon juice, formic acid and paraformaldehyde to produce optimal tissue transparency with minimal tissue deformity. We extended the time for gold chloride impregnation 1.5 fold. Gold chloride was reduced in the labrum using 25% formic acid in water for 18 h and in the capsule using 25% formic acid in citrate phosphate buffer for 2 h. Citrate binds gold nanoparticles, which minimizes aggregation in the tissue. We stored samples in fresh ultrapure water at 4°C to slow reduction and to maintain color contrast in the tissue. Tissue samples were embedded in Tissue Tek and sectioned at 80 and 100 µm instead of using glycerin and teasing the tissue apart as in Gairns' modified gold chloride method. We attached sections directly to gelatin subbed slides after sectioning with a cryostat. The slides then were processed and coverslipped with Permount. Staining consistency was demonstrated throughout the tissue sections and neural structures were clearly identifiable.

Key words: capsule, fibrous tissue, gold chloride, labrum, nerve endings, shoulder, stain

2.2 Introduction

Gold chloride staining can provide excellent staining of myelinated axons and sensory nerve endings (Boyd 1962, Freud 1884, Gairns 1930, Kahlden 1894, Mann 1929). Although it has been considered a myelin staining method (McNally and Peters 1998), it also stains blood vessels, neurofilaments, elastin and collagen containing structures (Hogervorst and Brand 1998), which often makes it difficult to identify nerve endings specifically. Although the technique has shown promising results, it often has produced inconsistent staining because of inadequate penetration of solutions (Vilensky et al. 2002).

In some studies, Ruffini (type 1 nerve ending) and Pacinian (type 2 nerve ending) corpuscles in different tissues were stained by gold chloride; however, the appearance of these sensory nerve endings varied among reports (Adachi et al. 2002, Bresch and Nuber 1995, Dhillon et al. 2011, Gazza et al. 2006, Georgoulis et al. 2001, Guanche et al. 1999, Hogervorst and Brand 1998, Hasegawa et al. 1999, Katonis et al. 1991, Raunest et al. 1998, Vangsness et al. 1995, Yahia et al. 1988, Zimny et al. 1989). The conflicting identification of Ruffini and Pacinian corpuscles using gold chloride staining is believed to occur because of differences in tissue section thickness (25–100 μm) and infrequent use of serial sections (Hogervorst and Brand 1998).

Several modifications have been made to the gold chloride staining method (Boyd 1962, Silverberg et al. 1989, Zimny et al. 1989). These modifications have improved and standardized some aspects of gold chloride staining, but they have not achieved sufficient dependability to ensure proper identification of sensory nerve endings. We developed major modifications of the Gairns' gold chloride staining method (Gairns 1930) that produce consistent and reproducible staining throughout a tissue sample and among different samples. In addition, our modifications enabled the identification of type 1 and type 2 nerve endings in fibrous tissue including the cadaveric shoulder capsule and the labrum.

2.3 Materials and Methods

2.3.1 Reagents

Normal saline was purchased from Baxter Corp. (Deerfield, IL). Sucrose (FW = 342.3), sodium citrate dihydrate (FW = 294.1), 88% formic acid (ACS reagent grade), xylene (histological grade; FW = 106.17), Permount, absolute ethyl alcohol (ACS reagent grade), and 70 and 100% ethyl alcohol (histological grade) were obtained from Fisher Scientific (Pittsburgh, PA). Sakura Tissue Tek O.C.T. compound, sodium phosphate monobasic anhydrous (ACS reagent grade, FW = 137.99) and sodium phosphate dibasic anhydrous (ACS reagent grade, FW = 141.96) were purchased from VWR (Radnor, PA). Aqueous gold chloride (5%) was obtained from Salt Lake Metals (Salt Lake City, UT). Paraformaldehyde (PFA), 1 N sodium hydroxide, porcine skin gelatin (type A), chromium III potassium sulfate dodecahydrate (ACS reagent grade FW = 499.4) and 70% nitric acid (ACS reagent grade, FW = 63.01) were obtained from Sigma (St. Louis, MO). Pure lemon juice purchased from a local store.

Anhydrous sodium phosphate monobasic and dibasic salts were used to prepare 0.2 M phosphate buffer (PB). All sucrose solutions and 4% PFA were prepared in 0.1 M PB diluted from 0.2 M PB. To prepare 4% PFA, sodium hydroxide was added to raise the pH of the PB slowly after powdered PFA was added to allow the PFA to dissolve. A lemon juice solution was prepared using three parts lemon juice, 0.6 parts 4% PFA and 0.4 parts 88% formic acid. Gold chloride (5%) was diluted to 1% using ultrapure water. Alternatively, 1% gold chloride can be purchased from Salt Lake Metals (Salt Lake City, UT). A 0.1 M citrate solution was prepared in ultrapure water. Citrate PB (pH 7) was prepared from 6.5 ml 0.1 M citrate solution and 43.6 ml 0.2 M PB. Absolute ethyl alcohol was added to dry ice to make the freezing bath. In addition, 70 and 100% histological grade ethyl alcohols were used for slide processing. A 2 M nitric acid solution was prepared using 70% nitric acid and water to clean further the pre-cleaned slides.

Gelatin chromium solution for subbing gelatin slides contained 5 g gelatin and 0.5 g chromium III potassium sulfate dissolved in ultrapure water.

2.3.2 Materials

Plastic molds and 75 x 50 mm pre-cleaned glass slides were obtained from Fisher Scientific. Gelatin coated 75 x 25 mm slides were obtained from Lab Scientific (Livingston, NJ). Glass jars that hold approximately 50 ml of solution were used.

2.3.3 Tissue Preparation

The glenoid capsule is thin, loose fibrous tissue (Hyde 2007, Tovin and Reiss 2007), whereas the labrum is dense cartilaginous fibrous tissue (Hill et al. 2008).

The capsule and labrum specimens were harvested from the anteroinferior and posteroinferior regions of shoulder pairs of three male and four female human cadavers. The cadavers ranged in age from 57 to 64 years.

The average size of the tissue specimens of interest was for the capsule 2 x 50 x 50 mm (thickness, width, and length) and for the labrum 9 x 21 x 15 mm (thickness, width, and length). The capsule and labrum are attached in the glenohumeral joint; the labrum serves as an attachment for the capsule. For convenience during staining, the attachment of the capsule and labrum were retained; thus, the maximal tissue length was 65 mm, 50 mm contributed by the capsule and 15 mm by the labrum. The two tissues are not separated until the reduction step of gold chloride staining.

The capsule is 2 mm thick; this thickness is not sufficient to achieve the desired stain color for tissue background and nerve endings. Therefore, additional tissue, primarily, muscle, was allowed to remain attached to the capsule, which increased overall tissue thickness to 5–7 mm. The tissue thickness is important for determining the amount of gold required for the gold impregnation and reduction steps. We tested tissue samples at different thicknesses including 5, 7 and 9 mm and found 7 mm thickness to be optimal.

Immediately after harvest, the specimens were washed twice for 10 min, each time in fresh saline before staining.

2.3.4 Staining

Figure 1 shows the gold chloride staining procedure. Briefly, the tissue was dehydrated with sucrose, treated with lemon juice solution, impregnated with gold chloride and the gold chloride was reduced. For each of these steps, the tissue specimens were completely submerged in 50 ml of the solutions.

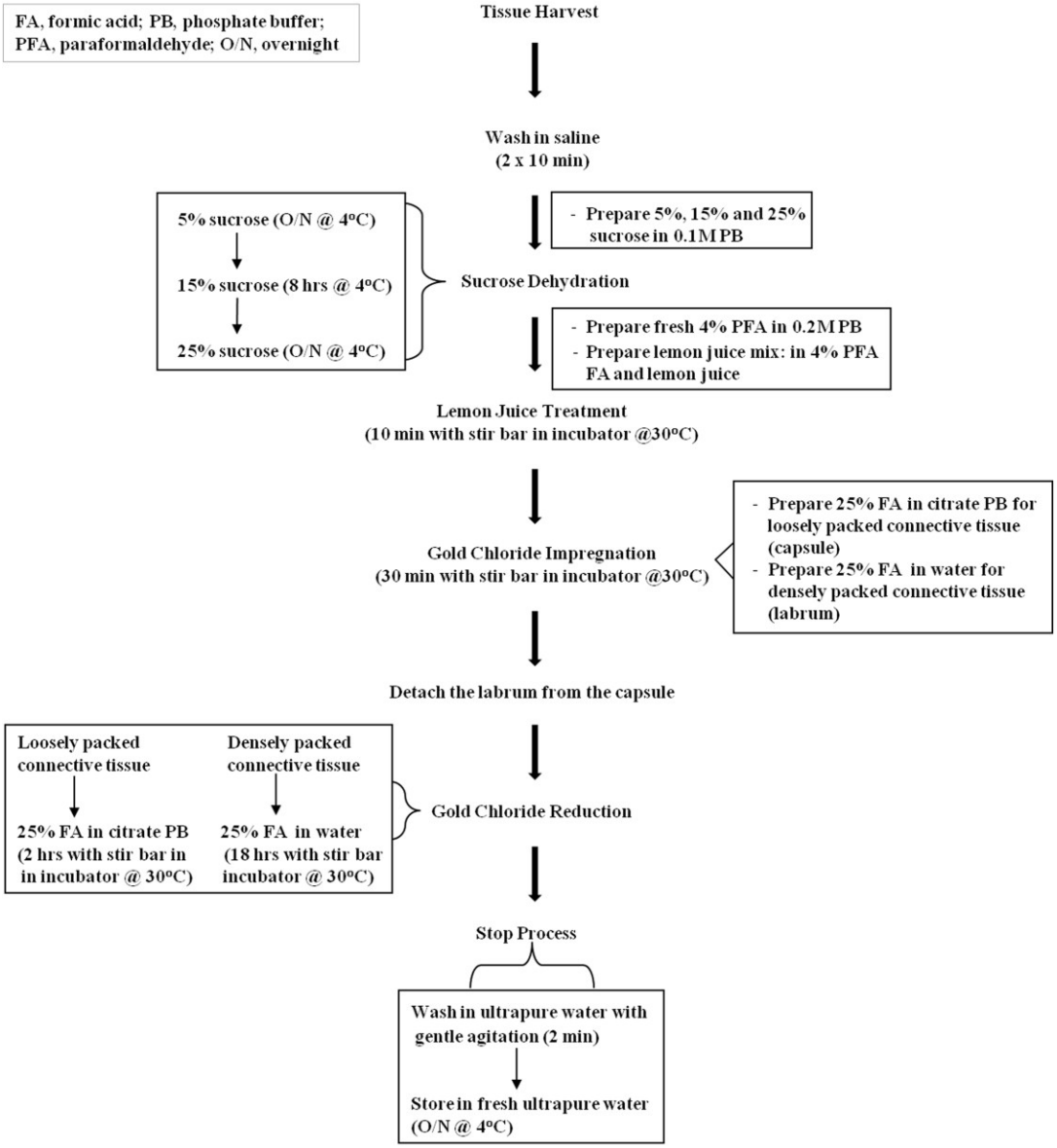

Figure 1. Flow chart for gold chloride staining procedure

Unlike other gold chloride protocols, our protocol included a reagent mixing rate of 400-450 rpm using a stir bar and controlled temperature in an incubator. The incubator not only allowed the temperature to be controlled, but also provided a dark environment. Stirring and controlled temperature were constant for each step of the staining process, except during sucrose dehydration. The procedure was carried out at both 20–22° C and 30° C.

2.3.4.1 Sucrose Dehydration

The saline washed specimens were towel blotted and dehydrated in 5, 15 and 25% sucrose at 4° C for a minimum of 8 h at each concentration.

Gairns' protocol (Gairns 1930) did not include sucrose dehydration. We tested staining with and without sucrose dehydration. Samples subjected to sucrose dehydration either before the lemon juice treatment or immediately after gold chloride reduction.

2.3.4.2 Tissue Transparency

After sucrose dehydration, the capsule and labrum were transferred to 50 ml of the lemon juice solution for 10 min in the dark. Gairns' protocol required soaking the tissue for 10 min in three parts 100% filtered lemon juice using freshly squeezed lemons and 1 part 88% formic acid in the dark at room temperature (Gairns 1930). Our specimens were tested under five different conditions: 1) three parts lemon juice and one part 88% formic acid (Boyd 1962, Gairns 1930, Silverberg et al. 1989), 2) lemon juice only (Silverberg et al. 1989), 3) three parts lemon juice and one part 4% PFA, 4) 3.6 parts lemon juice and 0.4 parts formic acid, and 5) three parts lemon juice, 0.6 parts 4% PFA and 0.4 parts 88% formic acid. Each lemon juice treatment was tested for 10, 15 and 20 min at both room temperature and at 30° C.

2.3.4.3 Gold Chloride Impregnation

The specimens were transferred to 50 ml 1% aqueous gold chloride for 30 min. The 1% aqueous gold chloride was used in the dark according to the original protocol (Gairns 1930). The specimens were not teased as they were in two protocols (Boyd 1962, Gairns 1930). Gold

chloride solution can be filtered for use a second time. Both fresh and filtered gold chloride solutions were used. Gold chloride solution was not re-used more than once.

2.3.4.4 Gold Chloride Reduction

The capsule and labrum were separated from each other and subjected to two different reduction processes. It is important to use ceramic forceps and blade to separate the labrum from the capsule, because gold reacts with metal. The capsule was immersed in 50 ml 25% formic acid prepared with citrate PB until axons appeared dark purple-black. Our protocol required 1.5 h when using fresh gold chloride and 2 h when filtered gold chloride was used. By contrast to the capsule, reduction of gold chloride in the labrum was performed for 18 h in 25 ml 25% formic acid prepared in ultrapure water.

Reduction of gold was tested under five different conditions including: 1) 25% formic acid in ultrapure water (Gairns 1930), 2) acidified water using acetic acid (pH 3.13) (Silverberg et al. 1989), 3) sodium hydroxide solution (7 drops of sodium hydroxide added to 100 ml water), pH 11.76, 4) 0.1 M citrate PB, then transferred to 25% formic acid in ultrapure water, and 5) 25% formic acid in 0.1 M citrate PB. It is important to note that the conditions for reduction in acidified water or sodium hydroxide solution, by contrast to the other three conditions, did not include controlled temperature and standardized stirring. Reduction was performed for 2, 4, 8, 12, 16, 18 or 24 h.

2.3.4.5 Stopping the Gold Chloride Reduction

When stained to the desired color, all specimens were washed in ultrapure water and stored in fresh ultrapure water at 4^{o} C overnight or until ready for embedding. In the original protocol of Gairns, tissue was towel blotted, then immersed in glycerin; there was no termination step for gold chloride reduction (Gairns 1930). In our protocol, we slowed gold reduction at 4^{o} C. After washing, samples were transferred to one of the following solutions: 1) 4% PFA for 2 min and 1% sodium thiosulfate for 2 min at room temperature, 2) 4% PFA for 2 min at room

temperature, or 3) water at 4° C overnight. Samples that were treated with PFA and/or sodium thiosulfate were transferred to water and stored at 4° C overnight.

2.3.5 Embedding

The labrum and capsule samples were examined after various embedding conditions and in various media including paraffin, gelatin, glycerin gelatin, or Tissue Tek. In earlier protocols, tissue samples were placed in glycerin (Gairns 1930, Boyd 1962). We ultimately chose Tissue Tek for embedding both labrum and capsule samples, although the embedding process differed depending on specimen size. Four freezing techniques were tested for the samples embedded in Tissue Tek. These techniques included using liquid nitrogen, methyl butane with liquid nitrogen, dry ice, or ethanol and dry ice. Ultimately, we selected ethanol and dry ice as the freezing technique for both the labrum and capsule samples.

2.3.5.1 Labrum Samples

Plastic embedding molds were filled carefully with Tissue Tek to avoid introducing air bubbles. The labrums then were towel blotted to remove excess water and immersed in Tissue Tek for at least 30 min. Subsequently, the molds were lowered into an ethanol and dry ice bath to snap-freeze the samples in the Tissue Tek. Care should be taken to prevent the ethanol from contacting the Tissue Tek or sample. Embedment of capsule tissue samples was more complex (see below).

2.3.5.2 Capsule Samples

Steps for embedding the capsule tissue was custom designed for and more complex than for the labrum described above (Table 1, Fig. 2). Glass jars were partially filled with Tissue Tek. The capsules were towel blotted and immersed in Tissue Tek for at least 30 min. Because the capsule samples were dome-shaped, they required flattening. To achieve optimal flattening, we removed the specimens from the Tissue Tek and conditioned them by applying downward pressure using an aluminum block. Downward pressure was applied three to five times on the

tissue with the surface of interest directed against a strip of polyethylene plastic covered with Kim Wipes. The Kim Wipes removed excess Tissue Tek. If noticeable Tissue Tek remains on the surface of interest after conditioning, a fresh Kim Wipe should be used to remove it. During each repetition of conditioning, the downward pressure was applied to the tissue until the capsule was completely spread, then the pressure was sustained for at least 5 sec. Once conditioning was complete, the samples were ready to be flattened permanently.

Table 1. Steps for tissue embedding.

Steps	Notes
1. Remove tissue from ultrapure water	
2. Blot tissue using Kim Wipe to remove excess water	
3. Transfer tissue samples to small jars containing Tissue Tek	30 min at room temperature
4. Start freeze bath using ethanol and dry ice	
5. Condition tissue samples	3–5 repetitions
6. Refer to the steps in Fig. 3A– D	
7. Wrap frozen block (Fig. 3D) in Parafilm and store @ -20^{o} C until ready for sectioning	For long term storage, samples shoul be stored at -80^{o} C

To flatten tissue permanently, we tested different techniques using a custom-made aluminum mold. Various attempts included: 1) pinning the tissue down to partially frozen Tissue Tek, 2) pressing the tissue against the bottom of an aluminum mold using a compression block (part V, Fig. 2A) and 3) using a compression block to press the tissue against polyethylene plastic that was customized to fit the base of the aluminum mold. The best results were obtained by pressing the tissue against polyethylene plastic using a compression block.

The tissue sample was placed in the custom made aluminum mold with the polyethylene plastic at the bottom (Fig. 2A-1) so that the tissue surface of interest was in contact with the plastic. The compression block was tightened against the tissue to produce maximal

flatness. The capsule was considered flat when at least ¾ of the length of the screws was screwed into the aluminum mold. The complete aluminum mold including the compression block then was lowered into an ethanol and dry ice freezing bath without allowing ethanol to flow into the mold (Fig. 2A-2).

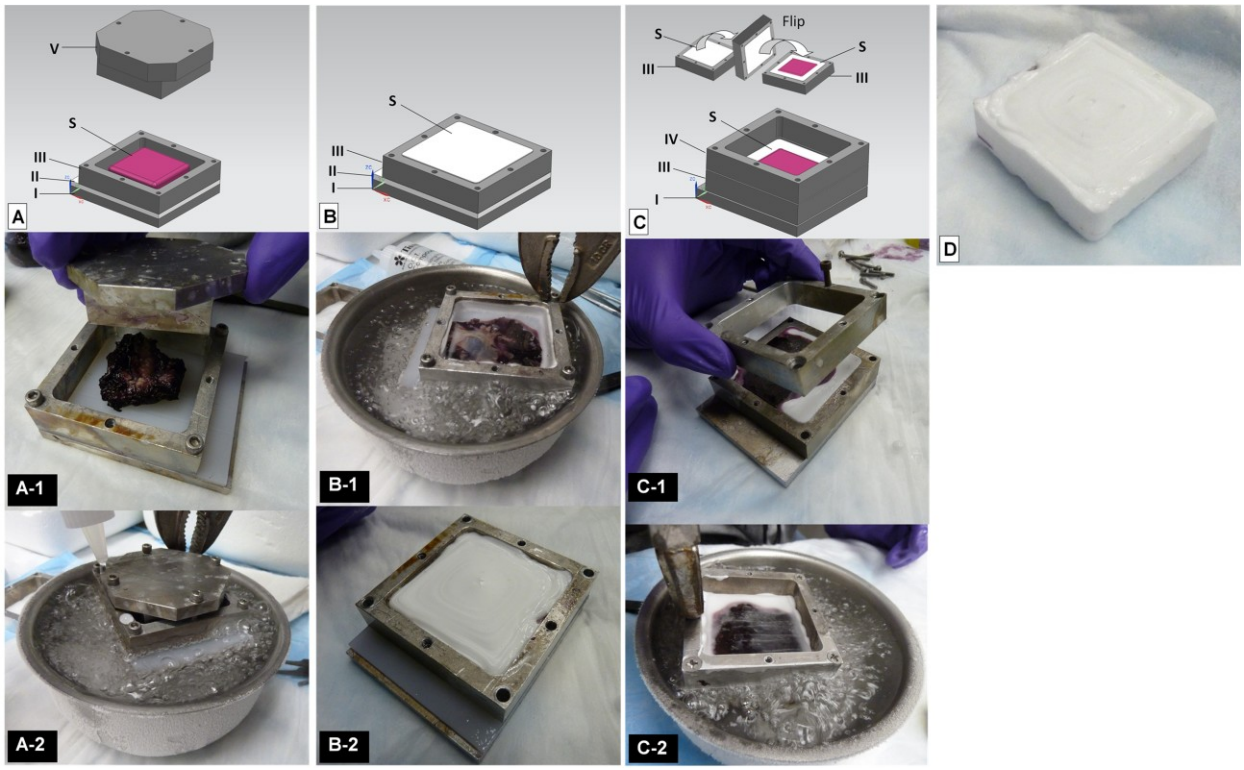

Figure 2. Materials and sample processing during embedding. A) Apparatus required for tissue flattening. I, base of aluminum mold; II, polyethylene plastic; III, aluminum mold; V, compression block; S, tissue specimen. A-1) Tissue surface of interest in contact with polyethylene plastic (part II) prior to flattening using the compression block (part V). A-2) Lowering of complete aluminum mold into ethanol and dry ice freeze bath. The white dots represent drops of Tissue Tek that indicate a completely frozen tissue surface. B) Parts required for embedding the exposed half of the tissue specimen. I, base of aluminum mold; II, polyethylene plastic; III, aluminum mold; S, tissue specimen. B-1) Lowering of complete aluminum mold filled with Tissue Tek into ethanol and dry ice bath. I, base of aluminum mold; II, polyethylene plastic; III, aluminum mold; S, tissue specimen. B-2) Mold containing half of specimen embedded and frozen in Tissue Tek. C) Parts S and III are flipped to expose the flattened surface of tissue (part S). I, base of aluminum mold; S and III, frozen specimen in an aluminum mold; IV, additional aluminum mold. C-1) Adding the additional aluminum mold (part IV) after parts S and III have been flipped. C-2) Lowering of complete mold into ethanol and dry ice freeze bath after a layer of Tissue Tek was added over the flattened tissue surface (part S). I, base of aluminum mold; S, frozen specimen; III, aluminum mold; IV, additional aluminum mold (see description in the capsule samples section**)**. D) Frozen tissue block after mold disassembly.

To determine whether the tissue was completely frozen, we applied two drops of Tissue Tek to the mold. When the Tissue Tek froze, the tissue surface of interest was completely frozen. The compression block then was removed and Tissue Tek was used to fill the area it had occupied, thus embedding the majority of the tissue. The tissue should remain flat against the plastic and should not be disturbed at any point during the remainder of the embedding process. After the mold was filled with Tissue Tek, it was lowered into the ethanol and dry ice freezing bath again (Fig 2B-1). When completely frozen (Fig. 2B-2), the portion of the mold that contained the partially embedded tissue specimen was separated from the base of the mold and flipped to expose the flattened surface of the tissue (Fig. 2C). Another aluminum mold, labeled as additional mold, was stacked on top of the flipped mold (Fig. 2C-1) and both were attached to the base without the plastic. The additional mold was another custom-made aluminum mold similar to the first except it was not designed to accommodate screws with a “round” head, but rather a “countersunk” head. A layer of Tissue Tek was applied to the exposed flattened part of the tissue and the mold was lowered into the ethanol and dry ice freezing bath (Fig. 2C-2) to complete the embedment. The aluminum mold then was disassembled and the block removed (Fig. 2D) and stored at -20^{o} C for the short term or at -80^{o} C for the long term.

2.3.6 Preparation of Gelatin Subbed Slides

Prior to sectioning the embedded samples, 75 x 25 mm gelatin coated and 75 x 50 mm pre-cleaned slides, were subbed with gelatin to improve tissue adherence. Several protocols were combined (Kubke 2011, Larison 2009, NIH) to establish our final protocol for subbing the slides. Slides were placed in glass racks with an open bottom, then placed in glass containers. Pre-cleaned slides were placed in dishwashing solution (approximately 80^{o} F) for 30 min to 1 h with gentle agitation, rinsed under running tap water for 15 min or until all traces of detergent were removed, then rinsed with running distilled water for 5 min. After rinsing, the slides were placed under a fume hood and the glass containers were filled with 2 M nitric acid. Slides

remained in the nitric acid solution overnight. The nitric acid then was removed and stored for re-use. Next, the glass racks were rinsed in running tap water for 5–10 min, then rinsed in running distilled water for 5–10 min. The glass containers were covered immediately with glass lids or foil to maintain lint-free slides and transferred to a biological safety cabinet, uncovered, and air dried overnight. Fresh gelatin chromium solution was prepared and slides were dipped in the solution for 1 min, then air dried for 10 min. This coating process was repeated three times. If air bubbles formed, the covered containers with gelatin subbed slides were placed in an incubator at 37° C for 30 min or until no air bubbles remained. Slides then were removed from the glass containers under a biological safety cabinet and air dried overnight. Once dry, the slides were ready for use. The 75 x 25 mm gelatin coated slides were rinsed with running distilled water for 5 min, air dried in the biological safety cabinet. The slides then were dipped in the gelatin chromium solution for 1 min and air dried for 10 min. This coating process was repeated twice.

