THE EFFECT OF THERAPEUTIC ULTRASOUND ON TENDON HEALING AND SENSORY NERVE REGENERATION AFTER ACHILLES TENDON RUPTURE

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written nor material which has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____(Signed)

YEUNG CHI KEUNG (Name of student)

LIST OF ABBREVIATIONS

Analysis of Variance (ANOVA)

Beam Nonuniformity Ratio (BNR)

Calcitonin Gene-Related Peptide (CGRP)

Center of Gravity (COG)

Centralized Animal Facilities (CAF)

Continuous Ultrasound (CUS)

Galanin (GAL)

Intra-Class Correlation (ICC)

Joint Position Sense (JPS)

Low Intensity Pulsed Ultrasound (LIPU)

Neuropeptide Y (NPY)

Phosphate Buffered Saline (PBS)

The Hong Kong Polytechnic University (PolyU)

Pulsed Ultrasound (PUS)

Range of Movement (ROM)

Spatial Averaged (SA)

Spatial Averaged Temporal Averaged (SATA)

Statistical Package for Social Sciences (SPSS)

Substance P (SP)

Tendon Calcaneus Complex (TCC)

Ultimate Tensile Strength (UTS)

Visual Analogue Scale (VAS)

ABSTRACT

Objectives

Though ultrasound therapy has been reported effective in promoting healing of Achilles tendon rupture in a number of animal studies, the underlying mechanisms of its effect are still not well understood.

Recent immunochemical studies on the injured rat's Achilles tendon showed in-growth of different types of sensory nerve fibers at the early stage of healing, suggesting the importance of the role of intact sensory function in tendon repair. Ultrasound is a form of mechanical energy that is transmitted through and into biological tissues as an acoustic wave. Thus it is important to relate the mechanical basis of ultrasound to the sensitivity of the tendon to mechanical stimuli through its sensory nerve fibers. Up to now there have been no reports addressing this relationship.

Therefore, ***the purpose of this study*** was to investigate the role of the sensory nerve in tendon healing and its response to ultrasound stimulation in enhancing tendon healing.

Methods

A series of animal studies were carried out on totally 128 rats. The outcomes of tendon healing were assessed biomechanically (80 rats) and histologically (24 rats). The in-growth of sensory fibers was evaluated by immunohistochemical analysis (24 rats). The tendon injury model was the right medial Achilles tendon hemi-tenotomy. Pulsed

ultrasound (PUS) was used for ultrasonic therapy (1 MHz, 2.5W/cm^2, duty cycle 20%, 5 minutes, 3 times per week). Animals with tendon injury were then randomly assigned equally into 4 groups: two neural intact groups received treatment of either sham PUS (control group) or true PUS (PUS group) to the injured area; the rats in the other 2 groups received an additional ipsilateral sciatic neurectomy (SN) and then the injured tendons were treated either by sham PUS (SN-control group) or true PUS (SN-PUS group). The animals were sacrificed at 2 or 4 weeks postinjury and bilateral Achilles tendons were harvested for biomechanical and histological analysis or immunohistochemical analysis.

Results:

Two-way Analysis of Variance (ANOVA) on the biomechanical testing data showed that the PUS group had significantly higher normalized UTS and stiffness than the control group ($p<0.01$) in animals with intact nerve supply. However, in animals with sciatic neurectomy, no significant difference was found in normalized UTS and stiffness between the SN-PUS and SN-control groups ($p>0.05$). It was also demonstrated that neurectomy retarded the recovery of stiffness of the injured tendon, since there was no significant difference in normalized stiffness value between 4 weeks and 2 weeks ($p>0.05$) in the animals with sciatic neurectomy. Besides, the UTS of the injured leg in SN-contol group was significantly lower than that of the control group at 4 weeks postinjury.

Histological analysis showed that in animals with intact neural supply, the collagen

matrix was denser and the alignment of collagen fiber bundles more regular in the PUS group. It was found that the scar in the PUS-treated group was more mature, as demonstrated by the lowered fusiform fibroblasts to tenocytes ratio and the significantly higher collagen matrix area when compared to the controls. However, in animals with sciatic neurectomy, the injured tendon was healed by scar tissue with inferior properties revealed by high cellularity, a lower portion of collagen matrix, and a higher fusiform fibroblast to mature tenocytes ratio throughout the 4 weeks. There was no significant difference in the morphological appearance of the healing area between the SN-PUS and SN-control groups.

Immunohistochemical analysis on tendons with intact neural supply revealed a significantly higher invasion of CGRP positive nerve fibers in the PUS group at 2 weeks postinjury, while this difference was not found in the 2 groups with sciatic neurectomy. In the two groups with sham PUS treatment (control and SN-control), the amount of invasion of CGRP positive fibers in injured tendons was significantly lower in the SN-control group. These results suggest a possible interaction between CGRP positive sensory nerve fibers and PUS in promoting tendon healing.

Conclusion:

Knowledge of the effects of therapeutic ultrasound on innervated and dennervated tendon models could help us to understand the possible mechanism of ultrasound-enhanced

tendon healing. Results of this study suggested that PUS can improve tendon healing only when there is an intact nerve supply. Denervation impacted the tendon healing. This study suggests the importance of the involvement of neurological components in ultrasound-enhanced tendon healing and the vital role of tendon innervation.

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AWARDS AND PUBLICATIONS

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Journals:

1. **Yeung CK**, Guo X, Ng GYF. Pulsed ultrasound treatment accelerates the repair of Achilles Tendon rupture in rats. Journal of Orthopaedics research, 2006;24:193-201.

2. **Yeung CK**, Guo X, Ng GYF. Effect of denervation on Achilles tendon healing, Journal of Biomechanics (submitted).

3. **Yeung CK**, Guo X, Ng GYF. Therapeutic ultrasound in soft tissue lesions: A systemic review by using meta-analysis. International SportMed Journal (submitted).

Conference proceedings:

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CHAPTER 1. INTRODUCTION

Therapeutic ultrasound has been used in physiotherapy as treatment modality for soft tissue injuries for more than 50 years (da Cunha et al., 2001; ter Haar, 1999). Its effects in promoting tendon healing have been suggested by several animal experiments (da Cunha et al., 2001; Ng et al., 2003; Saini et al., 2002). These studies reported that ultrasound could promote tendon healing in terms of increasing the breaking strength and collagen synthesis. The mechanisms underlying how ultrasound promotes tendon healing in living tissues remain unclear. Cellular studies *in vitro* tried to explain the physiological effects of ultrasound, like its mechanical effects on cell membrane permeability and collagen synthesis in stimulated fibroblasts (Dinno et al., 1989; Ramirez et al., 1999). However, the cellular responses to ultrasound in a bioenvironment inside a living animal may be different from the *in vitro* responses.

As we know, ultrasound is a kind of mechanical vibration which can propagate through a medium (Prentice, 2002). How this mechanical stimulation is “sensed” by the injured tissue and delivers its therapeutic effect is not, however, clearly understood. This study aimed to investigate how the mechanical stimulation from ultrasound interacts with the living animal to promote tendon healing. The author tried to relate this mechanical vibration to the neural components of the injured tendon – the sensory nerve fibers in the tendon. The study intended to examine whether the absence of sensory fibers would affect the injured tissue to “sense” the mechanical vibration and cause a decrease in tissue

response to ultrasound treatment. By knowing this relationship, researchers could further explain how therapeutic ultrasound affects the healing of injured tissue. The author tried to explore this relationship by using different outcome measures, like biomechanical tests and histomorphological analysis, as well as immunohistochemistry.

The purpose of this thesis is to present a series of studies to investigate the effects of therapeutic ultrasound on tendon healing, and its interaction with the presence of sensory nerve fibers in the injured tendon. The ultimate goal of animal studies in investigation of the efficacy of therapeutic ultrasound in animals is to infer the results to the human situation. The researchers shared this point of view and thus planned a preliminary, pilot study on the effect of therapeutic ultrasound on tendon healing in patients with Achilles tendon rupture, which is of great significance to the application of therapeutic ultrasound for the treatment of tendon healing in clinical practice.

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CHAPTER 2. LITERATURE REVIEW

2.1. Achilles tendon

2.1.1. The anatomy of the human Achilles tendon

The Achilles tendon is the strongest and thickest tendon in the body. It is a conjoined tendon of the gastrocnemius and soleus muscles (collectively known by some anatomists as *Tricep surae*), which together form the posterior, superficial compartment of the calf (Gray, 1999). There may also be a small contribution from the plantaris muscle, though this small muscle (muscle belly is usually 5 to 10 cm in length) is absent in 6 to 8% of individuals (Hollinshead, 1982) (Figure 2-1).

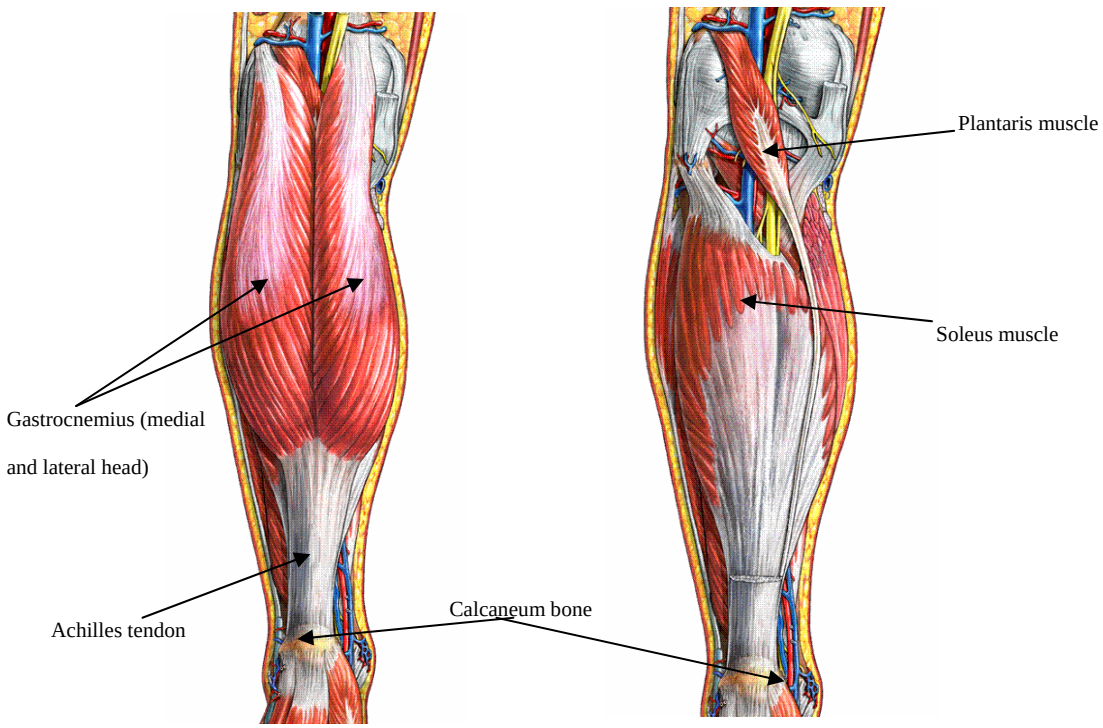

Figure 2-1. Anatomy of *Tricep surae* and Achilles tendon. (Adopted from A.D.A.M. Interactive Anatomy, 1997)

2.1.1.1 Gastrocnemius

The gastrocnemius is the most superficial muscle, a fusiform and two-joint muscle composed of medial and lateral heads. The medial head of the gastrocnemius rises from the popliteal surface of the femur behind the supracondylar ridge and the adductor tubercle, just above the medial femoral condyle. It is larger and extends more distally in the calf than the lateral head (Warwick and Williams, 1973). The lateral head is shorter and rises from the lateral surface of the lateral femoral condyle, above and behind the lateral epicondyle. Both medial and lateral heads also arise from the posterior capsule of the knee joint as well as the oblique popliteal ligament, and are attached to the condyles of the femur by strong flat tendons that extend for a short distance on the superficial surface of the muscles as an aponeurosis (Hollinshead, 1982). The muscle bellies extend to the middle of the calf, and at the point where the two muscles come together, form a tendinous raphe that becomes continuous with the aponeurosis on the anterior or deep aspect of the muscle (O'Brien, 2005). This is gradually incorporated with the tendon of the soleus into a broad, robust tendon in the posterior aspect of the lower leg.

2.1.1.2. Soleus

The soleus is a broad, flat pinnate muscle, which is wider and whose muscle fibers extend more distally than the gastrocnemius. It originates from the posterior surface of the

head and upper quarter of the posterior surface of the fibula, a fibrous arch between the fibula and tibia, and the oblique line and middle third of the medial border of the tibia. The muscle fibers end in posterior aponeurosis, anterior to the aponeurosis of the gastrocnemius. This muscle fuses with the gastrocnemius and forms the deepest portion of the Achilles tendon. The soleus muscle forms a broad tendon about midway down the leg, in a position deep to the tendon of the gastrocnemius. The proximal part of the soleus tendon glides freely deep to the gastrocnemius muscle, in order to allow independent movement of the two muscles (Hollinshead, 1982).

2.1.1.3. Plantaris

The plantaris originates from the distal part of the lateral supracondylar ridge, the popliteal surface of the femur, and the knee joint capsule. Its long tendon extends distally between the gastrocnemius and soleus muscles, and crosses obliquely from lateral to medial in a depression in the soleus muscle. It emerges on the medial side of the Achilles tendon, 12 cm from the Achilles insertion to the calcaneus. The insertion pattern of the tendon varies: 47% inserts into the medial aspect of the insertion site of the calcaneal tuberosity of the Achilles tendon, where fascial strands may extend to the medial border of the Achilles tendon; 36.5% inserts into the calcaneus 0.5 to 2.5 cm anterior to the medial border of the Achilles tendon; 12.5% demonstrates a broad insertion along the dorsal and

medial surfaces of the Achilles tendon; 4% inserts into the medial border of the Achilles tendon from 1 to 16 cm proximal to the Achilles insertion into the calcaneus (Cummins et al., 1946).

2.1.1.4. Achilles tendon

The Achilles tendon is approximately 15 cm long in an adult human. It starts at the musculotendinous junction of the gastrocnemius and soleus in the middle of the calf. The tendon is flattened at its junction with the gastrocnemius, gradually narrows and becomes rounded as it extends distally at approximately 4 cm from its insertion. Around this point, it flattens again and then becomes cartilaginous to insert into a rough area on the posterior surface of the calcaneus, distal to the postero-superior calcaneal tuberosity. On its anterior surface, it receives the muscle fibers from soleus almost to its insertion (O'Brien, 1992). The soleus and gastrocnemius muscles vary in their orientation and contribution to the Achilles tendon, and in the extent of their fusion. In 52% of subjects, the soleus contributes 52% to the Achilles tendon and its fibers form the anterior and medial portion of the tendon. In 35% of subjects, both gastrocnemius and soleus contribute equally. In 13% of subjects, the gastrocnemius forms two thirds of the tendon. In addition, the tendinous components of these two muscles are variable. The gastrocnemius component is the longer portion, contributing 11 to 16 cm. The soleus, in contrast, is shorter, containing a tendinous

component from 3 to 11 cm in length. Approximately 5 to 6 cm proximal to the calcaneal insertion, the independent tendons of the gastrocnemius and soleus fuse to become one tendon (Cummins et al., 1946; Schepsis et al., 2002).

The tendon fibers are not solely vertically oriented, but spiral at about 12 to 15 cm proximal to the insertion of the tendon, which is about the level where the soleus muscle begins to send out fibers to the Achilles tendon. The medial fibers rotate posteriorly and the posterior fibers rotate laterally. This rotation becomes more marked in the terminal 5 to 6 cm of the tendon. As the Achilles tendon descends, it twists through 90 degrees, with the gastrocnemius component mainly on the lateral and posterior part of the tendon. Twisting produces an area of stress within the tendon, which is most marked 2 to 5 cm above its calcaneal insertion (Barfred, 1971; Benjamin et al., 1986). The vascularity of this area is poor, and it is also a common site of tendinopathy and rupture (Jozsa and Kannus, 1997).

The width of the tendon at its point of insertion into the calcaneus varies from 1.2 to 2.5 cm. At this point, the tendon becomes broad distally and has a wide base triangular attachment. The inferior portion of the tendon above its attachment to the calcaneus has an area of fibro-cartilage. A retrocalcaneal bursa is present, just proximal and deep to the insertion, between the tendon and the posterior surface of the calcaneus. The posterior wall of the bursa is formed by the fibro-cartilage of the tendon. The anterior surface of the bursa is the cartilaginous layer, about 0.5 mm to 1 mm thick, located on the posterior aspect of

the calcaneus. Proximally, the superior border is formed by a synovial lining that separates the bursa from the proximal fat pad (Palastanga et al., 2006). This fat pad rests anterior to the tendon and occupies the Karger's triangle: the area between the Achilles tendon, the posterior tibia and the superior aspect of the posterior calcaneus. This fat pad separates the Achilles tendon from the deep flexors, like the flexor hallucis longus. Blood vessels also lie in the Karger's triangle and supply the Achilles tendon (Jones, 1994). Superficial to the tendon lies a subcutaneous tendo-Achillis, or retrotendo-Achillis bursa between the tendon and the overlying skin (Schepsis et al., 2002).

It was found that the Achilles tendon has a thick continuation of fibers of the tendinous insertion into the plantar fascia in the neonate. This continuity gradually diminishes with age and becomes solely a connection of the superficial periosteal fibers in the middle-aged foot (Snow et al., 1995).

Unlike other tendons around the ankle, which have a synovial sheath, the Achilles tendon is enveloped by a paratenon, a thin gliding membrane made up of a single layer of loose areolar tissue. It originates from the deep fascia of the leg, the fascia cruris. This tissue is richly vascularized and is responsible for a significant portion of the blood supply to the tendon (Carr and Norris, 1989). It also functions as an elastic tissue and allows the tendon to glide freely against the surrounding tissue, thus allowing a wide range of movement (ROM) (Kvist et al., 1987).

2.1.2. Structure of tendon

Healthy tendons are brilliant white in color and fibro-elastic in texture, showing great resistance to mechanical loads (Kannus, 2000). They appear white because they are mostly avascular (O'Brien, 2005). Tendons vary in their forms and the ways they attach to bones: they can be rounded cords, straplike bands, or flattened ribbons (Kannus et al., 2000), and the attachment is related to the function of the muscle. Muscles that create powerful force, like the calf, have short and broad tendons. On the other hand, the flexor muscles of the hand, which need to carry out subtle and delicate movements, have long and thin tendons (Kannus, 2000).

2.1.2.1. Constituents of tendons

Collagen fibrils, collagen fiber

Tendons consist of collagen (mostly type I collagen) and elastin embedded in a proteoglycan-water matrix. The dry mass of human tendons is approximately 30% of the total tendon mass, with water accounting for the remaining 70%. Type I collagen accounts for 65–80% and elastin approximately 1–2% of the dry mass of the tendon (Curwin, 1997; Hess et al., 1989; Jozsa et al., 1989b; Kirkendall and Garrett, 1997; O'Brien, 1997). These elements are produced by tenoblasts and tenocytes, which are the elongated fibroblasts and fibrocytes that lie within the collagen matrix (Hess et al., 1989).

Collagen is arranged in hierarchical levels of increasing complexity, beginning with soluble tropocollagen, a triple-helix polypeptide chain, which forms cross-links to create insoluble collagen molecules. The collagen molecules aggregate into microfibrils and then into ultrastructural units clearly visible with electron microscopy, known as the collagen fibrils (Kannus, 2000). The collagen fibrils in the Achilles tendon vary from 30 nm to 130 nm in diameter (O'Brien, 2005). A bunch of collagen fibrils forms a collagen fiber, which is the basic unit of a tendon that can be tested mechanically (O'Brien, 1997) and is visible under light microscopy (Curwin, 1997). The average size of the collagen fibers of the Achilles tendon is around 60 μm (O'Brien, 2005).

Collagen fibrils are not only oriented longitudinally, but also transversely and horizontally, with the longitudinal fibrils crossing each other, forming spirals and plaits (Chansky and Iannotti, 1991; Hueston and Wilson, 1972; Jozsa et al., 1991a). This complex ultrastructure of the tendons gives extra strength to the structure by providing a good buffer against longitudinal, transversal and rotational loads during movements and activities (Kannus, 2000).

In the resting state, the collagen fibers and fibrils of a tendon show a wavy configuration or crimps, which appear under the light microscope and more obviously under the scanning electron microscope as regular bands across the fiber surface. This configuration disappears if the tendon is stretched slightly, which results in straightening of

the collagen fibers (Hess et al., 1989)

A fine sheath of connective tissue known as the endotenon invests each collagen fiber and binds the fibers together. The endotenon is a thin reticular network of connective tissue inside the tendon that has a well developed crisscross pattern of collagen fibrils (Kastelic et al., 1978; Rowe, 1985). A bunch of collagen fibers forms a primary fiber bundle (subfascicle). The diameter of a subfascicle is about 15-400 μm. A group of primary fiber bundles forms a secondary fiber bundle (fascicle) with diameter ranging from 150 μm to 1000 μm. A group of secondary fascicles forms a tertiary bundle, and these tertiary bundles make up the tendon surrounded by the epitenon (Fig. 2-2). In a large tendon like the Achilles tendon, the diameter of the tertiary bundles could be up to 3000 μm. The epitenon is a relatively dense fibrillar network of collagen with strands of 8–10 nm in thickness. This network contains longitudinal, oblique, and transversal fibrils. Occasionally, the fibrils of the epitenon are fused with the superficially located tendon fibrils (Jozsa et al., 1991a).

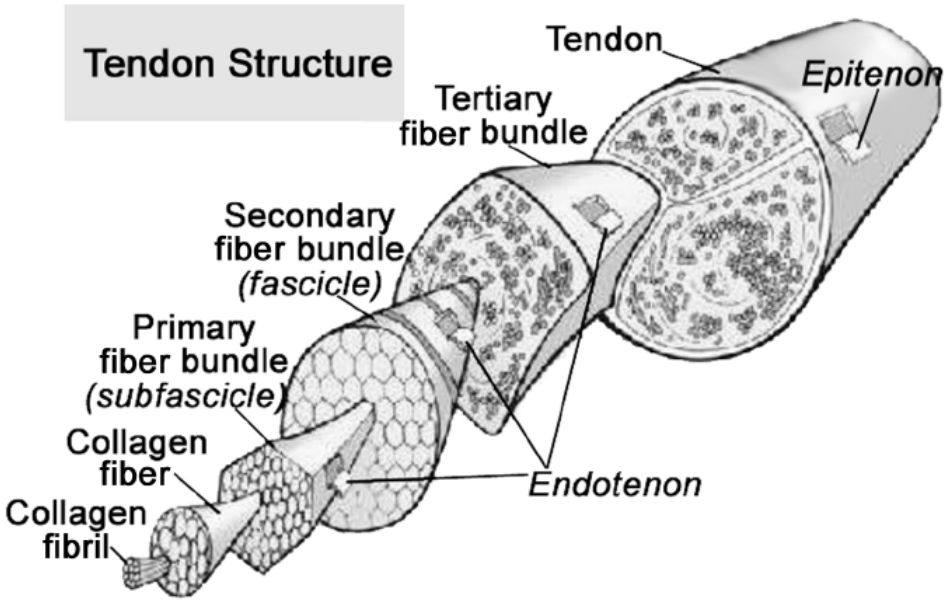

Figure 2-2. Structure of normal tendon (adopted from Sharma et al, 2005)

Molecular structure of collagen

The structural unit of collagen is tropocollagen, which is a long thin protein (280 nm long and 1.5 nm wide) that mainly consists of type I collagen. Tropocollagen is formed in the fibroblast as procollagen and then secreted extracellularly to become collagen by exocytosis. Collagen molecules consist of three polypeptide alpha-chains, each formed by amino acids. Two of the alpha-chains are identical (α-1) and one differs slightly (α-2). Each of these three chains forms a left-handed helix. The chains are connected by hydrogen bonds and wind together to form a ropelike, right-handed superhelix (Parry, 1988). Around two-thirds of the collagen molecules consist of 3 amino acids: glycine (33%), proline (15%) and hydroxyproline (15%). Glycine is found at every third residue, and enhances the stability by forming hydrogen bonds among the 3 chains (Borynsenko

and Beringer, 1989). Hydroxyproline is involved in the hydrogen bonding between the polypeptide chains, which increases the strength of the collagen (O'Brien, 2005).

2.1.2.2. Tendon cells

Within the extracellular matrix network, tenoblasts and tenocytes constitute about 90% to 95% of the cellular elements of tendons. The other 5% to 10% comprises chrondrocytes at the bone attachment and insertion sites, the synovial cells of the tendon sheath, and the vascular cells, such as the capillary endothelial cells and the smooth muscle cells of the arterioles in the endo- and epitenon.

Morphology and function of tendon cells

The morphological appearance and the cell to matrix ratio of the tendon vary with age. Tendons in new-born babies have a very high cell to matrix ratio. Tenoblasts are arranged in long parallel chains of different shapes and sizes: elongated, rounded, spherical, quadrangular or polygonal. In young individuals, the cell to matrix ratio is lowered and the tenoblasts are spindle-shaped. In adults, there is a further decrease in the cell to matrix ratio and the cells are mostly elongated (Ippolito et al., 1980).

Tenoblasts are immature tendon cells with numerous cytoplasmic organelles, reflecting their high metabolic activity. Besides the shape, the size of tenoblasts also varies,

with lengths from 20 μm to 70 μm and widths from 8 μm to 20 μm. The shape of nuclei is also different, from ovoid to very long spindle-shaped nuclei. As they mature, tenoblasts become elongated and transform into tenocytes. The length of tenocytes varies from 80 μm to 300 μm. Tenocytes have a higher nucleus-to-cytoplasm ratio than tenoblasts, with decreased metabolic activity shown by a marked decrease in the intracytoplasmic organelles responsible for protein synthesis. Tendon cells are active in energy generation through the aerobic Krebs cycle, anaerobic glycolysis, and the pentose phosphate shunt. They are active in energy production and in the biosynthesis of collagen and all components of the extracellular matrix network, like elastin fibers, proteoglycans and structural glycoproteins. (Kvist et al., 1987; O'Brien, 1997). With increasing age, metabolic pathways shift from aerobic to more anaerobic energy production (Hess et al., 1989; Kannus and Jozsa, 1991)

2.1.2.3. Extracellular tendon matrix

The extracellular tendon matrix is composed of the collagen fibers, elastic fibers, extracellular matrix, and anorganic components. Elastic fibers are rarely found in human tendons, and account for only approximately 1–2% of the dry mass of a tendon (Carlstedt, 1987; Hess et al., 1989; Jozsa et al., 1989b). The function of elastic fibers is not entirely clear, though they may contribute to the recovery of the wavy configuration of the collagen

fibers after the stretching of a tendon (Butler et al., 1978).

The tendinous ground substance that surrounds the collagen and tendon cells consists of proteoglycans (PGs), glycosaminoglycans (GAGs), structural glycoproteins, and a wide variety of other small molecules. PGs are strongly hydrophilic, enabling rapid diffusion of water-soluble molecules and the migration of cells. Their high fixed charge density and charge-to-charge repulsion force also provide the collagen fibrils with a high capacity to resist compressive and tensile loads (Jozsa et al., 1991b).

The concentration of GAGs is considerably smaller in a tendon than in cartilage or other types of connective tissue. Together with PGs, the considerable water binding capacity of these macromolecules improves the elasticity of a tendon against shear and compressive forces. They are also important for stabilization of the whole collagenous system of connective tissue and for maintenance of ionic homeostasis and collagen fibrillogenesis (Kannus, 2000).

Adhesive glycoproteins, such as fibronectin and thrombospondin, tenascin-C, and undulin, participate in the tendon repair and regeneration processes (Jozsa et al., 1991a; Lawler, 1986). These macromolecules seem to have the property to bind either macromolecules or cell surfaces together (Kannus et al., 1998).

2.1.2.4. Innervation of Achilles tendon

Tendons are innervated by nerves, which are mainly sensory in nature, deriving from neighboring muscular, cutaneous, peritendinous, or deep nerve trunks. These nerves consist of a longitudinal plexus with terminations lying near the myotendinous junction (Stillwell, 1957). The nerves to the Achilles tendon are received via nerve fascicles located subcutaneously, mainly from the sural nerve, and via nerves supplying the neighboring muscles, such as the tibial nerve and its branches (Stillwell, 1957, O'Brien, 1992). The nerves tend to form a longitudinal plexus and enter by way of the septa of the endotenon or the mesotenon if there is a synovial sheath. Branches also pass from the paratenon by way of the epitenon to reach the surface or the inner part of the tendon (Jozsa and Kannus, 1997; Ippolito and Postacchini, 1986).

There are four categories of nerve ending within tendons. Type I endings or Ruffini corpuscles, which are spherical or oval in shape and about 200μm by 400μm in diameter, are pressure receptors as they have a low-threshold reaction to pressure (Katonis et al., 1991). They are sensitive to stretching but adapt slowly (Jozsa et al., 1993).

Type II endings, which are onion-like Vater-Paccinian corpuscles, are also pressure sensors but they are primarily velocity receptors which have a rapid adaptation. They appear as oval or cylindrical in shape and about 150 μm by 250 μm in diameter. They work as dynamic mechanoreceptors at the beginning and end of the movement as they react to

acceleration and deceleration (Freeman and Wyke, 1967).

The type III or Golgi tendon organs are mechanoreceptors that work along with muscle spindles as tension receptors which are responsible for signalling position and response to both active contraction and passive stretching of the involved muscle-tendon units (Jozsa et al, 1988). They vary in total length from 800 μm to 1200 μm (Jozsa et al., 1993). They consist of unmyelinated nerve endings and are split into numerous branches that penetrate into the connective tissue septa located among the single collagen fasciculi of the tendon (Ippolito and Postacchini, 1986). They function as transducers that convert physical energy – pressure or tension – into afferent nervous signals (Jozsa et al., 1993). They are important in organizing the afferent sensory neuron system that controls the movement of the body via the central nervous system (Jozsa et al., 1988).

The type IV endings, also known as the free nerve endings, are the pain receptors, richly located in peritendinous tissues (Jozsa et al., 1993; Bjur et al., 2005). The tendinous free nerve endings of humans are about 1μm in diameter (Jozsa and Kannus, 1997).

A recent immunohistochemical study of normal human Achilles tendons found that immunoreactivity to sensory nerve markers (calcitonin gene-related peptides, CGRP, and substance P, SP) was present in the paratendinous loose connective tissue, and also in association with the blood vessels of the tendon tissue proper (Bjur et al., 2005). Besides, immunoreaction for CGRP in the fascicles was more marked than that for SP. A study

using prolonged eccentric exercise in rats to induce Achilles tendinosis also found an increased immunoreactivity to CGRP, which occurred in parallel with the occurrence of hypervascularization at 7–11 weeks after the start of eccentric exercise (Messner et al. 1999). CGRP was reported to be important in the regulation of angiogenesis and proliferation of fibroblasts and synoviocytes (Brain et al., 1985; Haegerstrand et al., 1990; Yule and White, 1999), while SP was reported to have effects on vasodilation and vascular permeability enhancement (Bolton and Clapp, 1986). These findings suggest that nerve fibers processing neuropeptides that are important for tendon healing are present in the Achilles tendon and may play a role in tendon healing (Ackermann et al, 2002; Bjur et al., 2005).

2.1.2.5. Blood supply of Achilles tendon

The human Achilles tendon is supplied by small branches of the posterior tibial, anterior tibial and peroneal arteries (Schmidt-Rohlfing et al., 1992). The tendon receives its blood supply from three regions: the musculotendinous junction, along the length of the tendon, and at its junction with the bone (O'Brien, 2005). The majority of the blood supply comes through the paratenon (Williams, 1986). Perimysial vessels from the muscle continue between the fascicles of the tendon and supply the myotendinous junction, where the blood vessels originating from the muscles are unlikely to extend beyond the proximal

third of the tendon (Carr and Norris, 1989; Peacock, 1959).

The blood supply from the osteotendinous junction is limited to the insertional zone of the tendon, although vessels from the paratendon communicate with periosteal vessels at the osteotendinous junction (Kannus et al., 2000). In the Achilles tendon, there is an area of low vascularity, about 2 cm to 6 cm above the bony insertion (Astrom and Westlin, 1994; Carr and Norris, 1989, Kvist et al., 1992; Zantop et al., 2003). In general, tendon blood flow decreases with increasing age and mechanical loading (Astrom, 2000).

2.1.3. Biomechanical properties of Achilles tendon

The basic function of tendons is to transmit the force generated from muscles to the bones, making joints and limbs move. They act as a buffer by absorbing external forces to limit muscle damage (Best and Garrett, 1994). In addition, tendons eliminate the need for unnecessary lengths of muscle between origin and insertion, thus enabling the muscle belly to be at a convenient distance from the joint (Elliott, 1965). Tendons exhibit high mechanical strength, which is capable of resisting high tensile forces with limited elongation. Tendons are not entirely inextensible, however, possessing some flexibility due to the presence of elastic fibers (Ippolito, 1986; O'Brien, 1992). Although tendons have good ability to withstand tensile or stretching forces, they are less able to withstand shear and compressive forces transmitted by the muscles (Hess et al., 1989).

The mechanical properties of tendons can be determined *in vitro* by tensile testing, which is a common method because the test simulates the way that loading is imposed on tendons in real life (Cuming et al., 1978; Johnson et al., 1994; Ng et al., 2003; Woo et al., 1980; Woo et al., 1982; Wren et al., 2001). A typical tensile testing machine is composed of an oscillating actuator and a load cell (Figure 2-3). It contains two clamps: a static one mounted on the load cell and a moving one mounted on the actuator. The isolated tendon specimen being studied is gripped by these two clamps and is stretched once the actuator is set to move. The load cell records the tension associated with the stretch applied, while the tensile deformation of the whole specimen is depicted by the displacement of the actuator. If deformation measurements are taken over a restricted region of the specimen, an extensometer is used.