2.3.7 Sectioning

The procedure for cryosectioning is given in Table 2. The frozen labrum tissue blocks were sectioned at 80 µm in the sagittal plane and capsule samples were sectioned at 100 µm in the contact surface plane. Sectioning of the labrum and capsule samples produced small and large sections, respectively. Therefore, the labrum sections were adhered to 75 x 25 mm gelatin subbed slides directly from the cryostat platform. Capsule sections were positioned, using forceps, on 75 x 50 mm gelatin subbed slides within the cryostat chamber, then the slides were moved outside the chamber so sections could dry on the slides. After sectioning, the slides were air dried at 4° C overnight.

Table 2. Steps for cryosectioning

Steps	Notes
1. Set cryostat parameters	Outside temperature = -25° C Chamber temperature = -20° C Thickness: 80 or 100 µm
2. Bind specimen to specimen disc in the cryostat chamber using Tissue Tek	Orient tissue as desired
3. Align tissue in cryostat	
4. Section tissue	Labrum samples, 80 µm; Capsule samples, 100 µm
5. Mount on gelatin subbed slides	
6. Air dry overnight at 4° C and 31–33% humidity	

In the original protocol (Gairns 1930), the tissue samples were embedded in glycerin, teased apart and viewed by light microscopy. After embedding, we stored the frozen blocks in the cryostat to permit their acclimation to the cryostat temperature (outside temperature = -25° C, chamber temperature = -20° C). Samples sectioned immediately after freezing were placed in the cryostat for 30 min, whereas samples stored at -80° C prior to freezing were placed in the cryostat for 1 h. Frozen blocks were sectioned at 50, 80, 100, 150 and 200 µm. Samples also were sectioned in the sagittal, coronal, transverse and contact surface planes. The sections were adhered to four types of slides including gold plus (75 x 25 mm), poly-L-lysine (75 x 25 mm), gelatin coated (75 x 25 mm) and gelatin subbed (75 x 25 mm and 75 x 50 mm).

2.3.8 Slide Processing

Steps for slide processing are given in Table 3. The day after sectioning, Tissue Tek was completely removed from the capsule sections by immersing them in two ultrapure water baths for at least 30 min (first bath, 15 min; second bath, 15 min) for labrum sections and at least 4 h (first bath, 3 h; second bath, 1 h) for capsule sections based on preliminary tests. The sections then were dehydrated in 70 and 100% ethanol for 1 min (labrum sections) or 2 min (capsule

Table 3. Steps for processing slides containing labrum and capsule sections

Steps	Labrum Sections	Capsule Sections
1. Place slides in ultrapure water at room temperature	30 min–1 h	4 h minimum, overnight maximum
2. Transfer slides to 70% ethanol	1 min	2 min
3. Transfer slides to 100% ethanol	1 min	2 min
4. Transfer slides to xylene	1.5 min	3 min
5. Transfer slides to fresh xylene	3 min	30-45 min
6. Coverslip using Permount mounting medium		
7. Store slides overnight @ 4° C with humidity of 31–33%		
8. Observe slides using light microscopy within 1 week of staining		

sections) in each solution. Two sequences of alcohol dehydration were tested: 70, 95 and 100% ethanol (Silverberg et al. 1989), and 70 and 100% ethanol; the 70 and 100% dehydration was more effective. The sections then were transferred to two baths of xylene. In the first bath, labrum sections were incubated for 1.5 min and capsule sections were incubated for 3 min. In the second bath, the labrum sections were incubated for 3 min and the capsule sections for 30–45 min. After dehydration, mounting medium was applied. Glycerol, glycerol-gelatin and Permount were tested as mounting media for coverslipping the slides. Permount mounting medium was the medium of choice and was applied to each section (Yahia et al. 1988). After coverslipping, the slides were stored at 4° C until analysis by light microscopy.

2.4 Results

2.4.1 General Procedures

The temperature in the room in which initial trials were performed fluctuated between 18 and 30° C. Temperatures below ambient (< 22° C) prevented complete tissue transparency and limited the reduction of gold chloride to free gold. Despite using a stir bar, fluctuating temperature caused inconsistent staining. Using a stir bar to control the mixing rate in the lemon juice treatment, gold chloride impregnation and reduction steps produced consistent penetration of solution and staining appearance. The lemon juice treatment resulted in inconsistent tissue transparency when a stir bar was not used. Use of a stir bar produced uniform gold chloride impregnation throughout the tissue sample (3 mm depth) as demonstrated by serial sections. Like the lemon juice treatment, complete gold chloride reduction was not consistent in the absence of a controlled mixing rate and temperature. Strict temperature control (27–30° C) and use of a stir bar to control the mixing rate in every step except sucrose dehydration produced uniform penetration of all solutions throughout the tissue samples as well as improved reproducibility.

2.4.2 Sucrose Dehydration

Sucrose dehydration prior to staining enabled smooth cutting of the labrum and capsule without altering the outcome of tissue staining. A specimen obtained after sucrose dehydration is shown in Fig. 3A. The timing of sucrose dehydration was important. The use of sucrose after gold chloride staining produced a pink background and pink myelinated axons. We were unable to differentiate blood vessels and nerve endings; fascicles, however, were identifiable. In the absence of sucrose dehydration, the tissue tore during sectioning.

2.4.3 Tissue Transparency (Lemon Juice Treatment)

Immersing the tissues in pure lemon juice mixed with formic acid as suggested in previous protocols resulted in good transparency, but also extensive tissue deformation. In

addition, staining intensity was decreased compared to the use of 100% lemon juice or 100%

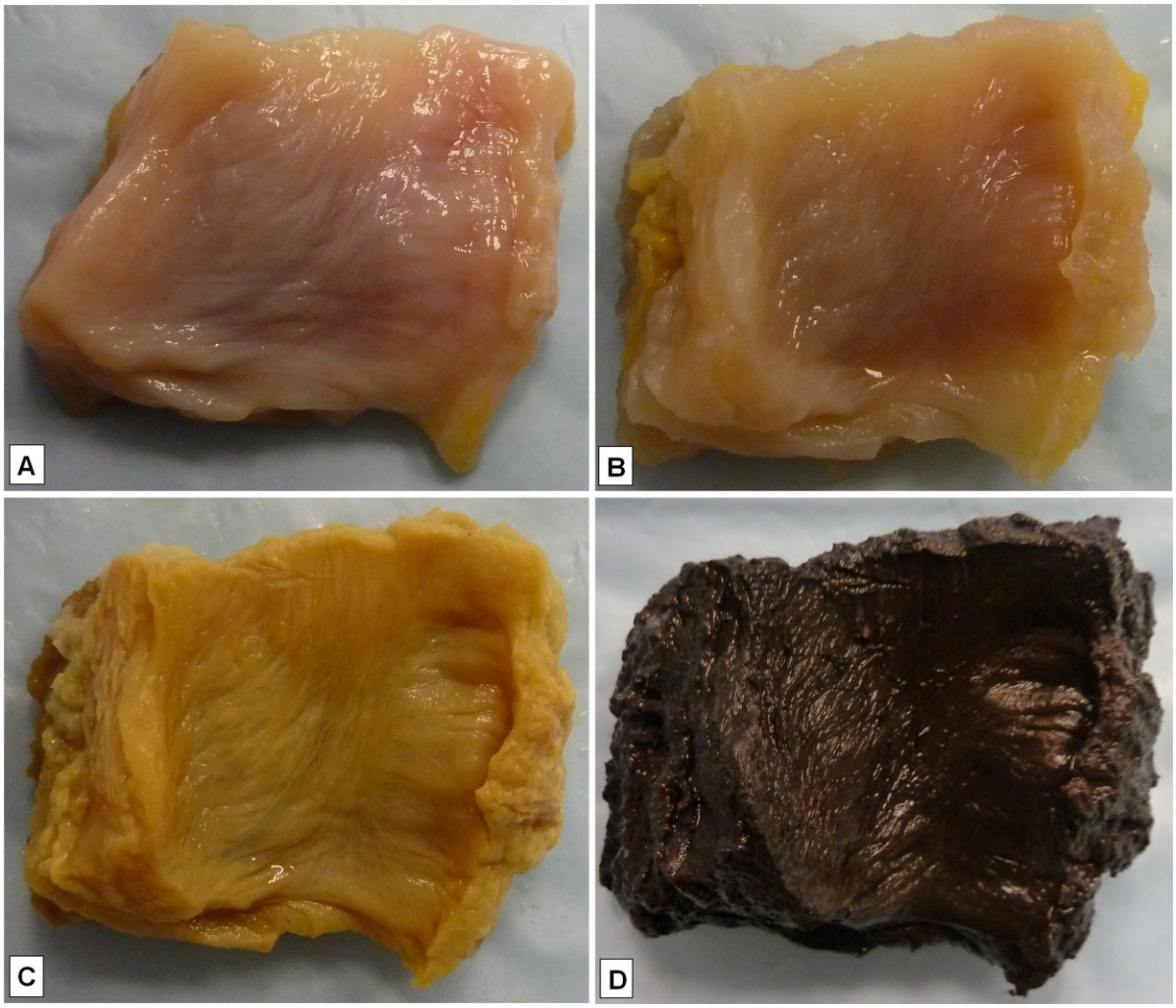

Figure 3. Appearance of the glenoid capsule after each step of the gold chloride staining. A) Harvested tissue after sucrose dehydration. B) Tissue after lemon juice treatment. C) Tissue after gold chloride impregnation. D) Tissue after gold chloride reduction.

lemon juice mixed with PFA and formic acid. Lemon juice alone, however, produced adequate tissue transparency. Pure lemon juice combined with 4% PFA and 88% formic acid produced adequate tissue transparency for optimal staining intensity and little or no tissue deformity (Fig. 3B). This treatment yielded the best results after 10 min exposure at 30^{o} C.

2.4.4 Gold Chloride Impregnation

Our protocol produced a uniform yellow appearance of the tissue (Fig. 3C). The intensity of the yellow color was greater when we used fresh gold chloride compared to gold chloride that

had been used earlier and filtered. In the latter case, we compensated for the differences in intensity in the gold chloride reduction step by adjusting the incubation time (see below) to achieve comparable intensity. Tissue samples 5–7 mm thick produced optimal staining results and required 30 min for gold chloride penetration and dispersal throughout the tissue.

2.4.5 Gold Chloride Reduction

The use of 25% formic acid in ultrapure water as a reducer caused more extensive deformity of the capsule than the labrum. Tissue specimens reduced in acidified water or sodium hydroxide solution suffered little or no tissue deformity, but showed minimal staining intensity. Samples that initially were placed in citrate PB then transferred to 25% formic acid in ultrapure water showed the greatest staining intensity of the entire tissue specimen for all structures (nerve endings, fascicles and blood vessels) compared to using acidified water or sodium hydroxide. The use of 25% formic acid in citrate PB produced optimal staining intensity of the myelinated axons and fascicles, and caused minimal tissue deformity as shown in Fig. 3D. The best staining results were achieved when the labrum was reduced in 25% formic acid in ultrapure water for 18 h and the capsule was reduced in 25% formic acid in citrate PB for 1.5 or 2 h. Capsule specimens exposed to fresh or filtered gold chloride were placed in reducing solution for 1.5 h and 2 h, respectively. Reduction of gold chloride in the capsule using 25% formic acid in citrate PB did not cause extensive tissue deformity, but did cause the tissue to become “dome shaped.”

2.4.6 Stopping the Gold Chloride Reduction

The use of 1% sodium thiosulfate caused a cloudy haze to form around the sensory nerve endings, which made identification of the components of the nerve endings difficult. Using 4% PFA following 1% sodium thiosulfate increased the clarity of the nerve endings. We have not tested the effects of 4% PFA on staining variability, however, and suggest that if 4% PFA is

used in this protocol, additional testing should be done. Storing the samples at 4° C dramatically slowed the reduction of gold chloride and was the best way to stop reduction.

2.4.7 Embedding

Paraffin, gelatin, and glycerin-gelatin blocks stiffened, but did not harden, without either formalin or freezing. We were unable to cut sections from the paraffin, gelatin and glycerin-gelatin blocks. Tissue Tek was the best embedding medium for both the capsule and labrum; its use minimized tearing of both tissue types. The aluminum mold we designed for flattening tissue together with the series of steps we developed to embed the capsule, consistently achieved complete or near complete flatness of the surface of interest. The labrum did not require flattening. An ethanol and dry ice bath froze both tissues uniformly without forming ice crystals, unlike using liquid nitrogen, methyl butane with liquid nitrogen, or dry ice alone.

2.4.8 Cryosectioning

Serial sections were collected from 56 capsule and labrum specimens. Infiltration of tissue specimens with Tissue Tek enabled easy sectioning of the tissue with few or no tears or air bubbles. Tissue samples cut at 50 μm did not show the entire structure of sensory nerve endings. Labrum and capsule specimens cut at 80 and 100 μm, respectively, showed both full and partial nerve endings. Sections were cut easily and allowed subsequent slide processing. For the labrum, the structures of interest were more easily identifiable when sectioned at 80 μm than at 100 μm. The 150 μm thick sections cut easily and showed the majority of the structures of interest, but did not permit identification in regions dense with these structures The 200 μm thick sections showed more distinctive structures of sensory nerve endings than 150 μm thick sections, but did not cut easily. Serial sectioning of the specimens allowed us to confirm the identities of most structures.

2.4.9 Slide Processing

The original Tissue Tek protocol required the sections to be immersed in water for 10 min, but this time was insufficient for complete removal of Tissue Tek from 80–100 µm thick sections. We compared incubation times in water ranging from 10 min to 5 h and found that 30 min to 1 h for labrum sections and 4 h for capsule sections were optimal.

2.4.9.1 Labrum Sections

Labrum sections detached from gold plus, poly-L-lysine or gelatin-coated slides after 20 min in ultrapure water regardless of surface area. Labrum sections remained attached to the gelatin subbed 75 x 25 mm slides for 30 min to more than 3 h while the Tissue Tek was dissolving in the water. After Tissue Tek removal, minimal curling occurred when the sections were incubated in 70 and 100% ethanol for 1 min and two baths of xylene for 1.5 and 3 min.

2.4.9.2 Capsule Sections

A large amount of residual Tissue Tek remained in the capsule sections after immersion in water for less than 3 h. Excess Tissue Tek on the slide appeared as a white haze across the sections and slide after exposure to ethanol and xylene. The capsule sections did not remain on pre-cleaned slides when immersed in water for 3 h, but they did remain attached to the gelatin subbed slides for more than 3 h. At 3 h, gel-like patches of residual Tissue Tek were observed throughout the capsule sections and the sections appeared distorted under the microscope. A minimum of 4 h was required for complete or near complete removal of Tissue Tek. Two alcohol (70 and 100%) baths instead of three (70, 95 and 100%), after Tissue Tek removal improved the clarity of the sections. To remove the gel-like patches completely, the sections were immersed in a second xylene bath for up to 45 min.

2.4.10 Consistency of Tissue Staining

We monitored labrum and capsule staining using light microscopy. Because we sectioned the labrum in a sagittal plane, more sections were collected to sample the entire

tissue width (21 mm). For this reason, some specimens yielded up to 263 sections. The sections for the labrum, unlike the capsule, showed patterned tricolor staining that enables one to differentiate the structural zones of the labrum. Moreover, the staining pattern appeared to be the same in all the sections. Figure 4A demonstrates eight serial labrum sections from 263 total sections.

The capsule thickness in the contact surface plane was 1.5–2.0 mm; we collected an average of seven 100 µm sections, which corresponded to a tissue depth of 0.7 mm. Once this depth was reached, the color of the stained tissue started to appear different from the previous

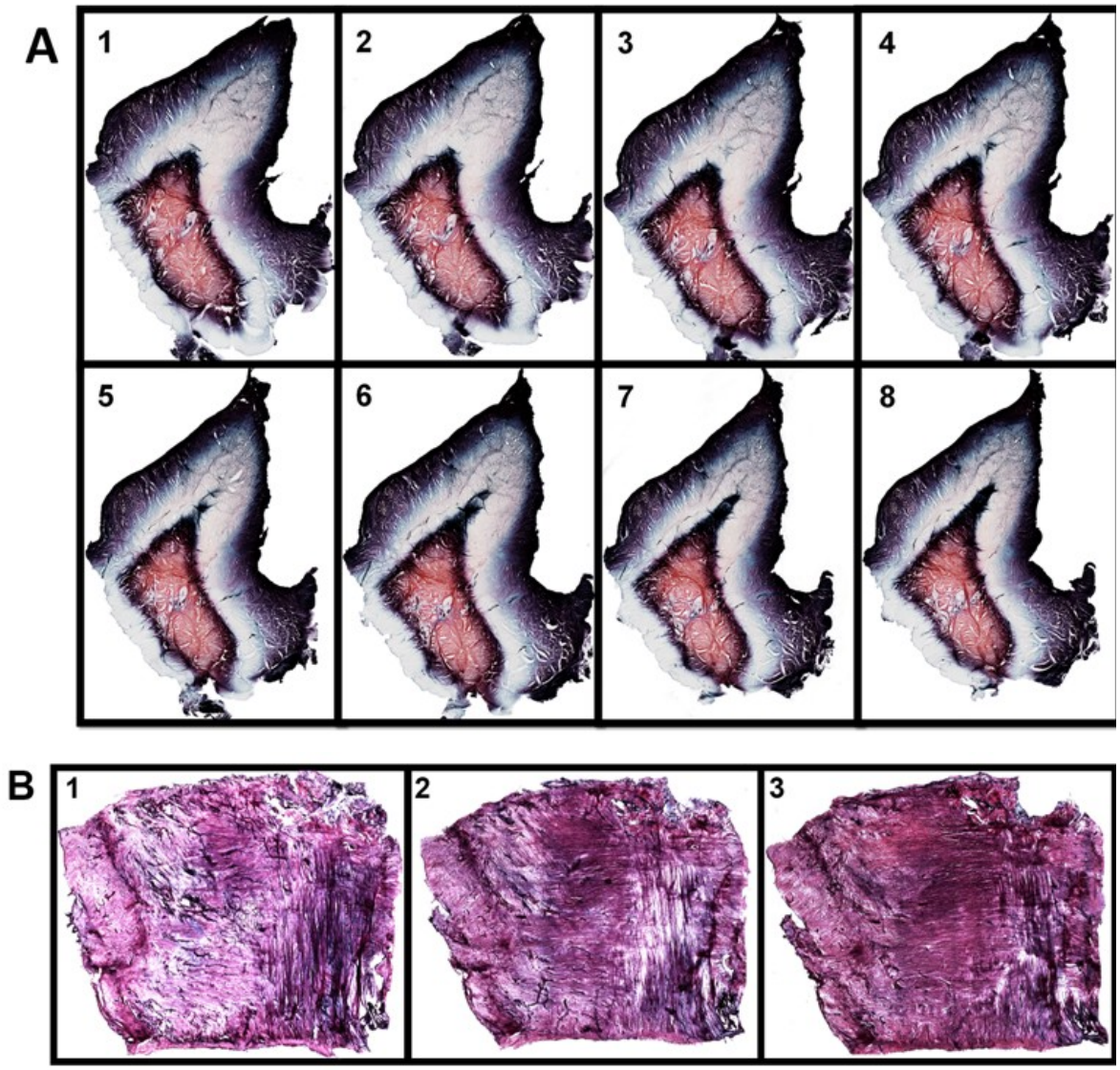

Figure 4. Representative serial sections of labrum (A, 10 X) and capsule (B, 4 X) showing consistent gold chloride staining.

seven sections in which the staining was less intense and lighter pink. Figure 4B shows consistent staining in three of the seven serial capsule sections.

2.4.11 Appearance of Neural Structures

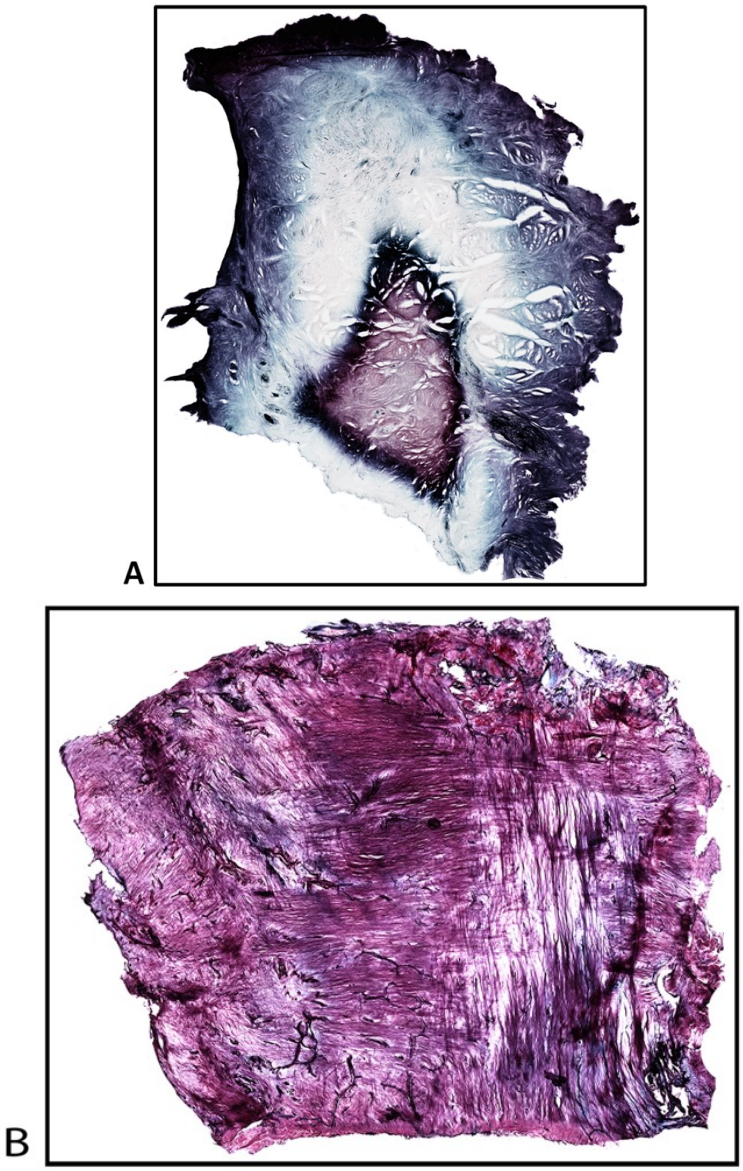

Figure 5. A) Section from labrum tissue reduced in 25% formic acid in ultrapure water showing the transparent to purple range of colors across three concentric layers; neural structures are stained black. B) Section of 100 µm thick glenoid capsule processed using 25% formic acid in citrate phosphate buffer showing pink to purple collagenous background and containing dark purple-black structures identified as type 1 and type 2 nerve endings. A, 10 X; B, 4 X.

Tissue samples that were reduced in 25% formic acid in ultrapure water without using a stir bar showed black myelinated axons, fascicles and blood vessels. The collagenous background appeared transparent. Staining was inconsistent among and throughout the samples. Type 1 nerve endings were easily identifiable. It was difficult, however, to differentiate type 2 nerve endings, fascicles and blood vessels when capsule tissue was reduced in 25% formic acid in ultrapure water. Tissue specimens reduced in acidified water showed results similar to 25% formic acid in ultrapure water, but less staining intensity. Reduction in sodium hydroxide resulted in a light blue collagenous background. The

capsules of type 2 sensory nerve endings and blood vessels stained darker blue than collagen, but not as dark as the myelinated axons. The entire structure of type 1 nerve endings stained dark blue and was easily identifiable.

Sections reduced in 25% formic acid in ultrapure water under controlled conditions (temperature and mixing rate) showed a color range within the background of the labrum. Myelinated axons were black and the external capsules of type 2 nerve endings were blue. The background showed three concentric layers. The outermost layer was pink-purple, the second layer was transparent, and the innermost layer was pink (Fig. 5A). When capsule tissue was reduced in 25% formic acid in ultrapure water under controlled conditions, flocculation developed and the desired color contrast between the tissue background and the structures of interest was not achieved by varying exposure time in the reducing solution.

Capsule tissue reduction in 25% formic acid in citrate PB produced optimal color contrast among the collagenous background, type 2 nerve endings, blood vessels and type 1 nerve endings. The use of citrate PB also eliminated flocculation. The collagenous background appeared transparent pink to purple and contained dark purple-black structures (Fig. 5B). The axonal tree-like formation and intertwined cylindrical endings of type 1 sensory nerve endings stained dark purple (Fig. 6A, B). The blood vessels stained darker purple-black with a lumen (Fig. 6C). Myelinated axons stained dark purple. The lamellae of cross sections of the external capsule of type 2 nerve endings showed a light pink concentric pattern and the entire corpuscle had an onion-like appearance (Fig. 6D–F). The lamellae were not defined clearly. Type 2 sensory nerve endings presented with an elongated, conical, pinkish external capsule and a dark purple inner capsule with nuclei in an orbital pattern (Fig. 6D, E, G) when their structure was preserved during sectioning.

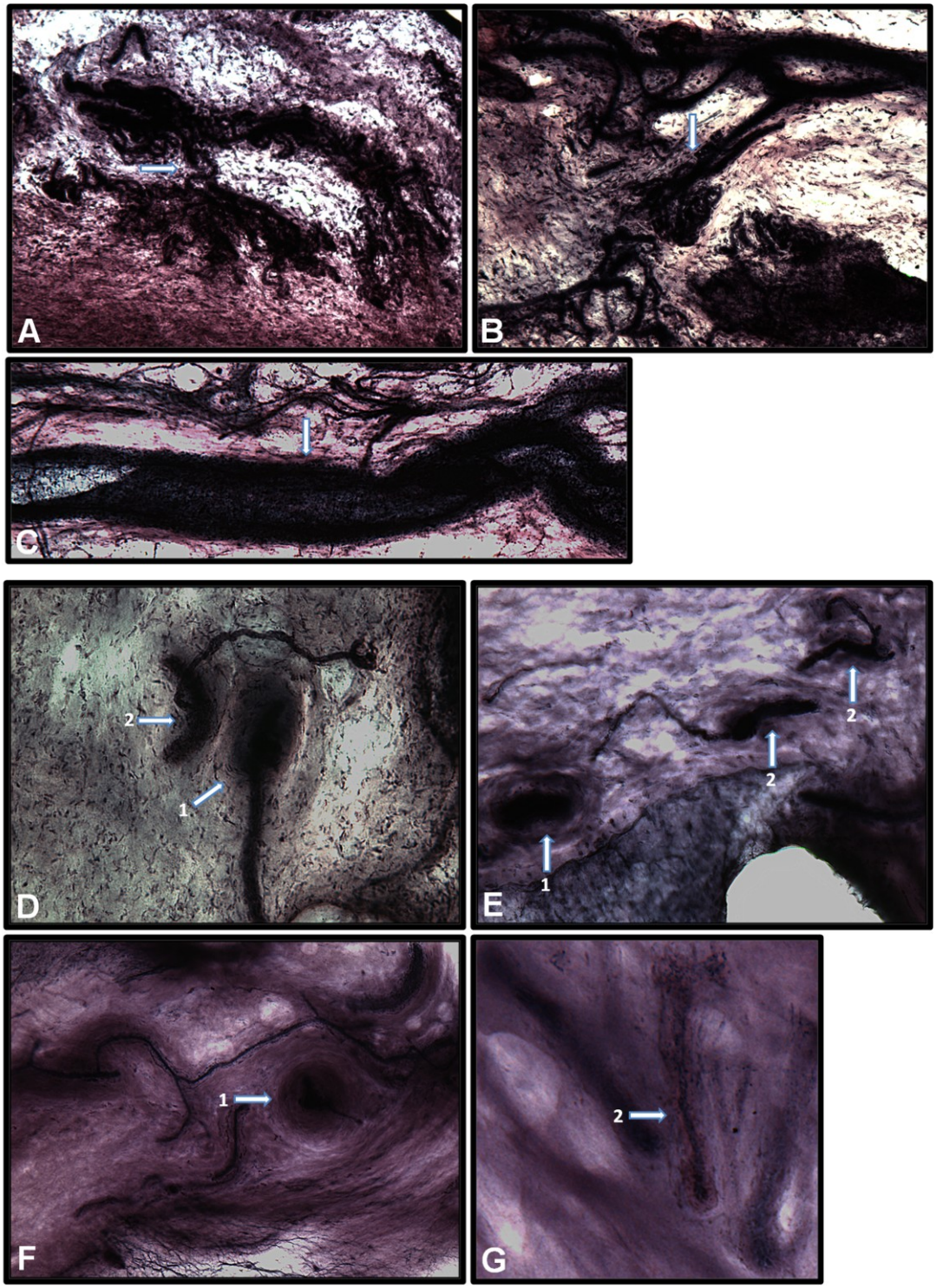

Figure 6. Glenoid capsule (white arrows). A) Type 1 sensory nerve ending innervating capsule tissue at the capsule-labrum junction. B) Type 1 sensory nerve ending innervating capsule tissue at the humeral attachment. C) Blood vessel. D–G) Different presentations of type 2 sensory nerve endings innervating capsule tissue including a cross-section of type 2 sensory nerve ending (1) and the entire corpuscle of type 2 nerve ending (2).

2.5 Discussion

2.5.1 General Observations

Two major difficulties often encountered with earlier staining protocols (Boyd 1962, Gairns 1930, Silverberg et al. 1989, Zimny et al. 1989) include intermittent failure to achieve either tissue transparency or gold reduction, and the absence of uniform staining throughout the depth of the tissue. Temperature and mixing rate of solutions are important for gold nanoparticle synthesis (Beesley 1992). Our results suggest that a temperature controlled environment (27–30° C) and a mixing rate of 400–450 rpm are necessary for consistent staining within the tissue and among samples.

2.5.2 Sucrose Dehydration

Because fibrocartilaginous tissues are difficult to section, we added a sucrose dehydration step to our protocol. Specifically, we used sucrose dehydration before the tissue transparency step, because it did not interfere with staining at this stage in our protocol. Sucrose is an excellent cryoprotectant, which prevents formation of ice crystals and preserves optimal morphology (Hayat 2000). We found sucrose dehydration to be especially beneficial when working with the labrum; the capsule was easier to work with and did not require additional treatment.

2.5.3 Tissue Transparency

The tissue transparency step is critical for resolving neural structures by light microscopy. Citric acid, a component of lemon juice, is essential for achieving transparency, but 100% lemon juice was more effective than citric acid alone (Silverberg et al. 1989). How transparency is achieved in tissue in unclear, but previous research on the browning of fruits and vegetables offers a clue (Pilizota and Subaric 1998). Tissue pigmentation results from an oxidative reaction. In addition to citric acid, lemon juice contains vitamin C, which is an anti-oxidant that prevents browning of fruits by binding oxygen. Apparently, in this staining process,

using lemon juice produced optimal tissue transparency that was caused by citric acid, an acidifier, and vitamin C, an anti-browning reagent. It also is important that in an acidic environment (pH < 3), enzymes responsible for browning are less active (Pilizota and Subaric 1998).

The tissue transparency step in gold chloride staining protocols includes exposure of tissue to both lemon juice and formic acid (Boyd 1962, Gairns 1930), which lowers the pH. When we exposed cadaver tissue to lemon juice only, the tissue color was lightened and under the acidic conditions (pH < 3), the tissue became transparent. The change in tissue pigmentation suggested that lemon juice and formic acid exert similar effects on tissue. We found that lemon juice and formic acid treatment for 10 min at 30^{o}C produces tissue transparency, but should be combined with 4% PFA to minimize tissue deformation.

2.5.4 Gold Chloride Impregnation

Gold chloride staining depends on deposition of gold chloride, a metallic salt, in the tissue (Mann 1929), a process known as gold impregnation. Boyd noted that the extent of staining depends on the extent of gold impregnation, which is related to the ratio of gold chloride solution to muscle tissue. In our protocol, the extent of gold impregnation depends primarily on the relation of gold to tissue thickness (Boyd 1962) and exposure time. To select the best conditions, we varied the tissue thickness and kept the volume of gold chloride solution constant.

Tissue thickness was critical for staining the capsule. Because the capsule tissue ranged from 1.5–2 mm thick, a layer of fat and muscle was left attached to increase overall tissue thickness, which helped produce optimal staining results. This was true for the reduction step as well. The best results were observed in tissue samples 5–7 mm thick. Boyd (1962) asserted that the duration of tissue exposure to gold chloride plays a negligible role in gold chloride staining.

To the contrary, we found that leaving the tissue in gold chloride for 10 min longer than in the original protocol improved its penetration throughout the tissue.

2.5.5 Gold Chloride Reduction

Gold chloride reduction is the most critical step for structure identification based on color differences among the tissue background, blood vessels, myelinated axons and sensory nerve endings. The type of reduction solution, duration of reduction and temperature together determine the best color contrast among structures. We achieved excellent color contrast when we used 25% formic acid in ultrapure water for 18 h for labrum tissue and 25% formic acid in citrate PB for 2 h for capsule tissue.

The mechanism of gold chloride staining of myelinated axons and sensory nerve endings is not clear. The chemistry of gold in the synthesis of gold nanoparticles and binding of gold to proteins, however, is better understood. Gold clearly plays a role in staining the proteins of myelinated axons and nerve endings. Mann (1929) observed that gold chloride staining was undependable. Freud (1884), in his research on nerve identification using gold chloride staining demonstrated that the intensity of the staining depended on the type of reduction solution used and the duration of exposure. In addition, Freud (1884) found that gold staining depends on the interaction of the metal with an organic compound such as citrate, sucrose, acetic acid, formaldehyde or formic acid. Organic acids, particularly formic acid, develop easily recognized images of the structure(s) of interest (Lenher 1913). This observation supports Gairns' use of formic acid in his method for gold chloride reduction (Gairns 1930).

When gold chloride is reduced, gold spheres (nuclei) are formed; the process is known as nucleation. As the concentration of gold increases, the atoms cluster to form particles. Flocculation often occurs during gold nanoparticle synthesis. Citrate, commonly used as a reducing agent, stabilizes gold particles by preventing gold nanoparticle aggregation and flocculation (Kumar et al. 2007). Earlier research has suggested that citrate ions have a high

affinity for ionic gold. The chelating head group of citric acid interacts with gold ions to form citrate-Au^{3+} complexes known as citrate-membrane assemblies (Gonzaga et al. 2008). In the presence of sodium citrate and an acid (e.g., tannic acid), the reduction of gold is improved (Beesley 1992). Therefore, the use of formic acid prepared in a citrate phosphate buffer not only yields the best staining results, but also minimizes flocculation in capsule tissue.

2.5.6 Stopping the Gold Chloride Reduction Reaction

As gold in tissue is reduced over time, its color continues to change until reduction is complete. Depending on the type of reduction solution used, the color varies, so the end colors differ also. We sought to achieve a specific color contrast between the collagen fibers and other structures of the tissue. To do this, it was necessary to stop the reduction process before it was complete. Although storing the samples in ultrapure water at 4°C did not stop the reduction completely, it did slow the process significantly, which allowed us to consistently obtain the desired staining contrast.

2.5.7 Embedding

The capsule, when removed from the reduction solution, shrank unevenly and was deformed into a “dome shape,” which made it difficult to embed. Conditioning, which included three to five repetitions of downward pressure applied to the tissue to achieve tissue flatness, reduced tissue stiffness and ultimately flattened the dome shape; complete or nearly complete tissue flatness (Fig. 3C-1) was achieved in this way. The plastic on the bottom of the aluminum mold prevented extraneous precipitation in the tissue that would occur if the tissue were pressed against metal. Embedding the capsule in a series of steps caused the tissue surface to remain flat during embedment for cryosectioning. In this way, the desired orientation was achieved without distorting shape of the tissue.

2.5.8 Cryosectioning

Two variables that contribute to different results among studies include the thickness of sections and examination of either random or serial sections (Hogervorst and Brand 1998). We selected 80 and 100 µm sections as optimal for labrum and capsule sections, respectively. To confirm the identification of structures, we suggest examining serial sections for anatomical examination.

2.5.9 Slide Processing

The time required for complete removal of Tissue Tek from labrum sections (labrum) varied depending on the surface area of the sections and the type of slide used. After Tissue Tek removal, shorter incubation times in ethanol and xylene for labrum sections compared to capsule sections prevented extensive curling of tissue. The extended periods in all solutions for slide processing improved the clarity of the sections and aided the identification of neural structures. The time may vary for different tissue types during each step of slide processing.

2.5.10 Appearance of Neural Structures

Type 1 sensory nerve endings have a tree-like structure with intertwined cylindrical endings known as capsules (Halata 1977). Type 2 sensory nerve endings include a neurite surrounded by an inner capsule consisting of specialized Schwann cells and squamous cells surrounded by an external collagenous capsule (Bell et al. 1994); however, not all type 2 nerve endings have an external collagenous capsule (Peterson et al. 1972). We identified the substructures of both type 1 and type 2 nerve endings using our staining protocol. We observed that the color contrast between type 1 and type 2 sensory nerve endings, collagenous background and blood vessels varied within the tissues we examined depending on the reducing solution used.

Gold chloride reduction in 25% formic acid in ultrapure water produced excellent results for the labrum, but the staining was inconsistent in the absence of consistent temperature and

mixing rate. Controlling these two factors maximized the color range in the tissues and produced the most favorable results.

Under controlled conditions, the labrum exhibited tricolor staining of the three concentric layers consisting of a superficial mesh, dense circumferential braided core and loosely packed peri-core zone (Hill et al. 2008). The background color of the labrum and capsule could be manipulated by changing the amount of time the tissue was exposed to the reducing solutions. Although we could change the background color of the capsule by reducing gold in 25% formic acid in ultrapure water, flocculation still occurred. Therefore, we used citrate PB instead of water. Use of citrate PB both eliminated flocculation and produced the best color contrast between the collagenous background and neural structures.

Gold chloride staining of myelinated axons and nerve endings has been used for many years and has undergone many modifications. Each modification yielded similar results, but none addressed the problem of inconsistent staining. We altered most of the original steps of the Gairns' protocol (Gairns 1930) to produce more consistent and optimal staining. Our changes included a consistent temperature, mixing rate, sucrose dehydration, altered lemon juice solution, 25% formic acid in citrate PB for reducing gold, the period that the specimens were in solution for each step, and a novel embedding process. Ultimately, our modified protocol enabled us to achieve consistent staining of nerve endings in both the labrum and capsule.

CHAPTER 3

Neuroanatomical Distribution of Mechanoreceptors and Fascicles in the Shoulder Capsule and Labrum

Preface: Chapter 3 experiments were carried out using our improved gold chloride staining protocol described in chapter 2.

3.1 Abstract

Background and purpose: Mechanoreceptive characteristics are often overlooked post injury and during treatment in the articular tissues of the shoulder. Yet, the distribution, location, and spatial arrangement of mechanoreceptors are important for neural signal conciseness and accuracy in proprioceptive information to yield functional joint stability. Awareness of the mechanoreceptor distribution and their associated fascicles in the shoulder glenohumeral joint stabilizers, capsule and labrum, may allow the retaining of proprioceptive integrity while restoring mechanical stability during surgery. We hypothesize there are mechanoreceptor and fascicular scarce regions that exist within the capsule and labrum that are more apt for surgery.

Methods: We determined the neuroanatomical distribution of mechanoreceptors and their associated fascicles in the capsule and labrum from eight human shoulder pairs using our improved gold chloride staining technique and light microscopy.

Results: A distribution pattern was consistently observed in the capsule and labrum from which we derived a neuroanatomical map. Both tissues demonstrated mechanoreceptor dense and scarce regions. Additionally, capsular fascicles were located in the subsynovial layer whereas labral fascicles were concentrated in the peri-core zone.

Interpretation: Based on our results, we suggest that the mechanoreceptor and labrum fascicular scarce regions may be more apt for surgical repair, thus allowing for neural structure preservation during surgery. Neuroanatomical mapping of mechanoreceptors and fascicles in the capsule and labrum could help guide the development of surgical techniques used to restore capsular and labral mechanical restraint, and in turn, reduce the rate of recurrent shoulder instability.

Keywords: Capsule, Labrum, Mechanoreceptors, Neuroanatomy, Shoulder, Surgery

3.2 Introduction

Mechanoreceptors play an important role in functional joint stability, but are not often considered when assessing possible causes of neuromuscular dysfunction. Specifically, articular mechanoreceptive nerve endings convey proprioceptive information (Riemann and Lephart 2002) that initiate reflexive muscular contractions. These contractions stiffen the joint, providing dynamic (functional) stabilization when required. The importance of mechanoreceptors in the glenoid capsule and labrum (static stabilizers) has been overlooked for many years when developing surgical techniques for restoring glenohumeral joint stability.

To date, surgery has been shown to be the most effective treatment for repairing the glenoid capsule and labrum while restoring mechanical stability of the shoulder compared to no surgery. Yet, there is still a high rate of recurrent instability (Robinson and Dobson 2004, Wang and Arciero, 2008). It has been suggested that surgery for restoring mechanical restraint, such as capsular shifts and plications, may disrupt mechanoreceptor structure, orientation, distribution, and concentration (Nyland et al. 1998, Steinbeck et al. 2003) as the capsule and labrum are mechanoreceptor containing tissues (Bresch and Nuber 1995, Guanche et al. 1999, Maass et al. 2001).

Mechanoreceptors have a receptive field with an assigned topographic location, and so the position of the receptive field is important for perceiving the location of mechanical stimuli in the body. Additionally, the spatial arrangement (distribution) of mechanoreceptors is essential for perceiving stimulus characteristics including the location and source of the stimulus, discrimination of the stimulus size and shape, and fine resolution of the stimulus. (Kandel et al. 2000)

Currently, it is believed that damage to the articular mechanoreceptors can result in proprioceptive deficits that lead to the onset of and recurrent glenohumeral joint instability (Hammer 2005, Lephart et al. 1994). More recently, the thought of preserving

mechanoreceptors during surgery has been gaining attention. Awareness of sensory nerve ending distribution and orientation could help guide surgical approaches to minimize mechanoreceptor damage, misalignment, and orientation.

Fascicles, from which axons project and terminally end as mechanoreceptors, are also found in the glenoid capsule and labrum. It is likely that the importance of these fascicles has also been overlooked. For instance, fascicles supplying the nerves of the knee, when stimulated, result in muscular contractions (Stewart 2003) suggesting that the anatomy and somatotopy of fascicles in other important tissues, such as the glenoid capsule and labrum may have clinical relevance. Fascicular damage has been observed during surgery and is thought to be the cause of “restrictive” clinical deficits such as muscle weakness specific to the damaged or misaligned fascicle (Stewart 2003). Although the clinical relevance of fascicles has not been well understood, their anatomical distribution should be considered.

The purpose of this study was to determine the neuroanatomical distribution of mechanoreceptors and fascicles in the glenoid capsule and labrum. In the course of the study, we sought to identify mechanoreceptor scarce regions that may be more apt for surgical repair. We conclude that neuroanatomical mapping can guide surgical intervention and ultimately help reduce the recurrence of shoulder instability.

3.3 Materials and Methods

Sixty-three glenoid capsule and labral specimens were harvested from eight cadaveric shoulder pairs (five fresh and three frozen) including four males (23, 55, 76, and 81 years of age) and four females (58, 62, 81, and 98 years of age). One of the cadaveric shoulders was found to be missing a labrum. The specimens were dissected from the most common region of injury (anteroinferior) and the least common region of injury (posteroinferior) as shown in Figure 1. Immediately following tissue harvest, the specimens were washed in saline for 10 min at room temperature. Subsequently, the specimens were stained using our modified gold chloride staining technique developed specifically for capsular and labral tissues (Witherspoon et al. 2013).

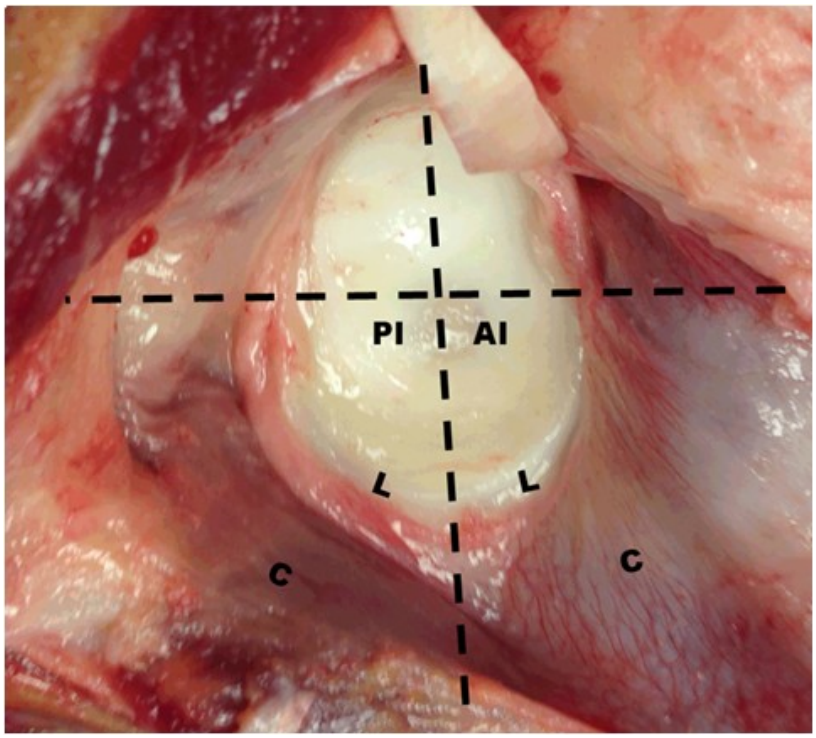

Figure 1. Image showing the anteroinferior (AI) and posteroinferior (PI) regions of the glenohumeral joint containing the capsule (C) and labrum (L) specimens harvested for this study.

3.3.1 Gold Chloride Staining

The following is a brief overview of the staining protocol (Witherspoon et al. 2013). The tissue specimens were blotted with Kim Wipes and placed in increasing sucrose concentrations (5%, 15%, and 25%) for a minimum of 8 hrs in each concentration at 4°C The remaining steps were performed at 30°C and a stir rate of 450 rpm. The specimens were then treated with 50 ml of solution containing lemon juice, paraformaldehyde and formic acid for 10 min. Next, the

specimens were transferred to 50 ml of 1% gold chloride and incubated for 30 min. The labrums were then separated from the capsules using ceramic instruments and exposed to different reducing solutions. The capsules were reduced in 25% citrate phosphate buffer for 1.5-2 hrs and the labrums were reduced in 25% formic acid for 18 hrs. Once the reduction step was completed, the specimens were stored in ultrapure water overnight.

3.3.2 Embedding

The next day, both capsule and labrum specimens were infiltrated with Tissue Tek and then frozen using an ethanol and dry ice bath. The embedding process used for capsular specimens was more complex than that for labral specimens. In order to section the capsules in the plane of the articular contact surface, we developed a novel embedding process in which the capsules were first flattened using a custom clamping device and then frozen (Witherspoon et al. 2013).

3.3.3 Sectioning and Slide Processing

The labrum was sectioned in the sagittal plane at 80 μm thickness and the capsule was sectioned in the contact plane at 100 μm thickness. These sections were then mounted to gelatin subbed slides and the slides were dehydrated using 70% ethanol, 100% ethanol and two xylene baths, sequentially.

3.3.4 Data Collection

Neural structures within the tissue were observed using light microscopy and images were collected. Because mechanoreceptors have specialized nerve endings of varying morphology, to avoid confusion regarding the different terminology used to describe the nerve endings, Freeman and Wyke (Freeman and Wyke 1967) developed a numerical classification system (types 1 through 4) that categorized mechanoreceptors based on the primary morphological features. We sought to identify mechanoreceptors having type 1 and type 2 nerve endings. A more descriptive morphology of the nerve endings identified in this study was

provided based on descriptions given by other authors (Freeman and Wyke 1967, Bell et al. 1994, Jozsa et al. 1993) that most closely resembled the sensory nerve endings in the capsule and labrum.

Distributional mapping of the mechanoreceptors and fascicles was determined after analyzing the serial sections. During image viewing, the characteristics and location of each neural structure were recorded on a diagram representative of the tissue of interest aiding in constructing the neuroanatomical map. The entire length and thickness of each labrum was mapped in this manner, whereas only the first 500 μm of thickness of each capsule, from the joint contact surface outward, was mapped.

3.4 Results

Two primary types of mechanoreceptors were observed in this study: slow adapting type 1 sensory nerve endings resembling Ruffini corpuscles and rapid adapting type 2 sensory nerve endings resembling Pacinian corpuscles. Type 1 and type 2 sensory nerve endings, as well as fascicles, were found in both the glenoid capsule and labrum. The fascicles consisted of a bundle of three to six axons, branched in a unidirectional or bidirectional manner, and were always accompanied by blood vessels. Fascicular characteristics varied between the capsule and labrum as described below.

3.4.1 Morphology of Nerve Endings

3.4.1.1 Glenoid Capsule

Type 1 sensory nerve endings (Fig. 2A), resembling Ruffini corpuscles, consisted of a bulb-shaped structure described as a hilum (Freeman and Wyke 1967) from which multiple axons projected. At the terminus of each projected axon were corpuscles, primarily shaped as globular tree-like structures. The globular corpuscle consisted of an extensive arborization similar to Ruffini endings reportedly found in the periodontal ligament (Maeda et al. 1999), but without an outer collagenous layer. The arborization included fine coiled capsules at the termini. Rarely observed ovoid shaped Ruffini corpuscles consisted of a collagenous external layer enclosing a small arborization.

Type 2 sensory nerve endings presented with subtle variations in appearance as shown in figures 2B-2E. However, they morphologically resembled the basic structure of Pacinian corpuscles as described by Bell et al. (Bell et al. 1994) and were identical to the structures observed by Boyd (Boyd 1962). These mechanoreceptors presented with elongated conical-shaped (Freeman and Wyke 1967) corpuscles. The nerve ending was connected to an axon that projected into the corpuscle where it terminated. The terminus, also called a neurite, was ensheathed by an inner capsule that appeared as a thick or thin organized orbital cloud

containing multiple nuclei. Surrounding the inner capsule was a collagenous layer known as the external capsule. When the corpuscle was cross sectioned, the external capsule had an onion-like structure. However, when the entire corpuscle was in view, it appeared ellipsoidally shaped. The type 2 nerve endings enwrapped the collagen fibrils of the capsule (Fig. 2B) and often appeared as a single corpuscle or bifurcated. They appeared to communicate with each other (Fig. 2C) and were often outlined by capillary blood vessels (Fig. 2D) similar to type 2 mechanoreceptors identified by Freeman and Wyke (Freeman and Wyke 1967).