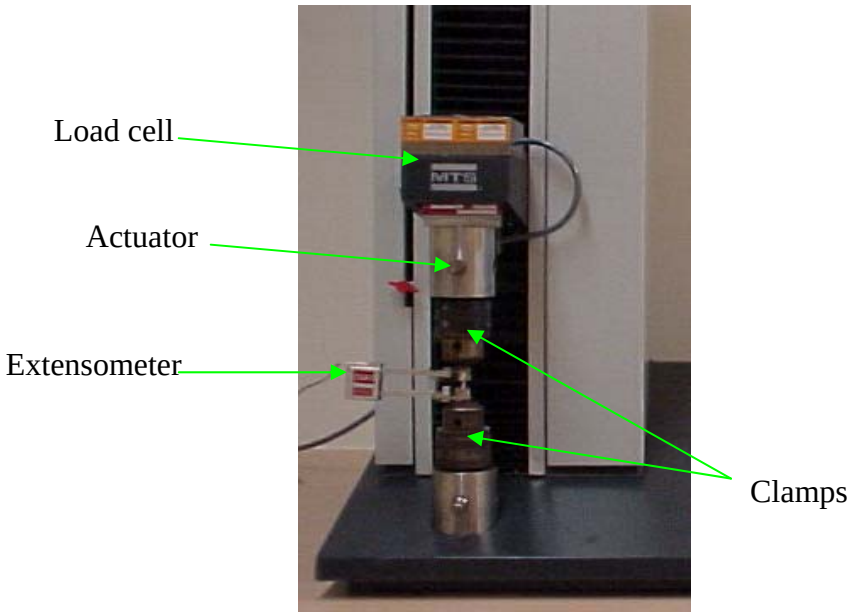

Figure 2-3. A typical tensile testing machine

A typical force-deformation curve of an isolated tendon is shown in Figure 2-4. The

slope of this curve relates to stiffness (N/mm), and the area under the curve relate to energy (J). Four different regions can be identified in the tendon force-deformation curve. Region I is the “toe” region, where the stiffness of the tendon gradually increases. The tendon is elongated mainly by reducing the crimp angle of the collagen fibers at rest, and there is minimal stretching of the fibers (Butler et al., 1978) (refer to section 2.1.2.1). Unloading of the tendon being stretched within this region can restore it to its initial length, while the loading is still within the tendon’s elastic limit.

Region II is where the stiffness remains constant as a function of elongation, and it is represented by a linear appearance of the curve. In this region, stretching is imposed in the fibers which have lost their wavy or crimped appearance (Diamant et al., 1972). The slope in this region is often referred to as the elastic stiffness of the tendon (Best and Garrett, 1994). The microfailure of fibers occurs at the end of this linear region, and thus the tendon stiffness begins to drop. Unloading at the end of region II may not restore the tendon’s initial length due to micro-failure of the fibers (Jozsa and Kannus, 1997; Maganaris and Narici, 2005).

Beyond the linear load is region III, where additional fiber failures occur in an unpredictable fashion (Butler et al., 1978). In this region, the collagen fibers slide past one another as the crosslinks fail (O’Brien, 1992). Once the maximum load is attained, complete failure occurs at region IV, with the load-supporting ability of the tendon totally

lost. Thereafter, the fibers recoil into a tangled bud at the ruptured end (Best and Garrett, 1994). The parameters that are measurable from this force-elongation curve include stiffness in region II, maximum load, time to maximum load, deformation to failure and energy to failure (Butler et al., 1978).

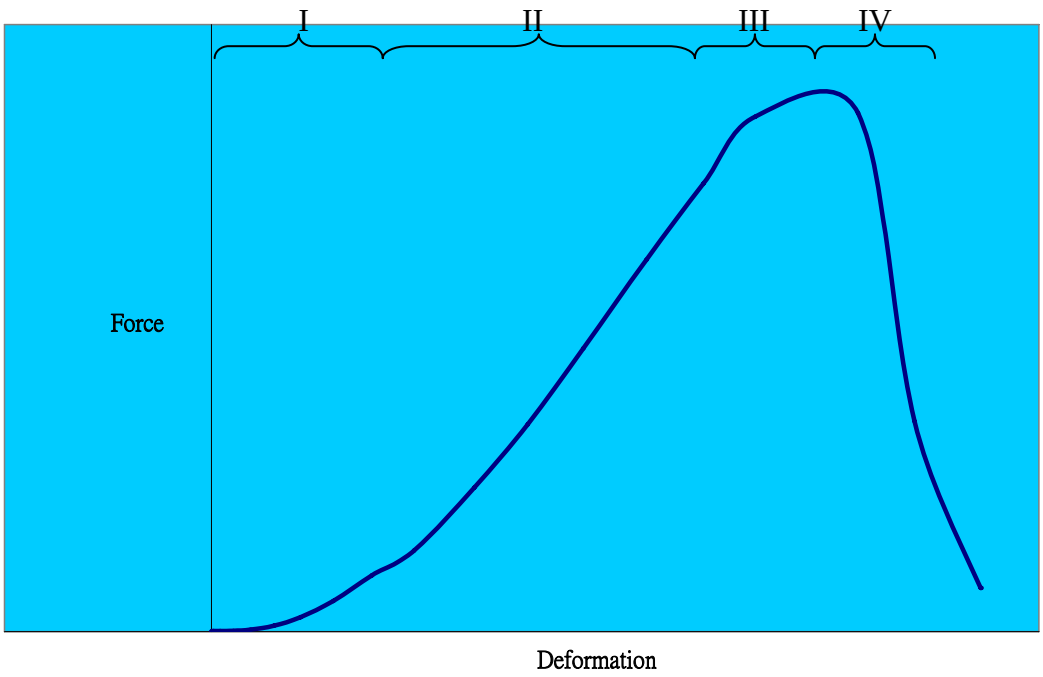

Figure 2-4. Typical force-deformation curve of a tendon under tensile loading which exceeds the tendon elastic limit. I. Toe region; II. Linear (stiffness) region; III. and IV. Failure region

However, the dimension of the tendon being tested could affect the shape of the force-elongation curve. For example, thicker tendons are stiffer than thinner ones, and shorter tendons are stiffer than longer ones (Butler et al., 1978). Thus the force-deformation curve reflects only the structural properties of the tendon. To compare the material properties of tendons with different dimensions, the force-deformation curve

will need to be adjusted by dividing the force by its original cross-sectional area so as to obtain the stress value (MPa), and normalizing the deformation by the tendon's original length to obtain the strain (%) value.

The shape of the stress-strain curve is similar to the force-deformation as illustrated above, but reflects the intrinsic material properties rather than the structural properties of the specimen (Maganaris and Narici, 2005). Variables taken from a stress-strain curve include Young's modulus (MPa), which is the stress divided by the strain of the specimen, ultimate stress (stress at failure) (MPa), ultimate strain (strain at failure) (%), and energy density (work done per unit volume of the tendon, J/cm^3).

Although determining the stress strain curve can reflect the material properties, accurate measurement of the cross-sectional area of a small tendon in a small animal, especially the injured tendon, may be difficult. Some studies with small animal models are more practical for studying structural properties than material properties. For example, research on the biomechanical properties of healing rat ligaments or tendons with different treatment modalities has measured the structural properties and tried to normalize the value of the injured leg to that of the uninjured, contralateral leg to get the percentage recovery of the structural properties of the healing tendon or ligament (Fung et al., 2002; Ng et al., 2003; See et al., 2004).

Tendons are viscoelastic materials, as they display force-relaxation and creep (Jozsa

and Kannus, 1997). Force-relaxation means that with the same degree of deformation, the load required to maintain that deformation decreases over time (Best and Garrett, 1994). Creep refers to an increase in length or deformation over time when the tendon is under a constant load (Jozsa and Kannus, 1997). As suggested by Parry (1988), the ability of a tendon to inhibit creep is directly related to the percentage of small-diameter fibrils present. Small fibrils have a greater interface with the surrounding matrix, therefore the area over which the shear stress exists at the fibril/matrix interface is much enhanced, increasing the ability to inhibit permanent creep (Craig et al., 1989; Parry, 1988).

Normal tendons are strong, with high tensile strength. The tensile strength of tendons is related to the thickness and collagen content, and a tendon with an area of 1 cm^2 is capable of bearing 500 to 1000 kg (Elliott, 1965; Shadwick, 1990). The peak strength of the Achilles tendon and the mechanical work by the calf muscles are 2233 N and 34 J in the squat jump, 1895 N and 27 J in the counter movement jump, and 3786 N and 51 J when hopping (Fukashiro et al., 1995). During strenuous activities such as jumping and weight-lifting, very high loads are placed on tendons (Zernicke, 1977, Scott and Winter, 1990). In the human Achilles tendon, forces of up to 9 kN, which corresponds to 12.5 times body weight, have been recorded during running (Komi et al., 1992). The tensile strength of healthy tendons increases during childhood and adolescence, is highest between 25 to 35 years of age, and slowly declines afterwards (Ippolito, 1986). Tendons in male

subjects show higher maximum rupture force, greater stiffness and a larger cross-sectional area than those in women. Younger tendons have significantly higher tensile rupture stress and lower stiffness (Thermann et al., 1995).

2.1.4. Functions of Achilles tendon

The gastrocnemius and soleus, via the Achilles tendon, function as the chief plantar flexors of the ankle joint. The calf muscles exert their force on the posterior part of the foot via the Achilles tendon during the propulsive phase of many activities, such as walking, running and jumping. The gastrocnemius muscle functions primarily as a plantar flexor of the ankle, while also producing flexion of the knee joint. The soleus muscle plays an important postural role by preventing the body from falling forward while standing (Palastanga et al., 2006).

2.2 Tendon injury

2.2.1. Epidemiology of Achilles tendon ruptures

There have been increasing clinical cases of Achilles tendon rupture in the last few decades, probably because of the increased popularity of recreational sporting activities in a predominantly sedentary population (Kannus and Natri, 1997; Leppilahti and Orava, 2003; Moller et al., 1996). Leppilahti et al. (1996) reported that the incidence in Finland had increased from 2 in 100,000 in 1986 to 12 in 100,000 in 1994. In Scotland, the incidence increased from 4.7 per 100,000 in 1981 to about 6 per 100,000 in 1994 (Maffulli et al., 1999). The incidence of Achilles tendon ruptures in Denmark increased from 18.2/100,000 inhabitants in 1984 to 37.3/100,000 in 1996 (Houshian et al., 1998).

Complete Achilles tendon ruptures are mostly associated with sports activities (Jozsa et al., 1989a; Leppilahti et al., 1996). Ruptures in association with racquet or ball games or other sports activities have been noted in 75% to 88% of cases (Cetti et al., 1993; FitzGibbons et al., 1993; Josey et al., 2003; Jozsa et al., 1989a; Maffulli et al., 2003; Moller et al., 1996; Nistor, 1981; Sölveborn and Moberg, 1994; Speck and Klaue, 1998). Among them, the distribution of Achilles tendon ruptures according to different sports varies from country to country according to national sporting traditions (Jarvinen et al., 2005). For example, Achilles tendon ruptures are common in soccer, tennis, track and field, indoor ball games, downhill skiing and gymnastics in northern and central Europe, while

American football, basketball, baseball, tennis and downhill skiing dominate the statistics in North America (Jozsa and Kannus, 1997; Maffulli, 1999).

Study statistics have found bimodal age distribution among patients with ruptures, whereby the first peak is in the fourth decade of life, followed by a second but lower peaks in the sixth to eighth decades of life (Leppilahti et al., 1996; Moller et al., 1996). The incidence of Achilles tendon rupture in men is about 1.7 to 7 times greater than in women (Jozsa et al., 1989a; Maffulli et al., 1999; Maffulli et al., 2003). Generally, Achilles tendon ruptures occur most commonly in men in their 4^{th} and 5^{th} decades, with an average age of between 30 and 40 in many studies (Schepsis et al., 2002). Patients are often those engaged in sedentary work and professional occupations (Inglis and Sculco, 1981; Jozsa et al., 1989a; Jozsa and Kannus, 1997).

2.2.2. Etiology of Achilles tendon ruptures

The etiology of Achilles tendon rupture remains unclear (Schepsis et al., 2002; Williams, 1986). Most patients do not have any symptoms, like tenderness or stiffness, before the rupture (Jozsa and Kannus, 1997; Maffulli, 1999; Kannus and Natri, 1997). Both intrinsic and extrinsic factors have been speculated: intrinsic factors include degenerative changes of the tendon, while extrinsic factors are mostly associated with activities of high force with loading higher than the limit of the tensile strength of the

tendon (Sharma and Maffulli, 2005). Histological studies have found that almost all ruptured Achilles tendons underwent degenerative changes before the injury episode (Jozsa et al., 1990; Kannus and Jozsa, 1991). These changes include hypoxic and mucoid degeneration, poor vascular supply, tissue and cell necrosis, calcification, tendolipomatosis, and irregular, degenerated collagen fibers at and around the rupture site (Kvist et al., 1992; Jarvinen et al., 2004; Maffulli et al., 2002; Tallon et al., 2001). As most patients suffering from Achilles tendon rupture were sedentary workers or professionals, it was believed that a sedentary lifestyle would decrease the circulation and nutrition to the tendon, and regeneration would not keep pace with the recurring microtrauma, thus leading to degeneration. These degenerative changes could predispose to traumatic tendon lesions on acute exertion (Jozsa et al., 1989a). About 80% of patients have Achilles tendon rupture at 3cm to 6 cm proximal to the calcaneal insertion, within the zone of poor vascularization, where poor blood supply may contribute to rupture (Jozsa et al., 1989a).

Besides the intrinsic factors, the rupture can be due to either indirect or direct trauma to the tendon. Direct contusions (while the tendon must be under high tension during impact) account for 5% of complete Achilles ruptures (Arner and Lindholm, 1959). Indirect trauma can be caused by three different mechanisms, during which uncontrolled contraction of the tricep surae muscle is usually present and may increase the tendon's tension: (i) pushing off with the weightbearing forefoot with simultaneous extension of the

knee joint, such as at the start of sprint running and jumping; (ii) sudden unexpected dorsiflexion of the ankle with the foot fixed on the ground, such as slipping on a stair, stumbling into a hole, or a sudden forward fall; and (iii) forceful dorsiflexion of a plantarflexed foot, such as when jumping or falling from a height and landing with the foot plantar-flexed (Arner and Lindholm, 1959; Jozsa and Kannus, 1997).

Other conditions, like local and systemic corticosteroid injection (Fisher, 2004), inflammartoy and autoimmune conditions (Dodds and Burry, 1984), genetically related collagen abnormalities (Dent and Graham, 1991), infectious diseases (Arner and Lindolm, 1959) and hyperlipidemia (Mathiak et al., 1999; Ozgurtas et al., 2003) are also known risk factors for tendon rupture.

The stereotypical clinical picture of Achilles tendon rupture is that the patient describes an audible snap and sudden pain, as if being kicked or hit from behind. Clinically, diagnostic tests for Achilles tendon rupture include the calf squeeze test (Thompson, 1962), the active plantarflexion test (Simmonds, 1957), the palpable gap test (Maffulli, 1998), magnetic resonance imaging and ultrasound (Laine et al., 1987; Marcus et al., 1989; Schepsis et al., 2002). Of these, the calf squeeze test is the most commonly used as it is convenient, simple to perform and reliable (sensitive up to 0.96) in diagnosing acute Achilles tendon rupture (Maffulli, 1998).

2.2.3. Response of tendon after injury

Tendons with injuries will undergo a complex healing process. Healing may occur in three ways: regeneration, repair by scar formation, or both. Regeneration, by definition, is a form of repair that produces new tissue that is structurally and functionally identical to normal tissue (Leadbetter, 1992), while scar repair is the product of connective tissue that has inferior functional and structural properties to the original tissue. Ideally, regeneration is better as it produces identical tissue. However, tendons are commonly healed by scar tissue formation (Jozsa and Kannus, 1997).

The healing process occurs in three overlapping phases: (1) the inflammation phase, usually from the first 24 hours of injury up to 3 days; (2) the proliferative phase, starting at 48 hours for up to 6 weeks; and (3) the maturation or remodeling phase, which is from the third to sixth weeks to one year (Enwemeka, 1989a; Houglum, 1992; Jozsa and Kannus, 1997; Sharma and Maffulli, 2005).

2.2.3.1. Inflammatory phase

Immediately after injury, the inflammatory phase begins, with blood plasma and tissue fluid leaking into the injury area, resulting in swelling and local temperature increase. This bleeding or leakage of fluid is stopped by the natural clotting mechanism, which is initiated by blood platelets (Houglum, 1992). Plasma protein and fibrinogen interact to

form large insoluble fibrin, forming the basic framework for a blood clot, which can be seen in the area within one hour after injury (Jozsa et al., 1989a). This clotting results in a tenuous, glue-like structure that provides a temporary fragile plug to restrain local hemorrhage and also serves to provide some tensile strength to the injury site during this early phase. Besides, mast cells, leukocytes and platelets migrate to the injury area and secrete chemotactic agents, histamine, fibronectin and bradykinin. Among them, the most important is histamine, which produces vasodilation and increases vascular permeability (Rang et al., 1995). Fibronectin is chemotactic for macrophages and leukocytes, while bradykinin also increases vascular permeability. Other cellular activities begin after a few hours, with the migration of polymorphonuclear leukocytes and monoctic cells and macrophage into the injured area. These inflammatory cells act to remove necrotic tissues and debris to prepare the area for the repair process. In addition, fibroblasts may be seen at 2 to 3 days after injury, although most of the proliferation of these cells occurs a few days later (Enwemeka, 1989a; Jozsa and Kannus, 1997).

2.2.3.2. Proliferative phase

Growth factors produced from platelets and macrophages stimulate the migration and proliferation of fibroblasts, myofibroblasts and endothelial cells in the injured area. The proliferation of fibroblasts and myofibroblasts is essential for synthesis of the extracellular

matrix which marks the beginning of the proliferative phase (Houglum, 1992). The morphology of the fibroblasts during tendon repair is different from that of normal tendons. They have a more prominent nucleus with well developed rough endoplasmic reticulum, indicating that they are in an active stage of synthesis (Enwemeka, 1989a). The fibroblasts produce glycosaminoglycans, as well as weak, thin, crosslink-deficient type III collagen (Jozsa and Kannus, 1997). With the production of collagen and the non-collageous matrix by fibroblasts, the original fibrin clot will be gradually replaced by a more permanent structure. Usually the extracellular matrix, combined with new blood capillaries and fibroblasts, is referred to as granulation tissue (Leadbetter, 1992). In the latter part of the repair phase, the immature type III collagen will be replaced by type I collagen, which makes the scar stronger and results in a more matured granulation tissue (Jozsa and Kannus, 1997).

2.2.3.3. Remodeling phase

The remodeling phase is characterized by collagen maturation (from type III to type I), decreased cellularity, better orientation of collagen fibers and denser collagen matrix (Leadbetter, 1992). Although the number of macrophages, fibroblasts, and myofibroblasts declines, fibroblast metabolism remains high and a higher proportion of type I collagen is present during this phase (Abrahamsson, 1991). With remodeling, the fibroblasts and

collagen fibers become better aligned to the direction of stress, with the fibroblasts assuming a spindle shape and gradually reverting to less active tenocytes (Enwemeka, 1989a). Tendon collagen becomes more densely packed with lower vascularity in the scar. The tensile strength of the regenerating tendon also increases. However, since the vascularity in tendons is lower than in muscles, the recovery of tendon strength is also slower. While some researchers have suggested that the strength of injured animal tendons takes 4 to 10 months to reach its final tensile strength (Houglum, 1992), there is no reliable scientific data on human tendons as most of the biomechanical studies of tendon healing have been performed on animal tendons (Jozsa and Kannus, 1997; Sharma and Maffulli, 2005).

Tendons can heal by intrinsic and extrinsic mechanisms: intrinsic healing means that the regeneration of tendons arises from the two ruptured ends, with the proliferation of epitenon and endotenon tenocytes for the synthesis of collagen and other extracellular matrices (Manske et al., 1984). Tendons undergoing intrinsic healing have better biomechanics and fewer complications. Tendons may also heal by invasion of cells from paratenon, tendon shealth and synovium. However, these tissues may intensely proliferate around the tendon and cause adhesion, which will impair the normal gliding mechanism of the tendon (Gelberman et al., 1984; Potenza, 1962).

2.2.4. Effect of neural involvement on tendon healing

Traditionally, the sensory nervous system has been thought to function as a receptive and afferent system, which detects changes in the external and internal environment, generates signals and reflexively activates the effector system to react to these changes (Holzer, 1988). However, with the discovery of different neuropeptides and their function in the regulation of different body responses, like the inflammatory response to injury, it is noticed that these sensory neurons not only serve a sensory role but also take part in local effector systems (Ambalavanar et al., 2006; Brain et al., 1985; Henderson et al., 2006; Schaffer et al., 1998).

2.2.4.1. Temporal appearance of neuropeptides in healing tendon

Studies by Ackermann et al. (2001 and 2003) have demonstrated that the healing of ruptured tendons is characterized by the appearance of sensory nerve fibers expressing different neuronal markers and neuropeptides in an orchestrated, temporal manner. These neuropeptides include CGRP, SP, neuropeptide Y (NPY) and galanin (GAL). The functions of CGRP and SP have been discussed in section 2.1.2.4. In addition, CGRP also potentiates the effect of SP, and they were found to be associated with the thermal nociception of healing tendons (Ackermann et al., 2003). It was found that the appearance of nerves with CGRP and SP immunoreactivity occurs as early as week 1 postinjury during

the inflammatory and early repair phase, and was found predominantly in the connective tissues adjacent to the rupture site near the blood vessel walls as well as the musculotendinous junction of the tendon. These suggest they may play a role in regulating angiogenesis and proinflammation. During the proliferative/regenerative phase (between 2 and 6 weeks postinjury), these nerve fibers progressively invade the rupture site as free nerve endings and exhibit their peak occurrence. They were observed among fibroblasts in the tendinous tissue, and may represent the stimulatory effect of these neuropeptides in the proliferation of fibroblasts (Nilsson et al., 1985; Yule and White, 1999). The appearance of CGRP and SP immunoreactive nerve fibers gradually decreased from week 6 onwards, suggesting that their functions are mainly in the early phase of the healing process. Generally, the appearance of CGRP was more marked than that of SP throughout the 16 weeks postinjury (Ackermann et al, 2001 and 2003), which reveals its relative importance and potentiation effect on SP.

Besides the CGRP and SP, the NPY and GAL immunoreactive nerve fibers both occur after week 4 postinjury as free nerve endings and near the newly formed blood vessels at the interface between the paratenon and the proper tendon tissue, which are of low occurrence or almost undetectable during the early phase of healing. It has been found that low levels of NPY promote vasodilation, while high concentration results in vasoconstriction (Zukowska-Groj et al., 1998). Similarity, GAL has been shown to inhibit

the effect of SP, which in turn suppresses inflammation and nociception (Heppelmann et al, 2000). All this implies that the neuropeptides may counteract each other in different phases of healing (Ackermann et al., 2003; Heppelmann et al., 2000).

Studies have shown that denervation affects bone, wound and ligament healing (Aro et al., 1981; Kim and Pomeranz, 1999; Ivie et al., 2002; Steinicki et al., 2000) and may relate to the absence or disruption of the supply of the above neuropeptides vital for tissue healing (Ackermann et al., 2001).

Studies on the effect of denervation on tendon healing are scarce. One recent study reported that denervation caused reduction in the load to failure of the Achilles tendon by 50% (Aspenberg and Forslund, 2000). Further studies with detailed biomechanical testing as well as histomorphological findings may help to explain the importance of neural involvement in tendon healing.

2.2.5. Management of Achilles tendon rupture

To date, there is still no consensus on the best way to deal with Achilles tendon ruptures. The common management of ruptured Achilles tendons can be divided into surgical and nonsurgical treatments. Operative treatment seems to have better functional outcome, with accurate restoration of tendon length and a lower rate of rerupture (Bruns et al., 2000; Cetti et al., 1993; Shields et al., 1978; Wong et al., 2002). Other authors have

suggested that non-surgical treatment produced as good a functional outcome as operative treatment, with fewer complications such as wound breakdown and infection (Nistor, 1981; van der Linden-van der Zwaag, 2004). Most surgeons prefer operative treatment in physically active or young patients, while conservative treatment may be more suitable for elderly and physically inactive patients (Maffulli, 1999; Movin et al., 2005). Despite the increased popularity of surgical treatment, the choice of operative versus conservative treatment for acute Achilles tendon rupture remains controversial (Bhandari et al., 2002).

2.2.5.1. Conservative treatment

Traditionally, conservative treatment for acute Achilles tendon rupture involved immobilization in a below-knee cast in the gravity equinus position for 4 weeks, followed by further immobilization, but in a more neutral ankle position, for another 4 weeks (Edna, 1980; Gillies and Chalmers, 1970; Jacobs et al., 1978; Lea and Smith, 1972; Lildholdt and Munch-Jorgensen, 1976; Nistor, 1976; Persson and Wredmark, 1979). After the period of immobilization, the patient may need to wear shoes with heel-lifts, with height around 2.5cm, for a further 4 weeks or until the patient can dorsiflex the ankle by about 10^{o} (Nistor, 1981). Comparable clinical outcomes as compared with surgical treatment following the above immobilization methods have been reported (Lea and Smith, 1972; Nistor, 1981). Patients under conservative treatment have a shorter morbidity and no hospital stay (Nistor,

1981). Some researchers have suggested that in closed acute rupture of the Achilles tendon, the paratenon should not be traumatized by surgical intervention as it is important to maintain a smooth gliding surface and to provide vascular supply to the injured tendon (Kader et al., 2005).

However, one major problem that exists for conservative treatment with immobilization is the higher incidence of re-rupture, which ranges from 10 to 35% (Gillies and Chalmers, 1970; Lea and Smith, 1972; Lildholdt and Munch-Jorgensen, 1976; Nistor, 1976; Persson and Wredmark, 1979), as compared with surgical treatment, which has a re-rupture rate of 0 to 6.25% (Cetti et al., 1994; Costa et al., 2003; Kangas et al., 2003; Mandelbaum et al., 1995; Speck and Klaue, 1998). One of the reasons for this may be the deteriorating effect on the tendon of prolonged immobilization. Murrell et al. (1994) reported that immobilization may have a detrimental effect on the mechanical recovery of the healing Achilles tendon.

Recently, there has been increased interest in the functional non-surgical treatment for Achilles tendon rupture. Carter et al. (1992) have developed a new functional brace which could allow immediate weight-bearing and early active plantarflexion with limited dosiflexion of the ankle joint. McComis et al. (1997) adapted their methods with a modified rehabilitation protocol to treat a small group of patients (n=15), and demonstrated good functional outcomes. Similarly, other treatment protocols for conservative treatment

were introduced, which include 3 to 4 weeks of cast immobilization and 4 to 5 weeks of removable orthosis (Eames et al., 1997; Saleh, 1992; Wallace et al., 2004; Wong et al., 2002). Patients treated with these protocols have a lower rate of re-rupture, with favorable recovery of joint functions. However, the evidence for the use of this functional non-operative treatment and other non-surgical managements is still limited, and no firm conclusions can be drawn (Khan et al., 2004).

2.2.5.2. Surgical treatment

With the advance of surgical techniques, surgery has been the choice of treatment for acute Achilles tendon rupture in the past 2 decades, especially for young, fit individuals and athletes (Khan et al., 2004; Maffulli, 1999). The techniques can be divided into open and percutaneous repair. For open repair, different operative techniques have been used, ranging from simple end-to-end suturing to complex repairs like the fascial turndown technique (Gerdes et al., 1992), tendon grafts or artificial tendon implants (Parsons et al., 1984), and augmentation or reinforcement techniques using the gastrocnemius muscle fascia (Moller et al., 2001a), the peroneus brevis tendon (Teuffer, 1974), the plantaris tendon (Lynn, 1966) or the flexor digitorum longus tendon (Mann et al., 1991).

In early diagnosed ruptures, the preferred method has been simple end-to-end Bunnell-, Kessler- or Krackow-type sutures with medial incision to minimize the chances

of sural nerve injuries and wound complications (Mandelbaum et al., 1995; Movin et al., 2005). Traditionally, after the operation, the leg is then immobilized in a below-knee cast with the ankle in the equinus position for 6 weeks without weight-bearing for 3 to 9 weeks (Kangas, 2003; Leppilahti et al., 1998). However, recent trends have tended to shift to more functional postoperative regimens, as reports have shown good results following early postoperative motion and weight bearing using a functional brace, orthosis, or posterior splints instead of cast immobilization. Weight-bearing is started on the day of operation subject to the patient's tolerance of crutches (Kader et al., 2005). Other techniques, like augmentation, tendon graft or other reinforcement techniques, are reserved for late-presenting or neglected ruptures, or re-ruptures (Moller et al., 2001a; Movin et al., 2005). As stated before, the rate of re-rupture in patients receiving surgical repair is lower when compared with patients receiving non-operative treatment. Functional brace regimens would further lower the re-rupture rate (Khan et al, 2004). However, other complications are common for surgical treatments. In a meta-analysis by Khan et al. (2004), surgical treatment was associated with a higher risk of other complications, including infection, adhesions, and disturbed skin sensibility.

Due to the occurrence of wound complications which may affect the outcome of surgical treatment, surgeons tried to minimize the wound site by the percutaneous repair technique introduced by Ma and Griffith (1977). Their technique involved a total of 6

small incisions bilaterally to the tendon, with sutures criss-crossing through this incision and the tendon and tied on the tendon surface. They reported only 2 minor non-infectious skin complications and no re-rupture. Percutaneous repair techniques were found to have a lower complication rate as reported by Khan et al. (2004), while other studies demonstrated that the rate of re-rupture is greater than that after open operative repair, as the repaired tendon is usually weaker than that repaired by open surgery (Maffulli, 1999, Movin et al., 2005).

Whether it is better to manage the acute Achilles tendon rupture by conservative or operative treatment is still inconclusive: some may support the use of surgical treatment because of its lower risk of re-rupture and greater rate of resuming sports activities (Moller et al., 2001b), while other studies favor non-surgical management with fewer complications (Nistor, 1976; van der Linden-van der Zwaag, 2004). The ultimate goal is to restore the function of the tendon to sustain load during locomotion and other sporting activities, and to lower the chances of re-rupture. Therefore, researchers are tending to investigate other management methods, like physical modalities, growth factors, gene therapy and tissue engineering, to stimulate tendon healing with the aim of restoring the normal biomechanical properties of the tendon (Sharma and Maffulli, 2005). One of these modalities which has been investigated extensively is therapeutic ultrasound. We discuss the details of therapeutic ultrasound in the following sections.

2.3. Therapeutic ultrasound

Ultrasound is a modality that is used for a number of purposes, including diagnosis, destruction of tissue and as a therapeutic agent (Prentice, 2002). As a therapeutic modality, ultrasound has been used to treat a wide variety of disorders, from skin wounds to malignant tumors (Young et al., 1990; Quan et al., 1989). In clinical practice, especially in physiotherapy, therapeutic ultrasound is one of the most widely used therapeutic modalities for rehabilitation of different injuries, primarily to stimulate repair of injured soft tissues (ter Haar, 1999) and bony fractures (Heckman et al., 1994), as well as for pain relief (Dyson, 1989). It has become one of the most commonly used treatments in the management of tendon injuries (Takakura et al., 2002). Both laboratory works and experimental studies in animals have demonstrated a number of physiological effects of ultrasound upon living tissues, as well as its effects on enhancing the repair of skin, tendons, ligaments, and nerves. However, even given the promising results of laboratory-based animal studies, there have still been controversial findings regarding the therapeutic effect of ultrasound on soft tissue injuries from clinical studies (Gam et al., 1995; van der Windt et al., 2002).

2.3.1. Introduction to therapeutic ultrasound

Ultrasonic wave is a form of mechanical energy where the energy is transferred

between two points in a medium by means of wave motion (Prentice, 2002). It is a sound wave with frequencies greater than the audible frequency range of humans (>20kHz). In therapeutic ultrasound, the frequency range is between 0.75-3MHz.

2.3.2 Types of ultrasound wave

For medical applications, the most important mode of propagation of ultrasonic energy is the longitudinal wave, which travels in both liquid and solid matter. The other major propagation mode for sound is the transverse (shear) wave, which will not travel through fluids. In humans, since soft tissue may be considered as a semi-fluid as far as ultrasonic propagation is concerned, so transverse waves become important only if some solid, such as bone, lies in the path of the sound beam. The passage of the ultrasound wave though a medium made up of tiny particles will cause those particles to oscillate about their equilibrium positions. In a longitudinal wave, the oscillations are along the wave propagation direction, whereas in a transverse wave, the oscillations are perpendicular to the direction in which the wave is propagating. Along the successive longitudinal wave, there are regions in the fluid for which the particle density is greater than at equilibrium (compression), and there are regions in the fluid where the particle density is smaller than at equilibrium (rarefactions) (Figure 2-5).

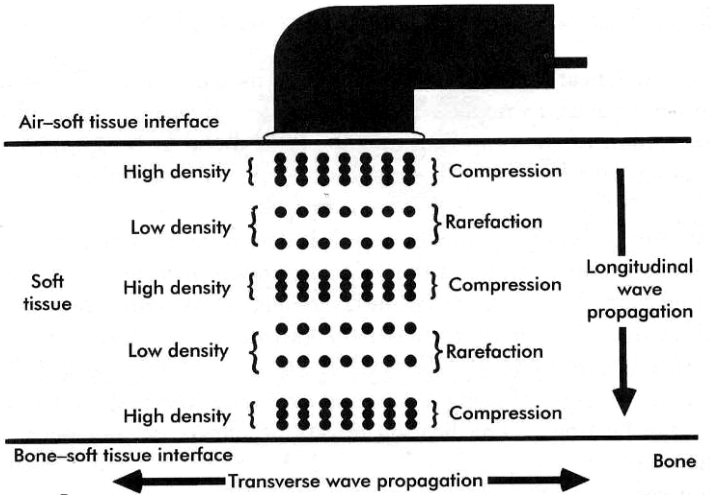

Figure 2-5. Graphic illustration of how ultrasonic wavestravel in biological tissues. Within soft tissue, ultrasound travels as a longitudinal wave (with regions of high molecular density-compression and areas of low molecular density-rarefaction). Transverse waves are found primarily in bone (Adopted from Prentice, 2002)

2.3.3. Ultrasound parameters

Various parameters are used to describe sound waves. The wavelength is defined as the distance between the two closest points on a wave which are performing the same motion at any instant in time. In a longitudinal wave, the wavelength, λ, is defined as the distance between adjacent compressions. The maximum displacement from the equilibrium position that the particles experience is known as the displacement amplitude ‘A’. The number of times that a particle undergoes one cycle of motion in unit time is the frequency ‘f’. The relationship between a wave velocity ‘c’ propagating through the medium at a particular wavelength and frequency is given by $c = f \lambda$.

2.3.3.1. Frequency

The frequency range of therapeutic ultrasound, as mentioned before, is between 0.75 and 3 MHz. The higher the frequency of the sound waves emitted from the sound source, the less the sound will diverge and thus a more focused beam of sound is produced. However, attenuation increases as frequency increases, so the greater the frequency, the more ultrasound energy is absorbed in the superficial tissue, and therefore the lesser the depth of penetration. Difference frequency of ultrasound have different half-value depth – the depth or distance at which half the initial energy has been absorbed. For example, for skin and tendon, the half-value depth of penetration to skin and tendon for 3 MHz are 4mm and 2 mm respectively, while for 1 MHz are 11.1 and 6.2 mm respectively (Hoogland, 1986).

2.3.3.2. Velocity

The velocity, which is the propagation speed of an ultrasound (US) wave, is dependent on the density and hardness of the conducting medium. Generally, the more rigid a material, the higher the velocity of transmission. For example, the average velocity of ultrasound in soft tissue is about 1540 m/sec, whereas the speed is as high as 4080 m/sec in bone.

2.3.3.3. Attenuation

Attenuation is a decrease in energy intensity as the ultrasound wave is transmitted through various tissues owing to either the absorption of energy by the tissue, or scattering and dispersion resulting from reflection or refraction (Kremkau, 2002). Tissues with high water content have a lower rate of absorption than those with higher protein content (Dyson, 1987). The energy absorbed may be converted to heat, thus resulting in local heating. The "heating depth" is dependent on the frequency of the ultrasound as well as the characteristics of the tissues through which the ultrasound is traveling (Demmink et al., 2003).

2.3.3.4. Mode of delivery - pulsed ultrasound (PUS) and continuous ultrasound (CUS)

Continuous ultrasound (CUS) refers to the continuous or uninterrupted emission of acoustic waves over time, whereas in pulsed ultrasound, there will be periodic interruption or pulsing of the ultrasound wave. In considering the energy output of CUS, one needs to consider the total power (W) delivered from the sound head, as well as the spatial averaged (SA) or W/cm^2 (total output/area of the sound head). Since the intensities of ultrasound waves recorded in different places on the entire surface of the sound head are different (according to the BNR, explained in the next section), the averaged intensity over a given area will represent the intensity of the ultrasound wave produced. This averaged intensity

is known as the spatial averaged (SA) intensity.

With PUS the intensity is periodically interrupted, with no ultrasound energy being produced during the off period. In this case, other parameters need to be considered. The first is duty cycle, which is defined as the percentage of time that ultrasound is being generated (pulsed duration) over one pulse period. For example, if the pulse duration is 5 msec and the total pulse period is 10 msec, the duty cycle is 50%. Thus the total amount of energy being delivered to the target tissues would be only 50% of the energy delivered if a CUS was being used. Therefore, if the duty cycle of the PUS is 50%, and the SA intensity is 1 W/cm^2, the temporal averaged intensity, which is the average intensity over time, should be smaller than 1. In this case, the spatial averaged temporal averaged (SATA) intensity should be 1 W/cm^2 x 50% = 0.5 W/cm^2.

CUS is used commonly if thermal effect are desired while PUS gives a reduced average heating of the tissue and thus considered to be non-thermal effect dominant. (Young, 2002).

2.3.3.5. Total acoustic energy

The total acoustic energy delivered per unit area during an application of ultrasound treatment is defined as energy fluence (Nyborg, 2006). In an exposure where CUS of intensity 0.5W/cm^2 is applied for 5 minutes, the energy fluence is 0.5x5x60=150J/cm^2. For

PUS, if the intensity of 2.5W/cm^2, duty cycle of 20% is applied for 5 minutes, the energy fluence is also equal to 150J/cm^2.

2.3.3.6. Beam Nonuniformity Ratio (BNR)

The degree of inhomogeneity of the ultrasonic beam intensity is measured as the BNR (Figure 2-6). The BNR is calculated as the ratio of the intensity of the highest peak to that of the averaged intensity of all peaks. The lower the BNR, the lower the intensity of the highest peak. This could reduce the probability that patients will experience uncomfortable sensations resulting from hot spots during therapy (Bélanger, 2002). The ideal ratio is 1:1, which is not achievable in real practice. Commonly, the BNR of ultrasound heads used in physiotherapy falls between 2 and 6, with 8 the maximum value recommended by the International Electrotechnical Commission.

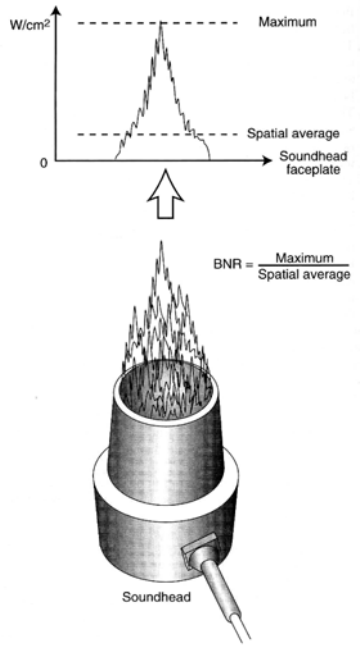

Figure 2-6. Ultrasound beam non-uniformity ratio (Adopted from Low and Reed, 1994)

2.3.4. Ultrasound generation

An ultrasound generator typically consists of a high frequency electrical generator containing an oscillator circuit and a transformer, which is connected to an ultrasound transducer through a coaxial cable (Figure 2-7). The control panel of an ultrasound unit usually consists of an intensity control, a timer that has preset values, a duty cycle control switch with a selector for continuous or pulsed mode, a power meter, and a switch to change the power display (either in W or in W/cm^2).

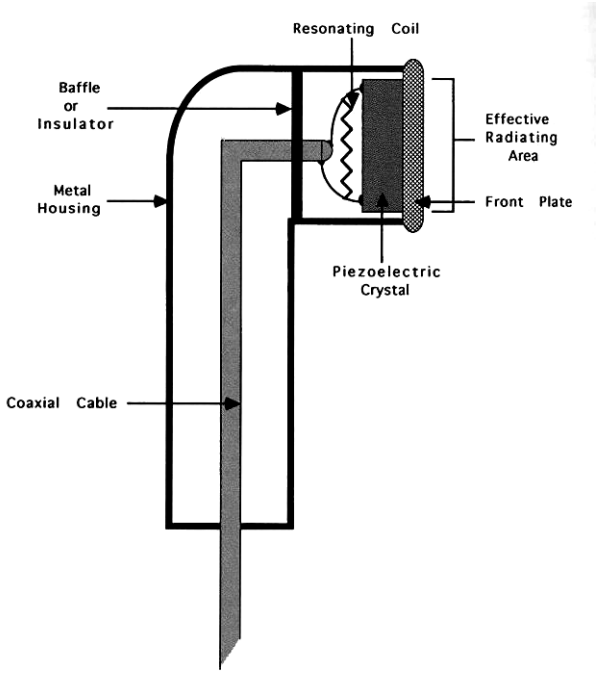

Figure 2-7. The structure of a transducer (Adopted from Prentice, 2002)

The transducer consists of a piezoelectric crystal, such as quartz, or synthetic ceramic crystals made of lead zirconate or titanate with specific thickness. When an alternating electric current has frequency identical to the resonance frequency as the crystal is passed

through the piezoelectric crystal, it will expand and contract. This process is known as the reverse piezoelectric effect (Low and Reed, 1994; Prentice, 2002). The vibration of the piezoelectric crystal will then result in the generation of ultrasound at a desired frequency (Figure 2-8).

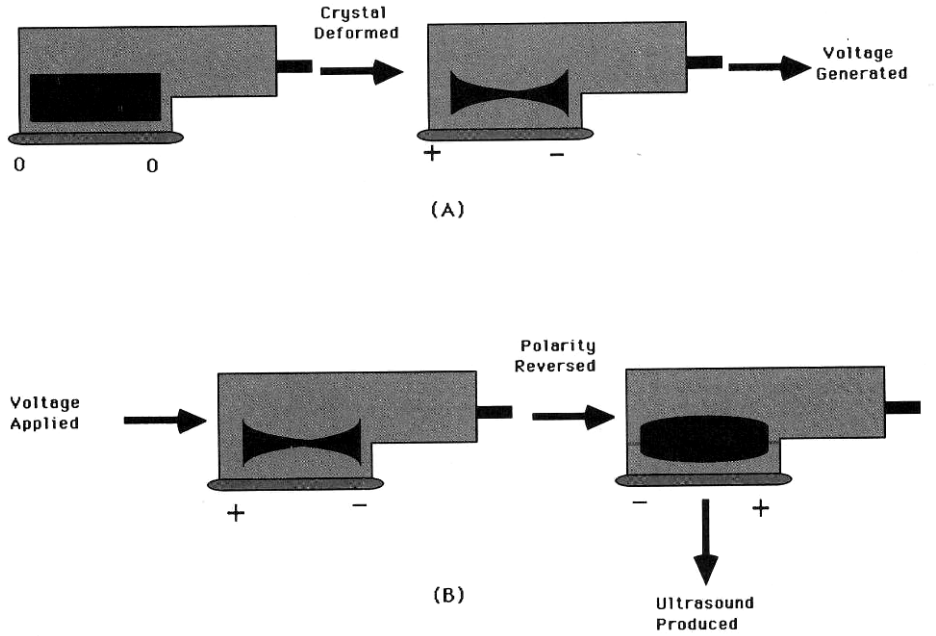

Figure 2-8. Ultrasound generation from the piezoelectric crystal (Reverse piezoelectric effect) (Adopted from Prentice, 2002)

2.3.5. Physiological effect of therapeutic ultrasound

The main physiological effects produced by therapeutic ultrasound can be divided into thermal and non-thermal effects. However, it is very difficult to identify the mechanisms involved in producing biological changes or to isolate non-thermal from thermal effects (ter Haar, 1999).

2.3.5.1. Thermal effects

Energy transported by an ultrasonic beam is attenuated as it passes through tissue. The energy loss may be due to scattering and absorption. The reason why the energy absorbed changes into heat may be explained as follows: As the ultrasonic beam passes through soft tissues, molecules are caused to vibrate under the repeated cycles of high- and low-pressure waves. As the intensity of the ultrasonic beam increases with more continuous emission of acoustic waves, the more vigorous the molecular vibration and, therefore, the more vigorous the microfriction between the irradiated molecules. This results in the generation of fictional heat in the tissue (Bélanger, 2002).

Most collagenous soft tissues are high in protein content, which have a high ultrasound absorption coefficient. This means that most of the ultrasound energy is absorbed by the collagen in soft tissue and is heated preferentially. It is believed that this heating effect could be therapeutic by enhancing cell metabolism, thus promoting soft tissue healing (Dyson, 1987). The therapeutic effects from the above thermal mechanism of ultrasound include an increase in the extensibility of collagenous structures such as tendons and scar tissue, a decrease in joint stiffness, pain relief, changes in blood flow and a decrease in muscle spasm (ter Haar, 1986).

However, most of the reports on the therapeutic benefits obtained from the thermal effects of ultrasound have been accompanied neither by accurately measured temperature

distributions, nor by rigorous dosimetry. In a human cadaver study which used an electric thermometer probe to measure temperature change, the authors found that a continuous mode of insonation delivered a greater and faster rise in temperature than a pulsed energy delivery for the same intensity at the same depth (Chambier et al., 2001). Besides, the smaller the frequency, the greater the increase in temperature. If the frequency and intensity remained the same, the heating decreased in function of depth. They also reported that the change in temperature caused by PUS is negligible at various depths. This implies that in order to achieve the therapeutic effect induced by an increase in tissue temperature, CUS is more effective than PUS. It was suggested that an increase in tissue temperature of 1°C will result in a 13% increase of the metabolic rate (Castel, 1993; Lehmann, 1982). A moderate heating of 2–3°C should reduce muscle spasms, pain and chronic inflammation, and promote blood flow (Castel, 1993, Draper et al., 1995; Maxwell, 1992), although randomized trials do not support the clinical relevance of such estimated heating effects (Beckerman et al., 1993).

2.3.5.2. Mechanical effects

The mechanical effects of therapeutic ultrasound on soft tissues can be attributed to two main processes, stable cavitation and microstreaming (Figure 2-9). Cavitation is defined as the formation, in fluids or solids, of cavities resulting from the formation of

microbubbles. These microbubbles can be formed either by an increased temperature at constant pressure (e.g. boiling) or by a decreased pressure at constant temperature (e.g. cavitation). Acoustic cavitation is triggered by the successive pressure waves (characterized by a cyclic drop in pressure) generated by therapeutic ultrasound devices. It begins when minute gas pockets with infiltrated fluid develop into microscopic bubbles that form cavities in such fluids and surrounding soft tissues. These microscopic bubbles expand and contract at the same frequency as the pressure waves generated by ultrasound.

Depending on the frequency and intensity level of the acoustic waves, stable and transient cavitations can occur in soft tissue. Bubbles which continue to pulsate in an acoustic field are called stable cavitations, while bubbles or growing nuclei whose amplitude of motion is so great that the bubble motion becomes unstable and the bubbles collapse are known as transient cavitations (Bélanger, 2002; Coakley, 1978). Transient cavitation may produce stresses that are great enough to disrupt cells, which is not desirable in promoting the healing of soft tissue injuries (Coakley, 1978). Transient cavitation is used by industrial ultrasound devices to clean surfaces when basic, manual methods of cleaning fail. Also, transient cavitation in soft tissues is very unlikely to be produced by therapeutic ultrasound, as the process is normally triggered at frequencies lower and intensities much higher than common therapeutic ultrasound devices (Bélanger, 2002).

Stable cavitation is characterized by non-implosive pulsations of microbubbles which trigger a related phenomenon called microstreaming. Microstreaming is the minute flow of fluid in the vicinity of the pulsating bubbles. Stable cavitation and microstreaming occur simultaneously in soft tissues, which are presumed to provide a mechanical energy level capable of altering cell membrane activity, which in turn could promote soft-tissue healing (Williams, 1983). It was reported in *in vitro* studies that the cavitation induced by therapeutic ultrasound could stimulate protein synthesis in human fibroblasts and neuroblastoma cells (Edmonds and Ross, 1988; Webster et al., 1978).

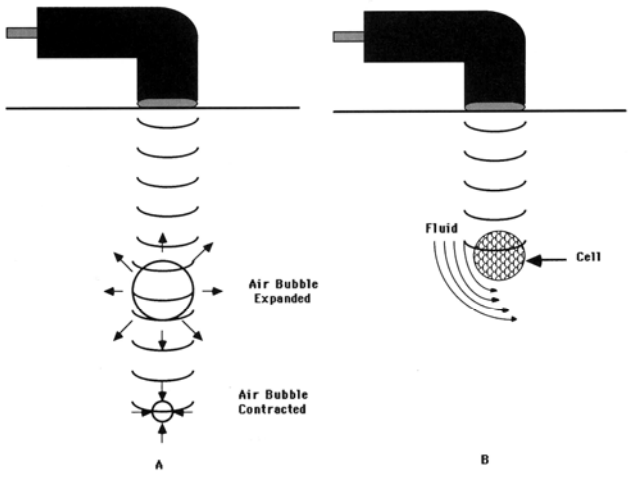

Figure 2-9. Cavitation (left) and microstreaming (right) (Adopted from Prentice, 2002)

It is suggested that in soft tissue healing, the non-thermal heating effect of ultrasound treatment is more important than its thermal effect (Dyson and Suckling, 1978). In clinical practice, during the early phase of tissue healing, inflammation, vasodilation and swelling may present. Clinicians usually choose to use ultrasound with pulse mode or with lower

dosage in order to reduce the potential heating effects, which may augment the inflammatory response. While there is a lack of studies confirming whether thermal or non-thermal effects or both would promote better tissue healing, Kopakkala-Tani et al. (2006) suggest that mechanical forces can stimulate the production of extracellular matrix molecules in tissue. They used PUS to stimulate proteoglycan synthesis in bovine primary chondrocytes and found a significant increase in the proteoglycan synthesis after 3-4 daily PUS. However, when they tried to measure the ultrasound-induced temperature rise during the PUS treatment and investigate the simulated, bare heating environment like that induced by PUS on proteoglycan synthesis, they found that there was no significant increase in proteoglycan synthesized by chondrocytes but only some heat stress responses. Similarly, Dyson et al. (1968) revealed that the most effective doses of ultrasound, which could promote wound regeneration, only caused a slightly increase in temperature. This may suggest that the non-thermal mechanical stimulation of ultrasound can stimulate tissue healing.

2.3.5.3. Physiological effect of ultrasound on soft tissue healing

Physiotherapists use therapeutic ultrasound to treat soft connective tissue injuries, which include tendons, ligaments, wounds, nerves and joint capsule injuries. The repair of such tissues consists of three overlapping stages: acute inflammation, proliferation and

remodeling. Cellular activities are different at different stages of healing, and the effects of ultrasound on these activities at different stages are also different.

Acute inflammation

Inflammation is an essential precursor of the proliferative stage of repair, which is generally short in duration (Enwemaka, 1989a). Immediately after injury, platelets and mast cells become activated and release materials which initiate repair. These materials include chemotactic agents, e.g. histamine, which attract polymorphonuclear leucocytes (PMLS) and monocytes to the site of the injury (Jozsa and Kannus, 1997). PMLS are responsible for the removal of tissue debris and pathogens from the wound, while monocytes will develop into phagocytic macrophages, which are responsible for releasing chemotactic agents and growth factors that are essential for the development of the new connective tissue to replace the injured material (Clark, 1985).

It has been demonstrated in an *in vitro* study that the cavitation and micro-streaming effects of ultrasound can change the permeability of the cell membrane, which can increase the transportation of calcium ions across the membrane (Dinno et al., 1989). The raised intracellular level of calcium ion can stimulate mast cell degranulation, causing the release of histamine, which is responsible for the production of vasodilatation, increased vascular permeability and the attraction of PMLS and monocytes essential for tissue repair (Dyson,

1987; Rang et al., 1995). It is also noticed that while therapeutic ultrasound can accelerate the resolution of the inflammatory response, it should not be considered anti-inflammatory. Recent *in viv*o studies have demonstrated that ultrasound, especially at high doses, could stimulate inflammation rather than anti-inflammation (Goddard et al., 1983; Leung et al., 2004).

Proliferative stage

The proliferative phase consists of the division and differentiation of cells attracted to the injury site, which produce a connective tissue matrix on which new blood vessels can grow. During this phase, the fibroblasts play an important role in the production of collagen (Kannus, 2000). Collagen is also a type of fibrous protein that gives soft connective tissue most of its tensile strength (Harvey et al., 1975). Evidence *in vitro* showed that ultrasound could stimulate collagen synthesis in tendon fibroblasts, which could eventually enhance the repair of injured tendons (Harvey et al., 1975; Ramirez, 1997). The newly formed collagen and connective tissue matrix, referred to as granulation tissues, is rarely as good as the uninjured tissue. This is because the collagen fibers at this stage do not have consistent organization or orientation, and because of the higher constitution of thin weak type III collagen (Jozsa and Kannus, 1997). In addition to stimulating the activity of fibroblasts, therapeutic ultrasound can also affect endothelial cell

activities, which could promote the formation of new capillaries and thus result in a faster restoration of circulation (Hogan et al., 1982).

Remodeling phase

During the remodeling phase, the type III collagen is replaced by type I collagen, and its arrangement also changes in response to the mechanical stresses to which the tissue is subjected. Application of ultrasound at this stage can promote a better alignment of collagen fibrils and prevent the formation of adhesions (Nussbaum, 1998). It is also used to increase the extensibility of mature collagens, such as those in the scar tissue (ter Haar et al., 1985).

2.3.6. In vivo studies of therapeutic ultrasound – tendon healing

Several *in vivo* studies have suggested that therapeutic ultrasound facilitates tendon healing in terms of the breaking strength, collagen synthesis and energy absorption capacity of the tendon (Nussbaum, 1998). Ng et al. (2003) investigated two dosages of ultrasound (1MHz, continuous mode, 1W/cm^2 or 2W/cm^2 for 4 minutes) on tendon healing in rats and found that both dosages significantly improved the breaking strength of the healing tendon when compared to the sham treatment group. Another two studies, using daily 5 minutes CUS at high (1W/cm^2) and low intensity (0.5W/cm^2) of ultrasound on a

rabbit model respectively (Enwemeka, 1989b; Enwemeka et al., 1990), found that CUS at lower intensity can improve not only the tensile strength and energy absorption capacity of the treated tendon, but also the tensile stress, which was not shown in those tendons treated with high intensity CUS.

Besides intensity, studies also reported the choice of using PUS for promote tissue healing. In an in-vitro study, collagen secretion from fibroblasts which were treated with 0.5W/cm^2 CUS increased 20%, while a 30% increase was recorded when the ultrasound was pulsed (0.5W/cm^2 SATA) (Harvey et al., 1975). Recently, da Cunha et al. (2001) compared the effect of CUS and PUS on the repair of the surgical ruptured Achilles tendon in a rat model, using the same SATA intensity at 0.5 W/cm^2 for 5 minutes of treatment (In their PUS parameter, spatial peak intensity is 2.5 W/cm^2, with 20% duty cycle and thus SATA intensity is 0.5W/cm^2) Their results demonstrate the superior beneficial effects of PUS on collagen synthesis, as well as on the organization and aggregation of collagen bundles during the early phase of tendon healing, while CUS induced a decrease in the ability to quicken the healing process. Studies from Byl et al. (1992, 1993) also demonstrated that PUS at 0.5W/cm^2 was the most effective dose for promote remodelling of incised lesions. Although all the studies mentioned above are different in terms of animal models and the methods of administering therapeutic ultrasound, they show that the administration of ultrasound treatment at low intensity (0.5W/cm^2) and pulsed mode for 5

minutes (energy fluence equal to 150J/cm^2) is effective to give a therapeutic effect in the early stages of healing.

However, there is some controversy surrounding the use of PUS for tendon healing. Several previous studies using PUS treatment have revealed no significant improvement in the biomechanical properties of tenotomized tendons. Roberts et al. (1982), using a rabbit model, found that no tenotomized flexor tendon in the treatment group had healed after six weeks of PUS treatment. Two studies investigating the effect of PUS treatment on sutured cockerel and chicken tendons (Gan et al., 1995; Turner et al., 1989), using PUS with a frequency of 3 MHz and a SATA intensity of 0.2 W/cm^2, reported no significant difference in the mechanical strength of the healing tendon between the treatment and control groups. Whether PUS could promote the improvement of biomechanical properties is still unclear. Also the mechanism whereby ultrasound treatment can enhance tendon healing is still not well understood.

Table 2-1 summarizes the *in vivo* studies which have investigated the effect of therapeutic ultrasound on tendon healing. In terms of the basic science research perspective, one common measure for tendon healing which many studies lack is the histomorphological study. Gan et al. (1995) and Saini et al. (2002) tried to assess the inflammatory infiltrate, fibroblastic activity and maturation of the collagen by 4 ordinal grades. However, their methods may have involved subjective assessment. Palmes et al.

(2002) used several criteria to assess the maturation of the scar tissue of a healing tendon: (i) the ratio between fusiform and flattened fibroblasts (tendocytes) in tendon tissue, (ii) the arrangement of fibers in tendon tissue, (iii) the expansion and composition of granulation tissue, (iv) the ratio between cell density and fiber density in tendon tissue, (v) the density of blood capillaries in tendon tissue, and (vi) the distribution of motile inflammatory cells. However, although they mentioned the above criteria, the tissue sections were assessed by a histologist subjectively. Generally, quantitative methods for histomorphological assessment of healing tendons after ultrasound treatment are still lacking.

Table 2-1. Summary of *in-vivo* studies on the effect of therapeutic ultrasound on tendon healing after rupture

.	**Animal model (N=treatment group(s) sample size, n=control group sample size)**	**Injury model**	**(i) Surgical repair (ii) Immobilization**	**US parameters (Frequency, intensity (SATA), duration, duty cycle)**	**Treatment frequency**	**Outcome measures**	**Results ***
Robert et al., 1982	Rabbits (N=7, n=7)	Flexor profundus tenotomy	(i) ✓ (ii) ✓	1.1 MHz, 0.8 W/cm^2, 5 minutes, PUS (duty cycle not mentioned)	Five daily treatments per week for six weeks	(i) breaking strength	(i) -
Stevenson et al., 1986	White Leghorn hens (N=22, n=11)	Complete transected profoundus tendon	(i) ✓ (ii) ✓	3 MHz, 0.75 W/cm^2, 5 minutes, CUS or PUS not mentioned	20 treatments daily, starting immediately after 4 weeks of immobilization	(i) functional recovery (toe flexion) (ii) gap formation (iii) tensile strength	(i) + (ii) +/- (iii) +/-
Frieder et al., 1988	Rats (group a N=4, group b N=4, n=3)	Achilles tendon partial rupture (Puncture with 20-gauge needle)	(i) × (ii) ×	Frequency not mentioned, 1.5 W/cm^2, 3 minutes, CUS or PUS not mentioned	Every other day for 2 weeks (group a) or 3 weeks (group b)	(i) tensile strength (ii) histology (number of fibroblasts and arrangement and orientation of collagen fibrils)	a(i) +/- a(ii) + b(i) + b(ii) +
Turner et al., 1989	Cockerels (N=13, n=5)	Flexor profundus tenotomy	(i) ✓ (i) ✓	3 MHz, 0.2 W/cm^2, 4 minutes, PUS, 20% duty cycle	Three treatments per week starting from post-op day 7, total 5 weeks	(i) mechanical strength (ii) propensity to form adhesions	(i) +/– (ii) +/-
Enwemeka, 1989b	Rabbits (N=12, n=14)	Achilles tenotomy	(i) ✓ (ii) ✓	1 MHz, 1 W/cm^2, 5 minutes, CUS	Daily for 9 days from post-op day 1	(i) tensile strength (ii) tensile stress (iii) energy absorption capacity	(i) + (ii) +/- (iii) +
Enwemeka, 1990	Rabbits (N=10, n=14)	Achilles tenotomy	(i) ✓ (ii) ✓	1 MHz, 0.5 W/cm^2, 5 minutes, CUS	Daily for 9 days from post-op day 1	(i) tensile strength (ii) tensile stress (iii) energy absorption capacity	(i) + (ii) + (iii) +
Jackson et al., 1991	Rats (N=27, n=27)	Achilles tendon partial rupture (Puncture with 18-gauge needle)	(i) × (ii) ×	Frequency not mentioned, 1.5W/cm^2, 4 minutes, CUS or PUS not mentioned	Daily for 8 days from post-op day 1 and then on alternating days until post-op day 21	(i) breaking strength (ii) collagen synthesis	(i) + (ii) +
Gan et al., 1995	Chickens (Group a: N=20, Group b: N=10, n=17)	Zone 2 flexor tenotomy	(i) ✓ (ii) ✓	3 MHz, 0.2W/cm^2, 3 minutes, PUS, 25% duty cycle	Ten daily treatments starting on day 7 (treatment group a) or day 42 (treatment group b)	(i) ROM (ii) scar maturation (iii) tensile strength	a(i) + a(ii) + a(iii) +/- b(i) +/- b(ii) + b(iii) +/-
da Cunha et al., 2001	Rats (Group a: N=15, group b: N=15, n=15)	Achilles tenotomy	(i) × (ii) ×	a) 1 MHz, 0.5 W/cm^2, 5 minutes, CUS b) 1 MHz, 0.5 W/cm^2, 5 minutes, PUS 20% duty cycle	Daily for 14 days	(i) organization of collagen fibers (birefringence)	a (i) +/- b (i) +
Saini et al., 2002	Dogs (N=3, n=2)	Achilles tenotomy	(i) ✓ (ii) ✓	Frequency and duty cycle not mentioned, 0.5 W/cm^2, 10 minutes	Daily for 10 days from post-operative day 3	(i) weight-bearing (ii) ultrasonography (iii) histomorphology	(i) + (ii) + (iii) +
Ng et al., 2003	Rats (Group a: N=10, group b: N=10, n=10)	Achilles tendon hemitransection	(i) × (ii) ×	a) 1 MHz, 1 W/cm^2, 4 minutes, CUS b) 1 MHz, 2 W/cm^2, 4 minutes, CUS	Six daily treatments per week (total 22 sessions)	(i) function by Achilles functional index (ii) vascoelasticity (iii) stiffness (iv) UTS	a(i) +/- a(ii) +/- a(iii) +/- a(iv) + b(i) +/- b(ii) +/- b(iii) +/- b(iv) +

2.3.7. Neural involvement – new perspectives in tendon healing and its relationship with ultrasound treatment

As mentioned in section 2.2.4., immunochemical study of the regeneration of nerve fibers in the ruptured rat's Achilles tendon showed an early in-growth of new sensory nerve fibers expressing different neuropeptide positivity, like CGRP, as early as 1 week postinjury (Ackermann et al., 2002). The authors presumed that this in-growth of nerve fibers provides a delivery system for sensory neuropeptides which may be required for tissue repair. They also suggested that early nerve regeneration is necessary for the normal neovascularization and proliferation of different cell types in tendon repair. Therefore, nerve regeneration may also be a critical process for normal tendon healing. Since therapeutic ultrasound is a type of mechanical vibration, when we study the mechanism by which ultrasound can improve tendon healing, we may also need to consider whether there may be some relationship between this early invasion of sensory nerve fibers and the mechanical effects of ultrasound treatment in the early stages of tendon healing, say for example, the present of this sensory nerve fibers in healing tendon is crucial for "sensing" the mechanical vibration generated by therapeutic ultrasound, which have still not been investigated.

Evidence shows that ultrasound at low intensity does promote peripheral nerve regeneration (Crisci and Ferreira, 2002; Hong et al., 1988; Lazar et al., 2001; Mourad et al.,

2001). Therefore, it is reasonable to hypothesize that ultrasound may able to promote the invasion of sensory nerve fibers inside the healing Achilles tendon. Studying the nerve regeneration pattern may be a good perspective for future research aimed at investigating the tendon healing processes.

2.4. Summary of literature review

The above literature review reveals several areas which are still lacking in research on the effect of therapeutic ultrasound on Achilles tendon healing. They are summarized as follows:

(i). Effect of PUS on Achilles tendon healing in terms of biomechanical properties as well as histomorphology;

(ii). Effect of denervation on tendon healing and its interaction with PUS's mechanical stimulus, which may explain the importance of neural involvement in tendon healing;

(iii). Whether there is a relationship between neuropeptides containing sensory nerve fibers and ultrasound treatment, for example, may ultrasound influence the invasion of this sensory nerve fibers into healing tendon, which may explain the therapeutic effect of PUS on tendon healing.

Based on the above areas, it is necessary to carry out a series of studies to fill in the missing areas in the research on therapeutic ultrasound as a treatment for Achilles tendon rupture, which is important for researchers to explain how ultrasound treatment could enhance the healing of injured tendons and what the underlying mechanisms may be. These findings would be clinically significant as they could help us to determine the possibility of using therapeutic ultrasound as a form of treatment option or as a supplement to conservative/surgical treatment during the rehabilitation of patients with ruptured

Achilles tendons.

2.5. Aims of thesis

This thesis aims to investigate the effect of therapeutic ultrasound on Achilles tendon rupture and the possible underlying mechanisms in animal studies. Based on section 2.4., the following research questions arise:

(i). Is PUS effective to improve Achilles tendon healing in terms of biomechanical properties as well as histomorphology?

(ii). Is sensory nerve fibers important for tendon healing and is it related to the tendon response to the mechanical stimulus from PUS?

(iii) Is there any relationship between neuropeptide-containing sensory nerve fibers, particularly the CGRP-containing sensory nerve fibers, and PUS treatment on Achilles tendon healing? Would invasion of this sensory nerve fibers be affect by PUS treatment?

The coming chapters of this thesis present a series of studies that were conducted to answer the above research questions: The first study investigated the effect of PUS on tendon healing in terms of recovery of biomechanical properties as well as histomorphology by animal model (Chapter 3). To investigate whether the therapeutic effect of PUS may interact with the neural components, the above comparisons were made between the PUS and sham PUS groups, where denervation of Achilles tendons was present in the rats of both groups (Chapter 4). In addition, to explain the relationship between neuropeptide-containing nerve fibers – particularly the CGRP-containing sensory

nerve fibers, which were found abundantly in healing tendons – and PUS on Achilles tendon healing, immunohistochemical analysis on healing tendons which had received PUS treatment or denervation or both was conducted (Chapter 5).

The thesis is concluded with a general discussion of the findings in view of the possibility of applications of ultrasound treatment on patients with tendon injury in future (Chapter 6).

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CHAPTER 3. THE EFFECT OF THERAPEUTIC PULSE ULTRASOUND ON THE HEALING OF INJURED TENDON

3.1. Introduction

There has been considerable debate regarding better treatment for acute ruptures of the Achilles tendon. Open repair is thought to be capable of restoring the normal tension and length of the tendon with a lower rate of re-rupture (Bruns et al., 2000; Cetti et al., 1993; Shields et al., 1978; Wong et al., 2002). However, the disadvantages of surgical intervention include wound infection, adhesion and higher costs (da Cunha et al., 2001; Paavola et al., 2002; Wong et al., 2002).