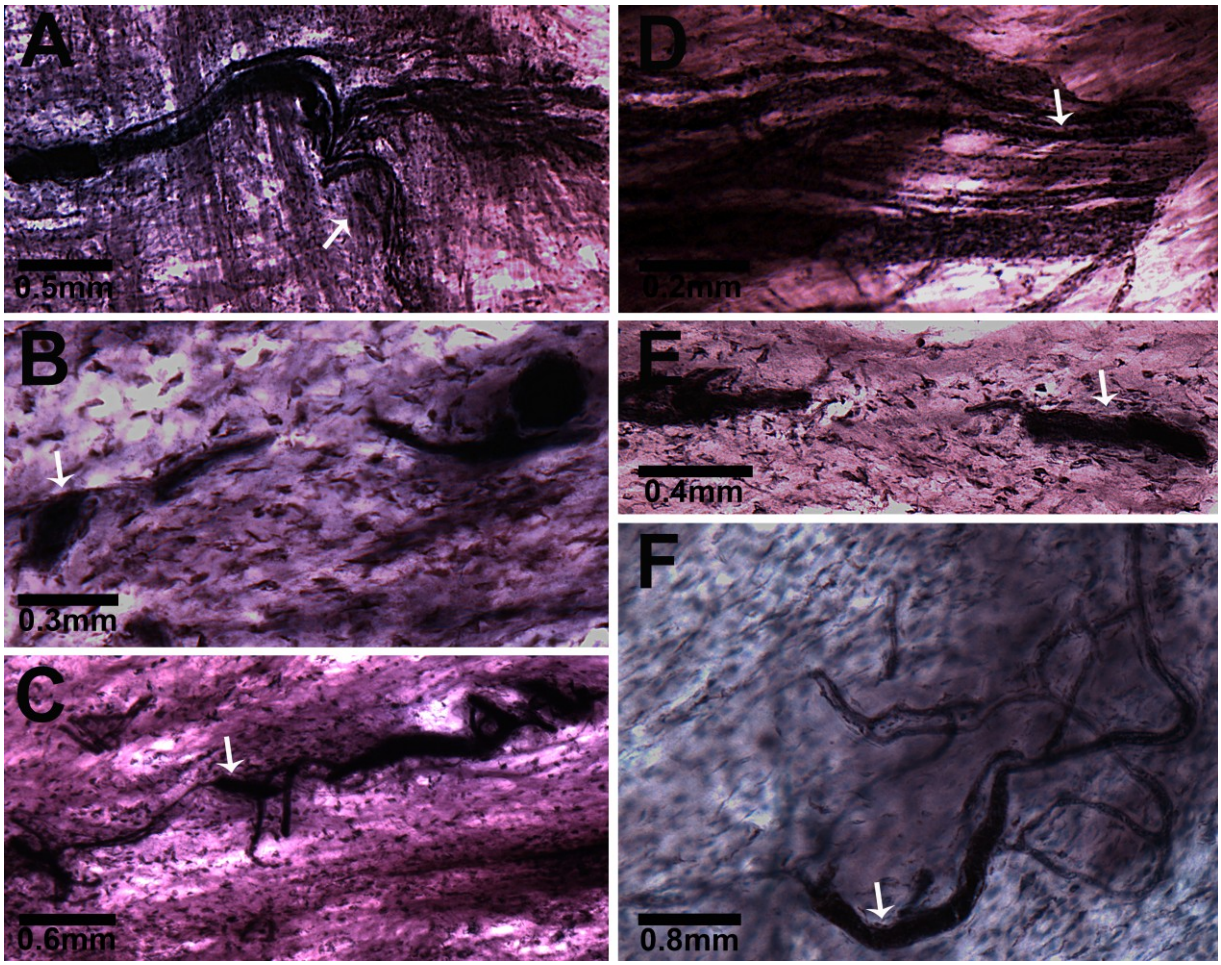

Figure 2. Images of neural structures in the glenoid capsule (indicated by arrows). A) Image of a type 1 nerve ending characteristic of the globular tree-like structure. B-F) Images of type 2 nerve endings showing subtle morphological variability. B) A single corpuscle enwrapping collagen fibrils and synapsing with another nerve ending. C) A chain of type 2 nerve endings. D) A single corpuscle outlined with capillaries. E) Encapsulated sensory nerve ending. F) Image of a complex branched encapsulated nerve ending.

In addition to the commonly described type 1 and 2 sensory nerve endings, another structure was identified that was described by Peterson et al. (Peterson et al. 1972) as simple, branched and complex encapsulated nerve endings. These nerve endings seemed to have a similar appearance to that of type 2 receptors but without a noticeable inner capsule. Some of the encapsulated endings appeared coiled. A complex encapsulated nerve ending is shown in Figure 2F.

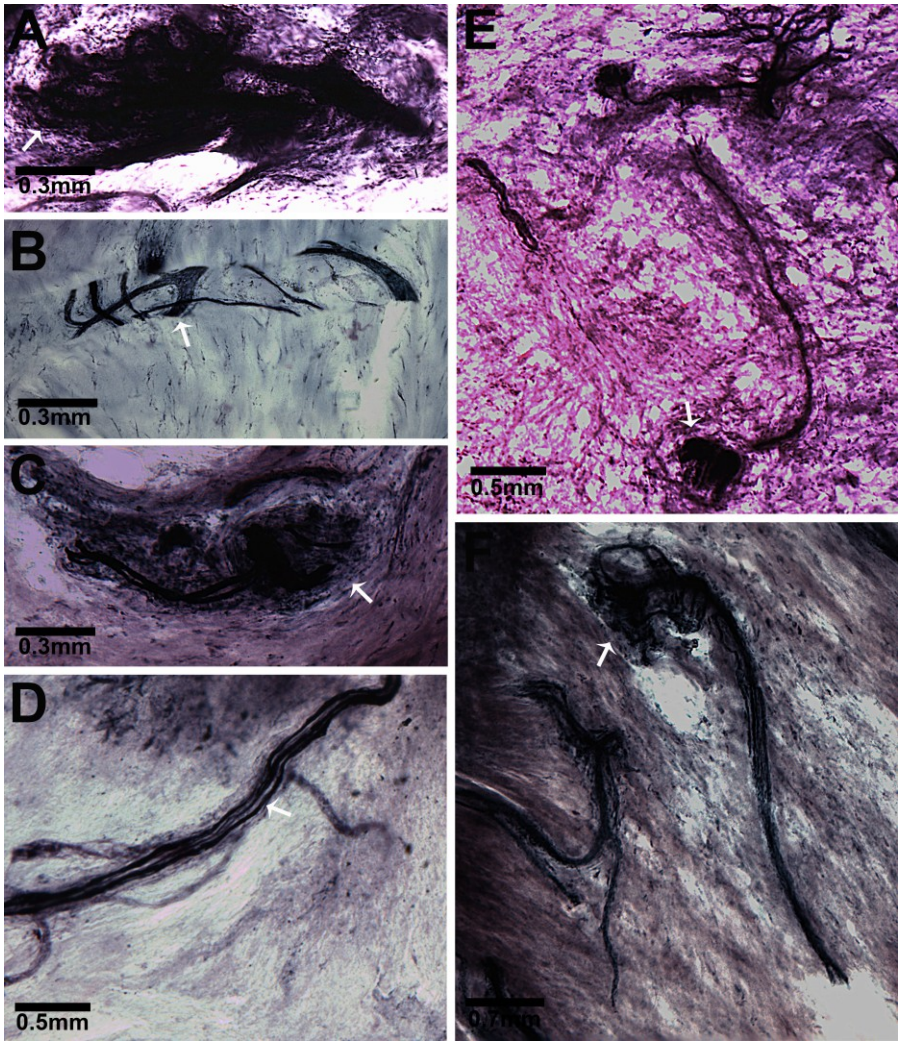

Figure 3. Images of neural structures in the glenoid labrum (indicated by arrows). **A)** A type 1 nerve ending with globular tree-like structure. **B)** A type 1 nerve ending resembling a Golgi Tendon Organ-like Ruffini. **C)** A Krause-like nerve ending (type 2). **D)** Fascicle consisting of several axons at the capsulolabral junction. **E)** A small encapsulated globular tree-like structure (type 1) with an external collagenous ring. **F)** A small encapsulated globular tree-like structure (type 1) without the external collagenous ring.

3.4.1.2 Glenoid Labrum

Type 1 sensory nerve endings in the labrum had similar morphology to those in the capsule except the globular tree-like structure was less intertwined (Fig. 3A). There were very few globular type 1 nerve endings encapsulated in an external collagenous layer (Fig. 3E) and unencapsulated (Fig. 3F). The globular

type 1 nerve endings were identified in older specimens (>76 years of age), while in the younger specimens they were scarce. The most commonly reoccurring nerve endings observed were Golgi Tendon Organ-like (GTO-like) Ruffini endings (Fig. 3B) as described for the meniscus (Zimny 1988) and anterior cruciate ligament (Raunest et al. 1998). Type 2 sensory nerve endings were identified as Krause-like structures (Fig. 3C), but they were rarely observed.

3.4.2 Distribution of Nerve Endings and Fascicles

Within the capsule and labrum, the sensory nerve endings were distributed throughout the tissue in different patterns. In the capsule, the axons and their associated type 2 nerve endings were primarily oriented medial to lateral and spatially arranged in the inferior to superior direction. Type 1 nerve endings were positioned inferior to superior at the most medial and lateral aspects of the capsule. In the labrum, sensory nerve endings were generally positioned in four areas of the peri-core zone relative to the dense core. The patterns did not vary with age; however, the number of nerve endings did vary such that the sensory nerve endings appeared distributed intermittently.

3.4.2.1 Glenoid Capsule

Type 1 nerve endings appeared as clustered groups of two or three found consistently at the capsulolabral and capsulohumeral junctions. They were more concentrated in the superficial layer (contact surface) and reached a depth of 200μm. These endings were oriented inferior to superior relative to the glenoid cavity.

Type 2 nerve endings were oriented medial to lateral relative to the glenoid cavity. The type 2 nerve endings became less concentrated at 500μm depth. Similar to type 1 nerve endings, type 2 nerve endings also appeared in groups of two and sometimes three. The corpuscles were found wrapped around the collagen fibers, synapsed longitudinally and distributed homogenously. Tissue specimens that had dense horizontal collagen fibers near the humeral attachment site contained type 2 nerve endings oriented parallel to the glenoid cavity.

Figure 4A outlines the distribution pattern of type 1 and type 2 nerve endings that we observed in the capsule.

Fascicles were located in the subsynovial layer of the glenoid capsule and branched in a bidirectional pattern, inferior to superior and medial to lateral. The fascicles appeared as a lattice network and were outlined with blood vessels. Fascicular axons projected toward the articular contact surface and ended as type 1 nerve endings. Fascicular axons directed away from the glenohumeral contact surface terminally ended as type 2 sensory nerve endings starting at a depth of 200μm.

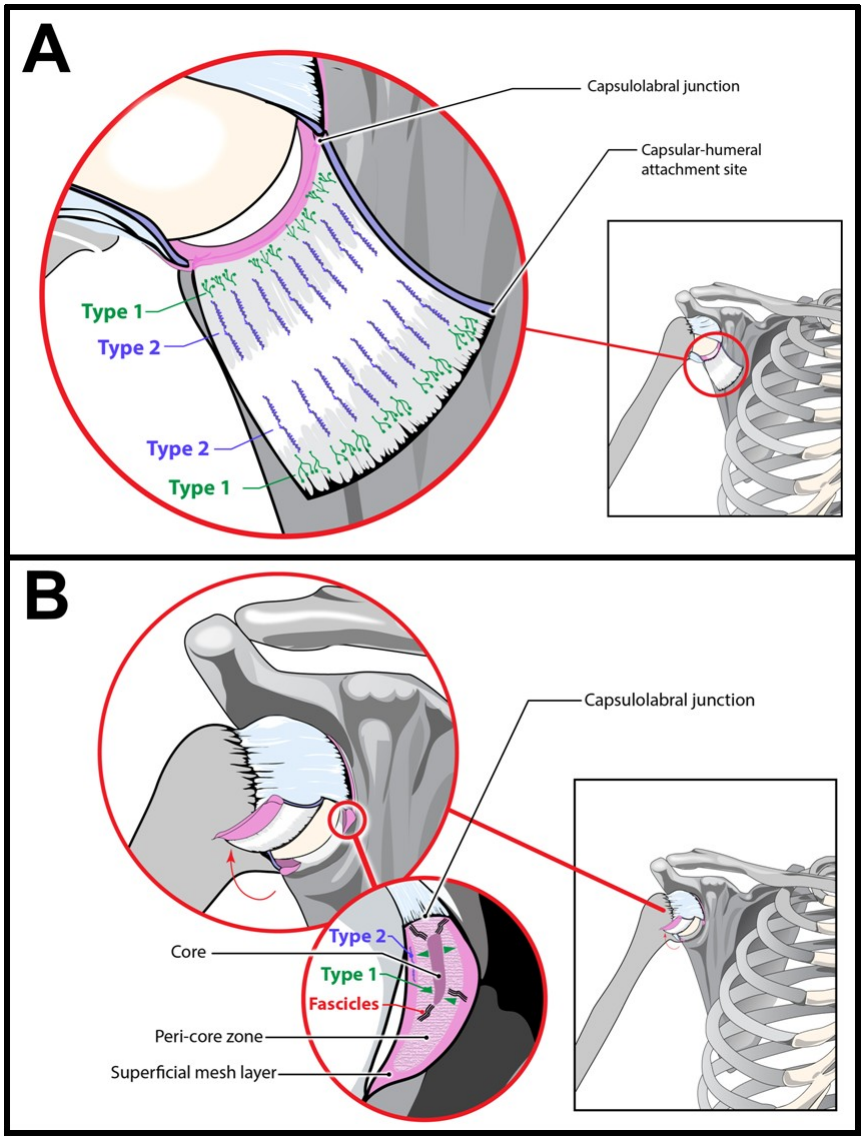

Figure 4. A) Sketch demonstrating the distribution of type 1 and type 2 nerve endings in the glenoid capsule. **B)** Sketch demonstrating the distribution of fascicles, type 1 and type 2 nerve endings in the glenoid labrum.

3.4.2.2 Glenoid Labrum

GTO-like Ruffini endings were sparsely distributed in the peri-core zone of the labrum, but were observed in larger numbers than the fascicles. These structures were positioned diagonally to the labrum core adjacent to the fascicles at four points appearing perpendicular to the glenoid rim. Type 2

nerve endings were rarely found, but when observed they were primarily found near the contact surface, close to the capsular attachment site. The distribution of nerve endings and fascicles in the glenoid labrum are outlined in Figure 4B.

The fascicles were generally positioned between adipose cells and consisted of bundles of three to six axons. The fascicles were more clearly identifiable in the labrum (Fig. 3D) than in the capsule. The more superior portion of the anteroinferior labral specimens contained more adipose cells, and thus fascicles, than the inferior portion. Adipose cells appeared intermittently in the peri-core zone. Individual axons extended from the fascicles within the adipose cells ending as GTO-like Ruffini endings. Fascicles were also located at the capsulolabral junction and gave rise to GTO-like Ruffini- and Pacinian-like nerve endings. Fascicles located closer to the contoured superficial layer of the labrum periodically ended as Krause-like nerve endings and tree-like Ruffini corpuscles.

3.5 Discussion

3.5.1 Nerve Ending Distribution and Function

Theoretically, the location and distribution of capsular type 1 and type 2 nerve endings support their ability to register sustained stretch, especially at extreme ranges of motion and changes in tension, respectively. Additionally, the random dispersal of simple, branched, and complex encapsulated nerve endings supports their ability to respond to “maximal variations in tension and pressure” (Peterson et al. 1972) that occur in areas exposed to the greatest amount of stress. In the labrum, the function of GTO-like Ruffini endings has yet to be determined. However, since we observed GTO-like Ruffini endings in the labral peri-core zone, and this labral region functions to absorb compressive forces (Hill et al. 2008, Nishida et al. 1996), we propose that GTO-like Ruffini endings are activated in response to sustained compressive forces. Since the distribution pattern of the capsular and labral mechanoreceptors seems to support their perceived function, it is reasonable to conclude that capsular and labral mechanoreceptors are important for glenohumeral joint function.

3.5.2 Nerve Ending Distribution and Surgery

Surgical techniques for restoring mechanical restraint to the glenohumeral joint have been developed with little consideration for preserving mechanoreceptors due to the lack of knowledge concerning their anatomical distribution. This may be one of the contributing factors to the reoccurrence of shoulder instability. We suggest that neural structures and their associated topography should be taken into consideration during surgical planning, based on our observation of a consistent distribution pattern and the proposed relationship between mechanoreceptor location and function. The results of this study imply the need to preserve and realign the neural structure during surgery.

Our study revealed several important findings that might be considered when planning or executing shoulder surgery. Fascicular and mechanoreceptor dense regions started at the

surface of the capsule and became less concentrated deeper into the tissue (~500μm) toward the ligaments and muscles. This suggests the most critical regions of concern regarding neural organization and concentration lie closest to the humeral head. Type 2 nerve endings, through the depth of the tissue, were more concentrated medial and lateral relative to the glenoid cavity and less concentrated in the mid region of the capsule thereby making the mid region of the capsule potentially more acceptable for surgery. Our findings also show that capsular fascicles appeared as a network throughout the surface of the glenoid capsule similar to the capsules of other synovial joints. The capsule of synovial joints has three layers including the synovia layer (direct contact with humeral head), subsynovial layer, and dense fibrous layer. The subsynovial layer is a loose connective tissue that contains both nerves and blood vessels supplying the synovium and dense fibrous connective tissue. (Buckwalter 1999) Since the fascicles appeared as a lattice network throughout the capsular surface, it would appear to be nearly impossible to avoid damage to these structures and restore their orientation and alignment with capsular tightening procedures if the subsynovial layer is disturbed. The distribution of these fascicles would seem to support the use of methods such as thermal capsulorrhaphy for capsular tightening, but this technique has proven to have the highest instability recurrence rate (Sullivan et al. 2008).

Within the labrum, the fascicles were oriented parallel to the glenoid rim primarily at the capsulolabral junction. The attachment sites for the capsule and for the glenoid rim (region closest to the capsule) to the labrum were two regions that were consistently concentrated with fascicles and type 1 nerve endings. These regions appear to be the most vulnerable areas for labral repair surgery. However, the peri-core zone at the capsulolabral junction was less concentrated with fascicles and sensory nerve endings making this area more acceptable for anchoring the labrum during surgical repair.

3.5.3 Limitations

In this study, we lacked information whether the cadavers used had previous shoulder injuries or were diagnosed with shoulder instability. Therefore, we were unable to determine the neural structure and distribution pattern in relation to injury. We were able to verify that the shoulders used in the study did not have any indication of shoulder surgery. Interpretation of the results from this study should be limited to the general distribution pattern of the neural structures within and between capsular and labral tissue specimens.

3.5.4 Future Directions

Further studies should be performed to determine the influence of surgical techniques on neural communication between the glenoid capsule, labrum, and surrounding musculature. In addition, neural structures, orientation, and distribution should be studied pre- and post- injury to gain insight into the effect of injury on articular mechanoreceptive characteristics. These neurological changes, following injury, may contribute to some aspects of faulty proprioception and contribute to recurrent instability. The suggested studies might further examine the effect of neural injury in the capsule and labrum on functional stability.

3.5.5 Concluding Remarks

This is the first study, to our knowledge, that has illustrated a neuroanatomical map of the anteroinferior and posteroinferior glenoid capsule and labrum. Additionally, we have identified mechanoreceptor scarce regions that may be more apt for surgical repair. Our findings add to the neuroanatomical understanding of the glenohumeral joint stabilizers primarily responsible for the reoccurring unstable shoulder.

CHAPTER 4

The Relationship Between Glenohumeral Joint Laxity and Mechanoreceptors in the Glenoid Capsule and Labrum: An Exploratory Study

Preface: While creating a neuroanatomical map (chapter 3), we also assessed the number of mechanoreceptors in the tissues of interest presented in chapter 4.

4.1 Abstract

Mechanoreceptors have sensory nerve endings that register stimuli for regulating neuromuscular control, and ultimately give rise to dynamic glenohumeral joint stabilization. A loss of neuromuscular control leads to excessive glenohumeral joint laxity resulting in shoulder instability and recurrent instability post treatment. Shoulder instability, characterized by excessive joint laxity, is the most common shoulder injury and the recurrence rate post treatment is 15-20%. Yet, the relationship between mechanoreceptors and glenohumeral joint stability has not been well studied.

Mechanoreceptors have been identified in both the capsule and labrum, two of the joint's most important static stabilizers. Injury to the joint can take the form of microtears in these stabilizers resulting in excessive joint laxity. We sought to explore whether the amount of glenohumeral joint laxity varies with the number of sensory nerve endings as the number of mechanoreceptors may be a key component in shoulder instability occurrence as well as recurrences. We hypothesized that the number of sensory nerve endings would decrease as joint laxity increased.

We used five fresh and three frozen cadaveric human shoulder pairs aged 23 and 55 to 98. The skin, subcutaneous tissue, and muscle were removed from the shoulders, scapula, and brachium leaving the capsule and labrum attached. The scapula and distal humerus were fixed to a customized base in neutral position (20^{o} abduction, 0^{o} rotation). With a materials testing machine, translation was applied in the posterior and anterior directions. Subsequently, the anteroinferior and posteroinferior capsule and labrum tissues (63 specimens) were removed and stained using our improved gold chloride staining technique for the identification of sensory nerve endings using light microscopy.

There was an average of 652 total number of sensory nerve endings in capsule. More specifically, within the anteroinferior and posteroinferior capsule, there was an average of 260

and 223 sensory nerve endings, respectively. The labrum, on the other hand, consisted of an average of 81 and 88 total sensory nerve endings anteroinferior and posteroinferior. Paired t-test analyses revealed a strong positive correlation between total neural count (sum of sensory nerve endings in the anteroinferior and posteroinferior capsule and labrum tissues) and total joint laxity (sum of anterior and posterior joint laxity). Further analyses revealed age, gender, and injury influence the relationship between neural count and joint laxity. We demonstrated that the potential relationship between neural count and joint laxity exists suggesting that mechanoreceptors should be studied in greater detail relative to joint instability. This study also suggests that the number of mechanoreceptors may play an important role in joint stabilization. For this reason, the loss and preservation of mechanoreceptors should be taken into the account following injury and when planning for surgery.

4.2 Introduction

Although proprioception is a mechanism required for protecting joints and maintaining joint stability, mechanoreceptors have been the least considered structures during proprioceptive dysfunction. Proprioception refers to the afferent information signaled from mechanoreceptors originating from the “internal areas” of joints (Nagai et al. 2007). Neural conduction studies have demonstrated joint proprioceptive deficits in the presence of injury, but the structures responsible for signaling proprioceptive information to the muscles have just recently been gaining greater attention and appreciation. Mechanoreceptors have different types of sensory nerve endings that register and signal information about joint position and different types of movement such as acceleration and deceleration. There are two major types of mechanoreceptors identified in the glenoid capsule and labrum, specifically type 1 and type 2 sensory nerve endings. The glenoid capsule and labrum are the two innermost static stabilizers of the glenohumeral joint that have direct contact with the humeral head. These structures are thought to be primarily responsible for mechanical restraint; however, the presence of mechanoreceptors distributed evenly throughout the capsule and labrum implies they also play a role in joint proprioception and functional stability (Witherspoon et al. 2013).

Mechanoreceptors transduce mechanical deformation of the tissue, in response to joint position and movement, to electrical signals (Warner et al. 1996). The location and distribution of the mechanoreceptors within the tissue represent a receptive field. The number of sensory nerve endings within the receptive field may account for the strength and conciseness of the signal. Neural count has been performed in the anterior cruciate ligament of rats and sheep (Kanemura et al. 2002; Raunest et al. 1998), hips of human subjects (Moraes et al. 2011), and shoulders of marsupials (Maass et al. 2001). However, the relationship between neural count and organ function has been more so studied in the brain rather than in joints. For instance, a small study showed a greater number of neurons in the prefrontal cortex of autistic children

compared to controls (Courchesne et al. 2011). Theoretically, a greater number of nerve endings within the stabilizers of the shoulder would yield more accurate information in response to what is occurring at the joint. However, in the presence of injury these detailed characteristics may be disrupted secondary to damage incurred by the mechanoreceptors.

Excessive joint laxity results from repetitive microtrauma, trauma, and damage to neural receptors (Lephart et al. 2002). Yet, neural count and its relationship to joint stability are rarely determined in the musculoskeletal joints of humans. Therefore, we sought to explore the relationship between neural count and glenohumeral joint laxity in human cadaveric shoulders.

4.3 Methods

4.3.1 Shoulder Preparation

Five fresh and three frozen cadaveric shoulder pairs with varying ages (23 and 55 to 98 years of age) for a total of sixteen shoulders were used in this study. The fresh shoulders were procured from the bodies within twelve hours of receipt whereas the frozen shoulders that were previously separated from the body were thawed. Immediately following shoulder separation from the fresh bodies and the thawing of frozen shoulders for mechanical testing, the musculature and subcutaneous tissue were removed from the scapula and humerus while the capsule and labrum remained intact.

The scapula of the shoulder complex was then secured to a custom base fixture. The humerus was fixed in a neutral position (20^{o} abduction and neutral rotation) relative to the medial border of the scapula (Borsa et al. 2000). We then identified and marked the location of the greater tubercle and drilled a hole anterior to posterior through the humerus. The glenoid tuberosity was used as the anterior reference point. After hole was drilled through the humerus, the shoulder was fixed in position and the humeral head was seated in the glenoid fossa. The test position was similar to the position used by clinicians with Load and Shift Stability test, except anterior and posterior translation was determined with the cadaveric shoulder in a horizontal plane.

4.3.2 Measurement of Joint Laxity

The testing fixture was then attached to the materials testing machine using C-clamps and a rod from the testing machine was placed through the hole. The fixture was designed to allow for anterior and posterior translation of the humeral head on the glenoid fossa for joint laxity measurement. Ten loading cycles (1 cycle: posterior->neutral->anterior) were performed to condition the specimens. Anterior-posterior joint laxity was extracted from the mean of the following five cycles. Laxity zones were measured using a custom Matlab routine.

4.3.3 Capsule and Labrum Tissue Preparation

While we were unable to obtain previous history of shoulder injury, the uninjured and injured groups were determined by observation following mechanical testing. To do so, the shoulders were further dissected exposing the capsule and labrum. Tissue injuries within the injured group included capsular tears, labral tears, tissue calcification, and tissue degeneration. The capsule and labrum were then harvested from the most common area of pathology (anteroinferior) and the least common area of pathology (posteroinferior). Subsequently, the tissues were immediately washed in two saline baths for 10 min each.

4.3.4 Gold Chloride Staining

Nerve endings in capsule and labrum were visualized using gold chloride staining that we modified specifically for these tissues (Witherspoon et al. 2013). In brief, following the saline wash, the specimens underwent sucrose dehydration with increasing sucrose concentrations (5%, 15%, and 30%). The specimens were transferred to lemon juice treatment solution containing lemon juice, formic acid, and paraformaldehyde for 10 min. The specimens were impregnated with 1% gold chloride for 30min. The capsule and labrum were then placed into separate containers, and the gold chloride was reduced in the capsule and labrum using different reducing solutions. For gold chloride reduction, the capsule was treated in 25% formic acid made in citrate phosphate buffer for 1.5-2 hrs while labral specimens were treated in 25% formic acid made in ultrapure water for 16 hrs. Once the gold chloride was reduced within the tissue specimens, the tissue was washed in ultrapure water for 2 min and transferred to fresh ultrapure water overnight.

4.3.5 Tissue Embedding and Sectioning

The tissues were blotted using a KimWipe, to remove access of water, and then infiltrated with Tissue Tek for a minimum of 30 min. Capsular specimens were flattened and frozen using our custom made device and novel embedding process. Labral specimens were

frozen in plastic molds. Freezing was achieved using an ethanol and dry ice bath. The frozen molds containing capsular tissue were sectioned at 100μm thickness whereas the labral specimens were sectioned at 80μm thickness. The sections were directly adhered to gelatin subbed slides. These slides were then processed in 70% and 80% ethanol followed by two xylene baths. The sections were analyzed using light microscopy.

4.3.6 Neural Count

The number of type 1 and type 2 sensory nerve endings (neural count) was determined using the rare event protocol neurostereological principles. The counting criteria included nerve endings with characteristic form and the use of serial sections to avoid counting a mechanoreceptor more than once. Our capability to clearly identify the beginning and end point of each mechanoreceptor was verified.

4.3.7 Statistical Analyses

Paired t-test analyses were used to analyze the relationship between neural count and joint laxity. ANCOVA analyses were used to determine whether age, gender, and/or injury influenced the relationship between neural count and joint laxity. Total neural count referred to the sum of sensory nerve endings in the anteroinferior and posteroinferior capsule and labrum. Total capsule or total labrum neural count refers to the sum of sensory nerve endings in the anteroinferior and posteroinferior regions of the tissue of interest. Total joint laxity referred to the sum of anterior and posterior joint laxity. Anterior and posterior joint laxity were not used independently to reduce potential error in the amount of joint laxity given there is no clearly defined mechanism designed for consistently defining neutral position.

4.4 Results

4.4.1 Number of Sensory Nerve Endings

4.4.1.1 General

The average total number of sensory nerve endings is summarized in Table 1. There was an average of 652 total sensory nerve endings. Anteroinferiorly, the capsule consisted of an average of 260 ± 117 sensory nerve endings and the labrum had 81.4 ± 90.0 sensory nerve endings. Posteroinferiorly, the capsule contained an average of 223 ± 79.6 sensory nerve endings and the labrum had 87.6 ± 58.4 sensory nerve endings. Within the capsule, compared to the labrum, there were a greater number of type 2 sensory nerve endings whereas the labrum consisted of a greater number of type 1 sensory nerve endings. The average percentage of anteroinferior and posteroinferior type 2 sensory nerve endings was 79.8% in the capsule and 36.7% in the labrum whereas there was an average of 63.3% type 1 sensory nerve endings in the labrum and 23.1% in the capsule (Fig. 1).

Table 1. Total neural count and neural count in the anteroinferior (AI) and posteroinferior (PI) glenoid capsule (C) and labrum (L).

Total Neural Count (N)				
		N	Mean	Std Dev
AI + PI		16	652	230
AI	C	16	260	117
	L	15	81.4	90.0
PI	C	16	223	79.6
	L	16	87.6	58.4

4.4.1.2 Gender and Injury Differences in Neural Count

The number of sensory nerve endings appeared to be similar between males and females (Table 2) as well as the injured and uninjured groups (Table 3). However, when observing gender differences in the injured and uninjured groups, there were apparent differences in the number of sensory nerve endings. Specifically, females presented with a greater total neural count ($\bar{y}$=783 ± 211) compared to males ($\bar{y}$=497 ± 70.5) in the uninjured

group whereas the opposite was true in the injured group (females, ȳ=543 ± 227; males, ȳ=785 ± 263). Essentially, the number of nerve endings decreased in the injured group compared to the uninjured in females, but increased in the injured group compared to the uninjured in males.

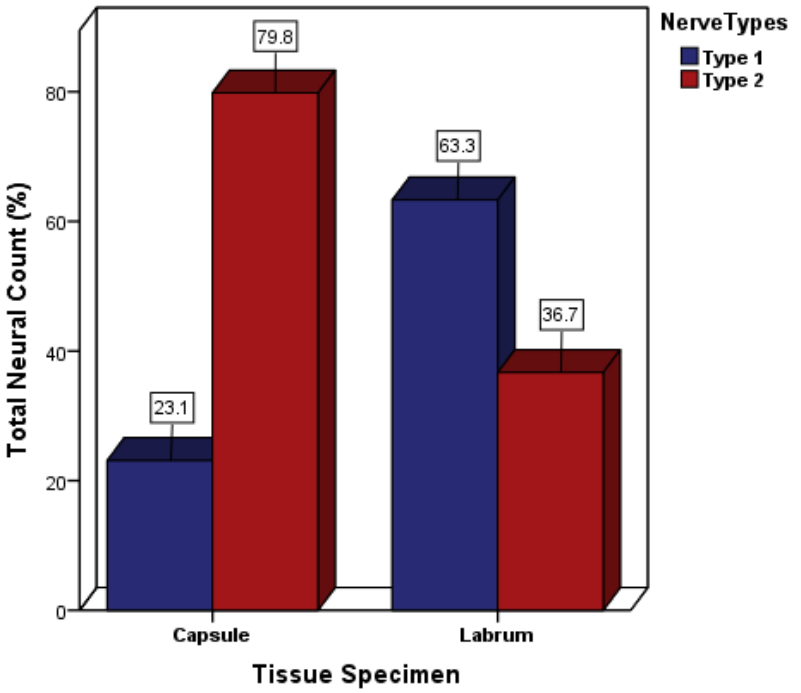

Figure 1. Percentages of type 1 and type 2 sensory nerve endings in the glenoid capsule and labrum.

Table 2. Total neural count between genders.

Total Neural Count			
Gender	**N**	**Mean**	**Std Dev**
Females	**8**	**663**	**240**
Males	**8**	**641**	**236**

Table 3. Total neural count between the uninjured vs injured groups.

Total Neural Count				
Group		**N**	**Mean**	**Std Dev**
Uninjured		**8**	**640**	**211**
Injured		**8**	**664**	**262**
Uninjured	**F**	**4**	**783**	**211**
	M	**4**	**497**	**70.5**
Injured	**F**	**4**	**543**	**227**
	M	**4**	**785**	**263**

4.4.1.3 Gender and Injury Difference in Joint Laxity

Gender and injury differences are outlined in Tables 4 and 5. There was greater joint laxity variability in females compared to males. Females also demonstrated greater amounts of joint laxity compared to males. Within the injured group, the shoulders had greater laxity values

than the uninjured group. Also within the uninjured and injured groups, the gender differences did not change.

Table 4. Total joint laxity between genders.

Total Joint Laxity				
Gender	**N**	**Mean**	**Std Dev**	**Variance**
Females	**8**	**27.0**	**7.68**	**58.9**
Males	**8**	**21.8**	**7.04**	**49.5**

Table 5. Total joint laxity between the uninjured vs injured groups.

Total Joint Laxity				
Group		**N**	**Mean**	**Std Dev**
Uninjured		**8**	**21.0**	**6.58**
Injured		**8**	**27.8**	**7.44**
Uninjured	**F**	**4**	**25.3**	**5.34**
	M	**4**	**16.8**	**4.86**
Injured	**F**	**4**	**28.8**	**10.1**
	M	**4**	**26.8**	**5.04**

4.4.2 Neural Count and Joint Laxity

Paired t-test analysis showed a significantly strong positive relationship (r=0.646, P=.009) between total neural count and joint laxity with the exception of an outlier (Fig. 2). This relationship appeared to be determined by the total capsule neural count (r=.710, p=.003; Fig. 3). The relationship between total labrum neural count and joint laxity was less apparent (r=.105, p=.709; Fig. 4). ANCOVA analyses revealed age, gender, and injury as three confounding variables. Gender and injury each showed a significant main effect (Gender, p=.009; Injury, p=.011) on the relationship between neural count and joint laxity. However, due to the small sample size we were unable to determine whether the strength of the relationship between total neural count and joint laxity changed when taking each of these variables into consideration simultaneously. ANCOVA results also showed a significant interaction (p=.037) between age and total neural count when studying the relationship between neural count and joint laxity. Age ($\geq$ 55 years of age) demonstrated a significantly negative correlation (r=-.921, p<.001) with total neural count (Fig. 5) and a negative trend with joint laxity.

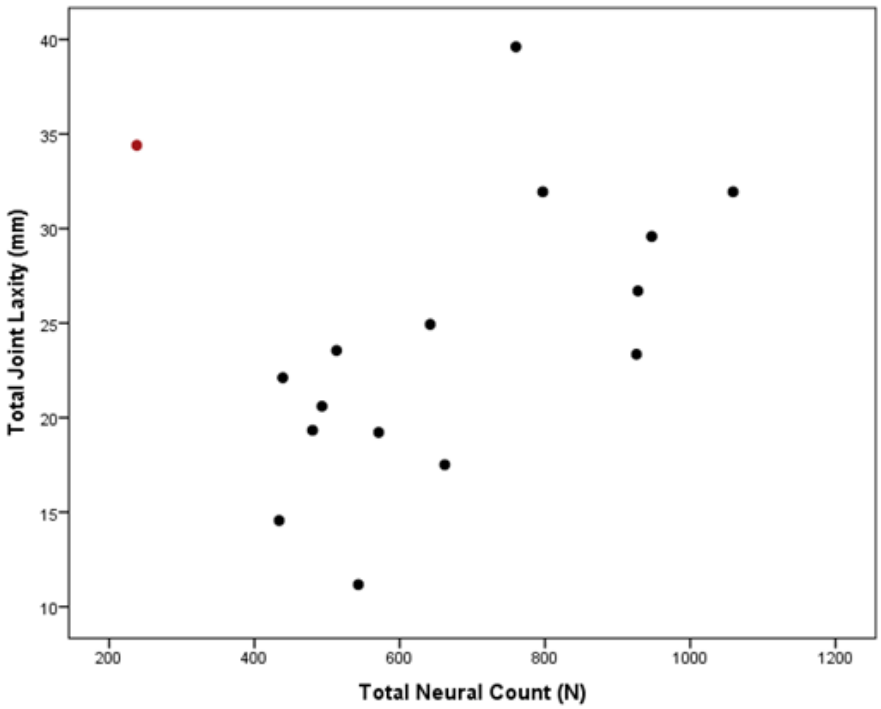

Figure 2. Relationship between total neural count and joint laxity. Red represents the 98 year old female, which is an outlier**.** The 98 year old was missing a labrum in the right shoulder.

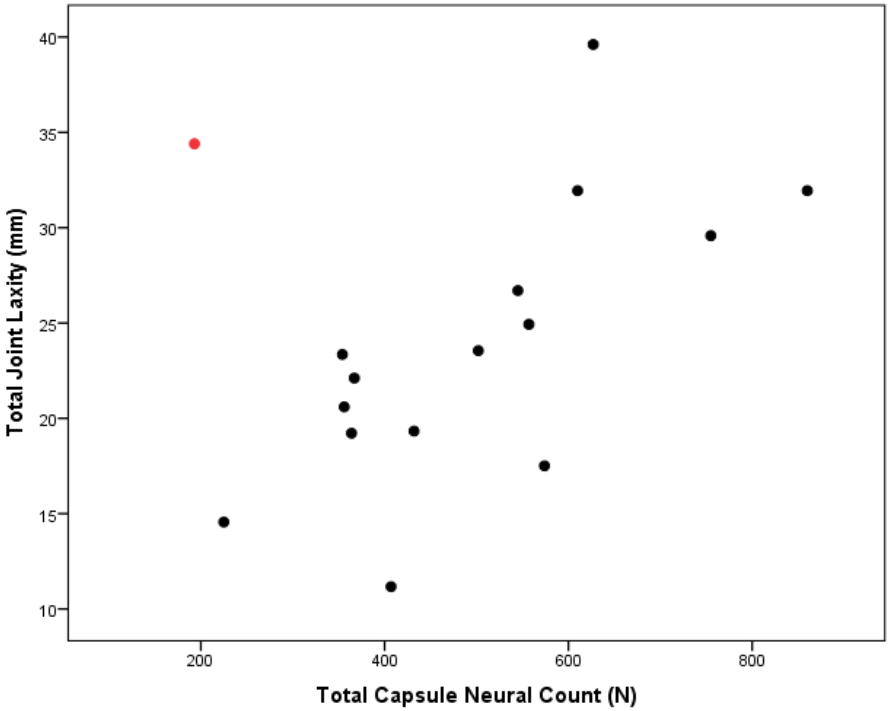

Figure 3. Relationship between total capsule neural count (anteroinferior (AI) and posteroinferior (PI) regions) and joint laxity.

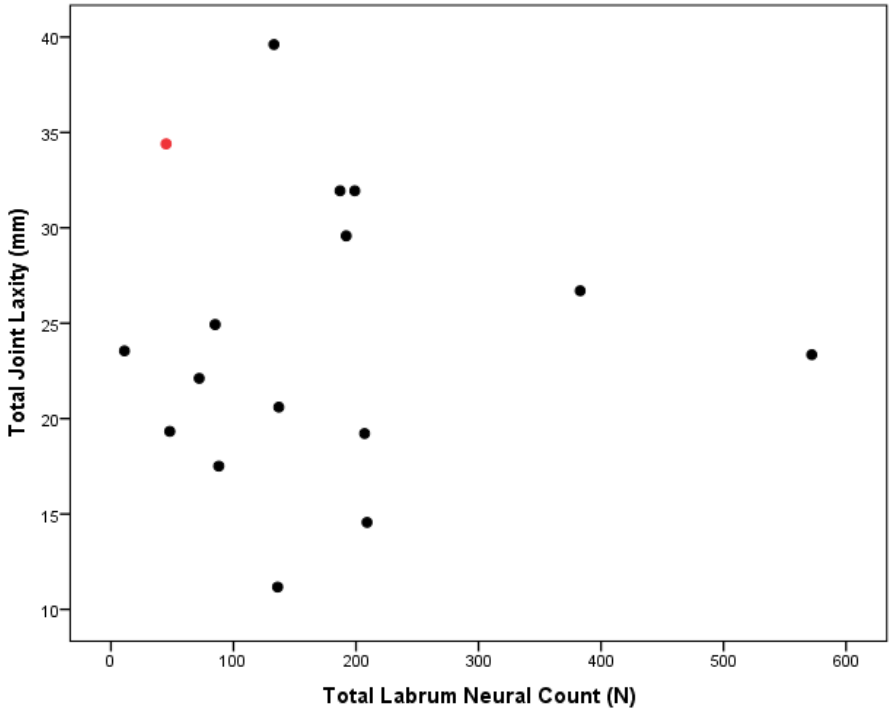

Figure 4. Relationship between total labrum neural count (anteroinferior (AI) and posteroinferior (PI) regions) and joint laxity.

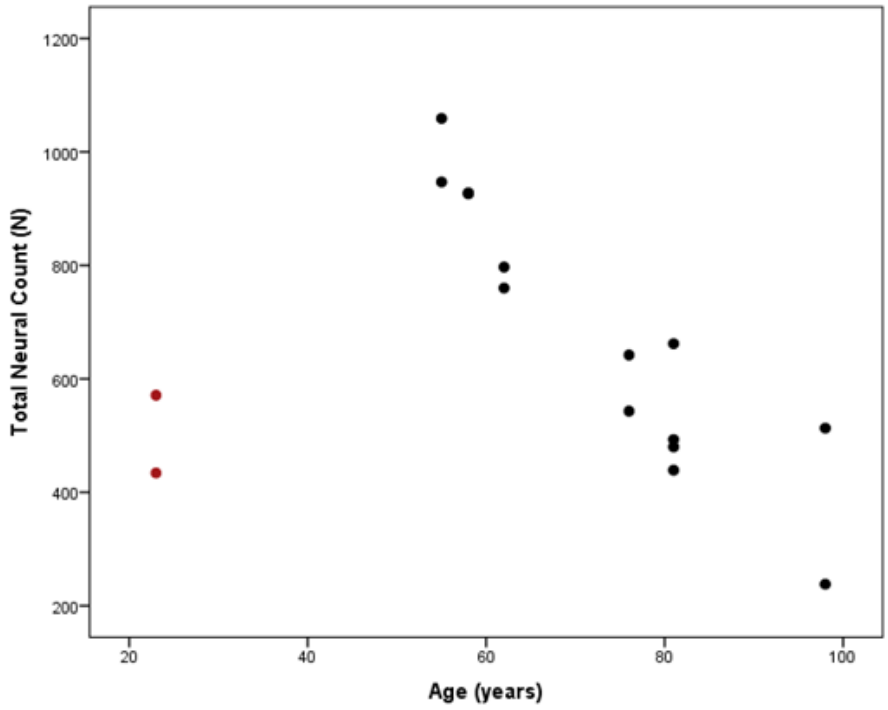

Figure 5. Relationship between total neural count and age. Red represents the 23 year old male, which is an outlier**.** The 23 year old died from a brain tumor whereas the aging (55-98 years of age) cadavers suffered from natural causes and other diseases such as Alzheimer's.

4.5 Discussion

Mechanoreceptors continue to gain a greater appreciation and have been identified in several articular tissues including ligaments (Rein et al. 2013), capsules (Backenkohler et al. 1996, Guanche et al. 1995, Maass et al. 2001, Solomonow et al. 1996), tendons/muscles (Guanche et al. 1995), and labrums (Bresch and Nuber 1995). To date, researchers have identified at least four types of sensory nerve endings in articular tissues including types 1 through 4. These mechanoreceptors are responsible for transducing mechanical deformation into electrical signals yielding a muscular response for neuromuscular control during movement and ultimately functional joint stability. This mechanism is otherwise known as joint proprioception such that joint position and different types of joint movement are registered by the sensory nerve endings and, in turn, elicit a neuromuscular response that results in the stiffening of the joint (joint stiffness) providing functional stabilization.

Injury to the glenohumeral joint disrupts the aforementioned mechanism resulting in proprioceptive deficits that lead to increased joint laxity (Lephart et al. 1997). With increased joint laxity, there is a concomitant decrease in joint stiffness (Borsa et al. 2002). There are a variety of factors that may contribute to proprioceptive deficits. These factors may include alterations in mechanoreceptor structure, reductions in the number of sensory nerve endings, changes in mechanoreceptor distribution, and the occurrence of mechanoreceptor neurogenesis (Nyland et al. 1998, Tibone et al. 1997). Within the capsule and labrum, we observed both type 1 and type 2 sensory nerve endings. Similar to marsupials (Maass et al. 2001), type 2 sensory nerve endings were the dominant type of mechanoreceptor in the shoulder joint capsule compared to type 1 sensory nerve endings. Our results showed a plausible relationship between total neural count and total joint laxity that was strongly determined by type 2 sensory nerve endings in the anteroinferior and posteroinferior joint capsule. It is important to note that type 2 sensory nerve endings were dispersed in a pattern

throughout the majority of the capsular surface area from medial to lateral (Witherspoon et al. 2013). This is the region where the humeral head makes the greatest amount of contact with translational movements. Type 1 sensory nerve endings were only located near the articular surface of the humerus and the capsulolabral junction (Witherspoon et al. 2013). Together, these data support the relationship between total neural count and joint laxity being strongly determined by the type 2 sensory nerve endings in the joint capsule.

Based on our results, glenohumeral joint laxity increased, as the number of sensory nerve endings increased. However, this did not hold true when comparing the uninjured and the injured shoulders. In the presence of injury, we expected the number of sensory nerve endings to be less with a greater amount of joint laxity. The number of mechanoreceptors did not appear to vary between the injured and uninjured groups; however, the amount of joint laxity did vary. Specimens within the injured and uninjured groups appeared to have a similar number of nerve endings except the injured group presented with a greater amount of joint laxity. A few speculations may be drawn from these results. It has been previously noted that mechanoreceptors most likely do not regenerate (Hogervorst and Brand 1998), and so adaptation for the number of sensory nerve endings required to accommodate increased joint laxity in the injured group does not occur. This phenomenon would theoretically account for proprioceptive deficits post treatment. Another possible mechanism would be the disruption of the functional capability of the sensory nerve endings. In this case, there would be increased joint laxity in the injured group while the number of sensory nerve endings remains constant. Lastly, capsular elongation may also occur in response to joint injury (Matsen et al. 2009, Omoumi et al. 2011, Pagnani and Warren 1994). Tightening the capsule has been shown to improve joint proprioception (Blasier et al. 1994). For these reasons, instead of there being a malfunction in the sensory nerve ending itself, there is no sufficient capsular tension for stimulating the sensory nerve endings leading to an increase in joint laxity. In this scenario, the

number of sensory nerve endings remains constant. All above listed possibilities warrant further studies to analyze the relationship between neural count and joint laxity in deeper details. These studies should include a larger sample population, neural signal conductivity, proprioception measurements, capsular measurements, and a neural count. Gender should also be taken into consideration. The consistency in neural count between the uninjured and injured groups with greater amounts of joint laxity in the injured group was only true within genders, but not between genders. However, due to limited number of subjects, there is not enough data to make conclusive remarks. Our results did show that gender and injury are confounding variables when studying the relationship between neural count and joint laxity.

Age was another confounding variable that influenced the relationship between neural count and joint laxity. Neural count and joint laxity demonstrated a positive relationship whereas there was a negative relationship between aging and neural count and a negative trend with joint laxity. Previous studies have shown that the number of sensory nerve endings decrease with age in the larynx (Yamamoto et al. 2003). However, it is not known what occurs on a functional level with age-related changes. Another study showed there were age-related changes in cognitive related motor tasks but not with proprioceptive control in individuals with presbyopia (Boisgontier et al. 2012). In support, it was demonstrated that proprioceptive deficits in the elderly exist in the subjects that had to rely solely on reactive proprioceptive input compared to preselected movements (Stelmach and Sirica 1986). These studies together, show that proprioceptive deficits may occur at the cognitive level or at the peripheral level. For this reason, age should be considered when assessing joint laxity relative to proprioceptive deficits. Additionally, it should be determined whether proprioceptive deficits are cognitive or peripheral joint related.

In summary, we have observed a potential relationship between neural count and joint laxity that should be further explored. This relationship should be studied in greater detail with a

larger sample size and controlled for age, gender, and injury. Results from such studies will advance research about proprioception and its relevance to joint instability. Ultimately, these studies would aide in the development of improved treatment approaches thus better quality outcomes for shoulder instability occurrence and recurrence.

4.5.1 Limitations

A larger sample size would have statistical models that take age, gender, and injury into consideration simultaneously when studying the relationship between neural count and glenohumeral joint laxity. Accurate history of injuries in shoulders under investigation may have provided insights into effects of injury types on neural count. Unfortunately, we had no previous knowledge regarding the shoulder history of the human cadaveric shoulders used within our study.

4.5.2 Future Directions

In the future, a larger study should be performed incorporating both an uninjured and injured aging population with an equal representation of males and females. This will allow for an analysis of the relationship between neural count and joint laxity with the incorporation of age, gender, and injury. Additionally, a more detailed study should be performed assessing all the aforementioned variables including a proprioception.

CHAPTER 5

The Relationship Between Glenohumeral Joint Stiffness and Collagen Content in the Glenoid Capsule and Labrum: An Exploratory Study

Preface: Chapter 4 presents the neural tissue properties of our study including neural count, and its relationship to joint laxity. Another tissue property of interest was the collagen content and its relationship to joint stiffness, which is reflected in chapter 5.

5.1 Abstract

In the shoulder, the capsule and labrum are static stabilizers that rely on their extracellular matrix to withstand mechanical forces during glenohumeral translation. The collagens are one of the main molecules that make up the extracellular matrix and are responsible for the tissue strength. Moreover, collagen is the primary load carrying molecule of fibrous connective tissues. The tissue strength of the capsule and labrum contributes to joint stability by providing mechanical restraint to resist glenohumeral translation. During injury, the amount of collagen, collagen fiber size, and collagen type ratios vary. In turn, these changes result in weakening of the tissue that may not only lead to decreased tissue stiffness, but also joint stiffness. We believe there is a relationship between the amount of collagen that makes up our tissues of interest (capsule and labrum) and glenohumeral joint stiffness.