Cast immobilization is the most common method for conservative treatment of Achilles tendon rupture (Bruns et al., 2000; Inglis et al., 1976; Möller et al., 2001; Nistor, 1981). Treatment usually consists of 8 weeks' below-knee cast immobilization (Bruns et al., 2000; Inglis et al., 1976; Möller et al., 2001; Wong et al., 2002). However, there are drawbacks to this management, such as the fact that it requires a longer duration for immobilization, thus resulting in atrophy and weakness of the calf muscles (Bruns et al., 2000; Kangas et al., 2003). Recently, functional bracing has been used as an alternative to immobilization in conservative treatment, allowing early mobilization during rehabilitation (Eames et al., 1997; McComis et al., 1997; Saleh et al., 1992; Wallace et al., 2004). The treatment protocols include 3 to 4 weeks of cast immobilization and 4 to 5 weeks of

removable orthosis (Eames et al., 1997; Saleh et al., 1992; Wallace et al., 2004) or cast immobilization in the early phase of healing followed by the use of dosiflexion-restricted orthosis, which allows early ankle movement (McComis et al., 1997). These resulted in good functional outcome (Saleh et al., 1992), low re-rupture rate (Eames et al., 1997; Saleh et al., 1992; Wallace et al., 2004), and an even faster healing rate than those managed by operative repair of acute Achilles tendon rupture (Wallace et al., 2004).

Recently, there have been an increasing number of reports suggesting that CUS promotes tendon healing after open repair in terms of recovery of the biomechanical properties of healing tendons. Ng et al. (2003) compared low dose (1 W/cm^2) with high dose (2 W/cm^2) CUS on tendon repair after partial transection of the Achilles tendon in a rat model. They found that both low and high dose CUS could improve the UTS of injured tendons when compared to a control group. However, there was no significant difference between the two dosages. Two studies have been carried out by Enwemeka and colleagues (1989b, 1990) comparing the effect of daily application of CUS at higher (1 W/cm^2) and lower intensity (0.5 W/cm^2) on rabbit tendon healing. Their results demonstrated that low intensity CUS improved not only the tensile strength and energy absorption capacity of the treated tendon, but also the tensile stress, which was not shown in those treated by higher intensity CUS. They concluded that the beneficial effects of ultrasound were more promising when it was applied at lower intensities.

More recently, it has also been found that PUS was more effective than CUS in promoting tendon healing (da Cunha et al., 2001; ter Harr, 1999). Da Cunha et al. (2001) compared the effect of CUS and PUS, using comparable SATA intensity at 0.5 W/cm^2, on the healing of ruptured Achilles tendons after surgical suture in a rat model. They demonstrated that PUS produced a superior beneficial effect with respect to collagen synthesis, with better organization and aggregation of collagen bundles. However, these results were not supported by previous studies, which used different protocols and found no significant difference in the mechanical strength of the healing tendon between the PUS treatment and control groups (Gan et al., 1995; Turner, 1989).

It is unknown whether PUS can supplement immobilization in the repair of ruptures of the Achilles tendon under conservative therapy. If PUS is able to accelerate tendon healing, the duration for immobilization could be reduced so that a reduction of associated complications is expected. The purpose of the present study was therefore to determine the effect of PUS on the repair of partially ruptured Achilles tendons in a rat model.

3.2. Methods

3.2.1. Animal model

Fifty-two male, adult Sprague-Dawley rats (mean body weight 397.6 ± 55.13 g, range 278.7-510.1 g) were used in the study. All the animals were mixed breeds and were obtained from the Centralized Animal Facilities (CAF) at the Hong Kong Polytechnic University (PolyU). The animals were also kept at the animal house in the CAF. Each animal was labeled and randomly allocated into the designated group upon arrival. All the surgical operations and treatments were performed in the operation room of the CAF. The biomechanical tests were performed in the Orthopaedic Laboratory, Department of Rehabilitation Sciences at PolyU.

3.2.2. Ethical considerations

Ethics approval was obtained from the Animal Subjects Ethics Sub-committee of PolyU before conducting the experiment. The approval form is attached (Appendix I). A licence to conduct animal experiments was also endorsed by the Department of Health of the Hong Kong Government. A copy of the licence is attached as Appendix II.

3.2.3. Experimental design

The rats were randomly assigned into either the PUS treatment group or a control group which received sham PUS treatment (Table 3-1). A hemi-transection of the right Achilles tendon was performed on each rat to mimic a partial tendon rupture. In addition, a patella tenotomy was also performed on the right knee in order to decrease loading on the right limb (Kimmel et al., 1999). Different groups of rats were euthanized 2 and 4 weeks after surgery. Ten rats per group served for testing the biomechanical properties of scar healing, while the tendons of another 3 rats in each group were harvested for histological analysis.

Table 3-1. Details of the animal groupings

Group	Surgery on right hindlimb	Treatment	Time of sacrifice (weeks post-surgery)
PUS	Medial transection of Achilles tendon and 5 mm resection of patella tendon	PUS	2 weeks (n=13) 4 weeks (n=13)
Control		Sham PUS	2 weeks (n=13) 4 weeks (n=13)

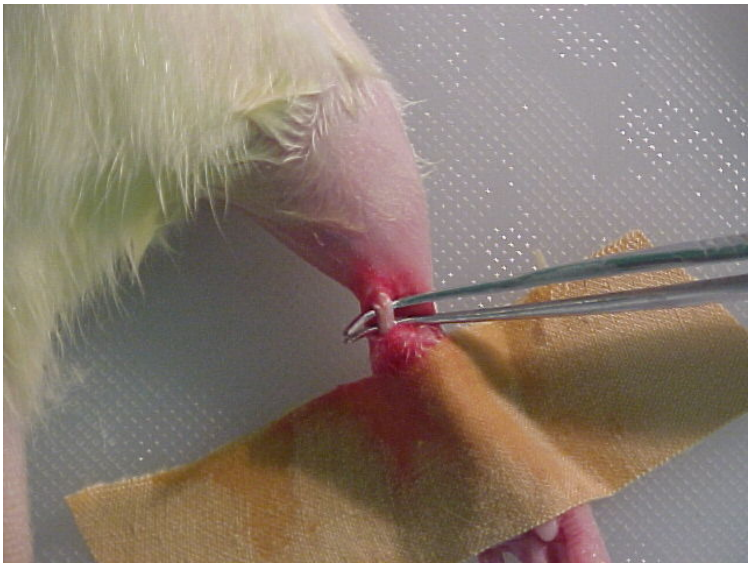
3.2.4. Injury model

All surgical procedures were carried out under general anesthesia in a sterile condition with intra-peritoneal injection of chloral hydrate (Riedel-de Haen, Germany). The dosage of the drug applied was calculated as 1 ml per 100 g of animal's weight. The skin at the incision site was shaved before surgery.

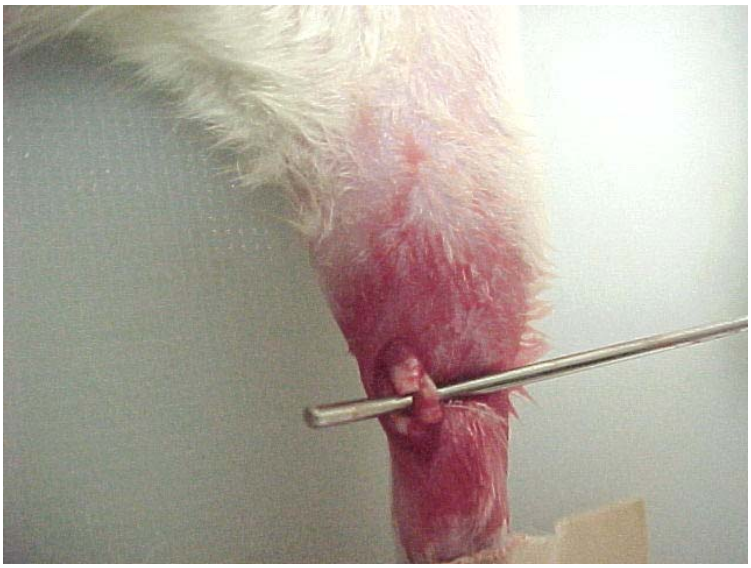
To create a partial tenotomy of the Achilles tendon, a longitudinal incision was made on the medial aspect of the right calf and the skin was retracted to expose the Achilles tendon. By blunt dissection, the medial and lateral portions of the Achilles tendon and the plantaris tendon were identified and separated with a probe. The medial Achilles tendon was then transected at the mid-point (0.5 cm above the calcaneal insertion) with the lateral Achilles tendon left intact in order to prevent retraction of the severed ends (Ng et al, 1996; Ng et al., 2003). The severed ends remained unsutured during healing (Figure 3-1).

Patella tenotomy was performed through a longitudinal incision on the antero-medial aspect of the right knee. The patella tendon was isolated by blunt dissection with a probe. The patella tendon was then resected by cutting out a 5 mm section of the tendon (Figure 3-2).

After the surgery, the animals were kept in 20 cm x 24 cm x 40 cm cages and allowed cage activity under a 12-hour daylight cycle. Temperature and relative humidity were maintained at about 21°C and 80% respectively. Food and water were given *ad libitum* throughout the study.

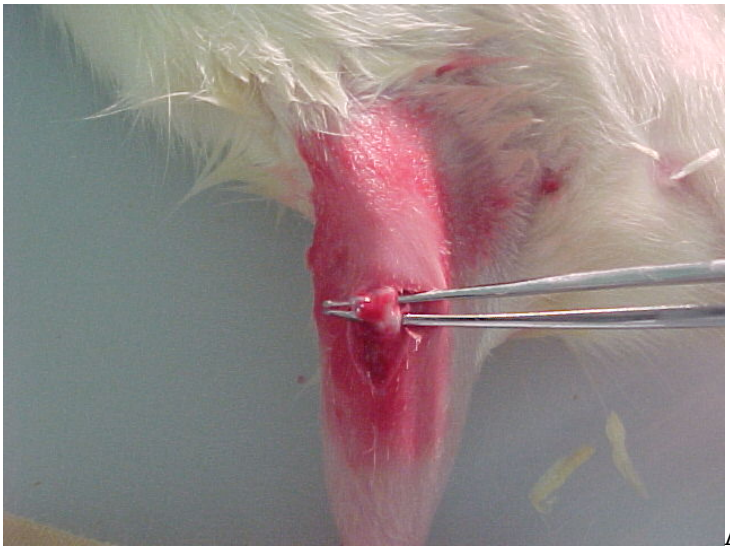


A



B

Figure 3-1. Hemi-transection of rat Achilles tendon. (A) Isolation of the whole Achilles tendon. (B) Transection of the medial part of the tendon with the lateral portion left intact


A

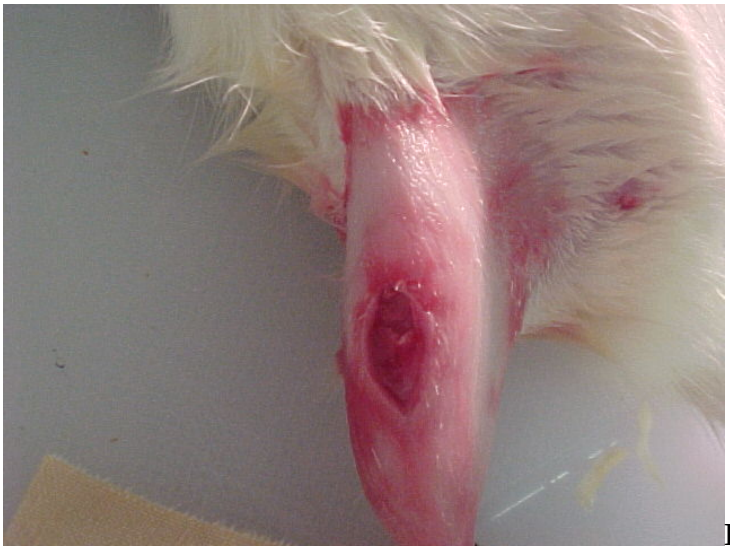

B

Figure 3-2. Patella tenotomy. (A) Isolation of the patella tendon from the surrounding soft tissue. (B) After 5 mm resection of the patella tendon

3.2.5. Ultrasound treatment

All the animals received either true or sham PUS treatment according to their group designation. PUS treatment was applied by using an ultrasound machine (Enraf Nonius, Sonopuls, Model no. 434, Holland) with a 0.8 cm^2 treatment head (Figure 3-3). The machine was calibrated with an ultrasound wattmeter (UW-II Bio-Tek, VT, USA, Figure

3-4) before the first application and then weekly afterwards (Artho et al., 2002). The animals in the PUS group received PUS at 1 MHz, 2.5 W/cm^2, 20% duty cycle (SATA of 0.5 W/cm^2) for 5 minutes. From our literature review, this PUS parameter was effective for promote tendon healing. Water was used as the coupling agent and the ultrasound head was held stationary 1 cm perpendicularly above the skin covering the ruptured ends of the Achilles tendon (Jackson et al., 1991). During the treatment procedure, the rat was held in a plastic restraining cone with only the left leg submerged in a plastic tank containing water at about 25 °C. Treatments were carried out 3 times per week, starting from post-operation day one. Animals in the control group were handled in the same manner but without exposure to PUS.

A

B

Figure 3-3. Ultrasound treatment unit. (A) An Enraf Nonus-Sonopuls, Model no. 434 therapeutic ultrasound machine and (B) a 1 MHz, ERA 0.8cm2 transducer

A

B

Figure 3-4. Ultrasound calibration unit. (A) An Bio-tek ultrasound wattmeter and (B) setup during calibration of the ultrasound

3.2.6. Biomechanical testing

To observe the effects of early and prolonged ultrasound treatment, 10 rats from each group were euthanized at the end of 2 or 4 weeks postinjury respectively, with an overdose of intra-peritoneal chloral hydrate. Bilateral lower limbs were harvested by hip joint disarticulation and the leg specimens were sealed in a plastic bag, labeled and then stored in a freezer at -40 °C. The preparation and mechanical testing procedures were based on previously established protocol (Ng et al., 2003). All tests were carried out by a single operator to avoid interpersonal variations. The specimens were thawed at room temperature for around 6 hours before testing. All soft and hard tissues were carefully dissected from the calcaneus-Achilles tendon complex (Figure 3-5). The lateral portion of the Achilles tendon was removed at the musculo-tendinous junction, leaving the medial portion of intramuscular tendinous fibers, the medial Achilles tendon and the calcaneus complex. The intramuscular tendinous fibers were secured between two pieces of white labeling tape with quick setting gel super glue (Aron Alpha, Toagosei Co. Ltd.). Extra care was taken to prevent the glue from running down the tendon (Figure 3-6).

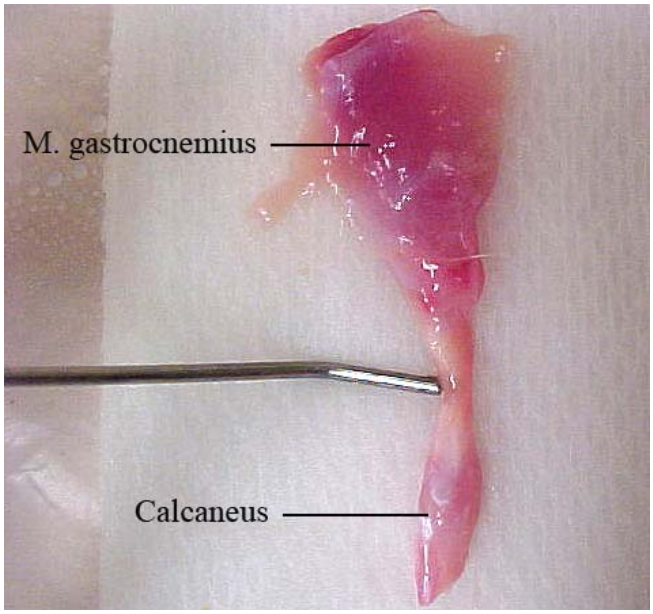


Figure 3-5. An injured Achilles tendon with medial gastrocnemius muscle. The tip of the probe indicates a scar at the middle portion of the whitish Achilles tendon

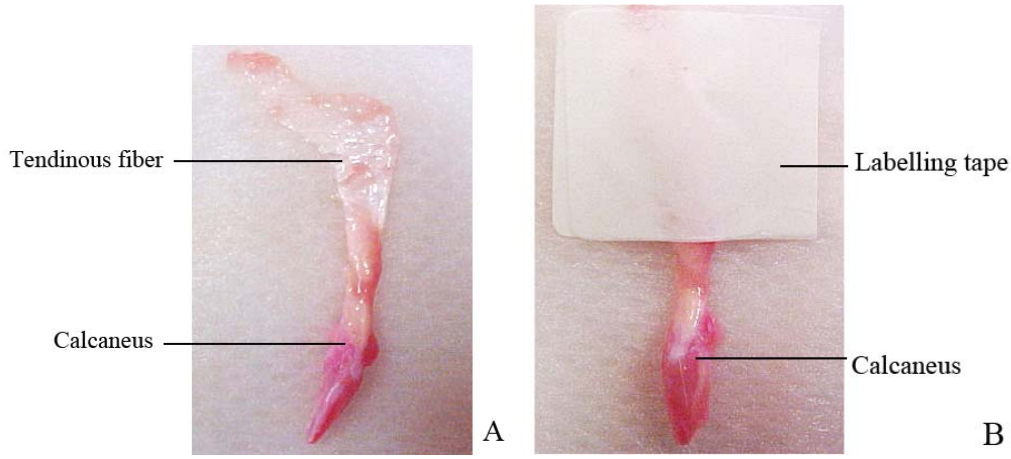


Figure 3-6. The tendon-calcaneus complex. (A) After removing all the muscle fiber from the musculotendinous junction of the Achilles tendon. (B) Two pieces of labeling tape cover the whole musculotendinous junction anterior-posteriorly, with quick setting glue mounted in between

The tendon length of the taped tendon-calcaneus complex (TCC) was measured with a pair of vernier calipers for later calculation of the strain value for the preconditioning and load relaxation testing. The tendinous portion of the construct was wrapped in gauze soaked with normal saline during the fixation period to prevent dehydration of the

specimen. Prior to mounting the specimens, the load cell and the extensometer of the MTS machine were calibrated by running the built-in protocol (Figure 3-7).

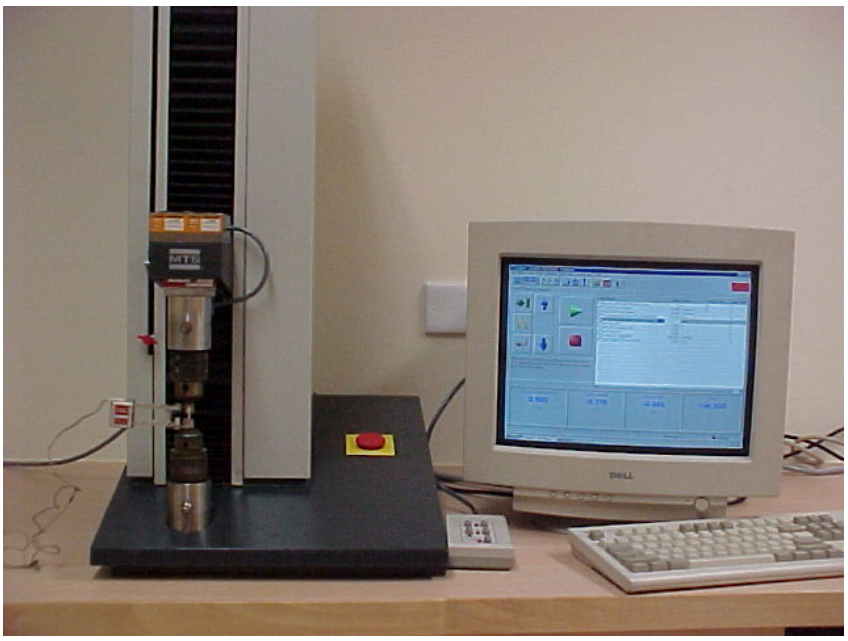


Figure 3-7. An MTS Synergie 200 machine and computer with software "Testwork 4 universal testing" installed.

The TCC was then mounted onto the adapter grips of an MTS Synergie 200 machine (MTS Systems Corporation, Minnesota). To measure the local strain of the tendon, an extensometer (MTS Model no. 634, 12F-24, MTS Systems Corporation, Minnesota) was attached to the interface of the adapter grips (Figure 3-8). Room temperature was controlled at around 25 °C and the specimen was kept moist with normal saline solution throughout the test.

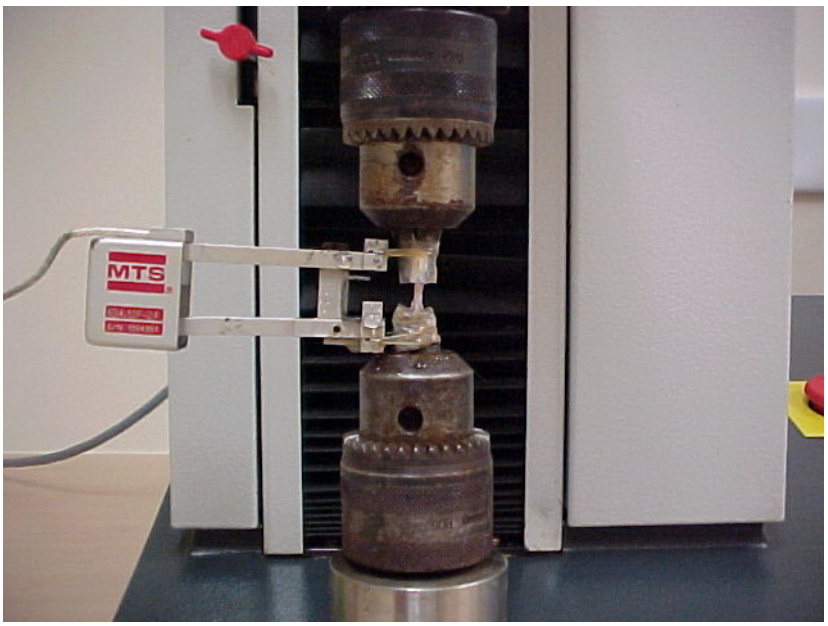


Figure 3-8. Setup for biomechanical testing of the specimen. The TCC is mounted on the adaptor of the MTS system. An extensometer is attached to the tip of the adaptor grip to measure the local displacement.

3.2.6.1. Preconditioning of thawed specimen

To minimize the deep freeze effect on the tendon tissue, each specimen was pre-conditioned with 10 oscillation cycles of 2.5% strain at a rate of 10 mm per minute before testing to failure (Woo et al., 1986).

3.2.6.2. Load relaxation testing

After preconditioning, the specimen was elongated to 2.5% strain for 5 minutes (Ng et al., 1996; Ng et al., 2003). The loads were recorded throughout these 5 minutes at a sampling rate of 5 Hz. Finally, the load relaxation property, representing the viscoelastic behavior of the specimen (Ozkaya and Nordin, 1999), was measured using equation 3.1;

an example of the load relaxation curve recorded by the machine is illustrated in Figure 3-9.

$$(Initial\ Load - Final\ Load)/Initial\ Load \times 100\% = Load\ relaxation \quad (3.1)$$

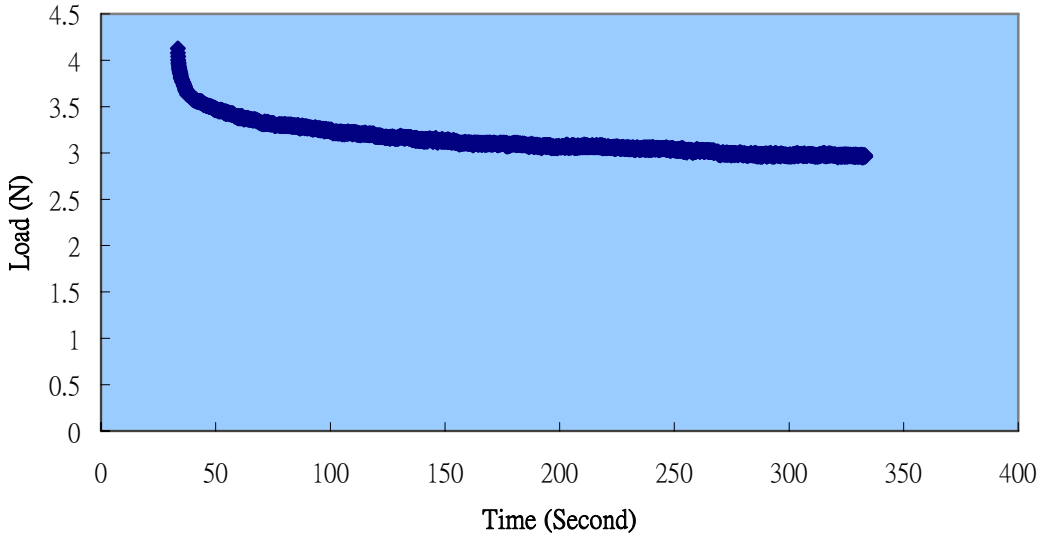


Figure 3-9. Example of load relaxation curve

3.2.6.3. UTS and structural stiffness

Each specimen was unloaded for 5 minutes after viscoelasticity testing to allow it to return to its original length. It was then subjected to the failure test at a loading rate of 500 mm/minute (Fung et al., 2002) while the load and displacement data were recorded at a sampling rate of 50 Hz. The load-displacement curve was plotted. The maximum load recorded represented the ultimate tensile strength (UTS), and the gradient in the linear

portion immediately beyond the toe region represented the structural stiffness of the specimen (Figure 3-10).

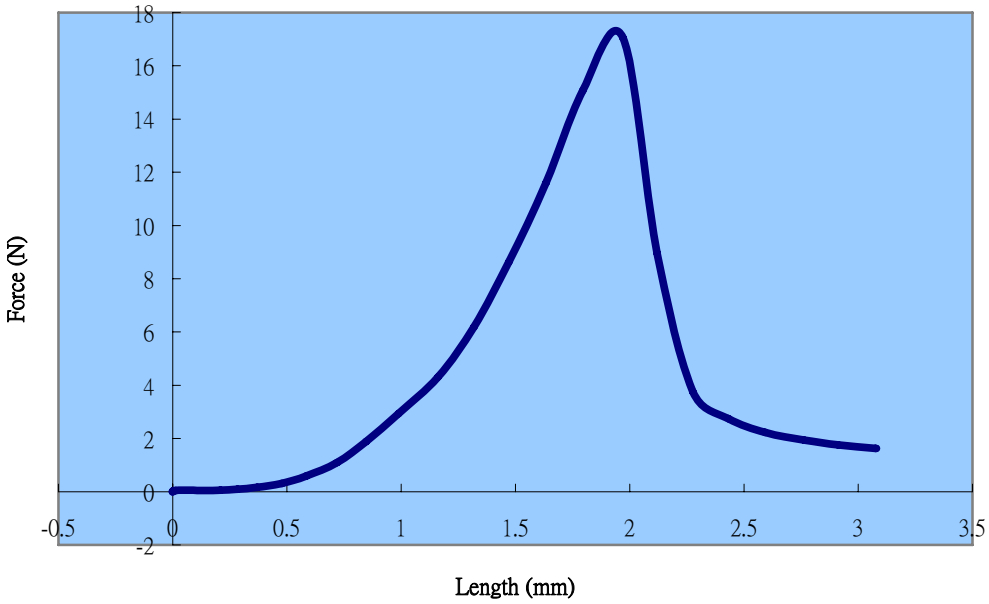


Figure 3-10. Example of load deformation curve

Finally, each of the above three values (UTS, stiffness and load relaxation) of the injured Achilles tendon was normalized against that measured from the contralateral healthy Achilles tendon of the same animal to calculate the percentage of normal strength restored by the injured Achilles tendon at each time point (Fung et al., 2002). This method of calculation was adopted to minimize the individual variations between different rats.

3.2.7. Histological analysis

Three rats from each group were euthanized at the end of 2 or 4 weeks postinjury. All rats were anaesthetized by intra-peritoneal injection of choral hydrate (1 ml/100g). Intra-arterial perfusion with phosphate buffered saline (PBS) was performed followed by perfusion with 4% phosphate buffered paraformaldehyde solution in pH 7.4. The Achilles tendons were dissected bilaterally and immersed in 4% phosphate buffered paraformaldehyde solution for 2 hours at room temperature. All specimens were rinsed in PBS and then soaked for at least two days in 20% sucrose solution. The tissues were cut on a Leitz cryostat to a section thickness of 5 μm. The frozen sections were mounted directly on SuperFrost/Plus glass slides.

The sections were stained with Ehrlich's haemotoxylin/eosin Two sections, one from dorsal and one from ventral aspect, were selected from each specimen and one low (100x) and two high (400x) power photomicrographs were taken of each section of the injured region The sections were then assessed, using the software analySIS® version 3.2 (Soft Imaging System GmbH, Germany) for cellular details and matrix composition with the following criteria (Palmes et al., 2002):

(1) Ratio between fusiform fibroblasts and flattened fibrocytes (tendocytes) in the tendon tissue. The fibroblasts mainly occur in two forms in tendon: (a) long, thin and spindle shaped form, which is metabolically less active and can mainly be found in mature

tendon. (b) Ovoid, plump shaped form, which is metabolically more active and can be found in active repairing tendon. Thus scar tissue with higher fibroblast to tendocyte ratio is in active stage of repairing. On the other hand, scar with lower ratio was less active or if found in later stage of healing (4-6 weeks postinjury, which is more close to the remodeling stage of tendon healing), lower ratio may mean more mature of the scar tissue. We differentiated them by their morphological appearance and count the total number of fibroblasts and tendocytes on each image manually, while those fibroblasts at the edge of the image which could not differentiate clearly would not be counted.

(2) Fibroblasts to collagen matrix area ratio = area occupied by fibroblasts in relation to the total collagen area of each image filed. Usually in a normal tendon, it is hypocellular. If this ratio is high, the scar may hypercellular or the collagen content is less, or both, which means less mature of the scar. Using the software, by selecting the specific colour threshold, the total area occupied by fibroblasts/collagen matrix could be calculated.

(3) Collagen fiber arrangement in tendon tissue. Collagenous bundles were thick, closely packed, oriented parallel to longitudinal axis in normal tendon. (Amiel et al., 1984). We assessed each section subjectly on the collagen fiber bundle alignment.

(4) Total area of collagen matrix in each image filed. It reflects tissue ability to withstand high stress. In general tissues with greather tensile strength are those with the highest collagen content (Parry et al., 1988).

All the sections being assessed were labelled with number, while the assessor did not know which treatment group the section belonged to during the assessment in order to eliminate bias.

3.2.8. Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare the normalized load-relaxation, stiffness and UTS values, as well as the mean total collagen matrix area, mean cell area to collagen matrix area, and mean fusiform fibroblasts to tendocytes ratio, between the treatment and sham treatment group and across time. The Statistical Package for Social Sciences (SPSS) for Microsoft Windows version 14.0 was used in the data analysis. An α level of 0.05 was set for all statistical comparisons.

3.3. Results

After 2 and 4 weeks post surgery, the injured tendons on the PUS and control groups showed thick fibrous scars, indicating that the injured sites were repairing. Generally, the scar in the 2-week group was thicker and more extensive when compared with the 4-week group. Macroscopically, there was no obvious difference between the PUS and control groups.

3.3.1. Biomechanical test results

The results of the biomechanical tests are shown in Figures 3-11 to 3-13. The mean

normalized UTS of the PUS group at 2 and 4 weeks postinjury were 48.9±18.39% and 77.1±15.31% respectively, significantly higher than in the control group (mean normalized UTS at 2 and 4 weeks were 30.4±15.46% and 54.3±18.40%) in the same time frame ($p<0.01$). When compared across time, the mean normalized UTS in the 4-week group was significantly higher than that in the 2-week group ($p<0.01$) (Figure 3-11).

Similar to the results of UTS, the PUS group also had a significantly higher value in mean normalized stiffness (2 weeks: 62.5±32.46% and 4 weeks: 92.5±31.12%) compared to the control group (2 weeks: 34.8±16.22% and 4 weeks: 65.0±25.48%) at the same time postinjury ($p<0.01$), while those in the 4-week group also had significantly higher mean normalized stiffness than the 2-week group compared across time ($p<0.01$) (Figure 3-12).

When the normalized load relaxation results of the PUS groups (2 weeks: 145.4±39.17% and 4 weeks: 123.9±31.89%) were compared to that of the control groups (2 weeks: 122.7±28.76% and 4 weeks: 125.6±32.13%), there was no significant difference among groups and across time ($p>0.05$) (Figure 3-13).