The aim of this study was to determine collagen content in the glenoid capsule and labrum and to investigate its relationship with glenohumeral joint stiffness. Thirty-two capsule and 31 labrum specimens were analyzed. These tissues were dissected from the most common (anteroinferior) and least common (posteroinferior) regions of injury in eight shoulder pairs that were removed from four male and four female human cadavers.

The results from this study showed gender differences in the total collagen content and joint stiffness as well as a significantly positive relationship with collagen content in specimens $\geq$ 55 years of age. Within genders, the amount of collagen content varied between the uninjured and injured groups. However, the negative trend observed between total collagen content and joint stiffness did not change in relation to gender or injury differences. Instead, this relationship became significant in the injured shoulders only.

Based on our observations, the collagen content may not only directly affect tissue stiffness, but also joint stiffness. Given these findings, the changes in collagen content of the glenoid capsule and labrum should be considered when joint stiffness is compromised.

5.2 Introduction

Fibrous connective tissues are composed of a rich collagenous extracellular matrix (Eleswarapu et al. 2011) where collagen is considered to be the main load carrying molecule (Holzapfel 2001) strongly influencing tissue mechanical behavior. A good indicator of tissue mechanical behavior is joint stiffness (McQuade et al. 1999); however, the relationship between tissue collagen composition and joint stiffness is not well studied, although these are two important components that influence joint stabilization.

The glenoid capsule and labrum are fibrous connective tissues that rely on their tissue material properties (i.e. collagen composition) to provide mechanical restraint thereby resisting excessive glenohumeral translation (Sein et al. 2010) Glenohumeral joint stabilization is maintained by a balance between inherent joint laxity (glenohumeral translation) and joint stiffness whereby the amount of laxity is regulated by available joint stiffness. Instability, on the other hand, is characterized by excessive glenohumeral translation. Yet, a study has shown that there is a concomitant decrease in joint stiffness with increasing joint laxity (Borsa et al. 2002). In the presence of injury, collagen composition of the joint components and joint stiffness are compromised (Riley 2008)

Injury to the capsule and labrum disrupts the ability of the fibrous tissues to resist glenohumeral translation. This, in turn, leads to increased glenohumeral translation, clinically described as excessive joint laxity, with a concomitant decrease in joint stiffness (Borsa et al. 2002, Crawford and Sauers 2006). Healing of fibrous connective tissues, in the presence of injury, includes collagen degradation and collagen synthesis otherwise known as collagen turnover (Colditz 2002, Potach and Grindstaff 2008).

Previous research has shown that tissue tensile strength is directly proportional to collagen synthesis (Delforge 2002). Given that the amount of synthesized collagen contributes to tissue strength (Delforge 2002) and the amount of stiffness within a joint is considered a good

indicator of tissue mechanical properties (Borsa et al. 2002), we sought to determine whether there is a relationship between capsular and labral collagen content and glenohumeral joint stiffness. Our findings add to understanding glenohumeral joint stabilization and the major contributors.

5.3 Methods

5.3.1 Shoulder Preparation

This study included five fresh and three frozen human cadaveric shoulder pairs obtained from four males and four females with age ranging between 23 and 98. Information about the specimens is provided in Table 1. The fresh shoulders were tested the day they were acquired (within 24-36 hrs of death) and frozen shoulders were tested the same day they were thawed. The skin, subcutaneous tissue and musculature were removed from the humerus and scapula while leaving the capsule and labrum intact. The capsule and labrum were then grossly examined for the presence of injury. These observations are included in Table 1. Following tissue removal, ¾ of the clavicle was sawed off. The scapula was then secured to a custom base fixture. Before securing the distal humerus, the humerus was aligned in neutral (20^{o} abduction and 0^{o} rotation) position parallel to the medial border of the scapula. Once neutral position was established, the distal humerus was secured to the fixture to prevent any rotation, and a hole was drilled through the humerus anterior to posterior using the greater tubercle as a reference point.

5.3.2 Measurement of Joint Stiffness

The custom base fixture was designed to allow displacement of the humeral head in the sagittal plane and was mounted horizontally to the loading cell of the materials testing machine using C-clamps. We used humeral head displacement trials to derive joint stiffness measurements. Prior to starting the displacement trials, the humeral head was gently pressed into the glenoid fossa and locked into place. A rod from the MTS was then placed through the drilled hole and secured. Subsequently, the humeral head was translated until a maximum of 80N was reached in the posterior and anterior directions. Ten loading cycles (1 cycle: posterior->neutral->anterior) were performed to condition the specimens. Anterior-posterior translations were extracted from the mean of the following five cycles. Stiffness measurements were then

derived from the extension zone of the anterior-directed and posterior-directed translations. At the end of the experiment, the shoulder specimens were removed from the MTS and dissected further.

5.3.3 Capsule and Labrum Tissue Preparation

Capsule and labrum tissues were dissected from the anteroinferior and posteroinferior regions of the glenohumeral joint. Following dissection, the tissues were washed in fresh saline twice for ten minutes and the excess fluid was then removed using Kim Wipes. Afterwards, the tissue specimens were stored at -80^{o} until ready for use for collagen extraction and quantitation.

5.3.4 Collagen Extraction from Capsule and Labrum

Prior to starting collagen extraction, 1.5 mm long stir bars were added to 2.0 ml round bottom Eppendorf tubes (one per tube). The dissected tissue wet weight was then determined (Table 2). The tissues were finely diced on a plastic strip with a scalpel into very small pieces ($\leq$ 1mm) and placed into the stir-bar containing tubes. Collagen extraction was performed in accordance with the Sircol Collagen Assay kit (BioColor Life Science Assays, UK) manual with minor changes. The changes (see below) were introduced due to capsule and labrum being enriched fibrous tissues and obtained primarily from the aging population (thus stiffer tissue) while the Sircol procedure was developed for collagen isolation either from cultured cells or tissues/cartilages from young specimens (thus softer tissue) which are easier to homogenize and extract proteins from.

An acid-pepsin digest was used to extract collagen from the capsule and labrum. In order to determine the best acid-pepsin conditions for extracting collagen from our tissues of interest, the tissue samples were tested in different concentrations of pepsin in the acid-pepsin solution including 0.1 mg/ml (Sircol manual), 0.5 mg/ml, 1.0 mg/ml, and 1.5 mg/ml. Pepsin was dissolved in 0.5 M acetic acid. We found that 0.5 mg/ml and 1.0 mg/ml pepsin concentrations

were most optimal for the capsule and labrum, respectively, and we used these concentrations in extracting collagen from all specimens.

The acid-pepsin solution was prepared using 0.5 M acetic acid and lyophilized pepsin (Sigma Aldrich). The acid-pepsin solution (1.5 ml) was added to the 2.0 ml tubes containing the stir bars and tissue. Then the tubes, in groups of four, were placed in small glass jars (50 ml) and the jars were placed on a stirring plate (four jars per plate) at 4^{o}C. Tube contents were stirred vigorously at 600 rpm for 24 hrs.

We found that the 24 hr stirring of tissue in the acid-pepsin solution did not extract all collagen. Therefore, fresh acid-pepsin solution was prepared the next day and 0.5 ml of the fresh solution was added to each tube. The samples were then returned to 4^{o}C and stirred vigorously for another 24 hrs. Thereafter, the acid-pepsin extracts were centrifuged at 12,000 rpm for 60 min at 4^{o}C. If the tissue did not pack tightly at the bottom of the tube, the supernatant and loosely packed tissue were transferred to a new tube and recentrifuged. The supernatant was pipetted into new 2.0 ml tube. The average amount of supernatant (acid-pepsin extract) recovered was 1.5 ml. Collagen protein within the acid-pepsin extract was then isolated and concentrated as per Sircol Collagen Isolation and Concentration Protocol (Sircol manual).

5.3.5 Collagen Isolation and Concentration

Acid Neutralizing Reagent (one tenth of the acid-pepsin extract volume) and Collagen Isolation and Concentration Reagent (twice the amount of Acid Neutralizing Reagent) were added to the acid-pepsin extract. The tubes were then inverted using a microtube rotatory device (Fisher Scientific) at a speed of 42 rpm at 4^{o}C for 10min. Subsequently, the tubes were placed in an ice water bath for 24 hrs to separate and concentrate the collagen at the bottom of the tube.

After 24 hrs, the samples exhibited two different layers, visible and invisible (Fig. 1). According to the Sircol manual, however, only an invisible layer should have been present (although, Sircol manual described using cultured cells and tissues). Following centrifugation (12,000 rpm at 4^{o}C) for 10 min, the visible layer formed a visible pellet that adhered to the tube. . For this reason, we then transferred the supernatant to a fresh tube for recentrifugation (12,000 rpm at 4^{o}C, 10 min). Following recentrifugation, the supernatant (volume excluding the combined volume of the Acid Neutralizing Reagent and Isolation and Concentration Reagent) was removed slowly using a pipette with gel loading tips. This constituted concentrated collagen solution, referred to in the Sircol manual as invisible liquid pellet). 100 µl of the collagen solution was pipetted into a new 1.5 ml tube in duplicate for collagen quantitation. The remainder was stored at -20^{o}C.

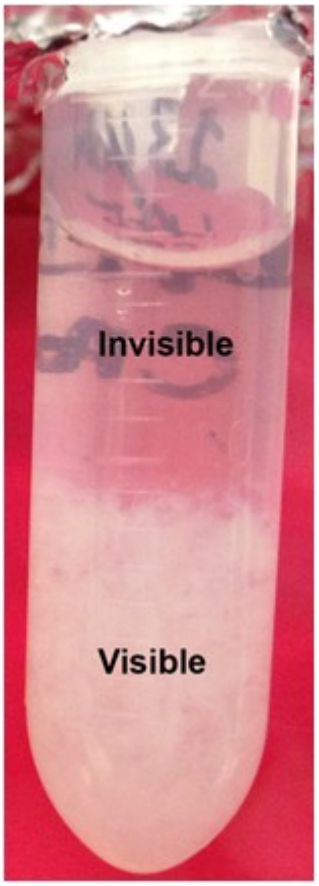

Figure 1. Visible and invisible layers in the sample at the collagen isolation and concentration stage following 24hr incubation in an ice water bath.

5.3.6 Quantitation of Collagen

The Sircol Collagen Dye Assay kit was used. This assay is a quantitative dye-binding method for measuring concentrations of soluble collagen types I to V without distinguishing the types. The dye reagent, containing Sirius Red in picric acid, binds to the Gly-X-Y motif (a motif characteristic for collagens) resulting in development of red color that can be quantitated using spectrophotometry. In a 1.5 ml tube, to 100 µl of concentrated collagen, 1000 µl of the Sircol Dye Reagent was added and the content of the tube was gently mixed for 30 min using a mechanical shaker. Once the dye was bound to collagen, the samples were washed using an Acid-Salt Wash Reagent and then 250 µl of the

Alkali Dye Reagent was added to the dye bound collagen. Next, 200 µl of each mix was transferred to a 96-well plate. The intensity of the resulting color was measured at 555 nm using SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Collagen concentration in each sample was determined using a standard curve prepared using the collagen standard supplied with the kit. The range of collagen concentrations in the standard curve was from 5 to 30 µg/µl. Collagen content in each capsule or labrum specimens was calculated considering extracted collagen concentration and the amount of tissue used for the extraction, and was expressed in ng of collagen per mg of tissue.

5.3.7 Statistical Analysis

Once collagen content was determined for each sample, we performed paired t-test analyses to assess whether there was a relationship between total collagen content, total joint stiffness, and age. Observational and mean comparative analyses were run to identify gender and injury differences in collagen content and joint stiffness. ANCOVA analyses were performed to determine the influence of age, gender, and injury on the relationship between total collagen content and joint stiffness. Total joint collagen content refers to the sum of collagen in the capsule and labrum anteroinferior and posteroinferior. Total capsule and total labrum collagen content refers to the anteroinferior and posteroinferior regions of the capsule or labrum. It is important to note that the collagen data from this study are solely based on acid-pepsin soluble collagen. Total joint stiffness was calculated as the sum of anterior and posterior stiffness. More in depth analyses were run to determine whether the relationships were consistent anterior and posterior in the capsule and in the labrum. Although the dissected regions of the capsule and labrum were anteroinferior and posteroinferior, these regions were associated with anterior and posterior stiffness, respectively.

5.4 Results

5.4.1 Specimen Characteristics

Specimen characteristics are presented in Table 1. After gross evaluation, eight shoulders were considered uninjured and the other eight were labeled as injured. The injured group included injury to the capsule, labrum, or both. Injury specifics are indicated in Table 1.

Table 1. Specimen characteristics including specifics on shoulder injuries.

Age	Gender	Side	Tissue Injury	Injury Site (Capsule, Labrum or both)
23	Male	Left	No	
		Right	No	
55	Male	Left	Yes	Labrum (detached)
		Right	Yes	Capsule (torn)
76	Male	Left	No	
		Right	Yes	Capsule (rotted) Labrum (calcified)
81	Male	Left	Yes	Capsule (frayed)
		Right	No	
58	Female	Left	No	
		Right	No	
62	Female	Left	No	
		Right	Yes	Labrum (frayed)
81	Female	Left	Yes	Capsule (heterotropic ossification)
		Right	No	
98	Female	Left	Yes	Labrum (N/A)
		Right	Yes	Capsule (frayed, neg. pressure)

5.4.2 Capsule and Labrum Collagen Content

5.4.2.1 Collagen Isolation and Quantitation

Collagen is a difficult protein to isolate. A traditional approach to isolate proteins from cells or tissues typically includes protein extraction using detergent based buffer. Collagen does not extract well under those conditions, thus a specific protocol had to be developed. This protocol includes denaturing collagen in acid (Mu et al. 2007) with subsequent telopeptide proteolysis by pepsin (Chambers 2004), to facilitate its release from the tissue. Unfortunately, we were not able to find a protocol developed specifically for collagen extraction from our fibrous tissues of interest, glenoid capsule and labrum. Therefore, we selected to use the Sircol method as a starting protocol, and we tested and modified some of the steps. Specifically, we changed pepsin concentrations, incorporated additional centrifugation steps, and modified the amount of reagents added to the samples (Acid-Neutralizing Reagent, Isolation and Concentration Reagent) to increase collagen yield.

We encountered some challenges in collagen isolation from the capsule and labrum. Specifically, during the isolation and concentration step, after centrifugation, we observed an unexpected visible layer as shown in figure 1. We postulated that this layer consisted of glycosaminoglycans and proteoglycans which are ground substances in the extracellular matrix to which collagen adheres. A guanidine hydrochloride extraction, used to extract glycosaminoglycans and proteoglycans prior to collagen extraction, did minimize the visible layer but greatly reduced the amount of measurable acid-pepsin soluble collagen. Therefore, we abandoned the guanidine hydrochloride extraction. Instead, we performed an additional centrifugation and pelleted the visible layer. After recentrifugation, removal of the supernatant was cumbersome since the collagen layer was not visible, and appeared to be the same consistency as the supernatant. Superficial to the supernatant were floating particles that were

difficult to remove from solution and care had to be taken not to transfer them with the supernatant.

We made an interesting observation about the 23 year old specimen. Following the isolation and concentration step, the collagen extracts presented with strikingly greater amount of visible layer, compared to other specimens, as shown in figure 1. We later noticed that this specimen collagen content was higher than one would expect based on the age as shown in figure 2. Also, the collagen content for this specimen was similar to the values for the 81 and 98

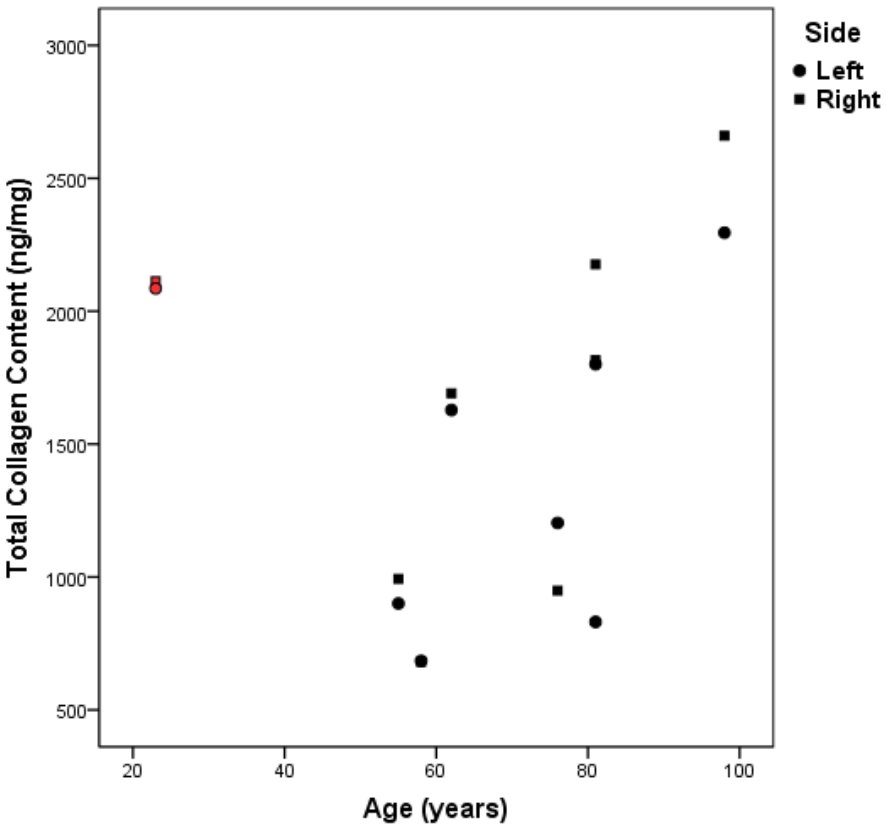

Figure 2. Relationship between total collagen content in left (circle) and right (square) shoulders and age. The red symbols represent the 23 year old male which was considered an outlier.

year old specimens; however, the amount of visible layer in the two older specimens was the same as in all other specimens and significantly smaller than in the 23 year old specimen. This

may suggest that the 23 year old specimen had some abnormalities in the collagen that may not be related to age but rather to other factor(s).

Initially, we sought to measure total collagen content using the Sircol Dye Assay and quantitate collagen type I and collagen type III using enzyme linked immunosorbent assay (ELISA). While we were able to quantitate total collagen, ELISA was more challenging. We employed commercial ELISA kits specific for collagen type I and collagen type III. Unfortunately, the sensitivity of the ELISA kits was not sufficient to quantitate these proteins in our extracts. The lowest concentrations of standards were 5 ng/ml and 40 ng/ml for collagens type I and III, respectively. Although we received readings from the microplate reader for some of our samples, they were lower than readings for the lowest standards on the standard curves. On the other hand, we did not have volumes of extracts sufficient for concentrating the extracts, due to small size of the specimens. Therefore, we were unable to evaluate collagen type levels in our specimens.

5.4.2.2 Collagen Content

Table 2 presents capsule and labrum tissue wet weight and collagen content. Capsule or labrum total collagen content refers to collagen content in both anteroinferior and posteroinferior capsule or labrum, respectively. Total collagen content refers to the sum of collagen content in the anteroinferior and posteroinferior capsule and labrum of each shoulder.

5.4.3 Relationship between Collagen Content and Specimen Characteristics

5.4.3.1 Age

Our sample population consisted of one 23 year old whereas the other specimens were $\geq$ 55 years of age. An observational analysis (Fig. 2) of total collagen content, and age revealed the 23 year old as an outlier such that the 23 year old exhibited similar amounts of collagen as the 81 and 98 year old. A more detailed analysis revealed the 23 year old had similar amounts of total collagen (Table 2) as the 98 year old exhibiting the highest amounts of collagen in the

Table 2. Capsule and labrum wet weight and collagen content, and total collagen content for tissue specimens dissected from the shoulders. LAI=Left Anteroinferior, LPI=Left Posteroinferior, RAI =Right Anteroinferior, RPI=Right Posteroinferior, N/A=Not Available

Age	Gender	Side	Specimen	Capsule Wet Weight (mg)	Capsule Collagen Content (ng/mg)	Total Capsule Collagen Content (ng/mg)	Labrum Wet Weight (mg)	Labrum Collagen Content (ng/mg)	Total Labrum Collagen Content (ng/mg)	Total Collagen Content (ng/mg)
23	Male	Left	LAI	99.6	846	1768	99.4	141	318	2086
			LPI	100.1	922		93.3	177		
		Right	RAI	99.8	747	1831	99.8	111	282	2113
			RPI	99.4	1084		99.8	171		
55	Male	Left	LAI	97.3	229	427	93.7	228	474	900
			LPI	99.5	198		99.4	246		
		Right	RAI	99.0	279	509	85.0	230	484	993
			RPI	99.6	230		100.1	254		
76	Male	Left	LAI	100.3	244	508	99.2	505	697	1204
			LPI	99.7	264		99.1	192		
		Right	RAI	99.0	191	576	100.1	163	373	949
			RPI	100.2	385		100.0	210		
81	Male	Left	LAI	99.9	291	505	100.1	148	325	831
			LPI	94.4	214		99.6	177		
		Right	RAI	99.1	405	874	43.8	389	942	1816
			RPI	100.3	469		31.9	553		
58	Female	Left	LAI	99.5	142	287	77.2	207	398	685
			LPI	100.0	145		68.2	191		
		Right	RAI	100.1	150	281	86.6	172	399	680
			RPI	100.0	131		45.6	227		
62	Female	Left	LAI	99.4	491	998	89.9	361	630	1628
			LPI	99.2	507		98.1	269		
		Right	RAI	99.8	393	865	77.3	334	826	1691
			RPI	99.4	472		46.8	492		
81	Female	Left	LAI	99.8	382	894	100.0	447	906	1800
			LPI	99.5	512		68.9	459		
		Right	RAI	99.5	602	1058	70.5	718	1118	2176
			RPI	100.2	456		30.1	400		
98	Female	Left	LAI	99.3	845	1489	N/A	N/A	807	2295
			LPI	98.1	644		25.4	807		
		Right	RAI	81.9	870	1549	39.1	464	1111	2660
			RPI	99.3	679		37.7	647		

capsule only. Yet, the lowest amount of collagen content was observed in specimens between 55 and 76 years of age. The direction of the relationship between the collagen content and age ($\geq$ 55 years of age) did not change when observed in the capsule and labrum separately. Since the 23 year old was a very strong influential factor presenting as an outlier, observational

analyses included the 23 year old whereas statistical analyses were run on specimens ≥ 55 years of age only.

Paired t-test analysis showed a strong positive relationship between total collagen content and age (r=0.739, p=0.003; Fig. 2). The strength of the relationship between age and total collagen content was strongly influenced by the collagen content of the anteroinferior and posteroinferior capsules.

5.4.3.2 Gender

Mean comparative analysis revealed no significant differences in total collagen content between males and females. In addition, there were no significant gender differences anteroinferiorly compared to posteroinferiorly in collagen content. The aforementioned results are outlined in Tables 3 and 4.

Table 3. Total collagen content between genders and the uninjured versus injured groups.

Total Collagen Content (ng/mg)			
		N	(Mean ± Std Dev)
Gender	Female	8	1702 ± 716
	Male	8	1361 ± 550
Injury	Uninjured	8	1548 ± 621
	Injured	8	1515 ± 704

Table 4. Capsule and labrum anteroinferior and posteroinferior collagen content between genders and the uninjured versus injured groups.

Collagen Content (ng/mg)					
Group		AI (Mean ± Std Dev)		PI (Mean ± Std Dev)	
		Capsule	Labrum	Capsule	Labrum
Gender	Female	484 ± 278	338 ± 218	443 ± 204	436 ± 213
	Male	404 ± 252	239 ±138	471 ± 344	248 ± 127
Injury	Uninjured	453 ± 267	325 ± 210	497 ± 346	273 ± 136
	Injured	435 ± 270	252 ± 157	417 ± 192	411 ± 229

5.4.3.3 Injury

There were no differences in total collagen content between the injured and uninjured groups (Table 3) except when gender was incorporated (Fig. 3). When gender was taken into consideration, differences were revealed in the injured group only (Fig. 3). Within the injured group, females presented with a higher total collagen content (p=.008) compared to males (Fig. 3).

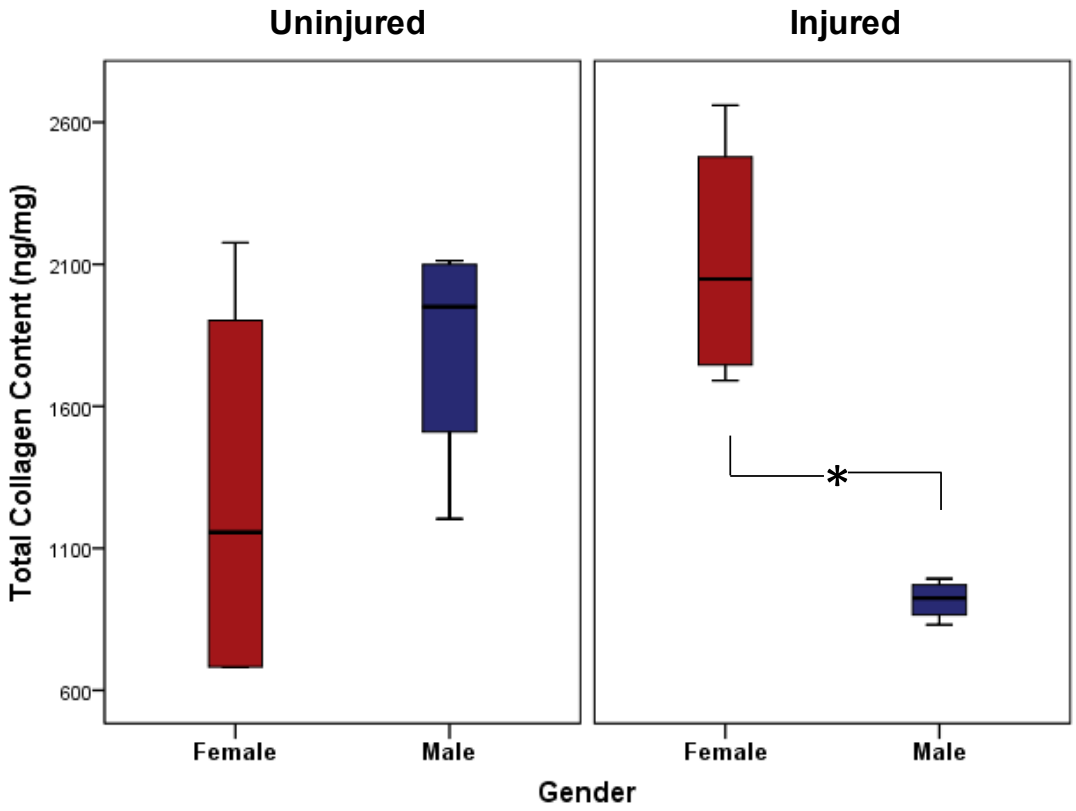

Figure 3. Mean differences in total collagen content between genders within the uninjured and injured groups. * indicates a significant difference between total collagen content.

Data for total capsule and total labrum collagen content in gender and in the presence or absence of injury are summarized in Figure 4. Within the uninjured group, males showed a trend to increase in total capsule collagen content compared to females (p=.056) whereas in the labrum, the amount of collagen content was similar. Females, in the injured group, presented with higher amounts of collagen in the total capsule (p<.001) compared to males. For injured

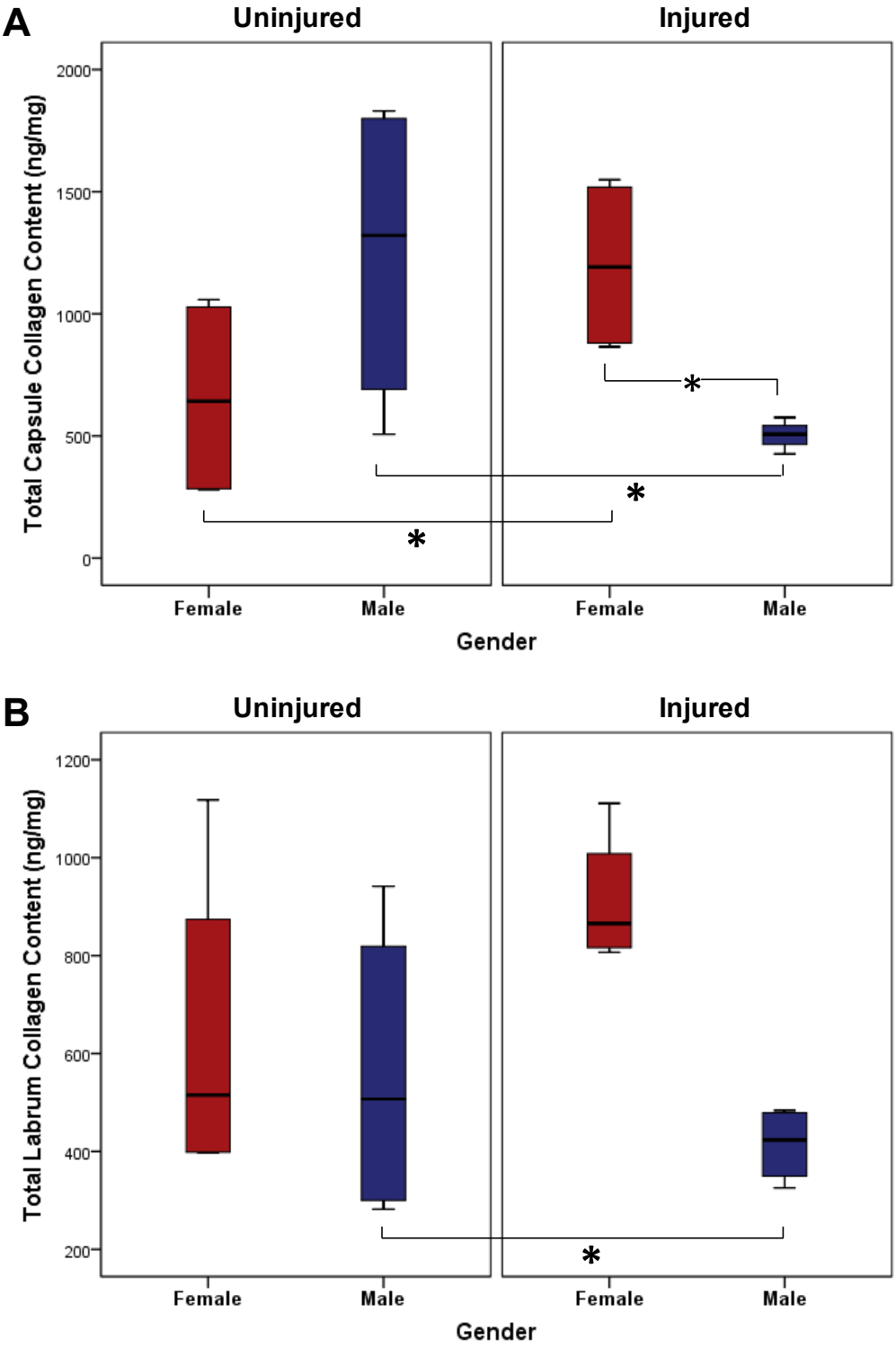

Figure 4. Mean differences in total capsule (A) and total labrum (B) collagen content between genders within the uninjured and injured groups. * indicates significant differences in collagen content between groups.

males, the collagen content for the total capsule (p=.001) and total labrum (p=.009) decreased compared to the uninjured group. Injured females, on the other hand, had higher collagen content (p=.030) in the total capsule compared to the uninjured females.

5.4.4 Joint Stiffness

Table 5 outlines anterior, posterior, and total (a sum of anterior and posterior) joint stiffness for each shoulder.

Table 5. Anterior, posterior, and total (anterior + posterior) joint stiffness for each shoulder.

Age	Gender	Side	Anterior Joint Stiffness (N/mm)	Posterior Joint Stiffness (N/mm)	Total Joint Stiffness (N/mm)
23	Male	Left	30.1	26.2	56.3
		Right	34.7	18.5	53.2
55	Male	Left	19.3	21.1	40.4
		Right	15.1	19.8	34.9
76	Male	Left	36.8	27.8	64.6
		Right	10.0	26.8	36.8
81	Male	Left	25.4	18.6	44.0
		Right	15.5	24.9	40.4
58	Female	Left	19.0	23.8	42.8
		Right	26.0	23.8	49.8
62	Female	Left	13.0	19.1	32.1
		Right	16.0	13.3	29.3
81	Female	Left	19.4	19.6	39.0
		Right	27.3	22.1	49.4
98	Female	Left	13.7	16.0	29.7
		Right	9.5	15.5	25.0

5.4.5 Relationship between Glenohumeral Joint Stiffness and Specimen Characteristics

5.4.5.1 Age

Unlike the relationship between collagen content and age, there was a negative trend (r=-.469, p=.067) between glenohumeral joint stiffness and age. This trend was strongly determined by anterior joint stiffness (r=-.489, p=.054). The direction of the relationship between stiffness and age was not influenced by gender or injury.

5.4.5.2 Gender

Males showed a trend to increase in total joint stiffness compared to females (Table 6). During anterior and posterior joint stiffness analysis (Table 7), males presented with more anterior stiffness and posterior joint stiffness in comparison to females. Within gender, there was a significant difference between anterior and posterior joint stiffness in males (p=.006).

Table 6. Total joint stiffness between genders and in the uninjured versus injured groups.

Total Joint Stiffness (N/mm)			
		N	(Mean ± Std Dev)
Gender	Female	8	37.1 ± 9.5
	Male	8	46.3 ± 10.5
Injury	Uninjured	8	48.6 ± 10.1
	Injured	8	34.9 ± 6.5

Table 7. Anterior and posterior joint stiffness between genders and in the uninjured versus injured groups. * indicates significant difference in anterior and posterior stiffness in males.

JointStiffness (N/mm)			
Group		Anterior (Mean ± Std Dev)	Posterior (Mean ± Std Dev)
Gender	Female	18.0 ± 6.0	19.1 ± 3.8
	Male	23.4 ± 9.5*	23.0 ± 3.7
Injury	Uninjured	25.3 ± 8.7	23.3 ± 3.2
	Injured	16.1 ± 5.3	18.8 ± 4.2

5.4.5.3 Injury

The uninjured group was shown to have a trend to increase (p=.336) in total joint stiffness compared to the injured (Table 6). Similarly, the trend to increase was noted in males (p=.309) and females (p=.524) (Fig. 5). A significant difference was observed between anterior and posterior joint stiffness in females of the uninjured group (Fig. 6)

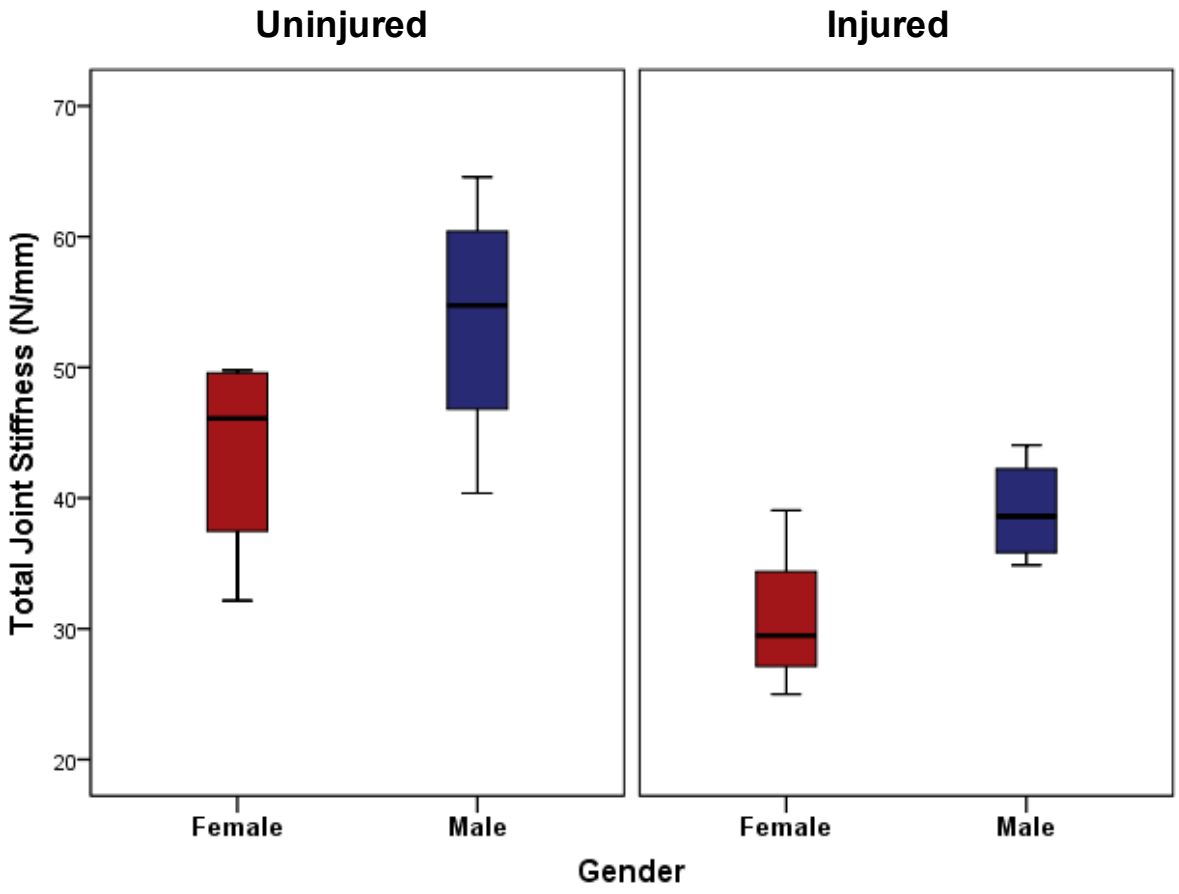

Figure 5. Total joint stiffness between genders within the uninjured and injured groups

5.4.6 Collagen Content and Joint Stiffness

A simple observational analysis showed a negative trend between total collagen content and joint stiffness. A more in depth observational analysis revealed the same negative trend in both the capsule and labrum independently. ANCOVA showed that gender potentially interacts with the relationship between total collagen content and joint stiffness (p=.057). Gender appeared to interfere with the direction of the relationship. Injury, on the other hand, did not

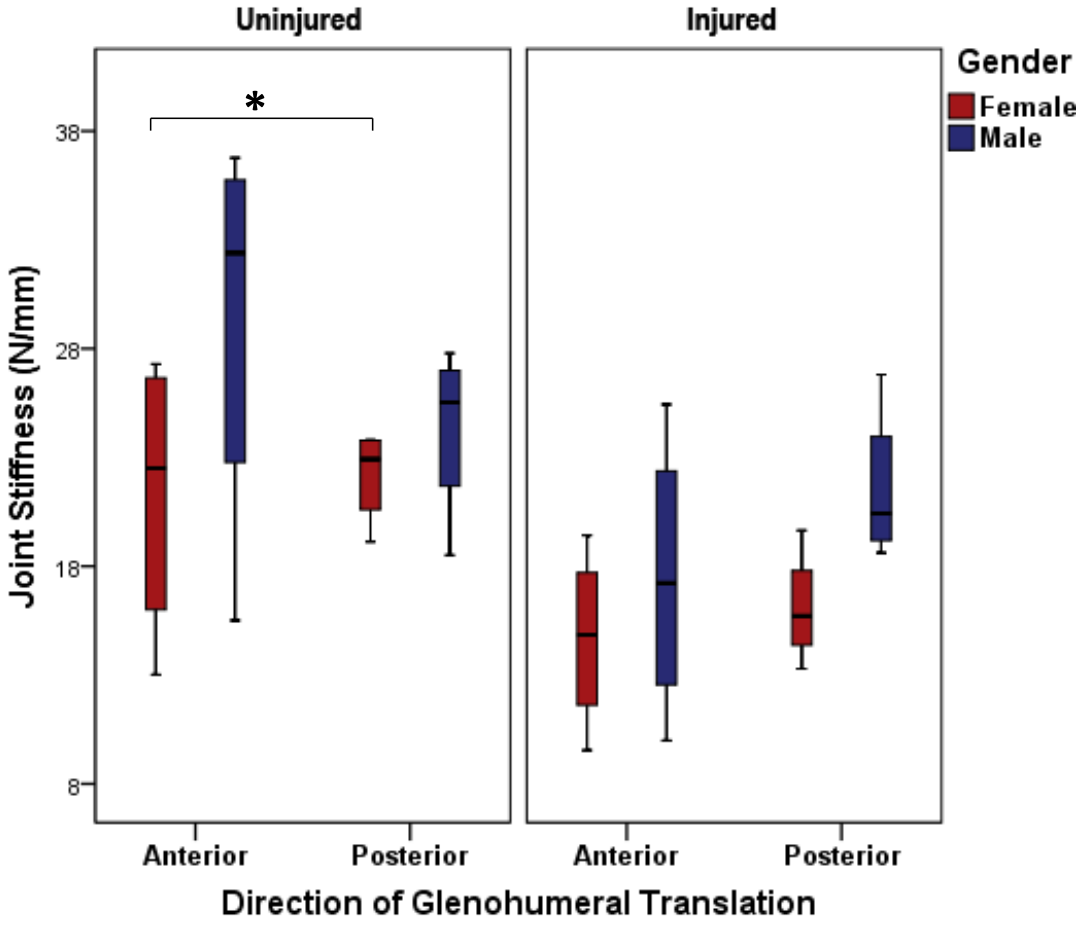

Figure 6. Anterior and posterior joint stiffness in males and females of the uninjured and injured groups. * indicates significant difference.

influence the direction of the relationship between total collagen content and joint stiffness, but paired t-test analysis revealed there was a significant strong negative relationship between total collagen content and total joint stiffness (r=-.814, p=.014) in the injured group (Fig. 7). The same results remained true for total capsule (r=-.803, p=.016) and total labrum (r=-.735, p=.030) collagen content with joint stiffness (Fig. 8).

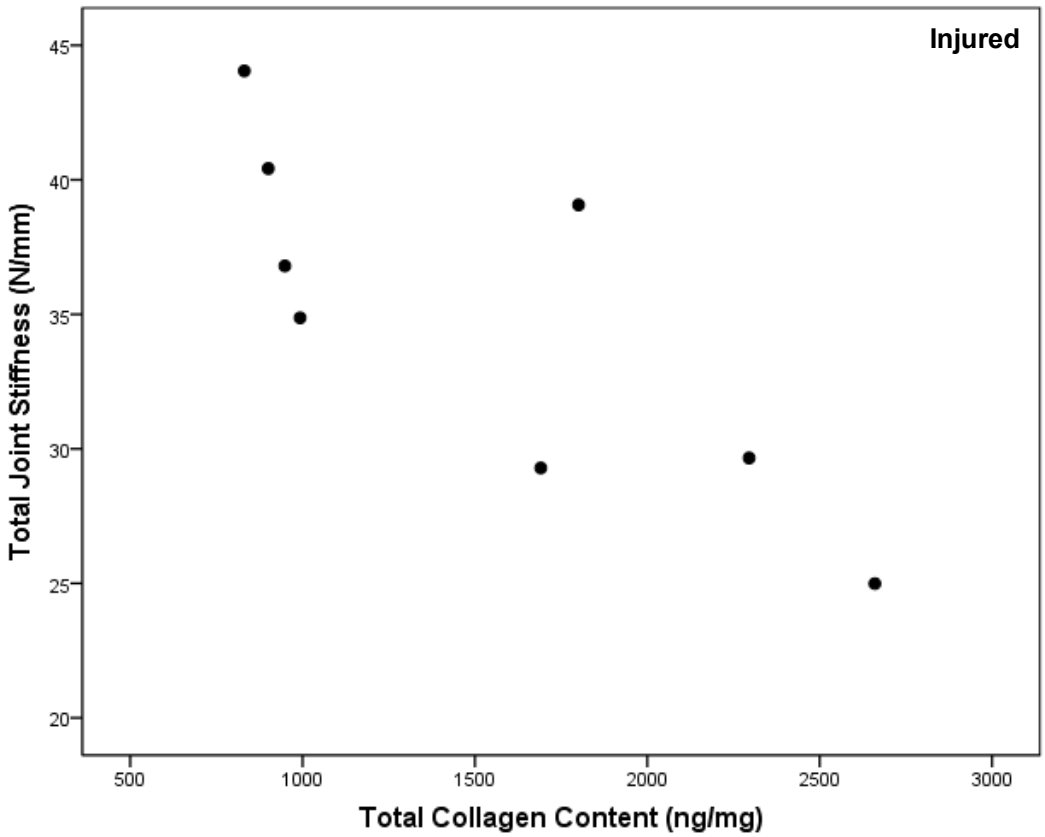

Figure 7. Relationship between total collagen content and glenohumeral joint stiffness in the injured group.

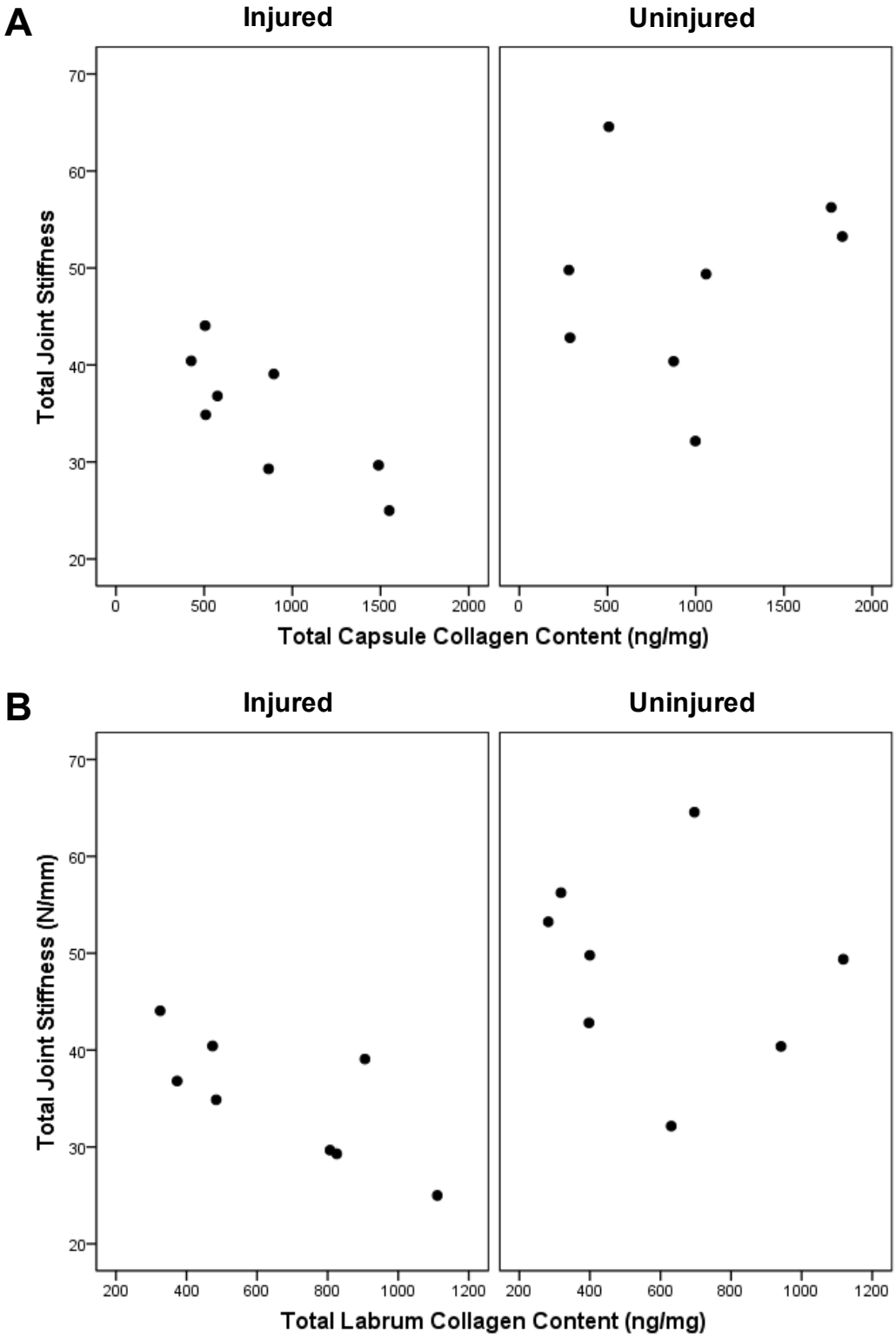

Figure 8. Relationship between total joint stiffness and collagen content of the total capsule (A) and total labrum (B) uninjured and injured groups.

5.5 Discussion

The relationship between tissue collagen and joint stiffness, to the best of our knowledge, has not been studied. Instead, research encompasses a more direct analysis, and so collagen is often studied relative to tissue stiffness (tensile strength) since collagen is responsible for tissue stiffness and tensile strength. This study provides information about the relationship between joint collagen content and joint stiffness as well as the influence of age, gender, and injury on this relationship.

In order to bridge the gap between fibrous tissues and their role in joint stabilization, an assessment is needed for the tissue properties of all joint stabilizers and how they relate to joint stabilization properties including joint stiffness and joint laxity. We primarily assessed the relationship between the combined joint collagen content of the anterior and posterior capsule and labrum with combined anterior and posterior joint stiffness. We also reported a more detailed analysis of the anterior and posterior characteristics of the capsule, labrum and joint stiffness.

Our findings showed there is a negative trend between collagen content and joint stiffness. As collagen increased joint stiffness decreased. There was no significance in this relationship. Interestingly, this relationship was influenced by age, gender, and injury. Due to small sample size, we were unable to study the degree of interaction between all the variables. Instead, we observed the relationship of age, gender, injury with both collagen content and stiffness independent of the other and together.

5.5.1 Age

Little is known about age-related changes in the capsule and labrum (Bobacz and Sunk 2006). Our study demonstrated a strong positive relationship between collagen content and age in specimens $\geq$ 55 years of age. Unlike collagen content, anterior-posterior joint stiffness decreased with increasing age. The 23 year old was a strong influential factor when studying

the relationship between collagen and age, but this was not the case with joint stiffness. This difference may be further explained by the biochemical and biomechanical changes in the collagen and tissues, respectively. Age-related changes in collagen have been well studied in different tissue types (skin, aorta, cervix, and tendon), but the relationship varies depending on the tissue (Cattell et al. 1996, Ding et al. 1997, Oxlund et al. 2010). Tendons are the most commonly studied articular tissue that most closely resembles our tissues of interest. Within tendons, there is increased collagen content, collagen cross-linking, and collagen fiber rigidity with increasing age (Kannus et al. 2005).