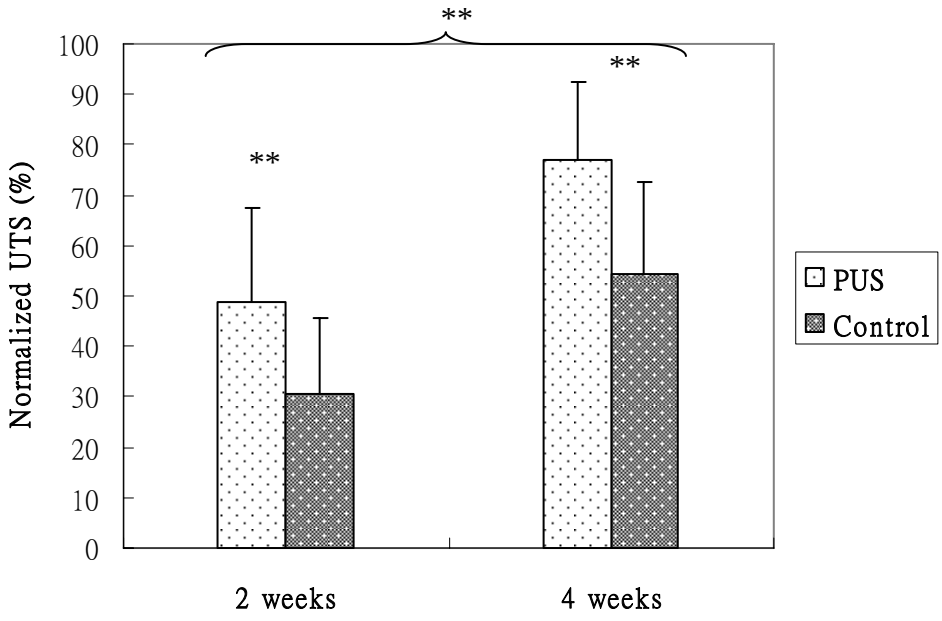


Figure 3-11. Results of normalized UTS values in the PUS and control groups at 2 and 4 weeks postinjury. **p<0.01

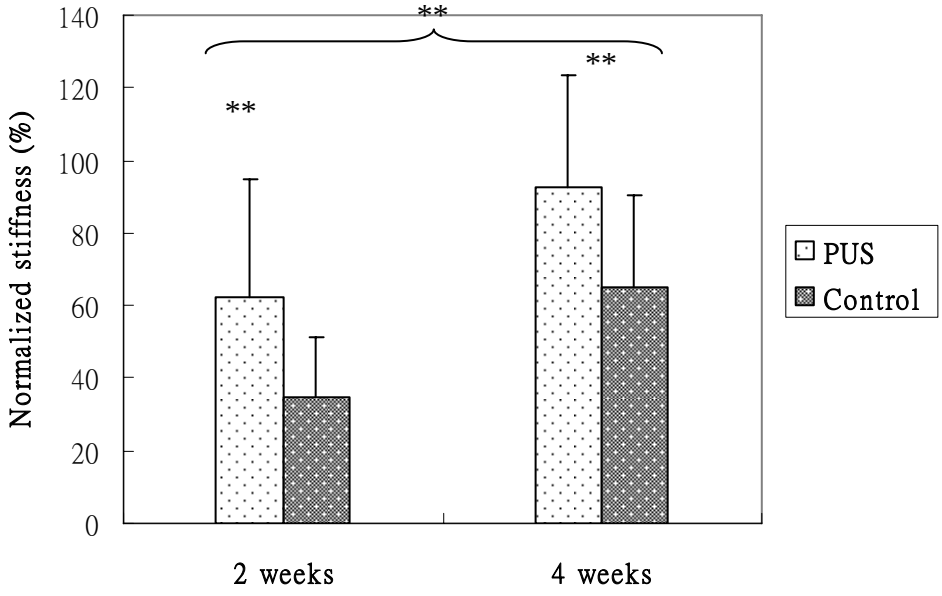


Figure 3-12. Results of normalized stiffness values in the PUS and control groups at 2 and 4 weeks postinjury. **p<0.01

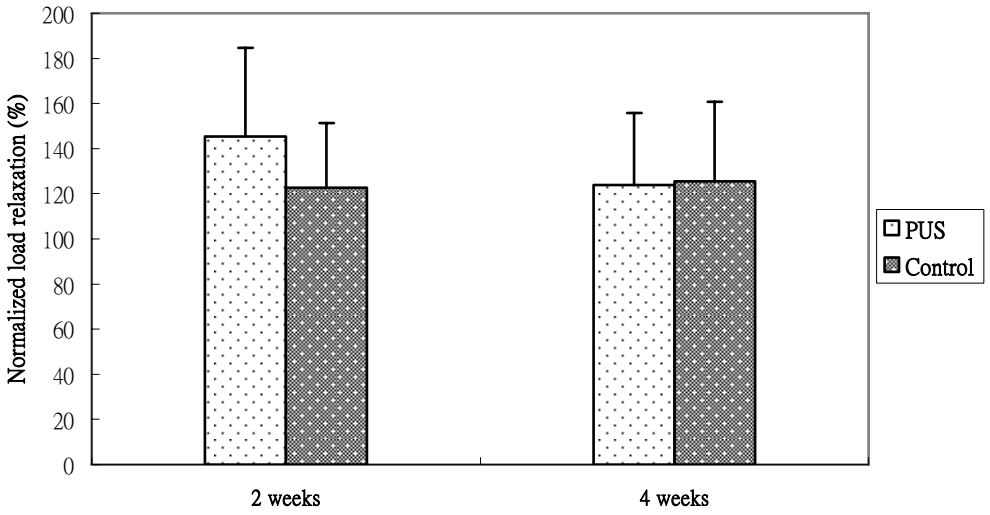


Figure 3-13. Results of normalized load relaxation values in the PUS and control groups at 2 and 4 weeks postinjury

3.3.2. Histological analysis results

Two weeks postinjury, the cut ends of the Achilles tendon in both the PUS and control groups were connected by scar tissue rich in multi-shaped fibroblasts. The ratio between fusiform fibroblasts and flattened fibroblasts in the PUS group was 4.35 ± 3.19, while in the control group the ratio was 6.37 ± 4.13. There was no significant difference between the 2 groups. The fibroblasts in the specimens from the PUS group looked more mature, with a well developed, active fusiform nucleus. The fibroblast to collagen matrix ratio in the specimens from the PUS group (0.18 ± 0.103) was slightly lower than that in the control group (0.25 ± 0.078), though not statistically significant ($p>0.05$). Although the collagen fiber bundles in the tendons of the PUS group were aligned irregularly, they

nonetheless appeared denser and the alignment was slightly better than that in the control group (Figure 3-14). The mean total area occupied by the collagen matrix in the PUS group (19502.3±1165.20 μm^2) was significantly higher than that of the control group (17339.47±953.04 μm^2) ($p<0.01$).

At 4 weeks postinjury, the scar was more mature in both groups, with a slightly lower fibroblast to matrix ratio, a denser collagen matrix and more closely packed, regularly aligned collagen fiber bundles than their 2-week counterparts. In the PUS group, the fusiform fibroblasts to flattened fibroblasts ratio at 4 weeks postinjury was 0.41 ± 0.191, compared to 4.35 ± 3.19 at 2 weeks postinjury. The same ratio in the control group at 4 weeks postinjury was 1.38 ± 1.628, which did not differ significantly from that of the PUS group. In the PUS group, the fusiform fibroblasts to tendocytes ratio decreased markedly, which meant that there was a higher proportion of tendocytes when compared to the 2-week group. This ratio in the PUS group was also slightly lower than that of the control group at 4 weeks postinjury, which was statistically not significant (0.13 ± 0.056 in PUS group, 0.21 ± 0.132 in control group). The scar in the PUS group appeared more mature, with more parallel-oriented collagen fiber bundles than that of the control group (Figure 3-15). The total collagen matrix area of each group was higher than that of the 2-week group ($p<0.01$). In addition, the total area of the collagen matrix in the PUS group (20758.4±1024.64 μm^2) was significantly higher than that of the control group

(19023.1±1125.44 μm^2) ($p<0.01$). The results of histological analysis are summarized in Figures 3-16 to 3-18.

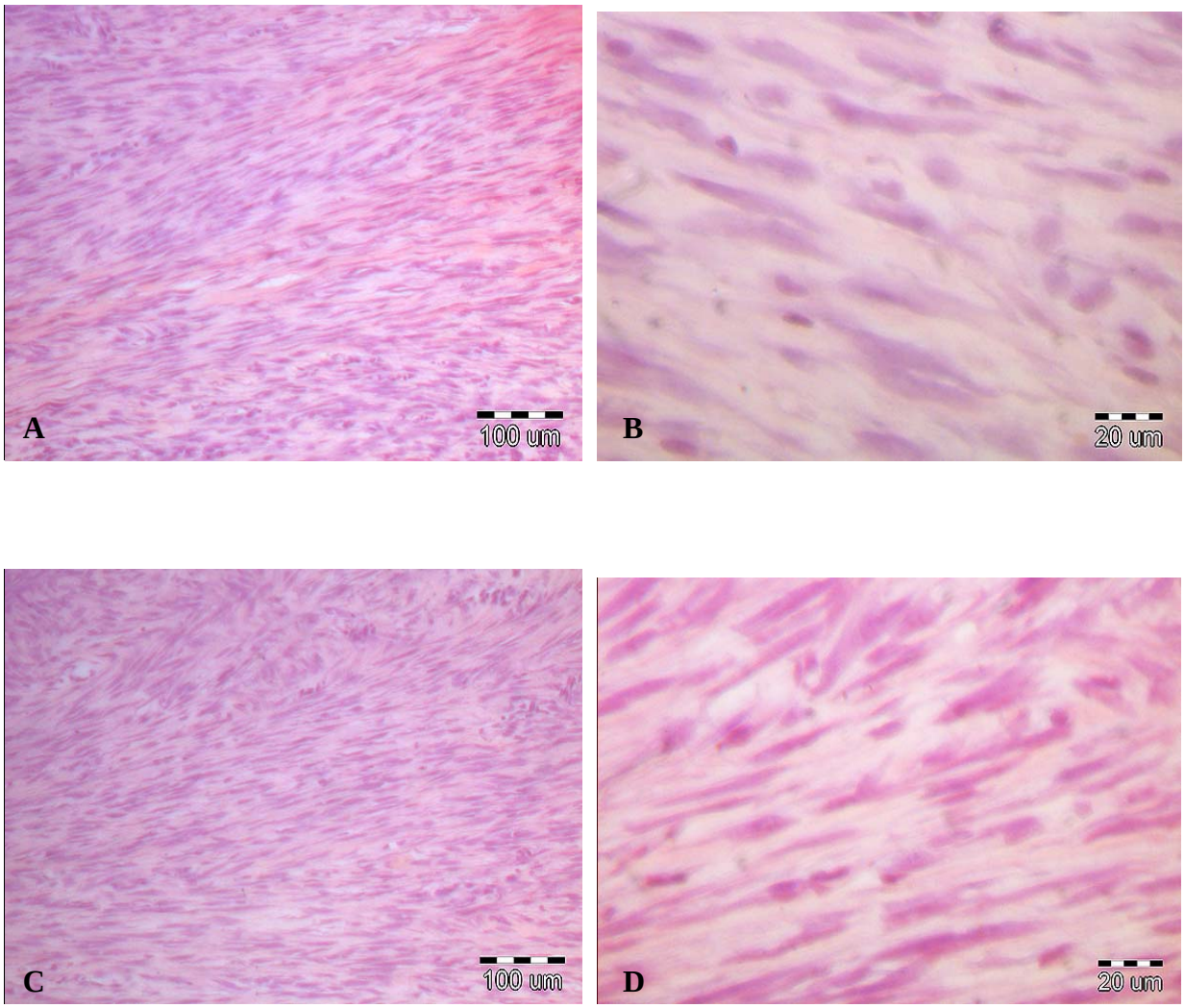


Figure 3-14. Histology of the Achilles tendon of rats in the PUS group (A: 100x; B: 400x) and control group (C:100x; D: 400x) at 2 weeks post-partial tenotomy

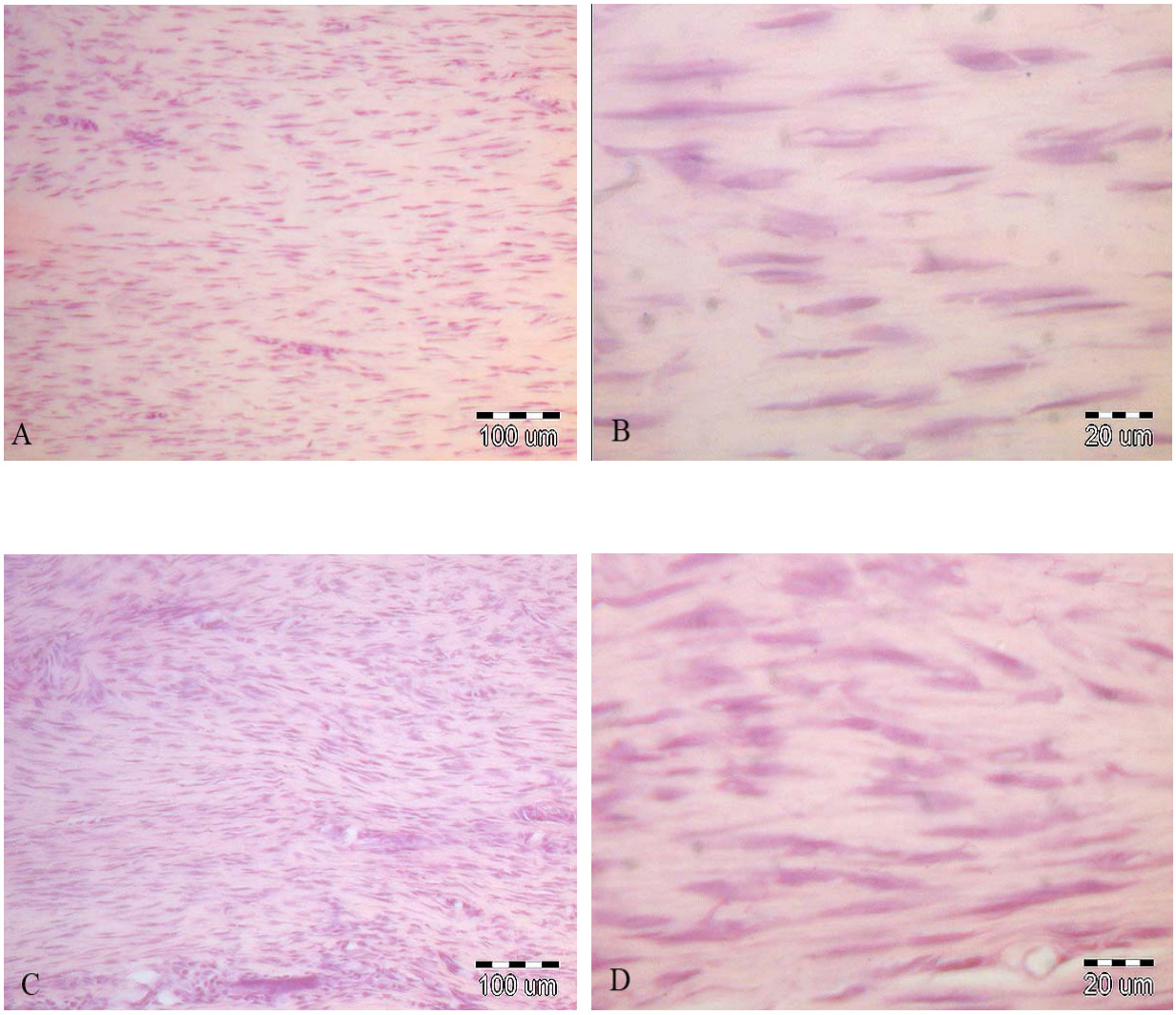


Figure 3-15. Histology of the Achilles tendon of rats in the PUS group (A: 100x; B: 400x) and control group (C:100x; D: 400x) at 4 weeks post-partial tenotomy

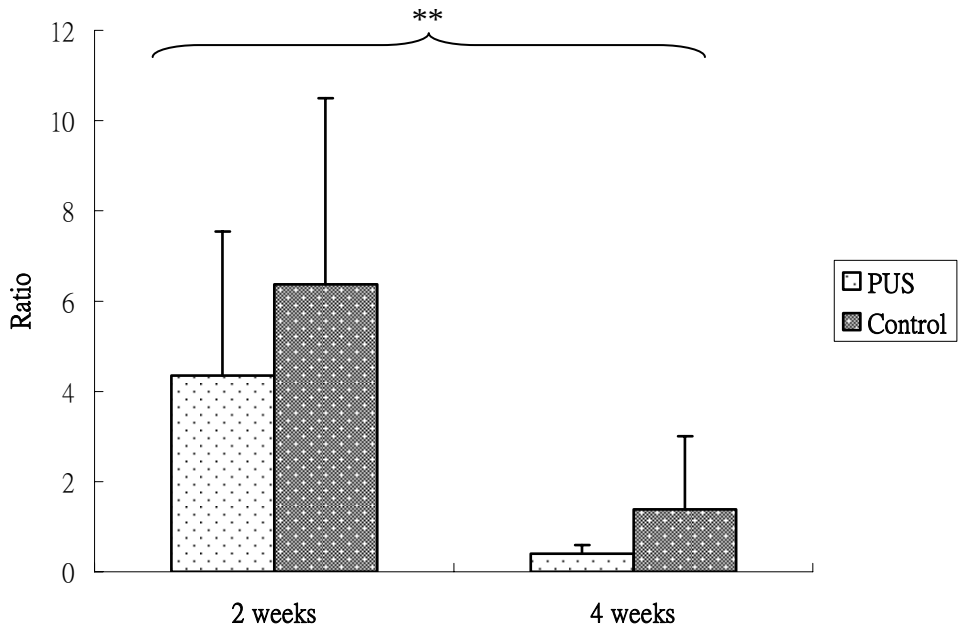


Figure 3-16. Results of mean fusiform fibroblasts to tendocytes ratio in the PUS and control groups at 2 and 4 weeks postinjury. **p<0.01

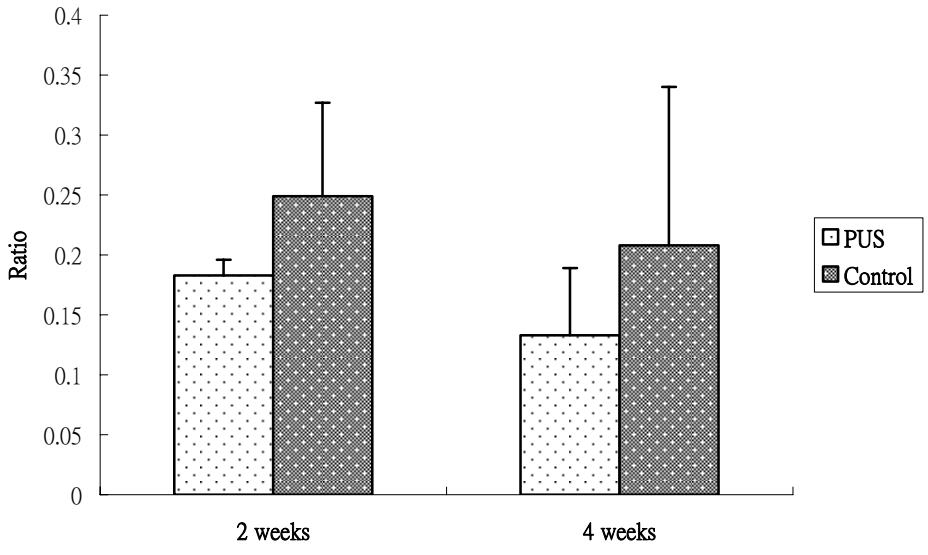


Figure 3-17. Results of mean fibroblast to collagen matrix area ratio in the PUS and control groups at 2 and 4 weeks postinjury

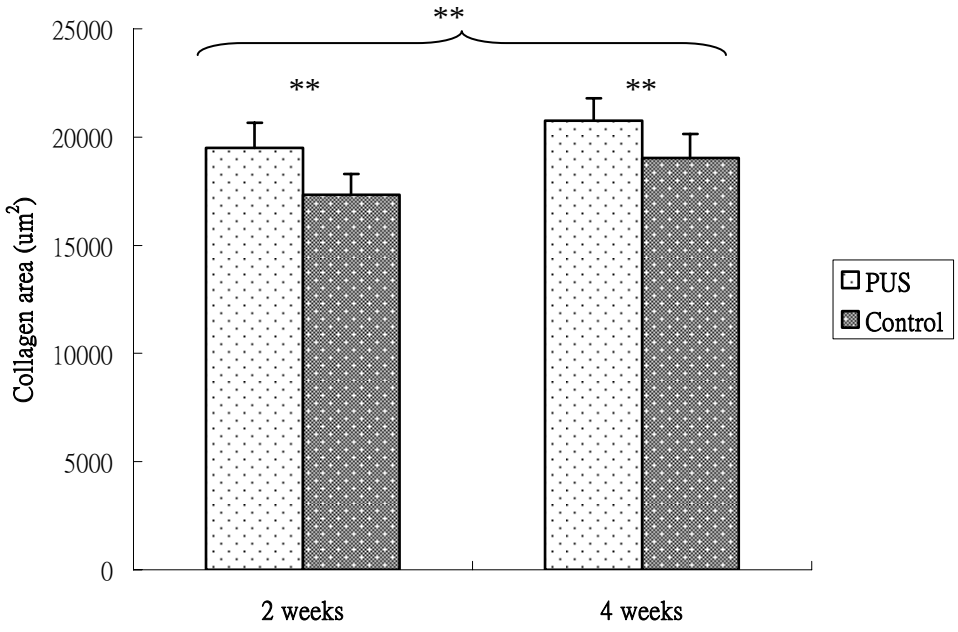


Figure 3-18. Results of mean total collagen matrix area in μm2 in the PUS and control groups at 2 and 4 weeks postinjury. **p<0.01

3.4. Discussion

The present study was designed to investigate the effect of PUS on the healing of partially ruptured tendons in a rat model. The results of our study showed that ruptures of the Achilles tendon could heal with scar formation under conservative management. PUS can accelerate the early healing process. During the proliferative phase, occurring within the first two weeks postinjury, fibroplasias and fibrillogenesis took place in the injured tendon (Enwemeka, 1989a). The results of biomechanical testing show that both the normalized UTS and stiffness of specimens in the treatment group were significantly higher than those in the control group ($p<0.01$). The ability of the tissue to withstand high stress levels is related to the percentage of collagen matrix in the tissue (Parry, 1988). A decrease in stiffness in the control group may also be correlated with an increase in the immature collagen matrix (Matsumoto et al., 2003). At 4 weeks postinjury, the cellularity of the scar had decreased and the collagen fiber bundles had become much denser as compared with the 2-week groups. This would indicate that the scar was undergoing maturation and remodeling (Jozsa and Kannus, 1997).

The mean normalized UTS and stiffness in the ultrasound treatment group were significantly higher than in the control group at both 2 and 4 weeks postinjury. At 2 weeks, the mean normalized UTS and stiffness of the PUS group were 48.9% and 62.5%, while at 4 weeks, the mean normalized UTS and stiffness of the PUS group reached 77.1% and

93.5% respectively. The findings of mechanical test were in line with the histological findings that a more mature scar tissue with a denser and more parallel arrangement of collagen fiber bundles was found in specimens which had received PUS treatment. In both the 2- and 4-week groups, it was found that the percentage of the recovery of stiffness was higher than that of UTS. It has previously been demonstrated that, in skin and ligament wounds, stiffness returns relatively faster than strength (Noyes, 1977; Zingg, 1975), and pulsed ultrasound may enhance the return of stiffness in the early stages of healing.

Similar to a previous study (Ng et al., 2003) which reported no significant difference in the load relaxation behavior between the ultrasound treatment and control groups, the results of this study also revealed that there was no significant difference in the load relaxation value between groups either at 2 or 4 weeks postinjury. This may be due to the fact that at 2 and 4 weeks, the basic fibrous structures have already been developed, resulting in a relatively constant tendon function under lower loads (Steiner, 1982).

Certain mechanisms of ultrasound are involved in its ability to promote tendon healing, like the increased concentration of calcium transport (Dinno et al., 1989; Dyson, 1987), the release of histamine and growth factors from mast cell and macrophages (Fyfe and Chahl, 1984), the proliferation of fibroblasts and protein synthesis (Harvey et al., 1975; Ramirez et al., 1997). However, several previous studies using PUS treatment revealed no significant improvement in the biomechanical properties of tenotomized tendons. Roberts

et al. (1982), using a rabbit model, found that no tenotomized tendon in the treatment group had healed after six weeks of PUS treatment. However, they had completely transected the tendon, repaired it with sutures, and immobilized the related joints after operation. Immobilization was found to have a detrimental effect on the mechanical recovery of the injured tendon (Gelberman et al., 1982; Murrell, 1994). In our study, the ankle joint of the rat was not immobilized, with loading on the injured limb reduced solely by a patella tenotomy. Two studies investigated the effect of PUS treatment on sutured cockerel and chicken tendons (Gan, 1995; Turner, 1989) and reported no significant difference in the mechanical strength between the treatment and control groups. In those studies, PUS with a frequency of 3 MHz and a SATA intensity of 0.2 W/cm^2 were applied. However, most therapeutic ultrasound studies with positive results on the mechanical properties of injured tendons used ultrasound with an intensity of at least 0.5 W/cm^2 SATA and at a frequency of 1 MHz (da Cunha et al., 2001; Enwemeka, 1989a; Enwemeka et al., 1990; Jackson et al., 1991; Ng et al., 2003; Saini et al., 2002). Less energy is attenuated by the surface tissues when using ultrasound at a lower frequency like 1 MHz, thus allowing the transmission of more energy to deeper tissues like the Achilles tendon (Cambier et al., 2001; Ward and Robertson, 1996). Studies using PUS which revealed no change or a decreased breaking strength in PUS-treated tendons may have used an intensity that was too low to induce therapeutic effects, or used a frequency other than 1 MHz.

As mentioned above, even after 4 weeks of PUS treatment, the UTS and stiffness of the injured tendon were about 77% and 94% respectively compared to the uninjured tendon. The tendon may require more time for further improvement in its mechanical properties. One study investigating the biomechanics of rat tendon healing found that, at the end of 4 weeks, the strength of the healing tendon was about 25% of normal (Steiner, 1982). Other studies using a sheep model to study the spontaneous repair of ruptured Achilles tendons found that, after 3 and 12 months, the total rupture force of the injured tendon was about 75.6% and 81.18% respectively (Bruns et al., 2000). Regardless of the surgical method or model used, these studies showed that a long period of time is required for recovery of the biomechanical properties of an injured tendon.

In the previous histomorphological study (Yeung et al., 2006), the authors tried to process the tissue sections by paraffin embedding methods. The process involved dehydration and could affect the interpretation of the amount of collagen matrix as the degree of dehydration may differ in different processing times or in tissues with different collagen content, which may affect the area ratio. We therefore adopted cryostat sectioning in this analysis, which involved less tissue processing and dehydration, and the results were more reliable.

In clinical situations, especially for elite athletes, the strength of an Achilles tendon after rupture and repair may not return to its original level, hindering the athletes' return to

their original level of activity even after a long period of time (Cetti et al., 1993). This may also explain the recurrence of Achilles tendon ruptures despite previous surgical treatment (Pajala et al., 2002). Furthermore, the period of prolonged rehabilitation may inhibit their training, potentially affecting their performance as well. The results of this study have important clinical implications because it has been suggested that PUS could enhance the recovery of mechanical properties as well as histomorphology in the early phase of healing. Early administration of PUS could promote a faster recovery of the mechanical strength of the injured tendon. However, the question of whether it can promote full recovery of the tendon needs a long follow-up period. Further studies with longer periods of PUS treatment are required to investigate the long-term effect of PUS on the biomechanical properties of the healing tendon.

3.5. Conclusion

It has been shown by the present study that administration of PUS at 1 MHz and 0.5 W/cm^2 SATA intensity can accelerate the healing of surgically transected Achilles tendons in rats, in terms of both biomechanical properties and morphological structure, at 2 and 4 weeks after injury. Studies with longer follow-up periods are needed in order to determine whether ultrasound can promote full recovery of the mechanical properties and morphological structure in a healing tendon.

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CHAPTER 4. THE EFFECT OF THERAPEUTIC PULSED ULTRASOUND ON THE HEALING OF DENERVATED, INJURED TENDONS

4.1. Introduction

Tendons are fibrous tissues that facilitate the stability and motion of joints. Significant dysfunction and disability may result from suboptimal healing of tendon injuries. Tendon healing is a complex process and its initiation, progression and termination are highly regulated by a large number and variety of external and internal factors such as mechanical loading, hormones and growth factors (Aspenberg and Forslund, 1999; Aspenberg and Forslund, 2000; Maffulli et al., 2002). Numerous studies have investigated the effect of mechanical stimuli on tendon healing (Murrell et al., 1994; Palmes et al., 2002). Murrell et al. (1994) found that immobilization of the ankle joints of rats has significant detrimental effects ($p < 0.001$) on the functional and mechanical recovery of Achilles tendon-calcaneal complexes. An experiment on mice found similar results, that postoperative mobilization induced a significantly more rapid restoration of load to failure and stiffness, as well as lower deflection in comparison to the immobilization group (Palmes et al., 2002). Clinically, recent studies suggest that early mobilization or weight bearing promote the functional outcome of patients with Achilles tendon rupture (Kerkhoffs et al., 2002; McComis et al., 1997). Previous studies have also demonstrated that PUS, a kind of mechanical energy generated by vibration of

piezo-electric crystals, could enhance the healing of injured tendons in terms of both the biomechanical properties (Yeung et al., 2006) and collagen synthesis (da Cunha et al., 2001, Yeung et al., 2006).

What are the mechanisms by which the healing process is regulated by mechanical stimuli? Accumulated data have suggested that innervation promotes tissue healing. The importance of innervation in promoting skin, bone and ligament healing has been shown in numerous studies. (Aro et al., 1981; Aro et al., 1985; Ivie et al., 2002; Kim and Pomeranz, 1999; Steinicki et al., 2000).

How the peripheral nervous system contributes to tendon healing remains unclear. Miscellaneous studies have revealed that the vascular responses involved during ligament and tendon healing are mediated by neurotransmitters or neuropeptides secreted by the nerve endings distributed in these connective tissues (Ackermann et al., 2002; Ferrell et al., 1997; McDougall et al., 1997). A neuropeptide, CGRP, was found abundantly within the sensory nerve of the Achilles tendon (Ackermann et al., 1999). CGRP was found to be a potent vasodilator that is important in the regulation of the proliferation of fibroblasts and synoviocytes (Brain et al., 1985; Haegerstrand et al., 1990; Yule and White, 1999). It has been reported that denervation impairs the healing of the rabbit collateral ligament (Ivie et al., 2002). Although tendons and ligaments are grossly similar in structure, a study using a rabbit model demonstrated that ligaments are more metabolically active than tendons

(Amei et al., 1984). Very little is known about the extent to which the healing process of tendons is affected by innervation. In addition, the role of innervation during tendon healing in response to the mechanical stimulus generated by PUS is also unclear. The aim of this study was to investigate the effect of denervation on tendon healing and the effect of PUS on the healing of denervated tendons.

4.2. Methods

Fifty-two male, adult Sprague-Dawley rats (mean body weight 417.9±56.62 g, range 293.8-579 g) were randomly assigned into 2 groups: the sciatic neurectomy with PUS treatment group (SN-PUS), and the sciatic neurectomy control group (SN-control) (Table 4-1). All the rats received a hemi-transection of the right Achilles tendon to mimic a partial tendon rupture (Ng et al., 2003). In addition, a right sciatic neurectomy was also performed. Different groups of rats were euthanized at 2 and 4 weeks after surgery. Ten rats per group served for assessing the biomechanical properties of the healing scar, while the tendons of another 3 rats in each group were harvested for histological analysis.

Table 4-1. Grouping details of animals that received sciatic neurectomy

Group	Surgery on rat right hindlimb	Treatment	Time of sacrifice (weeks post surgery)
SN-PUS	Medial transection of TA and 5 mm resection of sciatic nerve	PUS	2 weeks (n=13) 4 weeks (n=13)
SN-control		Sham PUS	2 weeks (n=13) 4 weeks (n=13)

4.2.1. Injury model

Hemi-transection of the right Achilles tendon was done following protocols described in chapter 3 section 3.2.4.

Sciatic neurectomy was performed through a 1 cm incision in the postero-lateral region on the upper part of the right thigh (Kimmel et al., 1999). The sciatic nerve was isolated from the adjoining tissues through dissection. A 5 mm section of the sciatic nerve was then resected (Figure 4-1).

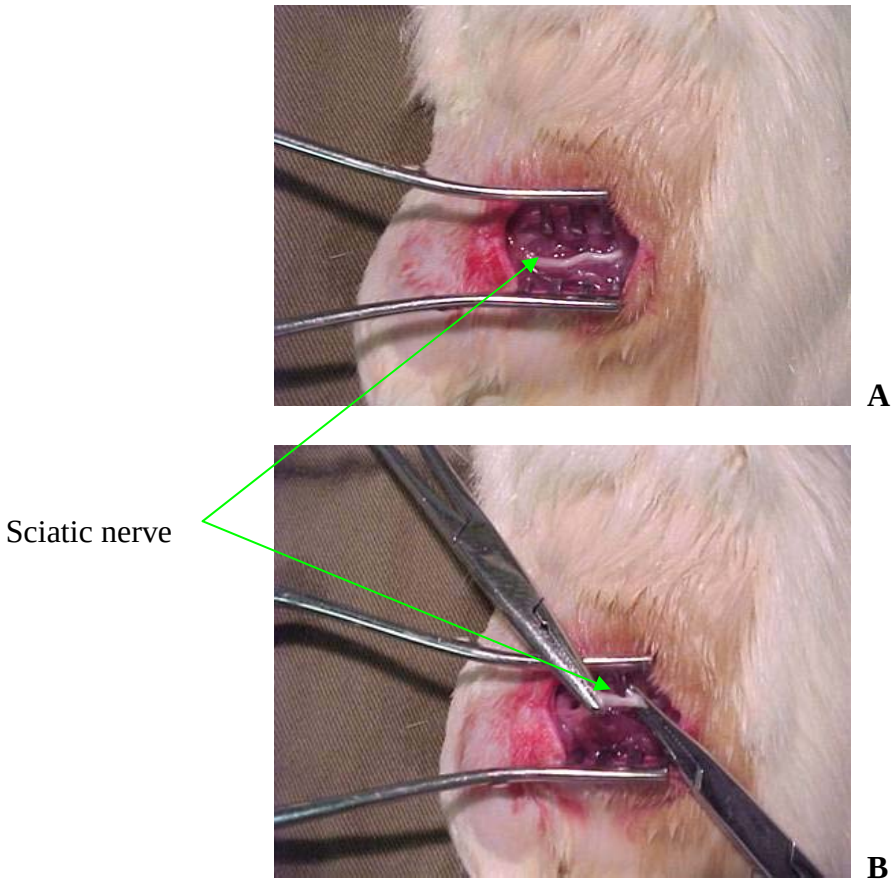

Figure 4-1. Sciatic neurectomy using a postero-lateral approach of rat's thigh (demonstration on left limb). (A) The sciatic nerve is exposed; (B) Resection of 5mm of the sciatic nerve

4.2.2. Ultrasound treatment

All the animals received either true or sham PUS treatment according to their group designation. All the treatment parameters and handling of animals were identical to the protocols presented in chapter 3 section 3.6.3.

4.2.3. Biomechanical testing and histological analysis

For the procedures of biomechanical testing and histological analysis, please refer to chapter 3 section 3.2.6-3.2.7.

4.2.4. Statistical analysis

Two-way ANOVA was used to compare the normalized load-relaxation, stiffness and UTS values, as well as the mean total collagen matrix area, mean cell area to collagen matrix area, and mean fusiform fibroblasts to tendocytes ratio, between the SN-PUS and SN-control groups and across time.

Besides, in order to further investigate the hypothesis that denervation may affect tendon healing and its response to the therapeutic mechanical stimulus from PUS, the data of control from the animals in this study were compared with those of the study in the previous chapter using the independent t-test, while comparisons were made between the two control groups (SN-control and control) and the two PUS groups (SN-PUS and PUS) at 4 weeks postinjury.

The SPSS version 14.0 was used in the data analysis. An α level of 0.05 was set for all statistical comparisons.

4.3. Results

At 2 or 4 weeks postinjury, all the injured tendons showed sign of repair with scar tissue between the ruptured stumps.

4.3.1. Biomechanical test results

4.3.1.1. SN-PUS and SN-Control group

The results of biomechanical tests between the SN-PUS and SN-control groups are shown in Figures 4-2 to 4-4. The mean normalized UTS of the SN-PUS group at 2 and 4 weeks postinjury was 28.3±13.47% and 44.7±15.79% respectively, while that of the SN-control group was 27.1±13.43% and 44.6±16.65% respectively. Significant differences in normalized UTS between groups were not found either at 2 or 4 weeks postinjury between the PUS-treated and control groups ($p>0.05$). However, the UTS of the injured tendons increased with time. The mean normalized UTS was significantly higher in the 4-week groups than in the 2-week groups ($p<0.01$), regardless of the presence of PUS treatment (Figure 4-2).

For normalized stiffness, no significant differences were found either between the different treatment groups or between different time points (SN-PUS 2 weeks: 44.9±33.73% and 4 weeks: 51.6±21.39%; SN-control 2 weeks: 43.8±29.72% and 4 weeks: 54.1±21.31%) ($p>0.05$). There was an increased normalized stiffness from 2 to 4 weeks in both the SN-PUS and SN-control groups. However, the differences were not of statistical significance ($p> 0.05$) (Figure 4-3).

Comparison of the normalized mean load relaxation between the SN-PUS (2 weeks 143.5±27.89% and 4 weeks 135.9±25.32%) and SN-control groups (2 weeks

131.8±46.50% and 4 weeks 131.5±24.52%) showed no significant difference between groups and across time ($p>0.05$) (Figure 4-4).

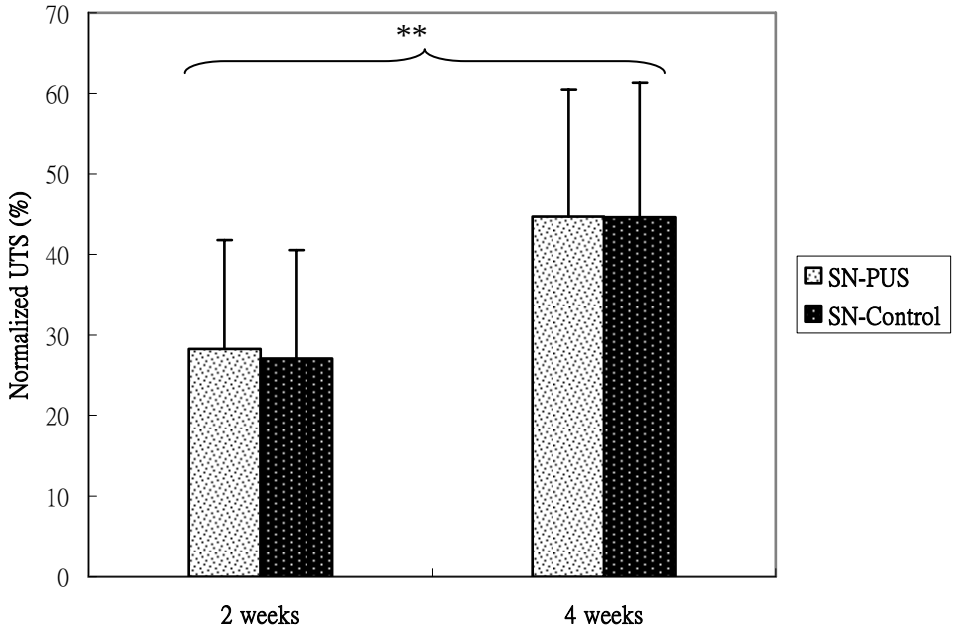

Figure 4-2. Results of normalized UTS values of the SN-PUS and SN-control groups at 2 and 4 weeks postinjury. **p<0.01

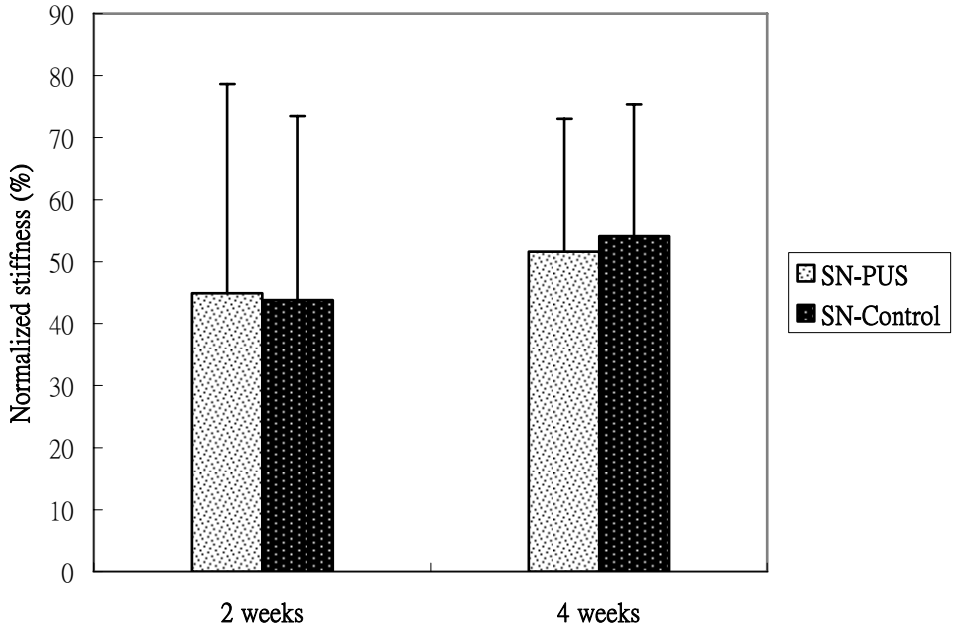

Figure 4-3. Results of normalized stiffness values in the SN-PUS and SN-control groups at 2 and 4 weeks postinjury

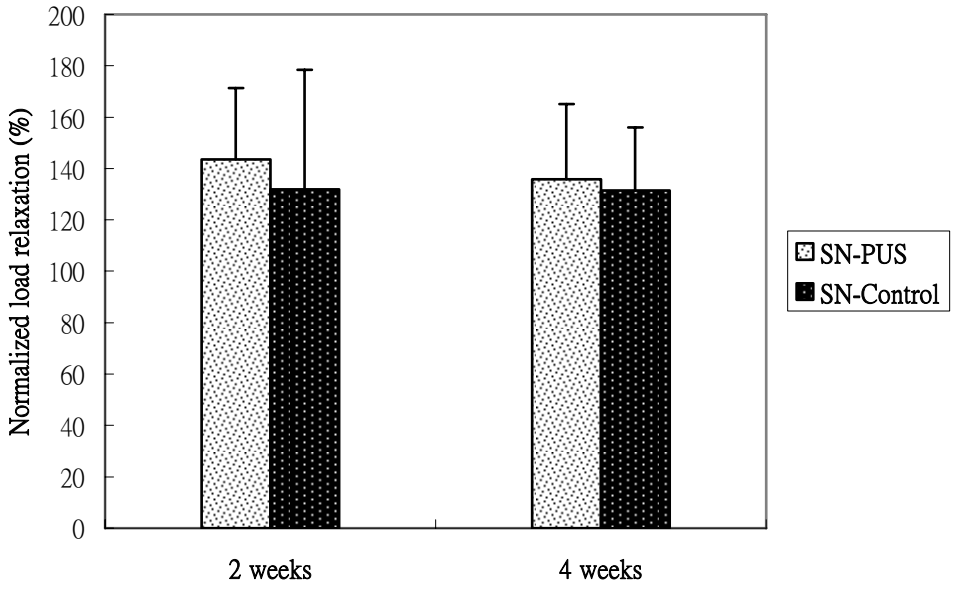

Figure 4-4. Results of normalized load relaxation values in the SN-PUS and SN-control groups at 2 and 4 weeks postinjury

4.3.1.2. SN-control and Control

When comparison on the mechanical testing results was made between the two sham-treated control groups (SN-control and control (from the previous chapter)), no statistical difference was found in normalized UTS, stiffness and load relaxation at 4 weeks postinjury. However, when we compare the UTS (in N) of the injured tendon between the two control groups, the control group had significantly higher values in UTS of injured tendon at 4 weeks postinjury ($p<0.05$) (Figures 4-5).

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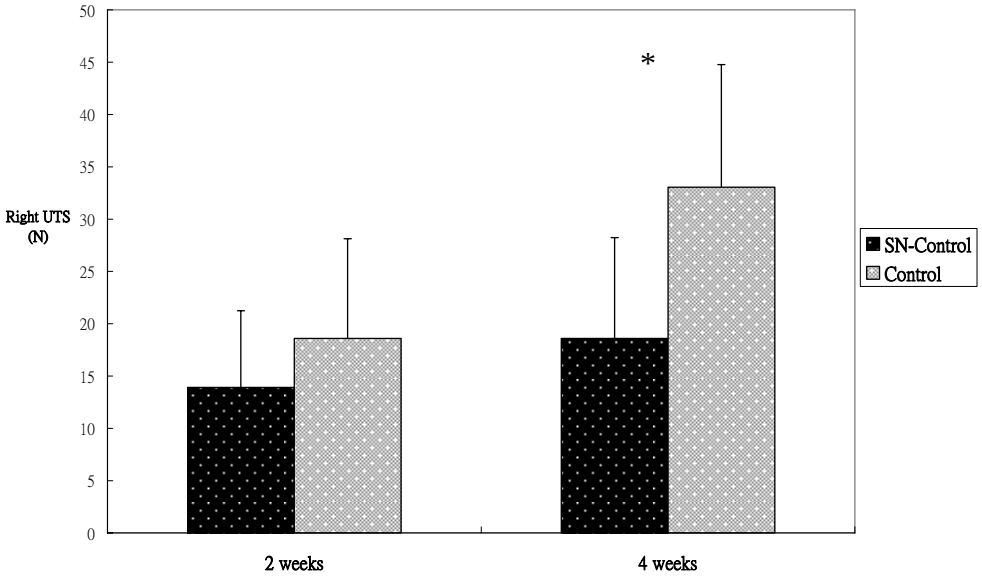

Figure 4-5. The UTS values of the injured tendon in the SN-control and Control groups at 4 weeks postinjury. *p<0.05

4.3.2. Histological analysis results

4.3.2.1. SN-PUS and SN-control

Two weeks postinjury, the scar of the tendons in both groups contained granulated tissue with a high density of irregularly-shaped fibroblasts. The fibroblasts appeared larger in size and more irregular in shape in the SN-PUS group. The ratio between fusiform fibroblasts and tendocytes in the SN-PUS group was 1.98 ± 0.652, while that in the SN-control group was 2.32 ± 1.175. There was no significant difference between the 2 groups. The fibroblast to collagen matrix ratio in the specimens from the SN-PUS group (0.329 ± 0.067) was slightly higher than that of the SN-control group (0.297 ± 0.103), but the difference was not of statistical significance ($p>0.05$). The collagen fiber bundles in the tendons of both groups were aligned irregularly, with lot of cells infiltrated (Figure 4-6).

There was no significant difference in the mean total area occupied by the collagen matrix between the SN-PUS group (16482.2±2060.50 μm^2) and the SN-control group (16278.3±1229.55 μm^2) ($p>0.05$).

At 4 weeks postinjury, the scar appeared more mature in both groups, with a slightly lower fibroblast to matrix ratio, a denser collagen matrix, and relatively more regularly aligned collagen fiber bundles when compared to the 2-week groups, and the alignment of collagen fiber bundles now appeared better in the SN-PUS group (Figure 4-7). In the SN-PUS group, the fusiform fibroblasts to flattened fibroblasts ratio was 2.54 ± 1.175, not significantly different from that of the SN-control group (2.73 ± 1.148) ($p>0.05$). In both groups, the fusiform fibroblasts to tendocytes ratio increased slightly from 2 to 4 weeks ($p>0.05$), which meant that there was a slightly higher proportion of fusiform fibroblasts at 4 weeks postinjury. The fibroblast to collagen matrix ratio was similar between the two groups (0.17 ± 0.108 in the SN-PUS group, 0.17 ± 0.098 in the SN-control group, $p>0.05$). When compared with specimens in the 2-week groups, the fibroblast to collagen matrix ratio of specimens in the 4-week groups was higher ($p<0.01$), regardless of the presence of PUS treatment. For the total collagen matrix area, the area in the SN-PUS group (17791.8±1299.40 μm^2) was similar to that of the SN-control group (17913.9±1270.07 μm^2) ($p>0.05$) at 4 weeks postinjury. No significant difference was found between the 2- and 4-week groups ($p>0.05$). The results of histological analysis are summarized in

Figures 4-8 to 4-10.

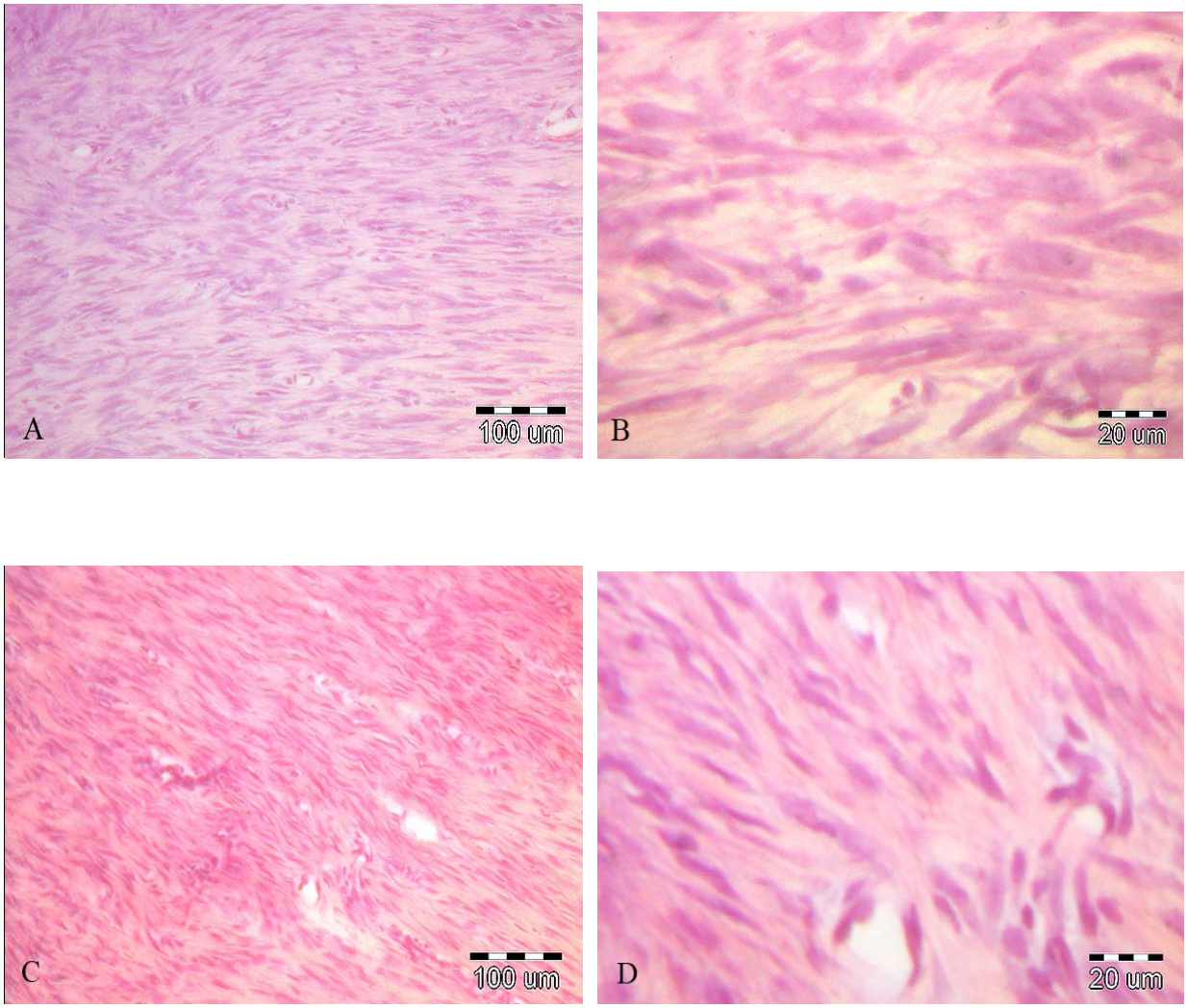

Figure 4-6. Histology of the Achilles tendons of rats in the SN-PUS group (A: 100x; B: 400x) and the SN-control group (C: 100x; D: 400x) at 2 weeks post-partial tenotomy and sciatic neurectomy

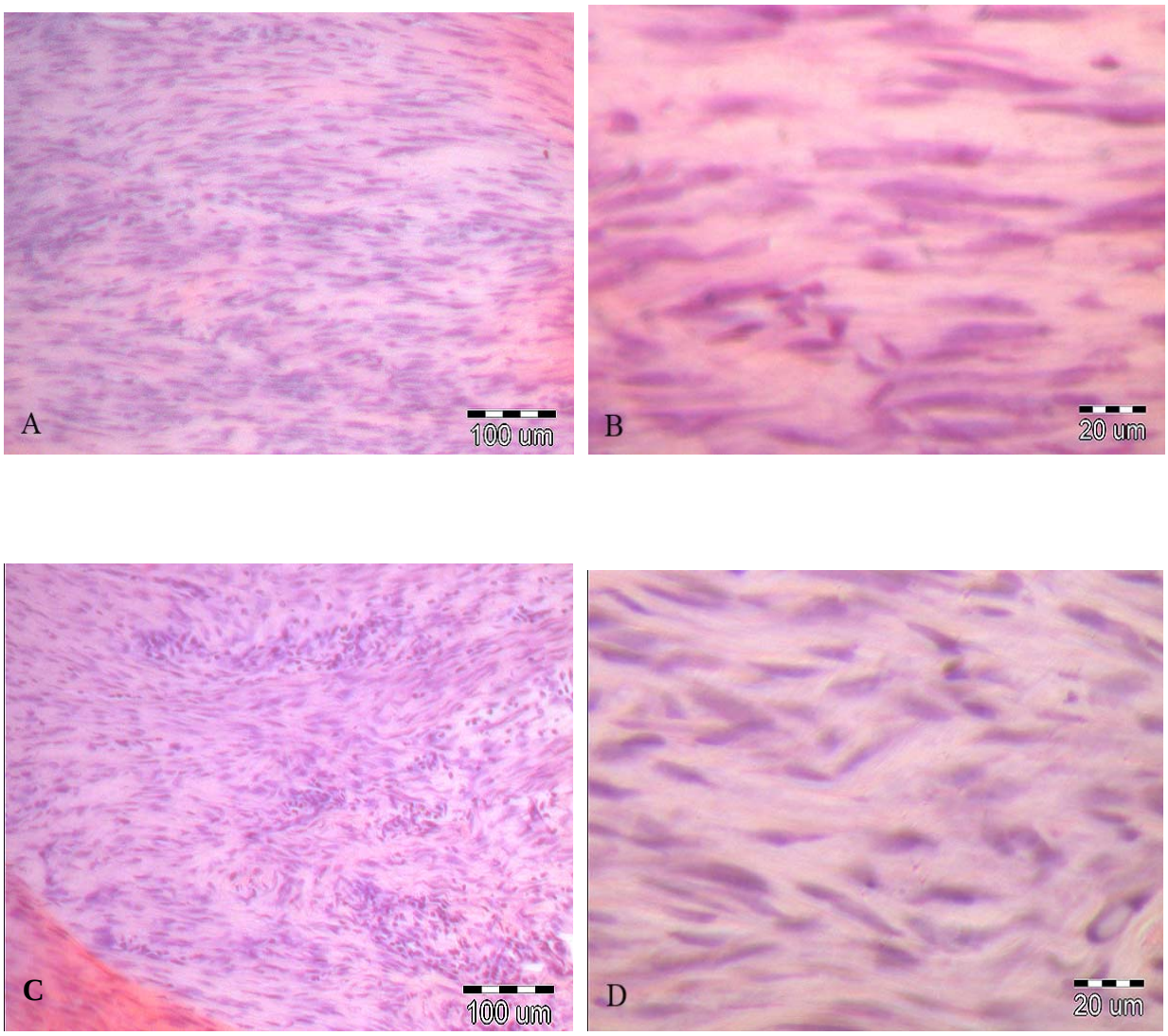

Figure 4-7. Histology of the Achilles tendon of rats in the SN-PUS group (A: 100x; B: 400x) and the SN-control group (C: 100x; D: 400x) at 4 weeks post-partial tenotomy and sciatic neurectomy

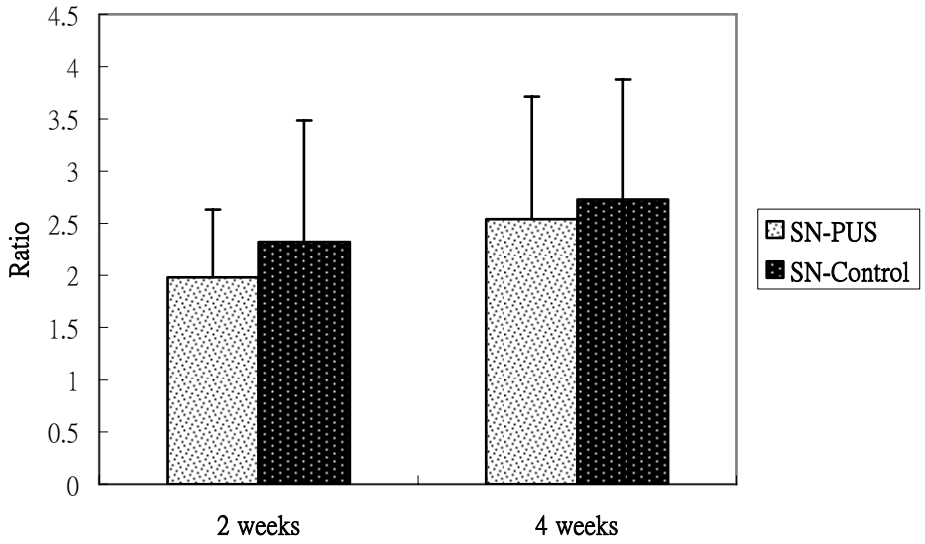

Figure 4-8. Results of mean fusiform fibroblasts to tendocytes ratio in the SN-PUS and SN-control groups at 2 and 4 weeks postinjury

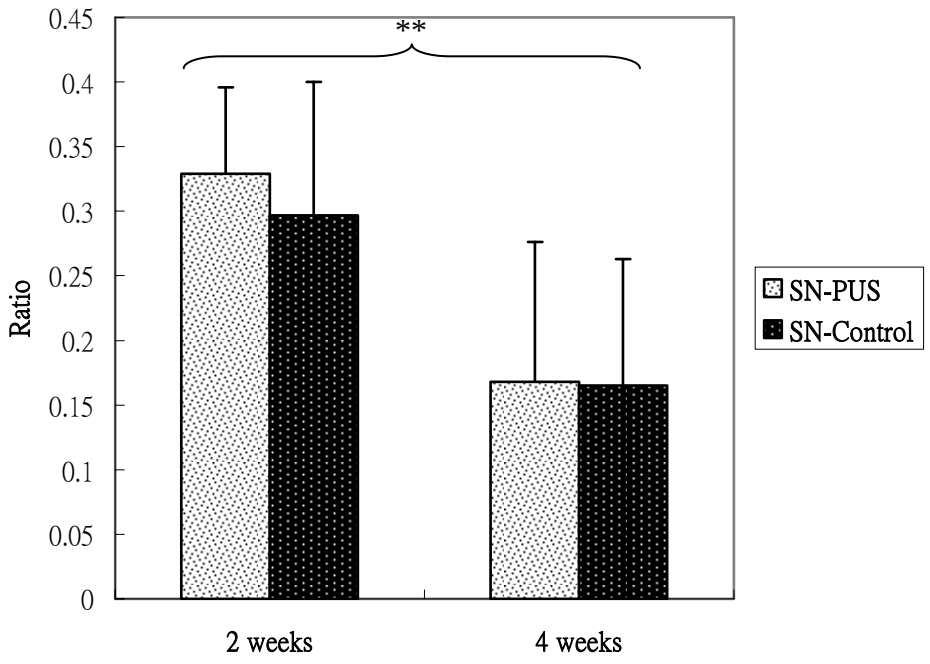

Figure 4-9. Results of mean fibroblasts to collagen matrix area ratio in the SN-PUS and SN-control groups at 2 and 4 weeks postinjury. **p<0.01

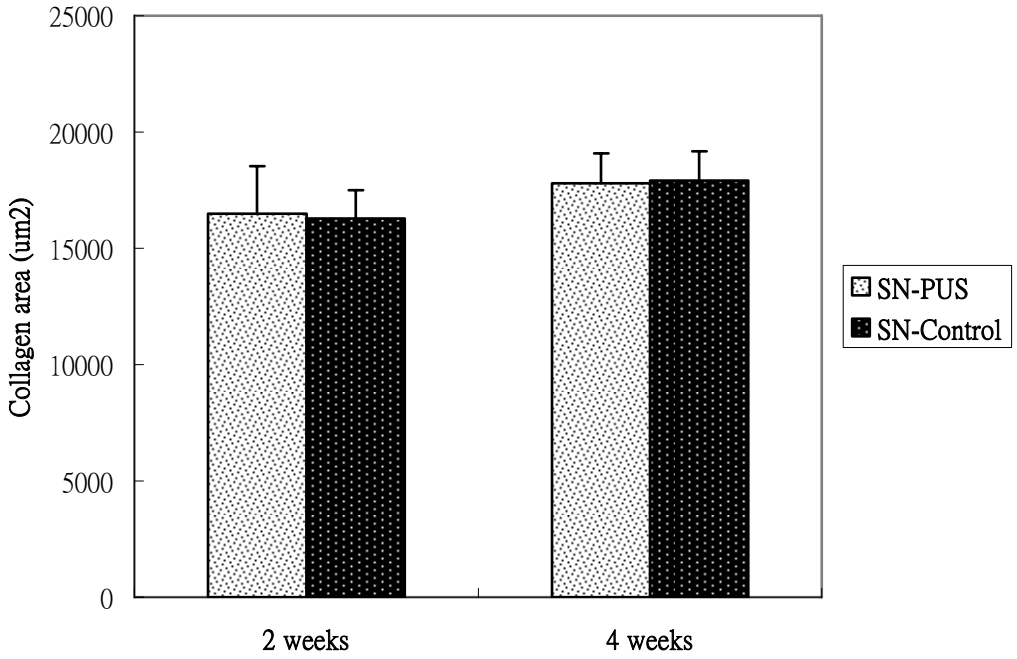

Figure 4-10. Results of mean total collagen matrix area in μm^2 in the SN-PUS and SN-control groups at 2 and 4 weeks postinjury

4.3.2.2. SN-control and Control

When comparison on the histological analysis was made between the two sham-treated control groups (SN-control and control), no statistical difference was found in fibroblast to tendocytes ratio and fibroblast to collagen matrix ratio. However, we found that the control group had significantly higher values in the total collagen matrix area, both at 2 ($p<0.05$) and 4 weeks postinjury ($p<0.01$) (Figures 4-11).

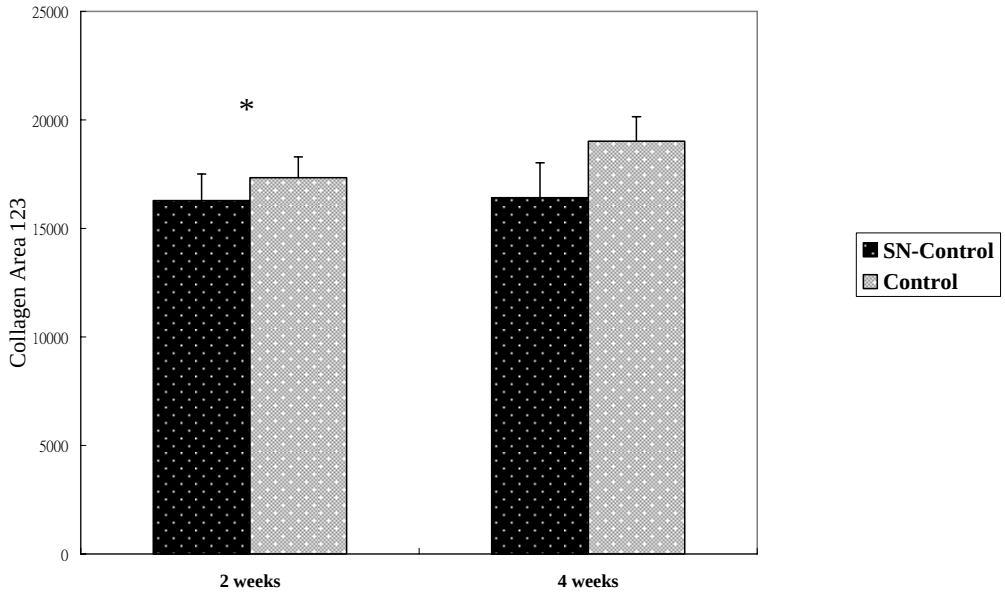

Figure 4-11. Results of mean total collagen matrix area in µm2 in the SN-contol and Control groups at 2 and 4 weeks postinjury. *p<0.05; **p<0.01

4.4. Discussion

The results of biomechanical testing and histological analysis of the present study were largely different from the findings of the previous study discussed in chapter 3, which studied the effect of PUS on the healing of neural intact tendons. By comparing the results of the two studies, we could probably find out the effect of denervation on tendon healing and the effect of PUS on the healing of denervated tendons.

4.4.1. Effect of denervation on tendon healing

The present study found that only the mean normalized UTS of the 4-week groups was significantly higher than that of the 2-week groups. However, the difference in stiffness between the 4-week and the 2-week groups was insignificant. Stiffness is a measurement of structural properties of large size and major load-bearing collagen fibrils in a repairing tendon under submaximal loading (Parry, 1988). It is also related to the proportion of different types of collagen fiber present (Robinson et al., 2004). This may imply that the differences in collagen types of scar tissues between the 2- and 4-week groups were small, and thus the maturation of scar tissue was relatively slow as compared to the neural-intact healing tendon, which showed a significant improvement in normalized stiffness from 2 to 4 weeks postinjury (Yeung et al., 2006). Further study on the collagen types of healing denervated tendons may be required to determine the

possible effect of denervation on tendon healing.

As mentioned, the mean normalized UTS value of the 4-week groups was significantly higher than that of the 2-week groups. The breaking strength of tendons depends on the percentage of collagen matrix in the tissue (Parry, 1988). The histological findings in this study agreed with the results of UTS, as the mean cell to collagen matrix ratio of the 4-week groups was significantly higher, possibly leading to an increase in UTS. However, the recovery of UTS and stiffness seemed to be slower than normal healing tendons, even when PUS was applied. At 4 weeks, the percentage recovery of UTS and stiffness of the SN-control group were 45% and 54%, slightly lower than in the healing, neural-intact tendons (the control group), which had percentage recovery of UTS and stiffness of about 54% and 65%, although no significant difference was found. However, when we perform addition comparison, using only the injured leg UTS, we could find that the mean UTS value of the control group was significantly higher than that of SN-control group, which is also supported by the results that the collagen matrix area was higher in control group when compare with SN-control. These results may imply that denervation, to a certain extent, could affect the recovery of the UTS and stiffness of healing tendons.

Evidence has shown the importance of innervation in promoting wound, bone and ligament healing (Aro, 1985; Ivie et al., 2002; Lusthaus et al., 1993; Madsen et al., 1998). Wounds in rabbits were shown to have lower mechanical strength and collagen content

after denervation (Lusthaus et al., 1993). Madsen et al. (1998) also reported that denervation induced a large but mechanically insufficient fracture callus. They suggested that nerve injury may result in defects in tissue composition or organization. Ivie et al. (2002) have demonstrated that the ultimate force at failure and average blood flow of the healing medial collateral ligament were both significantly decreased after femoral nerve transection. They reported that denervation decreased the UTS of the healing ligament by half.

Similar to our study, one recent study reported that denervation caused a reduction in the load to failure of the Achilles tendon by 50%, 2 weeks post-total transection (Aspenberg and Forslund, 2000). They suggested that the inhibition of muscle contraction could be one of the factors affecting the recovery of tendon strength. Their finding was further explained by Ackermann et al. (2002), who stated that following denervation, the loss of rapidly acting neurotransmitters with short-term effects in the peripheral nervous system may lead to muscle inactivity and thereby decreased tensile strength of the tendon. It has also been reported that decreased mechanical stimulus is associated with decreased synthesis of extracellular matrix proteins (Giori et al., 1993; Woo et al., 1982). Therefore, it is possible that the loss of mechanical stimulus after denervation may affect the recovery of injured tendons.

Another role of the peripheral nervous system in tissue healing which has been

recognized recently is its role in carrying slowly acting neuropeptides with long-term effects on vasoactivity, cell proliferation and synthesis of growth factors (Ackermann et al., 2001). Different neuropeptides have been shown to be involved in various cellular processes. CGRP is one of these neuropeptides, which is mainly found in the sensory nerve (Schäffer, 1998), include those in tendons (Ackermann et al., 1999; Ackermann et al., 2000). This neuropeptide is known to be a potent vasodilator and participates in the regulation of angiogenesis, and the proliferation of fibroblasts and synoviocytes (Brain et al., 1985; Haegerstrand et al., 1990; Schäffer et al., 1998). One immunochemical study of the regeneration of nerve fibers in the ruptured rat's Achilles tendon showed early in-growth of new sensory nerve fibers expressing CGRP immuno-reactivity as early as 1 week postinjury, corresponding to the inflammatory stage of the healing tendon (Ackermann et al., 2002). This increased immunoreactivity to CGRP was found mostly located in blood vessel walls surrounded by inflammatory cells, which complies with the proinflammatory role of CGRP (Ackermann et al., 2003). During the proliferative to regenerative phases (weeks 2-6), CGRP-expressing nerve fibers peaked in the rupture site of the proper tendinous tissue as well as the free nerve endings among fibroblasts in the tendinous tissue, which may indicate a stimulatory role of these neuropeptides on fibroblasts (Ackermann et al., 2003; Yule and White, 1999). This evidence shows that the in-growth of nerves provided a delivery system for sensory neuropeptides which may be

required for tissue repair. Early nerve regeneration is necessary for normal neovascularization and proliferation of different cell types in tendon repair. Thus denervation might inhibit these favorable cellular responses, in turn affecting the normal spontaneous healing of innervated tendons. Histologically, scar tissue in the denervated tendon contained cells which were more irregular in shape and grounded in collagen fiber bundles that were less organized. Besides, the fusiformed fibroblast to tenocytes ratio also did not change significantly across time. These findings may suggest that the differentiation of cells at different stages of healing was prohibited by denervation.