In theory, an increase in collagen content and cross-linking would allow for greater tensile strength and efficient tissue mechanical behavior. However, Birch et al. (2010), showed increases in type III collagen compared to type I collagen in the aging tendon (Birch et al. 1999). Type I collagen is responsible for tensile strength whereas type III collagen provides more tissue elasticity. We can speculate that if the composition of collagen types (specifically, type I and type III) is shifted with aging, while total collagen content in the 23 year old and in the 81 to 98 year olds were similar, the younger specimen would have more type I collagen and the older specimen would have more type III collagen. This may account for their similarities in total collagen content, but possible differences in collagen types and differences in joint stiffness. Although there is increased cross-linking with aging, it compromises the tissue mechanical properties including decreased tensile strength, maximal load, and maximal strain. For this reason, the increase in collagen cross-linking is suggested to be a maladaptive adjustment. An increase in rigidity of the collagen fibers also results in a concomitant decrease in tensile strength with aging. (Kannus et al. 2005)

Similar to tendon, the tensile strength of the capsule and labrum has been shown to decrease with increasing age as demonstrated by a decrease in the amount of force required to rupture the capsule and/or labrum (Hara et al. 1996). Not only is there a reduction in tissue

tensile strength, but there is also a decrease in tissue stiffness. These results have been observed in tendon as well as articular cartilage (Kannus et al. 2005, Kempson 1982). More so than tendons, the glenoid labrum tensile properties are more similar to articular cartilage (Smith et al. 2008).

Since collagen has been shown to become more rigid with increasing age while ligaments and tendons tend to become more lax with age, together, they are thought to void the effect of the other on tissue function resulting in little to no effect of age on joint stiffness. (Such et al. 1975) However, this may not be the case since our study showed a negative trend between joint stiffness and age that is close to being significant. Instead, the reduction in the amount of force required to rupture the glenoid capsule and labrum in conjunction with previous studies showing a reduction in tissue stiffness for similar articular tissues may carry over affecting joint stabilization properties such as joint stiffness. We believe this phenomenon could explain the negative trend between joint stiffness and age observed in our study.

5.5.2 Gender

Gender differences in collagen content of the glenoid capsule and labrum has not been previously documented. Our study showed that gender differences in collagen content were influenced by injury. Within the uninjured group, males exhibited a greater amount of collagen than females. Similar results were found in tendons and capsular ligaments (Hama et al. 1976, Kjaer and Hansen 2008, Lemoine et al. 2009). Conversely, within the injured group of our study, collagen content was higher in females than males.

Gender differences in collagen content of uninjured tissue suggest sex hormones may play a role in collagen formation. Since we observed opposite results in the injured group, this suggests gender and mechanisms associated with injury influence the collagen content pattern between genders. These assumptions would have to be further explored in the capsule and labrum, and with a larger sample size.

The relationship between gender and collagen content has not been well studied although the effects of sex hormones on collagen synthesis have been analyzed. Oestradiol decreases and testosterone increases collagen content in the aorta (Fischer and Swain 1980, Osakabe et al. 2001). Collagen type has also been shown to be influenced by gender. In kidneys, estradiol suppresses type I collagen synthesis. (Kwan et al. 1996) Mariotti et al. (2001) studied gender differences in type I and type III collagen production. The results from the cited study demonstrated significantly greater collagen type I and type III concentration in males than females in retrodiscal tissue. (Mariotti et al. 2000) If the same mechanisms play a role in the glenoid capsule and labrum, then the hormonal effects on collagen synthesis could account for the gender differences in collagen content of the uninjured group only.

In the presence of injury, there is a decrease in collagen content with an increase in collagen synthesis, especially type III collagen (James et al. 2008). Within our study, the differences in collagen content between genders in the injured shoulders may possibly be explained by tissue changes in collagen composition associated with tissue repair. The greater production of type III collagen in injured tissues in addition to the estradiol suppression of type I collagen, may explain females having a greater amount of collagen in the injured group. However, to support this suggestion, collagen types should be assessed in males and females within uninjured and injured groups.

Although the results for gender differences in collagen content were opposite for the uninjured and injured groups, these differences did not carry over to joint stiffness. Instead, male specimens exhibited slightly more joint stiffness than females in both groups. These results may be further explained by possible differences in collagen types since greater amounts of type III collagen compared to type I collagen weakens fibrous tissues (Riley 2011).

5.5.3 Injury

Our findings showed lower collagen content and joint stiffness in the injured group compared to the uninjured group. Similar findings for collagen content were observed in the tendon. Collagen content in the ruptured tendon was significantly less than the collagen content of the uninjured tendon. Also within the cited study, the amount of collagen was positively correlated ($p < .05$) to failure stress, but this was only true for ruptured tendons. Together, these results imply that mechanical weakening of the tendon and the changes in collagen content influence the pathophysiology of ruptured tendon. (Hansen et al. 2013) It was only the injured group in our study that showed a significant relationship between collagen content and joint stiffness where joint stiffness decreased with increasing collagen content.

When comparing the results from the previously mentioned study (Hansen et al. 2013) and our study, the amount of collagen appears to influence tissue tensile strength and joint stiffness differently. Increasing amounts of collagen in the ruptured tendon allowed the tendon to withstand greater failure stress (Hansen et al. 2013) whereas increasing amounts of collagen was accompanied by decreased joint stiffness (our study). For this reason, the relationship between joint stiffness and tissue tensile strength should also be studied in articular tissues. The differences may be due to collagen type, age, gender, and/or the phase of healing.

Decreased joint stiffness in the injured compared to the uninjured group in our study supports findings in the unstable shoulder. Clinically, joint laxity is assessed in the unstable shoulder. Injury incurred by the glenoid capsule and labrum results in increased glenohumeral joint laxity with decreased joint stiffness (Crawford and Sauers 2006, Marquardt et al. 2006). In essence, our findings are consistent with what we would expect to see in the pathologic shoulder.

5.5.4 Interactions

In summary, age, gender, and injury influence collagen content and/or joint stiffness. Since we had a small sample size, we were unable to statistically analyze the interactions of each variable with the relationship between collagen content and joint stiffness. However, our observational analysis did reveal interactions between age, gender, and injury and the impact of these interactions on the relationship between collagen content and joint stiffness. To strengthen our observations, as mentioned previously, a larger sample size incorporating each variable (age, gender, and injury) should be performed. Given the observed changes in collagen content relative to joint stiffness, especially in the presence of injury, collagen content and injury repair mechanisms related to collagen should be considered in the injured shoulder.

5.5.5 Limitations

Within our study, we were unaware of any diagnosed shoulder injuries. However, we did observe injury to half of the tissue specimens used in this study. Yet, we were unaware of the phase of healing. Another limitation includes the results from this study can only be generalized to acid-pepsin soluble collagen. Collagen types I and III have been shown to be the primary collagens extracted from tissues using an acid-pepsin digest. Lastly, we were unaware of any diagnosed diseased conditions per human cadaver.

5.5.6 Future Directions

In the future, a larger study should be performed controlling for age, gender, and injury. The study should also analyze collagen types, specifically, types I and III, and the phase of healing in the injured group. Long-term studies should assess the effect of treatment type on collagen remodeling including turnover rate as well as joint stiffness.

CHAPTER 6

Discussion and Conclusion

6.1 Discussion and Conclusion

6.1.1 Summary of Findings and Related Research

There is an ongoing interest in understanding the factors that contribute to shoulder instability, especially since the recurrence rate is still high post treatment. To date, the factors associated with the unstable shoulder that are of great interest to our work include: 1) microtears within the shoulder static stabilizers (glenoid capsule and labrum) identified as the most common pathological findings associated with instability (Matsen et al. 2009, Pagnani and Warren 1994, Rowe et al. 1984); 2) proprioceptive deficits clinically presenting as the loss of neuromuscular control (Lephart et al. 1997, Lephart et al. 1996); and 3) changes in the amount of glenohumeral joint laxity and stiffness, the two components of glenohumeral joint stabilization (Borsa et al. 2002). In theory, microtears within the glenoid capsule and labrum would affect their mechanoreceptor and collagen tissue properties thereby affecting their ability to contribute to stability of the glenohumeral joint.

Articular mechanoreceptors within joint stabilizers contribute to joint stability by stimulating a proprioceptive response that yields neuromuscular control during shoulder movements. A loss of neuromuscular control has been shown to result in increased joint laxity. Collagen is a tissue component primarily responsible for the capsule and labrum tissue strength. Consequently, the capsule and labrum are able to provide mechanical restraint against excessive glenohumeral translation contributing to joint stiffness and ultimately joint stability. We sought to analyze mechanoreceptor and collagen properties in the glenoid capsule and labrum, and whether there was a relationship between these tissue properties and joint laxity and stiffness.

To determine potential contributing factors to shoulder instability, we initially had to establish the amount of joint laxity and stiffness in cadaveric shoulders. We then determined the number of mechanoreceptors (neural count) and collagen content in the capsule and labrum.

While counting the number of mechanoreceptors, we also constructed a neuroanatomical map within the capsule and the labrum. A neuroanatomical map could assist with treatment of the unstable shoulder. Lastly, neural count and collagen content were correlated with joint laxity and stiffness. Variables that were not controlled, but considered, included age, gender, and observed injury.

6.1.1.1 Joint Laxity and Joint Stiffness

Our first goal was to obtain glenohumeral joint laxity and stiffness measurements. As joint laxity and stiffness together allow mobility and provide stability to the glenohumeral joint we sought to examine their relationship. We observed a significant inverse relationship ($r=-.824$, $p<.001$) where there was a concomitant decrease in joint stiffness with increased joint laxity in cadaveric human shoulders. The same results were observed previously in active men and women (Borsa et al. 2002). Clinically, joint laxity and stiffness are not considered simultaneously when diagnosing shoulder instability. Instead, only glenohumeral joint laxity is assessed. However, for the purpose of this study, we determined both joint laxity and stiffness. Our results showed that joint laxity was more variable in the injured compared to the uninjured group. Additionally, the joint laxity values within the interquartile range of the injured and uninjured groups overlapped (Fig. 1A). Together, this may suggest that a joint laxity assessment of the shoulder may or may not be indicative of capsule or labral injury. Joint stiffness, on the other hand, was most variable in the uninjured compared to the injured group. However, the joint stiffness measurements within the interquartile range of the uninjured group did not overlap with injured group (Fig. 1B). Together, these findings support the need to assess the amount of glenohumeral translation (joint laxity) present in the joint, in addition to the amount of force required to translate the humeral head (joint stiffness) when determining whether the amount of laxity within the glenohumeral joint should be considered as excessive. Finally, joint stiffness is

not the only variable that should be considered when assessing joint laxity, but also gender and injury.

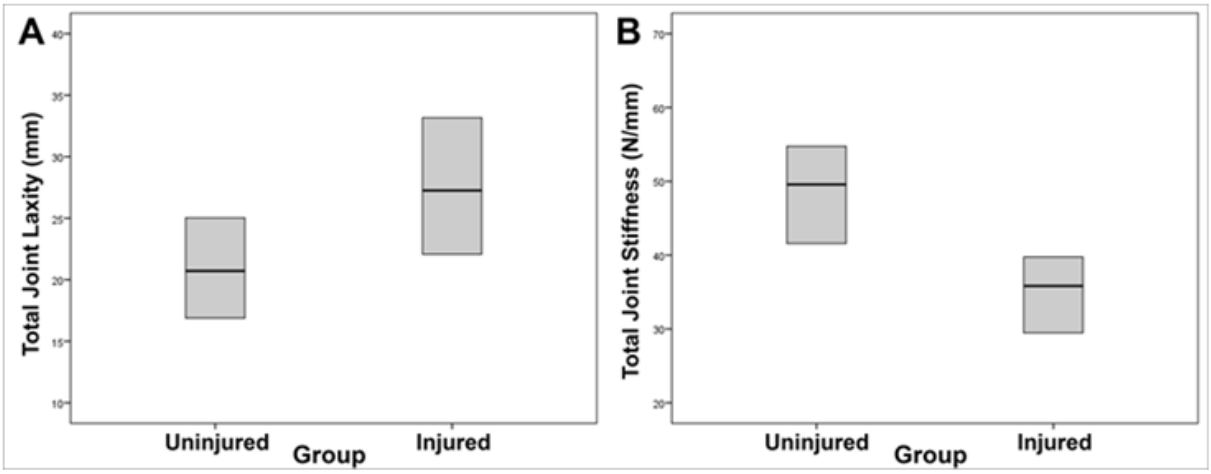

Figure 1. Boxplots showing variability in joint laxity (A) and joint stiffness (B) between the uninjured and injured groups**.**

Our data revealed gender differences similar to those found in another study (Borsa et al. 2002). Gender did not influence the direction of the relationship between joint laxity and joint stiffness, but rather the amount of laxity and stiffness. Females presented with greater laxity and lower joint stiffness compared to males. Such differences should be taken into consideration when determining whether a shoulder is unstable. Similar to gender, neither age nor injury influenced the direction of the relationship between joint laxity and joint stiffness. However, the data for total joint laxity and stiffness revealed greater laxity in the injured compared to the uninjured shoulders. We believe joint laxity and joint stiffness should be taken into consideration when assessing the unstable shoulder.

6.1.1.2 Mechanoreceptor Characteristics and Joint Laxity

The loss of neuromuscular control is a clinical symptom observed with neuromuscular dysfunction. To study mechanoreceptor characteristics that may affect joint laxity, we first developed a staining protocol that allowed for the identification of sensory nerve endings in the glenoid capsule and labrum. Mechanoreceptor characteristics have been overlooked in the capsule and labrum in relation to joint stabilization, more specifically, joint laxity. Our data

showed that within the capsule and labrum, the mechanoreceptors were distributed in a pattern that differed between tissues and varied by mechanoreceptor type. Identifying the distribution and location of mechanoreceptors helps determine their receptive field and topographical map that corresponds to different muscles surrounding the glenohumeral joint. Disruption of the distributional pattern may contribute to proprioceptive deficits or muscle weakness. The existence of a patterned distribution may suggest the importance of the orientation and positioning of mechanoreceptors in these tissues, and should therefore be considered and preserved during treatment.

Additionally, we observed a plausible relationship between neural count and joint laxity. We expected the number of mechanoreceptors to decrease with increasing joint laxity. Instead, the number of sensory nerve endings increased with increasing joint laxity. This positive trend could be due to mechanoreceptor adaptation in response to increased joint laxity or mechanoreceptor adaptation in response to injury that resulted in increased joint laxity. Mechanoreceptor adaptation refers to the regeneration of sensory nerve endings in response to changes in the external environment or intrinsic factors. An imbalance between the number of sensory nerve endings and joint laxity may result in proprioceptive dysfunction. Similarly to preserving the distribution pattern, it may also be critical to preserve the number of mechanoreceptors during treatment.

The relationship between neural count and joint laxity was affected by age, gender, and injury. Each variable demonstrated a significant main effect on this relationship. Age, additionally, demonstrated a significant interaction with neural count and joint laxity. Although we have identified a plausible relationship between neural count and joint laxity, a larger study should be performed controlling for age, gender, and injury to confirm this relationship.

Knowledge of the mechanoreceptor distribution and count, as revealed in this study, is a first step in understanding more contributing factors to joint stabilization. The results from our

study showed that there is a relationship between neural count and joint laxity. For this reason, mechanoreceptor preservation should be considered during treatment to prevent or reduce the rate of recurring instability.

6.1.1.3 Collagen Content and Joint Stiffness

Mechanoreceptors in glenoid capsule and labrum are embedded within the extracellular matrix collagen. Collagen is the ultimate capsular and labral tissue component responsible for the tissue strength as it is the primary load carrying molecule of fibrous tissues. The capsule and labrum rely on their collagen content to provide their tissue stiffness allowing these tissues to resist glenohumeral translation. The ability of these tissues to resist glenohumeral translation is their contribution to joint stiffness. Our findings showed a negative trend in tissue collagen content and joint stiffness relationship. This work demonstrated that tissue collagen content may contribute to joint stiffness. On the other hand, the decrease in joint stiffness with increasing collagen content may be indicative of collagen type redistribution, specifically when ratio of collagen type I (strength) to collagen type III (elasticity) decreases with increased production of collagen type III.

When comparing the injured group to the uninjured, there was a negative significant relationship between collagen content and joint stiffness with injury. This finding suggests that joint stiffness may only be affected when the capsular and labral collagen properties are compromised (injured). Tissue injury affects collagen remodeling process that involves collagen degradation and synthesis. After remodeling, tissue collagen properties are largely restored helping the tissue to regain its function. Disruption of collagen remodeling secondary to further injury does not allow for proper healing thereby making the tissue more susceptible to reinjury. Injury does not only affect the amount of collagen, but also influences the type of collagen produced.

Age, similar to injury, affected the relationship between collagen content and joint stiffness. There was a significant positive relationship between age and collagen content. This may be due to reinjury incurred in the tissues with aging. The increase in collagen content may be due to an imbalance between collagen degradation and collagen synthesis, which disrupts tissue strength and thus tissue function. Since age interacts with the relationship between collagen content and joint stiffness, a similar study would need to be performed controlling for age.

6.1.1.4 Collagen Content and Neural Count

Previous investigations have shown that mechanoreceptor size and type vary with loose/diffuse and dense connective tissue. “The more diffuse the joint capsule, the greater the presence of small, unencapsulated type 1 (Ruffini) and large type 2 (Pacini) mechanoreceptors. The more dense and regular the connective tissue (ligament-like), the greater the presence of relatively larger and more complex type 1 (Ruffini, resembling Golgi tendon organs) and smaller, developmentally simple, lamellated type 2 mechanoreceptors.” (Nyland et al. 1998) The aforementioned findings were similar to our observations in the glenoid capsule and labrum. In the flaccid glenoid capsule, the type 1 nerve endings were primarily unencapsulated and the type 2 nerve endings were larger than the type 2 nerve endings observed in the dense fibrous labrum. Additionally, type 1 nerve endings, observed in the glenoid labrum were primarily GTO-like Ruffini endings. Thus far, this was the only connection identified between mechanoreceptors and connective tissue. Interestingly, we observed another connection between mechanoreceptors and connective tissue, specifically, the collagen content. Our findings illustrated a significant positive relationship between the number of mechanoreceptors and collagen content. Based on our findings, an alteration in collagen content or composition, in response to joint injury may change the number of mechanoreceptors resulting in proprioceptive deficits associated with instability.

6.1.2 General Summary

The trends and relationships observed between joint laxity, joint stiffness, neural count, and collagen content demonstrate the importance of tissue properties in joint stabilization. However, we have run multiple small sampled exploratory studies. From each study, the results have continuously shown that tissue properties of the capsule and labrum should be considered when assessing and treating glenohumeral joint stabilization.

6.1.3 Limitations

Two major limitations of this study were the small sample size and the huge variability between ages. Shoulders to be included in the injured group were only identified during dissection. We were unable to retrieve records that demonstrated previous history related to diseases or shoulder conditions. We were also unaware of the dominant hand, which could possibly influence the outcomes of this study.

6.1.3.1 Joint Laxity and Joint Stiffness

No standard technique has been established for determining and maintaining neutral position of the glenohumeral joint when assessing the amount of anterior and posterior joint laxity independently. For this reason, we combined anterior and posterior joint laxity for this analysis. In the future, it would be beneficial to determine a way to not only record total joint laxity and stiffness, but to also record these values at the point of humeral head subluxation and dislocation. This would allow us to determine the amount of stiffness available in the joint relative to joint laxity pre and post-subluxation.

6.1.3.2 Neural Count and Joint Laxity

Although we were able to count the number of sensory nerve endings in the capsule and labrum, we were unable to identify whether these mechanoreceptors were degenerated. Sensory nerve endings were counted with the assumption that they were all “normal.” However, if the nerve endings were degenerated, then the number of “normal” nerve endings relative to

joint laxity may then change the relationship observed in this study. Future studies should assess the structure of nerve endings in the aging capsule and labrum, using the same staining protocol, to determine the percentage of degenerative nerve endings compared to healthy.

Age, gender, and injury presented as confounding variables within this experiment. However, we were unable to determine how these factors affected the relationship between neural count and joint laxity when taking each variable into consideration simultaneously due to the small sample size.

6.1.3.3 Collagen Content and Joint Stiffness

Collagen is a difficult protein to extract, especially from fibrous tissues such as the capsule and labrum. We chose to extract acid-pepsin soluble collagen and used it for our study; therefore, our results are limited to acid-pepsin soluble collagen only. Considering limited amount of tissue available for collagen extraction, we were not able to extract the amount of protein sufficient for concentrating, to enrich the collagen containing fraction, for using in ELISA to determine type I collagen and type III collagen. We performed ELISA using unconcentrated collagen and discovered that the available ELISA kits for both types of collagen were not sensitive enough to measure levels of collagen types individually. In addition, the specimens for collagen extraction were dissected from fresh and fresh frozen cadavers, although the effect of freezing on extractability of collagen is not known.

6.1.4 Clinical Relevance

6.1.4.1 Joint Laxity and Joint Stiffness

Clinical diagnosis of shoulder instability is often based solely on joint laxity. Our results have revealed a large variability in joint laxity with minimal variability in joint stiffness, and so a shoulder may appear to be unstable if a joint laxity assessment is the only test used. Determining the amount of joint stiffness and corresponding joint laxity available pre and post-subluxation would unveil ranges for stabilization and instability. Our body of work provided

values related to the static involvement of the glenoid capsule and labrum. In summary, we expected joint stiffness to vary with joint laxity.

6.1.4.2 Neural Count and Joint Laxity

Neuromuscular dysfunction is a clinical symptom associated with shoulder instability, in which the lack of neuromuscular control is apparent pre and post-treatment. Causal factors and how to address this deficit are still sought after. Knowledge of sensory nerve endings and fascicular distribution allows for the preservation of neural structure and organization during surgical treatment. The relationship between neural count and joint laxity further support the need to preserve the neural structures of the glenoid capsule and labrum.

It is understood that mechanoreceptors are responsible for the proprioceptive neurologic feedback mechanism. Interference with this feedback mechanism has been attributed to changes in neural conduction that resulted in delayed neuromuscular contractions surrounding the joint of interest (Myers and Lephart 2000, Hammer 2005). The delayed neuromuscular response could be due to disorganization or a change in the number of sensory nerve endings, especially since mechanoreceptors have a receptive field that corresponds to a given topographical location.

The results from this study may ultimately aide in the development of treatments for the prevention of recurrent instability. For this reason, this line of research should be further explored. Randomized controlled studies may be designed to determine the effects of surgery on the distribution patterns and joint proprioception. Future studies should also assess the effects of neural count on joint proprioception.

6.1.4.3 Collagen Content and Joint Stiffness

Oftentimes individuals are unaware of their initial tissue injury, and so the capsule and labrum incur further injury in response to functional tasks. Other contributors to reinjury may include inappropriate rehabilitation programs. For each phase of the collagen remodeling

process, different types of treatment are required to prevent further injury and assist with collagen fiber orientation (Potach and Grindstaff 2008). The significant relationship between mechanoreceptors and the collagen content within the glenoid capsule and labrum suggests it may be important to consider how treatments affect collagen content post injury because these treatments may also improve or worsen glenohumeral joint proprioception.

It has been previously suggested that type 1 nerve ending morphology may depend on the connective tissue loose versus dense texture (Nyland et al. 1998). If mechanoreceptors adapt to a changing environment, a collagenous environment that is not conducive for mechanoreceptor adaptation could negatively affect neural activation and signaling, count, branching, and size. Additionally, fascicles are organized parallel to the direction of the collagen fibers. As the collagen fibers changed direction, the fascicles branched in the direction of change appearing as a lattice network. The type of conservative treatment administered during the different phases of healing post-injury could influence the orientation of collagen fibers, and in turn, fascicular positioning (Potach and Grindstaff 2008). Since fascicles have an associated topographical location, a change in fascicular positioning may negatively impact signal conciseness. Essentially, all factors mentioned above could disrupt proprioceptive signaling, leading to recurrent shoulder instability.

6.1.5 Future Directions

6.1.5.1 Joint Laxity and Joint Stiffness

A study measuring joint laxity and joint stiffness in healthy and injured men and women should be performed in addition to measuring proprioceptive signaling. Such studies would assist with providing numerical ranges for diagnosing shoulder instability based on a laxity to stiffness ratio. The next phase would be to consider the different factors that may contribute to an unstable shoulder such as neural count and collagen content. Once the contributing factors

have been established, optimal treatment protocols should be developed to restore the properties of the contributing factors.

Future studies should also assess joint laxity and stiffness in an aging population (20-100 years of age). The same study should be performed in cadavers as well as human subjects. Cadaveric shoulders should be assessed with and without intact muscle. This would allow for determination of the amount of joint laxity and stiffness attributed to the capsule and labrum versus muscle. Additionally, when performing experiments related to instability, the subluxation point should be recorded. These results could reveal a numerical range for diagnosing instability.

6.1.5.2 Mechanoreceptor Characteristics and Joint Laxity

In combination with the joint laxity and joint stiffness studies, proprioceptive tests should also be performed in injured and uninjured groups. Muscular topographical identification associated with the fascicles and sensory nerve endings of the capsule and labrum would confirm the importance of the fascicles and sensory nerve endings. Additionally, this would help in determining the source of proprioceptive deficits related to instability. Future studies should also assess the effects of different surgical techniques on joint laxity, joint stiffness, and proprioception.

6.1.5.3 Collagen Content and Joint Stiffness

Collagen remodeling should be studied post-surgery and post-treatment. Different types of conservative treatment have been shown to stimulate proper collagen remodeling at different phases of healing (Potach and Grindstaff 2008). Therefore, the collagen content of the glenoid capsule and labrum should be compared in the uninjured and injured groups pre and post-treatment, in addition to assessing joint stiffness.

6.1.5.4 Mechanoreceptor Characteristics and Collagen Content

Collagen remodeling may not only be important for tissue strength, but also for mechanoreceptor regeneration and function. This phenomenon has not been studied, yet it should be. The relationship between the amount of collagen content and neural count supports the importance of both tissue properties. From the exploratory studies we have performed, it is evident that more research related to instability should focus on the tissue properties of the glenoid capsule and labrum and how these properties relate to clinical symptoms.

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