4.4.2. Effect of PUS on healing of denervated tendon.

As a form of mechanical stimulation, ultrasound could increase proteoglycan synthesis in bovine primary chondrocytes (Kopakkala-Tani et al., 2006). Its osteogenic effect could also enhance the remodeling of bone, which remodels in response to applied mechanical stimuli, like Low Intensity Pulsed Ultrasound (LIPU) (Nolte et al., 2001; Warden et al., 2000). Previous study has shown that PUS could improve the healing of partially ruptured tendons, indicating that PUS is also an effective stimulation to promote tendon healing (Yeung et al., 2006 and chapter 3). If PUS could promote the healing of neural-intact tendons, it may be possible for this effect to occur in denervated tendons. However, the results of this study showed that there was no significant difference in the

biomechanical performance or histo-morphological appearance of healing, denervated tendons that have received either PUS or sham PUS treatment. We recall from the previous section (4.4.1) that the loss of mechanical stimulus following denervation may cause deterioration in the strength of the healing tendon. However, even under the mechanical stimulus generated by PUS treatment, the strength of the denervated tendon was similar to that of the denervated tendon that received sham treatment. As the proximal supply of neuropeptides was cut due to denervation, the healing of injured tissue and the expression of neuropeptides was probably affected. Therefore, possible explanations for this phenomenon are that the tissue may lack some vital components, like neuropeptides, for normal healing and thus PUS has no effect on healing, or there may be some relationships between PUS treatment and the expression or regeneration of the neural components of healing tendons. If this relationship exists, it is reasonable to hypothesize that ultrasound may enhance the expression of neuropeptides vital for promoting tendon healing and thus lead to faster recovery of the healing tendon.

Similar to study one, there is one limitation on the histological analysis: the counting of different form of fibroblasts may subject to error due to the random orientation of fibroblasts in the tissue sections (Amiel et al., 1984), although careful inspection on the orientation of specimen during sectioning as well as persistent longitudinal orientation of collagen fiber bundles was done before counting. Further study using special staining

method, like immunochemical technique using proliferating cell nuclear antigen on the newly formed fibroblast may help to differentiate the fibroblast types.

In chapter 5, an immunohistochemical study was carried out to investigate the effect of ultrasound on the expression of CGRP in innervated and denervated tendons, in order to examine the relationship between the effect of ultrasound and nerve regeneration in the healing tendon.

4.5. Conclusion

The present study shows that denervation affected the healing of the injured tendon at 2 and 4 weeks postinjury. Besides, PUS treatment, which was shown to be effective in promoting tendon healing, made less of a contribution to the recovery of the denervated tendon in terms of its healing after partial injury. Further study on the relationship between neural components and PUS treatment may be required in order to explain this phenomenon.

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CHAPTER 5. IMMUNOHISTOLOGICAL STUDY OF THE EFFECT OF THERAPEUTIC PULSED ULTRASOUND ON SENSORY NERVE REGENERATION DURING ACHILLES TENDON HEALING

5.1. Introduction

CGRP is a sensory neuropeptide synthesized in the dorsal root ganglion, transported peripherally and costored in large electron-dense storage vesicles, the accumulation of which constitutes varicosal enlargements of the sensory axon. The release of CGRP from the nerve terminal was first demonstrated by Zaidi et al. (1985). Since then, much interest has been devoted to the efferent effects of CGRP-containing fibers.

Ackermann et al. (1999) demonstrated with immunohistochemistry and radioimmunoassay the presence of nerve fibers immunoreactive to SP, CGRP, neurokinin A, GAL and somatostatin in the Achilles tendon. Recent immunohistochemical studies have shown that tendon healing is accompanied by an invasion of nerve fibers containing sensory neuropeptides including CGRP (Ackermann et al., 2002; Ackermann et al., 2003; Bjur et al., 2005). The function of CGRP has been investigated extensively (Bolton and Clapp, 1986; Brain et al., 1985; Burssens et al., 2005; Haegerstrand et al., 1990; Nilsson et al., 1985; Yule and White, 1999). It has been suggested that the CGRP participate in the regulation of inflammation as well as fibroblast proliferation during wound healing, since their depletion impairs wound healing (Khalil and Helme, 1996; Smith, 2002). Besides skin wounds, CGRP has also been found to express intensively in the early phase of bone

and ligament healing (Cruise et al., 2004; Hukkanen et al., 1993; McDougall et al., 2000).

CGRP has been found to express intensively during the inflammatory, proliferative and remodeling phase after tendon rupture, with the peak occurrence in the inflammatory to proliferative phases (Ackermann et al., 2003). Increased expression of CGRP and SP has also been found in association with tendinosis (Sharma and Maffulli, 2005). Several experiments have demonstrated that CGRP has trophic and vasodilatory effects (Brain et al., 1985; Haegerstrand et al., 1990; Yule and White, 1999). It has been found recently that CGRP positive neural fibers were unmyelinated (C type) and small myelinated (A-delta type) primary sensory neurons which perceive pain and mechanical strain or stress (Brouns et al., 2006). This finding suggests an important pathway through which mechanical stimuli can regulate bone formation and remodeling in fracture healing.

PUS has been found to have an effect on promoting tendon healing (da Cunha et al., 2001; Yeung et al., 2006). The exact mechanism is still not well understood. Ultrasound is a form of mechanical energy that is transmitted through and into biological tissues as an acoustic pressure wave at frequencies above the limit of human hearing. The results of our previous study (Chapter 4) have also shown that denervation impairs not only tendon healing, but also the reactivity of tendons to PUS stimulation. Taken together, these findings imply that the innervation of CGRP positive fibers and the level of CGRP expression are important factors in the reactivity of a tendon to PUS stimulation.

The aim of the present study was to determine the relationship between (1) the level of expression of CGRP and PUS stimulation, and (2) the level of CGRP in the rupture area and the healing tendon.

5.2. Methods

Twenty-four male, adult Sprague-Dawley rats (mean body weight 335.5±71.09 g, range 252-461.3 g) were randomly assigned into 4 groups: the sciatic neurectomy with PUS treatment group (SN-PUS), the sciatic neurectomy with sham PUS treatment (SN-control) group, and the patella tenotomy with PUS treatment (PUS) and patella tenotomy with sham PUS treatment (control) groups. All the rats received a hemi-transection of the right Achilles tendon. The details of the surgery and ultrasound are the same as described in previous chapters (Chapters 3: 3.2.4-3.2.5 and 4: 4.2.1.1). The details of the groupings are summarized in Table 5-1.

Table 5-1: Details of the groupings of animals

Group	Surgery on right hindlimb	Treatment	Time of sacrifice (weeks post-surgery)
SN-PUS	Medial transection of TA and 5 mm resection of sciatic nerve	PUS	2 weeks (n=3) 4 weeks (n=3)
SN-control		Sham PUS	2 weeks (n=3) 4 weeks (n=3)
PUS	Medical transection of TA and 5 mm resection of patella tendon	PUS	2 weeks (n=3) 4 weeks (n=3)
Control		Sham PUS	2 weeks (n=3) 4 weeks (n=3)

5.2.1. Immunohistochemistry

All the rats were anaesthetized by intra-peritoneal injection of choral hydrate (1 ml/100 g) before euthanasia. Intra-arterial perfusion with PBS was performed, followed by perfusion with 4% phosphate-buffered paraformaldehyde, via cannulation of the ascending aorta through the left cardiac ventricle (Hukkanen et al., 1995). Next, the bilateral Achilles tendons (medial portion) were dissected and immersed in 4% phosphate-buffered paraformaldehyde for 2 hours at room temperature. All specimens were rinsed in PBS and then soaked for at least two days in 20% sucrose solution, pH 7.4 (Sigma Chemicals, St. Louis). The tissues were then mounted in OCT solution and cut on a Leitz cryostat to a section thickness of 15μm. The frozen sections were mounted directly on SuperFrost/Plus glass slides and stored in a freezer at -80 ºC before staining.

Immunostaining methods were based on indirect immunofluorescence labeling. The sections were left at room temperature for about 15-20 minutes before staining. They were then immersed and rinsed for 2 x 5 min in PBS. The sections were then incubated with 10% normal bovine serum (Santa Cruz Biotechnology, USA) in PBS and BSA (0.1g/ml) for 60 minutes at 37ºC, in order to block the non-specific bindings. Subsequently, sections were incubated with goat polyclonal primary antiserum for CGRP (1:250; Santa Cruz Biotechnology, USA) overnight in a humid atmosphere at 4 ºC. After incubation with the primary antiserum, the sections were rinsed in PBS 3 x 5 minutes. The sections were then

incubated with bovine anti-goat IgG-FITC (Santa Cruz Biotechnology, USA) for 1 hour at 37ºC. Afterwards, the sections were rinsed for 3 x 1 minute in water and then mounted with 90% glycerol in PBS with the cover slip on top. To demonstrate the specificity of the staining, controls were included with the omission of the primary antiserum, which was replaced by PBS. A Nikon epifluorescence microscope (Eclipse 80i Yokohama, Japan) was used to analyze the sections. A Spot Flex^{TM} Shifting Pixel color digital camera (Diagnostic Instruments Inc, USA) was used for photography.

5.2.1.1. Semi-quantitative analysis

Two sections from different levels (dorsal and ventral) of the injured tendon from each of the specimens were selected and stained, so that a total of 24 sections from the 2-week groups and 24 sections from the 4-week groups were stained. Staining was carried out simultaneously for all sections. Each section was assessed with a fluorescence microscope at low power (40x) immediate after the staining. A photo was taken of each section of the healing area around the rupture site (mid third of the tendon). Each slide was assessed and the area of positively stained nerve fibers was measured using software analysis® 3.2. A threshold of fluorescence intensity was set as a reference value. Only nerve fibers with fluorescence intensity above the reference value were counted. The results were expressed as the percentage of the fractional area occupied by CGRP positive

nerve fibers in relation to the total area of the tendon in each image. The percentage of each group at each time point (2 or 4 weeks) was averaged from six images of tendons harvested from three rats. There were 48 images in total for semi-quantitative analysis of innervation.

5.2.2. Statistical analysis

The mean percentage of fluorescent to total area was calculated for each group at 2 or 4 weeks postinjury. Statistical analysis was performed using non-parametric comparisons of the four groups at each time point by the Kruskal-Wallis one–way ANOVA. If the Krukal-Wallis test showed statistical significance, a further comparison between 3 pairs of groups (the SN-PUS and SN-control, PUS and control, SN-control and control) was conducted to test the hypothesis. Bonferroni correction of the p value ($p= 0.05/3=0.0167$) was used to protect from type I errors (Munro, 2005). Statistical significance was considered to be found when $p \leq 0.0167$.

5.3. Results

5.3.1. Two weeks postinjury

Generally, the occurrence of positive nerve fibers in the SN-PUS and SN-control groups was obviously less than in the PUS and control groups. In both the SN-PUS and SN-control groups, miscellaneous CGRP positive fibers could be seen near the peripheral part of the tendon tissue, but few were found inside the proper tendinous tissue or near the blood vessels (Figures 5-1 to 5-2). On the other hand, positive CGRP nerve fibers were shown abundantly in the proper tendinous tissue as free sprouting nerve fibers in the PUS group, and were relatively denser when compare to those in the control group (Figures 5-3 and 5-4). The results of Kruskal-Wallis one-way ANOVA on the fraction of area (%) occupied by CGRP immunoreactive nerve fibers showed a significant difference among groups. Post-hoc analysis showed that the mean fraction of area occupied by CGRP immunoreactive nerve fibers in the PUS group was significantly higher than that in the control group ($p=0.009$). In addition, the same fraction of area in the control group was also higher than that in the SN-control group ($p=0.002$). However, there was no significant difference between the SN-PUS and SN-control groups on the mean fraction of area occupied by CGRP immunoreactive nerve fibers ($p>0.0167$).

5.3.2. Four weeks postinjury

At 4 weeks, a slight increase was found in the neuronal immunoreactivity in the SN-PUS and SN-control groups, especially inside the proper tendinous tissue. The SN-PUS group showed a relatively higher density of positive nerve fibers (Figures 5-5 to 5-6). On the other hand, there was a marked decrease in CGRP positive nerve fibers in the proper tendinous tissue of the PUS and control groups, whereas the control group showed a slightly higher density of CGRP positive fibers. In both groups, some of the nerve fibers tended to shift to the peripheral region of the tendon, near the paratendon and surrounding loose connective tissues (Figures 5-7 to 5-8). Results of Kruskal-Wallis one-way ANOVA on the mean fraction of area occupied by CGRP positive fibers showed no significant difference among the groups. The results of the mean fraction of area occupied by CGRP positive fibers are shown in Figure 5-9.

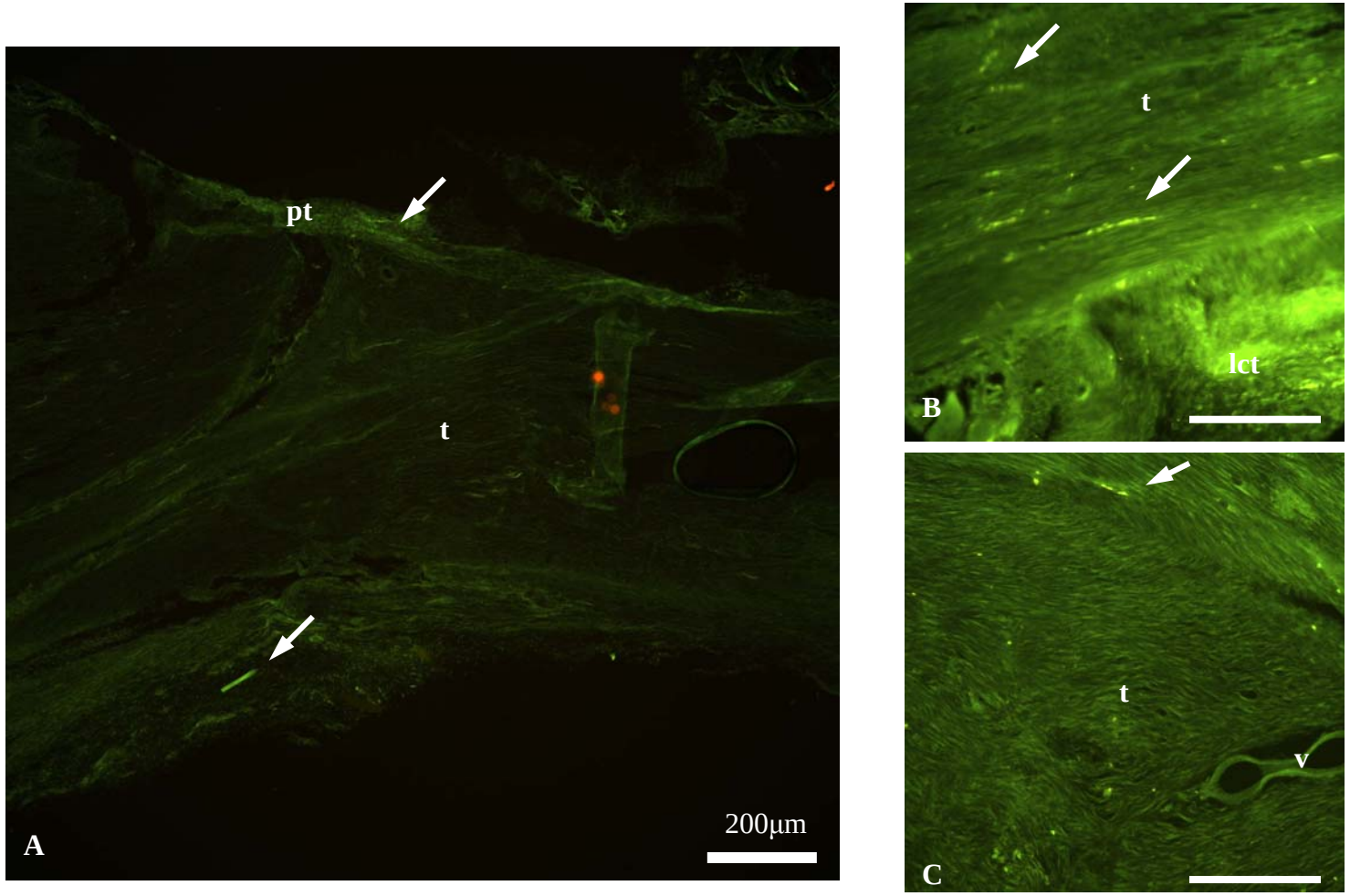

Figure 5-1. Immunofluorescence staining of the healing scar of tendon specimen from the SN-PUS group at 2 weeks postinjury. A: low power (4x). B and C: High power (20x). Close analysis found immunoreactive nerve fibers occurred near the peripheral part of tendinous tissue. Arrows denote positive nerve fibers; lct = loose connective tissue; pt = paratendon; t = tendinous tissue; v = blood vessels. Bar=200μm

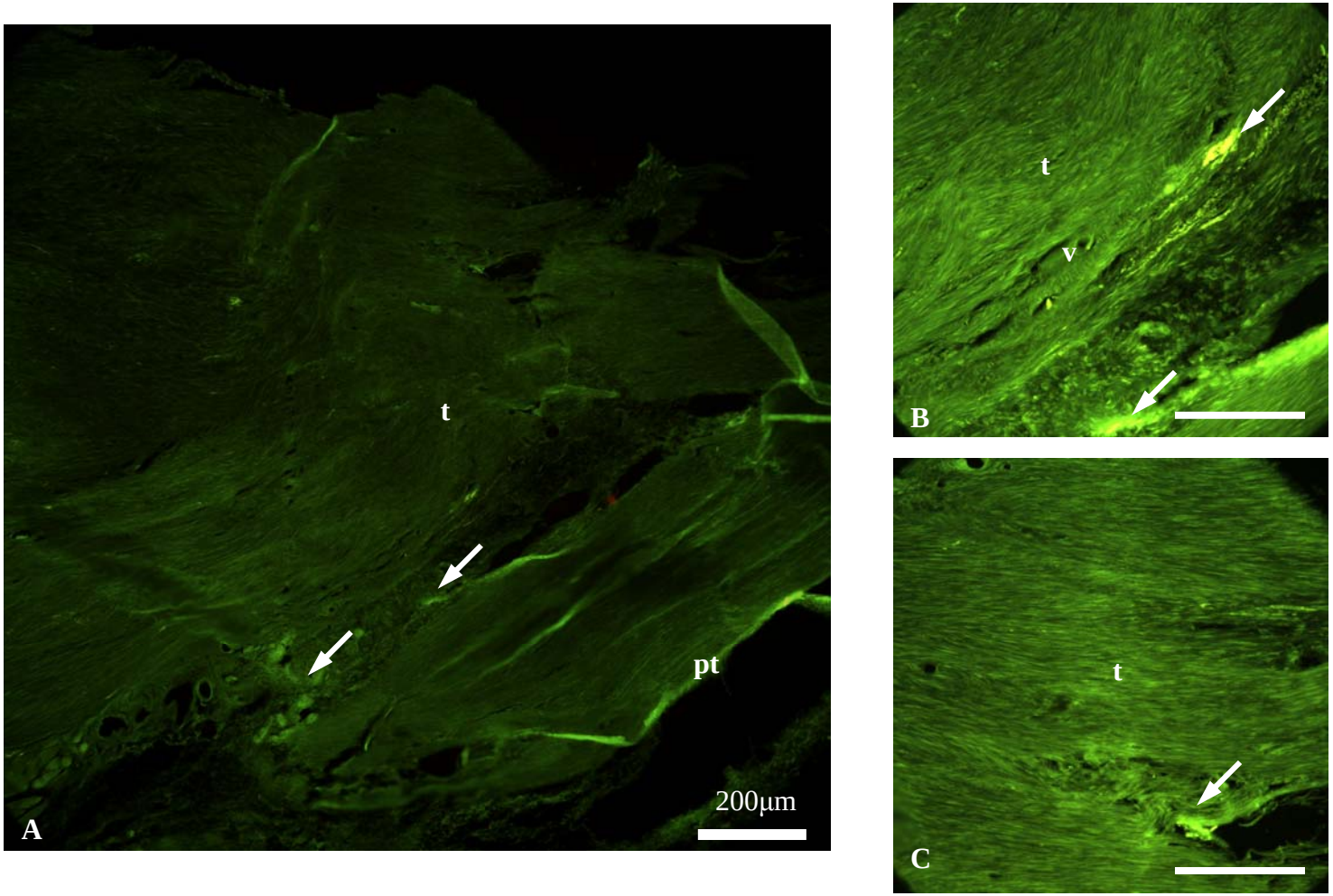

Figure 5-2. Immunofluorescence staining of the healing scar of tendon specimen from the SN-control group at 2 weeks postinjury. A: low power (4x). B and C: High power (20x). Similar to SN+PUS group, immunoreactive nerve fibers occurred near the peripheral part of tendinous tissue. Arrows denote positive nerve fibers; pt = paratendon; t = tendinous tissue; v = blood vessels. Bar=200µm

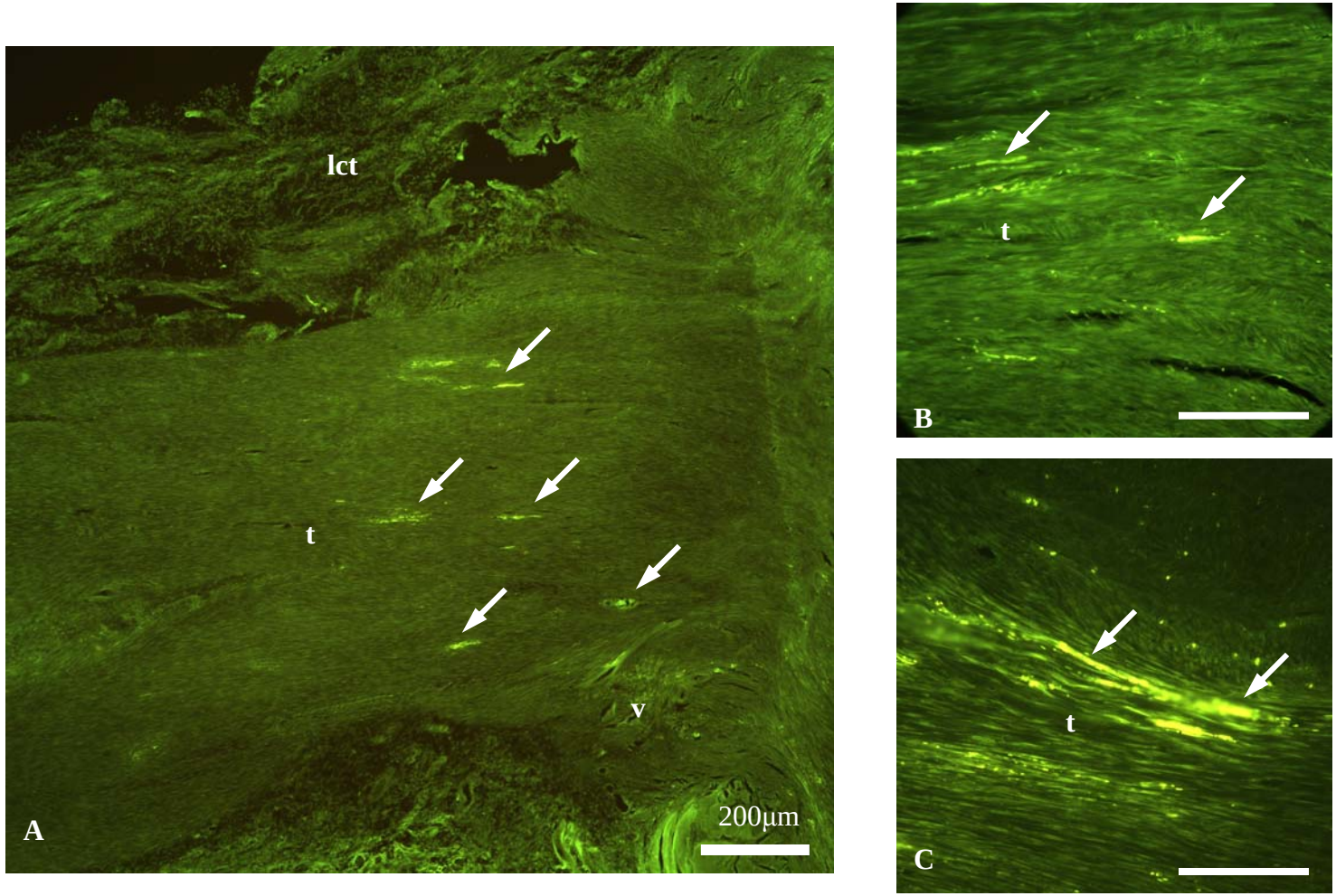

Figure 5-3. Immunofluorescence staining of the healing scar of tendon specimen from the PUS group at 2 weeks postinjury. A: low power (4x). B and C: High power (20x). Abundant of positive nerve fibers were found within the tendinous tissue. Arrows denote positive nerve fibers; lct = loose connective tissue; t = tendinous tissue; v = blood vessels. Bar=200µm

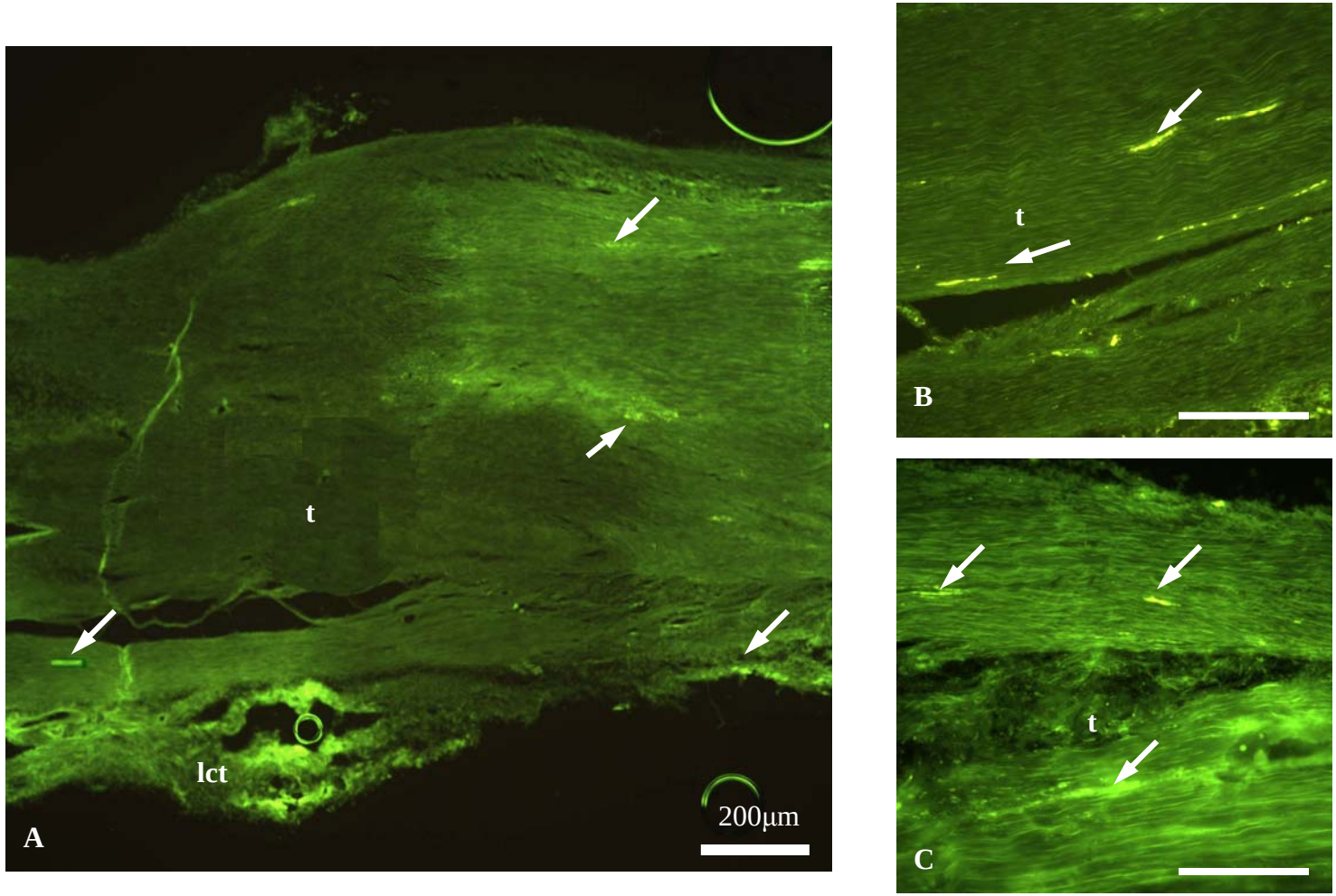

Figure 5-4. Immunofluorescence staining of the healing scar of tendon specimen from the control group at 2 weeks postinjury. A: low power (4x). B and C: High power (20x). A few positive nerve fibers were found invading the tendinous tissue and near the peripheral part of tendon. Arrows denote positive nerve fibers; lct = loose connective tissue; t = tendinous tissue. Bar=200µm

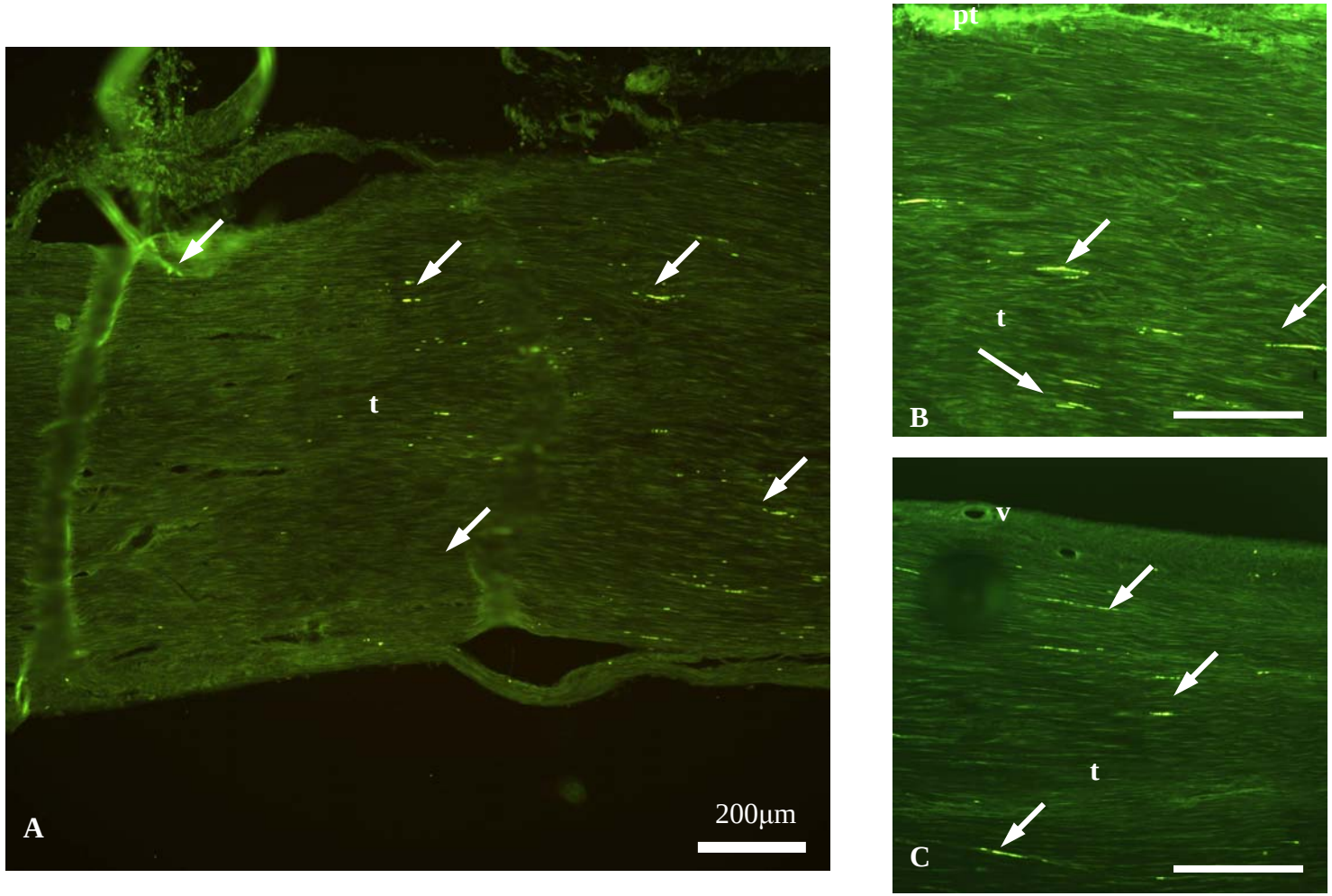

Figure 5-5. Immunofluorescence staining of the healing scar of tendon specimen from the SN-PUS group at 4 weeks postinjury. A: low power (4x). B and C: High power (20x). Compare to 2 weeks group, there was an increasing number of positive nerve fibers invading the tendinous tissue. Arrows denote positive nerve fibers; pt = paratendon; t = tendinous tissue; v = blood vessel. Bar=200μm

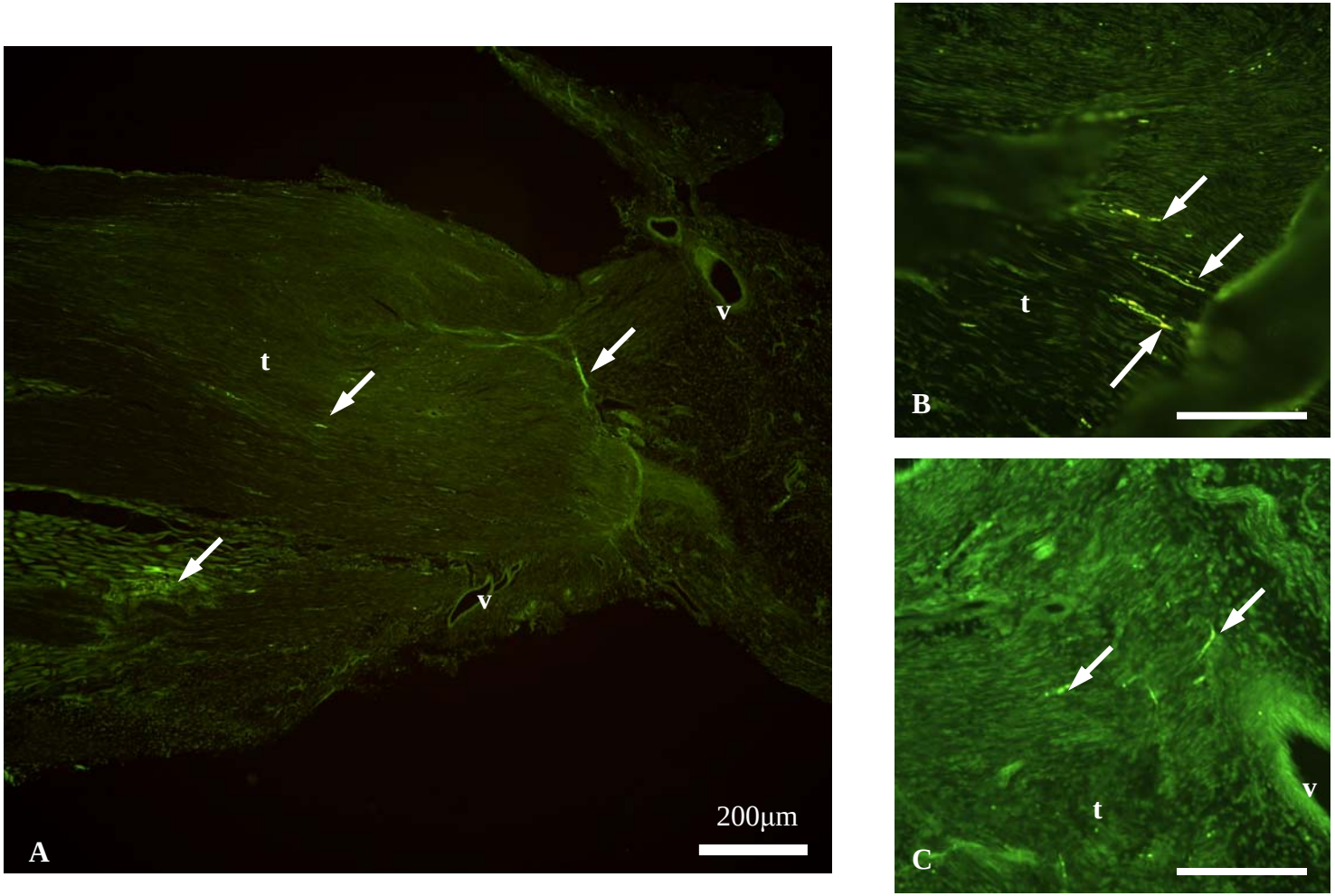

Figure 5-6. Immunofluorescence staining of the healing scar of tendon specimen from the SN-control group at 4 weeks postinjury. A: low power (4x). B and C: High power (20x). When compare to 2 weeks group, the number of immunoreactive nerve fibers were increased but still relatively less in density. Arrows denote positive nerve fibers; t = tendinous tissue; v = blood vessels. Bar=200µm

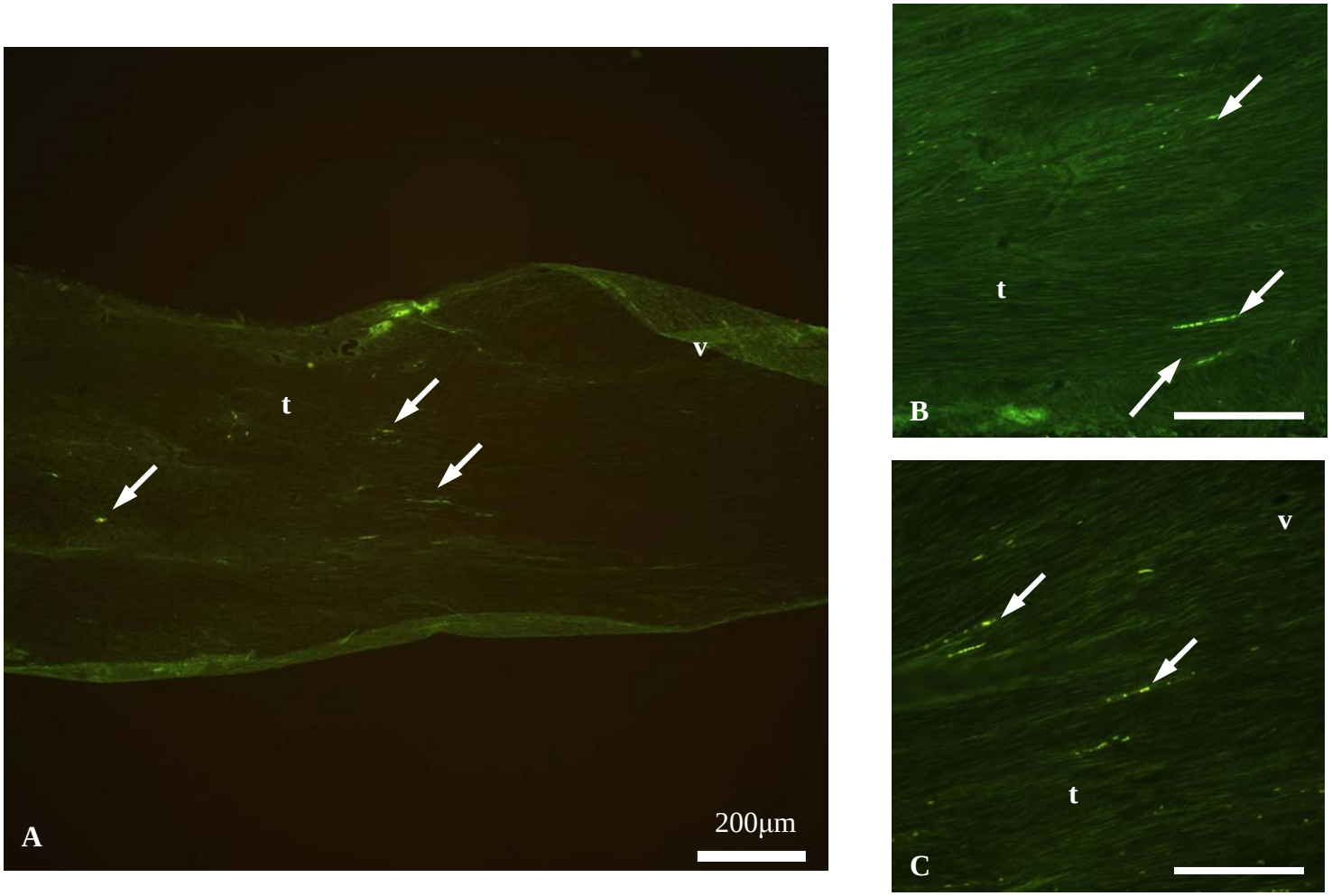

Figure 5-7. Immunofluorescence staining of the healing scar of tendon specimen from the PUS group at 4 weeks postinjury. A: low power (4x). B and C: High power (20x). When compare to 2 weeks group, the number of immunoreactive nerve fibers were markly decreased. Some fibers tended to shift to the peripheral part of tendon. Arrows denote positive nerve fibers; pt = paratendon; t = tendinous tissue; v = blood vessels. Bar=200µm

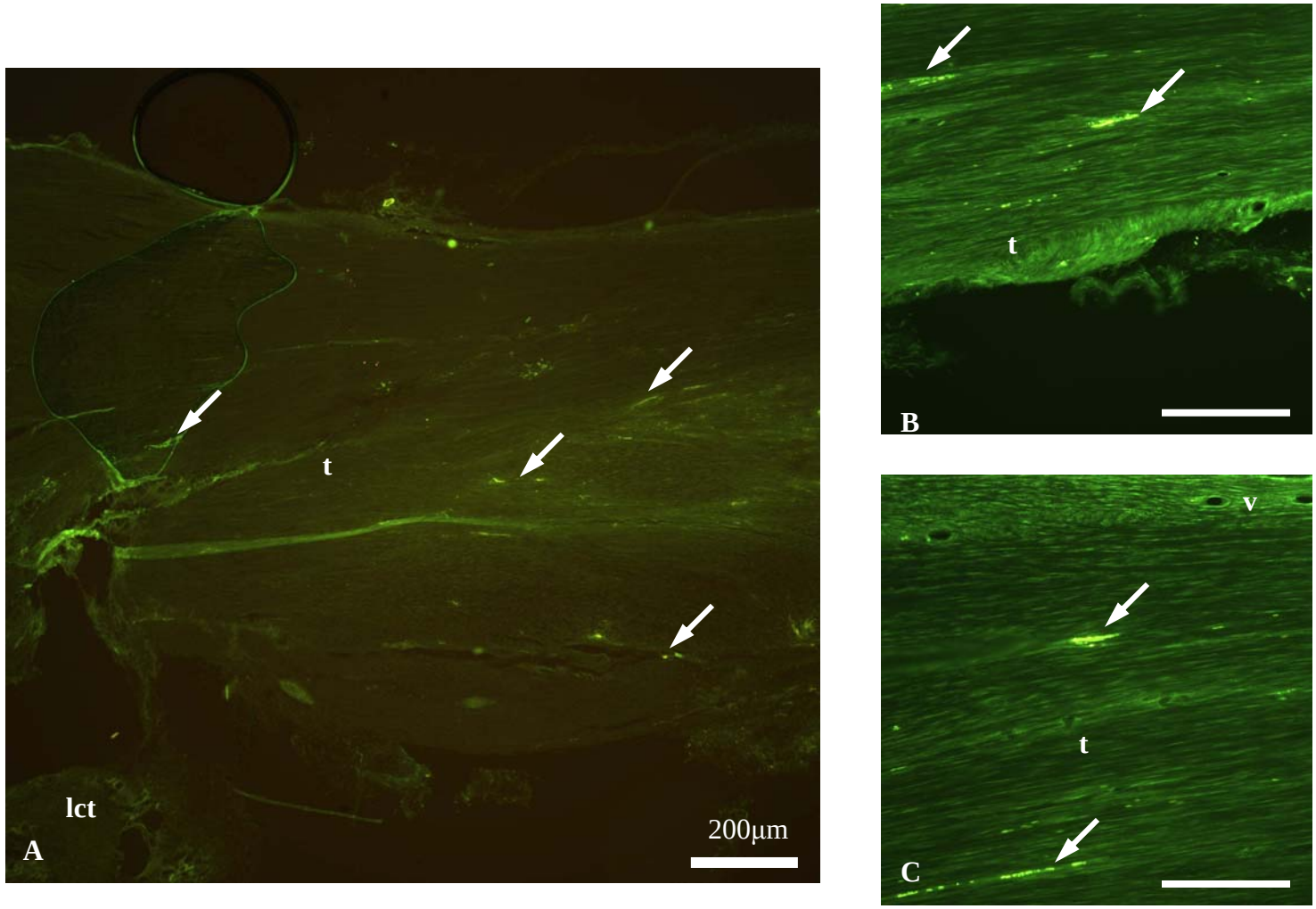

Figure 5-8. Immunofluorescence staining of the healing scar of tendon specimen from the control group at 4 weeks postinjury. A: low power (4x). B and C: High power (20x). When compare to 2 weeks group, the number of immunoreactive nerve fibers within the proper tendinous tissue were slightly decreased. Similar to PUS group, some fibers tended to shift to the peripheral part of tendon. Arrows denote positive nerve fibers; pt = paratendon; t = tendinous tissue; v = blood vessels. Bar=200µm

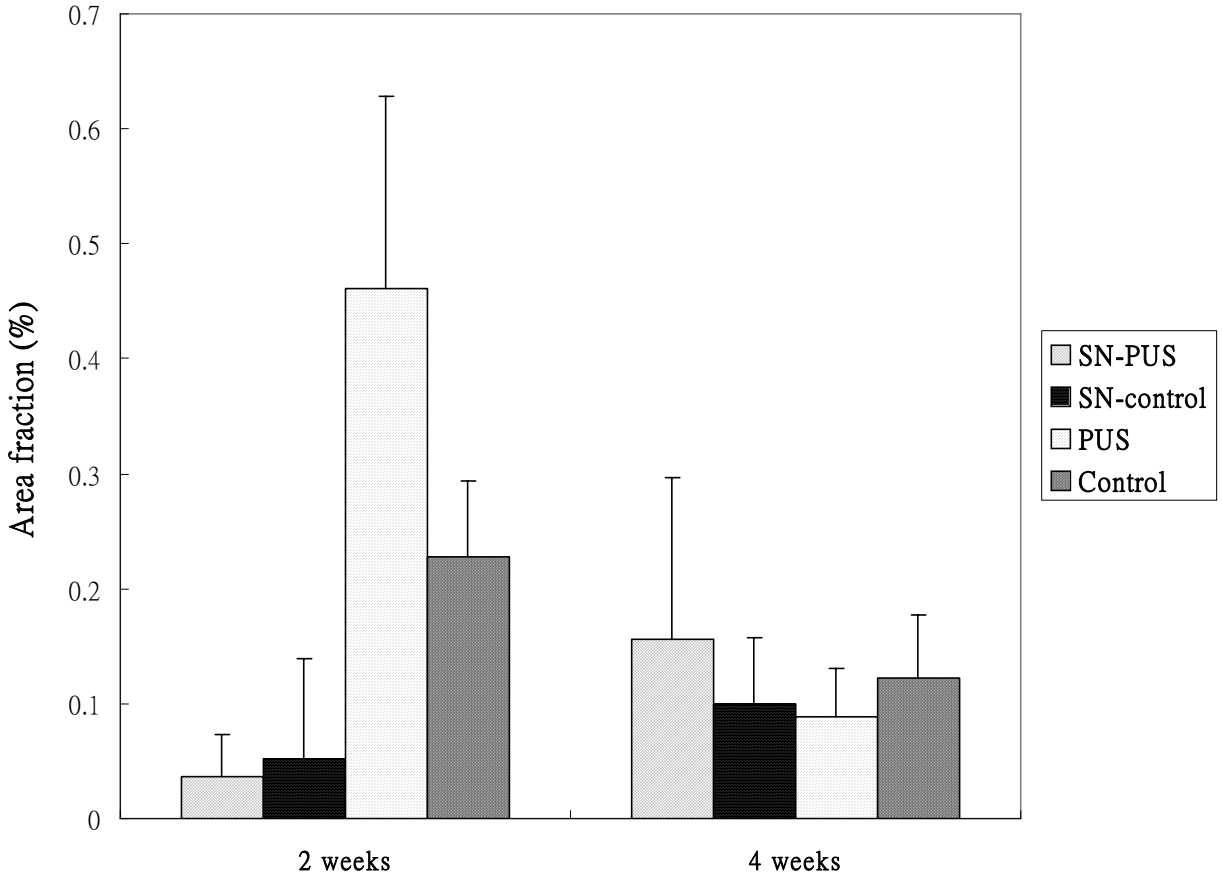

Figure 5-9. Bar chart representing the mean percentage fraction of area occupied by CGRP immunoreactive nerve fibers among 4 groups at 2 and 4 weeks postinjury. a:p=0.009; b:p=0.002

5.4. Discussion

This is the first study to investigate the effect of denervation and ultrasound treatment on the invasion of sensory innervation of healing tendons in animals. The results demonstrate that the healing tendon was characterized by the invasion of nerve fibers containing sensory neuropeptides CGRP, agreeing with the previous work by Ackermann et al. (2002 and 2003). Surprisingly, this invasion was found even in tendons where the proximal nerve was resected, although the nerve fiber expressions were fewer than those of the neural-intact tendons in the early phase of healing. It was also revealed that PUS treatment resulted in a higher concentration of CGRP in tendons at 2 weeks postinjury, which showed that PUS could promote the sensory nerve fibers' in-growth into the healing scar of the tendon. Since these neuropeptide-containing nerve fibers have been shown to be important for the regulation of tendon healing, it is reasonable to hypothesize that this promotion effect of nerve fibers in-growth is directly related to the early recovery of biomechanical properties and histomorphological appearance of the injured tendons that received PUS treatment.

5.4.1. Effect of PUS on sensory nerve in-growth

The results of this study show that PUS promoted the early in-growth of nerve fibers containing CGRP during the proliferative phase (2 weeks postinjury) of tendon healing.

This sensory neuropeptide has been found to have trophic effects which promote fibroblast proliferation (Haegerstrand et al., 1990; Yule and White, 1999), and the findings of the present study also show that the free nerve fibers were concentrated in the proper tendinous tissue, where fibroplasia and fibrillogenesis were taking place. This evidence further suggests that CGRP was involved in the proliferation of fibroblasts in the healing tendon. Another study reported that skin wounds in rats treated with CGRP exhibited faster healing by 10 days when compared to a control group (Khalil and Helme, 1996). This finding supports the role of CGRP in tissue healing. Previous work described in chapter 3 also found that injured tendons treated with PUS have a significantly higher UTS, stiffness and amount of collagen matrix at 2 weeks postinjury. This may also be related to the higher concentration of trophic CGRP neuropeptides during the proliferative phase.

Similar to the findings by Ackermann et al. (2002), the expression of these CGRP immunoreactive nerve fibers was temporal and decreased in the late phase of injury. It was also observed that the decrease in CGRP immunoreactive nerve fibers was more marked in the PUS group, and that the area fraction occupied by positive nerve fibers was relatively smaller than that of the control group, although statistically not significant. During the remodeling phase, the scar became more mature with decreased cellularity and vascularity, the importance of trophic and vasodilatory effects given by CGRP became less and thus they were gradually withdrawn, while their distribution was shifting from the central to the

peripheral part of the tendon. Biomechanically, the PUS-treated tendons still had significantly higher UTS and stiffness compared to the controls, possibly because the proliferation of fibroblasts and thus collagen synthesis were enhanced during the proliferative phase, and this carry-over effect further enhanced the strength of the remodeling tendon which was undergoing a transformation of type III collagen to type I collagen. Therefore, this PUS-treated tendon would have superior biomechanical properties while there was a marked drop in CGRP immunoreactive nerve fibers.

How PUS could promote a higher invasion of CGRP-containing nerve fibers may need further investigation, but there is some evidence that ultrasound at low intensity could promote peripheral nerve regeneration (Lazar et al., 2001), which may be related to the regeneration of sensory nerve fibers in tendons. Hong et al. (1988) conducted a study on the ultrasound thermotherapy effect on the recovery of nerve conduction in an experimental compression neuropathy model on the rat's tibial nerve. They used two different ultrasound dosages: 0.5 W/cm^2 and 1 W/cm^2, both continuous at 1 MHz for 1 minute 3 days per week over 1 month. They found a statistically significant improvement in the recovery rates of normal nerve conduction velocity in the ultrasound group of 0.5 W/cm^2 when compared to the control group, which was not found in the group with 1 W/cm^2. They concluded that low doses of ultrasound may facilitate the recovery of compression neuropathy. Similarly, another study by Mourad et al. (2001) found a

statistically significant acceleration in the recovery of gait in rats with complete crush injury of the sciatic nerve after ultrasound treatment (0.25 W/cm2, 2.25 MHz for 1 minutes), relative to a control group. More recently, use of LIPU was shown to enhance regeneration of the surgically transected sciatic nerve in rats (Crisci and Ferreira, 2002). They found that LIPU not only promoted the recovery of the A- and B-types of nerve fiber, but also resulted in a stronger metabolic activity of Schwann cells. Some of the proposed therapeutic effects on peripheral nerves are the acceleration of remyelination or axonal regeneration, the macrophage-led portion of the degeneration phase of peripheral nerve recovery, and the influx of nutrients to and toxins out of the injury site (Lazar et al., 2001). This may explain how ultrasound can promote nerve regeneration in tendons.

It is also worth considering that the action of CGRP is pro-inflammatory, as one of its actions is related to vasodilation. In this study, the ability of PUS to promote a higher invasion of CGRP-containing nerve fibers during the early phase of healing may also be related to recent studies demonstrating that ultrasound can stimulate inflammation rather than anti-inflammation (Goddard, 1983; Leung et al., 2004). However, further analysis of the expression of the nerve fiber during the beginning of tendon healing may be needed, probably within the first 3 days postinjury.

5.4.2. Denervation effects on the expression of CGRP

When the proximal peripheral nerve was being resected, the expression of CGRP-containing nerve fibers presented a different picture. Only a few of the CGRP positive nerve fibers could be found at the peripheral part of the tendon, near the paratendon. In the studies by Ackermann et al. (2003), some CGRP immunoreactive nerve fibers could normally be found at the paratendon and loose connective tissue around the uninjured tendon. Whether those positive nerve fibers were newly formed or already existed as mature fibers may need further investigation by other nerve markers, e.g. growth-associated protein 43 or protein gene product 9.5 (Ackermann et al., 2002), but the obvious decrease in invasion of sensory nerve fibers into the healing tendon apparently resulted from denervation. As hypothesized before, disruption of the proximal neuropeptide supply by denervation not only affected the delivery of trophic CGRP to the rupture site, but also related to the delay in healing of the tendon and resulted in a decrease in collagen synthesis. Eventually, the overall biomechanical properties of the denervated tendon were reduced. This was also suggested by another study reporting that peripheral denervation in rats reduced the load to failure of the Achilles tendon by half at 2 weeks postinjury (Aspenberg and Forslund, 2000). Similarly, a previous study of the rat medial collateral ligament has demonstrated that the ultimate force at failure and average blood flow of the healing medial collateral ligament were significantly decreased after femoral

nerve transection (Ivie et al., 2002). As stated above, CGRP is a potent vasodilator, and reduced blood flow in the healing ligament may relate to a deficit in this neuropeptide. All this evidence suggests that denervation affects tendon healing and results in scar tissue with inferior biomechanical properties, which may be related to the disruption in supply of the neuropeptides essential for tendon healing.

Regardless of whether denervated tendons have received PUS or sham PUS treatment, they show a slight increase in the invasion of CGRP immunoreactive nerve fibers during the remodeling phase. By visual inspection, this invasion of nerve fibers into the proper tendon tissue was less than that into the neural-intact tendon at 2 weeks postinjury. Presumably this new nerve fiber in-growth may be due to re-innervation from other sources of nerves innervating around the Achilles tendon. Achilles tendon innervation originates from 3 neighboring sources: the cutaneous, muscular and peritendinous nerve trunks (Ackermann et al., 2005). Although the main muscular and peritendinous supplies (mainly by the tibial and sural nerves) were disrupted, some cutaneous branches from the saphenous nerve (supplied by the femoral nerve) may have invaded the tendon (Green, 1963; Palmer, 1976), resulting in an increased appearance of CGRP immunoreactive nerve fibers. However, the marked reduction in concentration of neuropeptides in the early phase of healing already caused a delay in tendon healing, and thus in the previous studies (Chapter 4), the biomechanical properties and histomorphology of the tendon at 4 weeks

postinjury were still, to a certain extent, inferior to those of the neural-intact control group.

From the results of this study, the PUS treatment seemed to have no significant effect on the expression of CGRP immunoreactive nerve fibers in denervated tendons at either 2 or 4 weeks postinjury, even though it was found that PUS could enhance the invasion of CGRP immunoreactive nerve fibers in the early proliferative phase of neural-intact tendons. This could probably explain why there was no significant difference in biomechanical properties between healing, denervated tendons with PUS or sham PUS treatment. Apparently, the results of this study agree with the hypothesis, cited at the end of the previous chapter as "ultrasound may enhance the expression of neuropeptides vital for promoting tendon healing and thus lead to faster recovery of the healing tendon".

In this study, only one of the neuropeptides – CGRP – was investigated. As stated before, other neuropeptides may also be involved, like SP, GAL and NPY. As regards GAL and NPY, since they occur in the later stages of healing (from 4 weeks onward) and the main focus of this study was on the early phase of tendon healing, these two neuropeptides were not investigated. Reports have also found that CGRP facilitates the release of SP and potentiates the effect brought about by SP (Le Greves et al., 1985; Woolf and Wiesenfeld-Hallin, 1986), and CGRP is relatively more abundant in both normal and healing tendon tissue (Ackermann et al., 1999 and 2001). The results of this study also suggested that its expression in the early phase of healing may also be directly related to

the promotion effect brought about by PUS, or the inhibiting effects due to denervation, on the early phase of tendon healing.

In addition to tissue healing and vasodilation, CGRP and SP are also related positively to the nociception of the tendon, where neuropeptides GAL suppress the nociceptive effects (Ackermann et al., 2003). Many studies have been concerned with the effect of ultrasound treatment in modulating painful conditions after musculoskeletal injury (Gam and Johannsen, 1995; van der Windt et al., 1999). We also conducted a meta-analysis on the effect of therapeutic ultrasound in soft tissue healing, with pain as the common outcome measure being analyzed (Appendix III). We selected 6 out of 218 studies which were of good methodological quality and low bias. However, the results, which were similar to those of van der Windt et al. (1999), showed that ultrasound did not have a significant effect in relieving the pain associated with soft tissue lesions when compared with a placebo treatment group. One possible explanation for this may be that the level of CGRP is higher after ultrasound treatment, and is thus related to the presence of nociception and may not decrease the patient's pain threshold during the early phase of healing. The pain level did not decrease significantly between the treatment and placebo groups. However, with improvement in the biomechanical properties of the soft tissue, the functional outcomes of the injured soft tissue and related joints may improve. It is therefore necessary to conduct further well-designed trials with a large sample size and a

common outcome measure on function instead of pain in order to determine the role of therapeutic ultrasound in the treatment of soft tissue lesions.

5.4.3. Limitations

Similar to previous to previous two studies, since we would like to cause disruption of the sensory innervation of the Achilles tendon, so we performed sciatic neurectomy on the rat's hindlimb. In this case, we may affected the muscular contraction of the rats' hindlimb and thus the weightbearing status of the injured limb would change. In order to create a similar change in weightbearing status, we have performed patella tenotomy on the rat with intact sciatic nerve so that they could not weightbear on the affected limb. However, the muscular components of the injured limb being affected between innervated and denervated groups were diffenent. This may potentially affected our interpretions on the effect of denervation on tendon healing. Thus we choose to perform comparison mainly between the PUS treated group and its own control in order to draw the conclusion that PUS was effective only on neural intact tendon.

We suggested that if further study need to eliminate the sensory innervation of the injured tendon, one may try to cut off the sensory supply at the dorsal root ganglion, which is at the spinal cord of the rats. We haven't adopted this method as it is technically difficult to be perform in our laboratory setting.

5.4.4. Implications of present study

This is the first study to relate neural components and physical modality to the healing of tendons. Further study on the effect of CGRP or other neuropeptides on tendon healing in terms of biomechanical properties and histomorphology may be needed to confirm their roles in the regulation of different phases of tissue healing. This may also imply to the possibility of using a neural mediator, like CGRP, as another form of treatment for tendon healing, e.g. via injection or incorporated with PUS – sonophoresis.

5.5. Conclusion

The present study showed that PUS could enhance the invasion of CGRP immunoreactive nerve fibers into the injured site of a neural-intact tendon during the proliferative phase of healing. However, the effect of PUS was not shown in denervated tendons. Denervation also caused a marked reduction in the CGRP positive nerve fibers and may be related to the delay in tendon healing.

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CHAPTER 6. GRAND DISCUSSION AND CONCLUSION

6.1. Aims of thesis

Recalling the aims of the thesis as presented in the last section of Chapter 2, we intended to:

(i) Investigate the effect of PUS on tendon healing in terms of recovery of biomechanical properties and histomorphology by an animal model,

(ii) Investigate whether the therapeutic effect of PUS may be interact with neural components, and

(iii) Explain the possible relationship between neuropeptide-containing nerve fibers and PUS on Achilles tendon healing.

6.2. Effect of PUS on tendon healing

In this study, it was found that the normalized UTS and stiffness of the injured tendons of the PUS group were significantly higher than those of the control group (Chapter 3). This may also be related to the improved histomorphology of the healing scar: the results of histomorphological analysis indicated that the healing scar in the PUS-treated group was more mature, given that the observed fusiform fibroblasts to tenocytes ratio was slightly lower than that of the control group ($p>0.05$), while the amount of collagen matrix

was significantly higher in PUS-treated tendons ($p<0.05$). Both biomechanical and histomorphological analysis showed that PUS at the selected dosage (1 MHz, 0.5W/cm^2 SATA, duty cycle 20%, 5 minutes) could improve healing in a neural-intact tendon. A semi-quantification analysis was adopted in this study to measure the maturation of scar tissue, which could provide a more objective measurement of the histology of the healing tendon, in order to explain how PUS could improve the biomechanical properties of injured tendons.

6.3. Possible relationship between the therapeutic effect of PUS and the neural involvement

In this study, the author tried to compare the healing of the PUS and sham PUS groups, where the denervation of Achilles tendons was present (Chapter 4). However, with denervation, no significant difference in the results of the biomechanical test was found between the PUS- and sham PUS-treated tendons. Healing of tendons still could be observed by normalized UTS, where a significantly higher UTS value in the 4-week group was observed when compared with the 2-week group. Histologically, the injured tendon with sciatic neurectomy was healed by scar tissue with inferior properties, which was reflected by similar collagen matrix areas, as well as similar fusiform fibroblast to mature tenocytes ratios across time. The histomorphology of the PUS- and sham PUS-treated

tendons were also similar. The results reflect that neurectomy affects tendon healing, while the tendon may heal by scar tissue with inferior properties, both biomechanically and histomorphologically. Certainly some vital components for healing were lacking, like tensile loading during muscle action, or the recently discovered invasion of sensory nerves with neuropeptides, which may be involved in the regulation of tendon healing (Ackermann et al., 2003). Denervated tendons, besides healing in an inferior manner, also did not response to PUS treatment. There may presumably be some inter-relation of the neural component of the injured tendon and the mechanical stimulation from PUS. Further investigation of their relationship may help to explain the mechanism of how PUS treatment acts on living animals.

6.4. Relationship between neuropeptide-containing nerve fibers and therapeutic ultrasound in Achilles tendon healing

An immunohistochemical analysis – particularly the CGRP-containing sensory nerve fibers, which were found abundantly in the healing tendon – was carried out on injured tendons which had received PUS treatment or denervation or both (Chapter 5). It was demonstrated that during the proliferative phase of tendon healing, PUS could promote a higher invasion of CGRP-containing nerve fibers. As CGRP has trophic effects and aids in proliferation of fibroblasts, the increase in invasion of the PUS-treated tendon may be

related to the early recovery of biomechanical properties and faster maturation of scar tissue. The reaction of sensory nerve to PUS stimulation may provide one of the pathways that ultrasound regulate tissue repair, while further studies like investigating the expression of this sensory nerve fibers under PUS treatment in more time intervals is need to explain whether it is a causal relationship. On the other hand, denervation resulted in a marked decrease of CGRP-containing nerve fibers entering the injured tendon, and possibly resulted in the tendon being healed by a scar with inferior properties.

The outcomes of this study are the first to demonstrate the possible relationship between ultrasound and the invasion of sensory nerve fibers within the healing tendon. However, further to section 5.4.3., this study has other limitations: Firstly, only one type of neuropeptide – the CGRP, which may relate to the early healing of tendons – was investigated. Other neuropeptides like NPY and GAL may also play an important role, although they may have greater involvement during the later phase of healing (Ackermann et al., 2002; Ackermann et al., 2003). Besides, similar to the limitation to Chapter 4, which the author would like to address here, the denervation of the tendon was induced by resection of the sciatic nerve. The main drawback of this method was the paralysis of the muscle at the lower limb. A few studies have investigated the effect of joint disuse on tendons and found out that immobilization decreases the total weight of the tendon, stiffness, and tensile strength (Amiel et al., 1982; Tipton et al., 1975; Tipton et al., 1986;

Woo et al., 1982). Also in rabbits, 4 weeks of immobilization caused the formation of irregular and uneven collagen fibers and dilated veins and capillaries, and decreased the ultimate load and stiffness of healing Achilles tendons when compared to tendons in a neural-intact control group (Yasuda et al., 2000). However, in their model, joint immobilization was commonly used as a model of disuse. In this study, the ankle joint of the rat had not been immobilized; rather, it was allowed free movement of the denervated limb. Thus the conditions may have been different. Also, as the comparisons on the effect of PUS and sham PUS were done on two groups of rats with identical denervation and injury of the Achilles tendon, if PUS had its own effect on the healing tendon regardless of the presence of the intact nerve, the two groups should have different outcomes. That they had the same outcomes in terms of biomechanical properties, histomorphology and the expression of neuropeptide-containing sensory nerve fibers may be explained by the conceivable relationship between these sensory nerve fibers and PUS stimulation.

6.5. Conclusion

This is the first study to investigate the possible effect of denervation on tendon healing, and to attempt to explain the underlying mechanism of PUS on living animals by examining its relationship with the neural element, particularly the sensory nerve fibers in healing tendons. This is a first step to explaining the mechanism of ultrasound in

promoting tendon healing by studying how ultrasound interacts with healing tendons *in vivo*. These results suggested the possible involvement of neurological components in ultrasound-enhanced tendon healing and the vital role of tendon innervation. As stated in Chapter 5, this may imply the possibility of using a neural mediator, like CGRP, as a form of treatment for tendon healing, or incorporating it with PUS – sonophoresis – as another treatment alternative. Of course, these outcomes provide some direction for future research on ultrasound therapy and the clinical treatment of Achilles tendon rupture.

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APPENDICES

Appendix I. Ethical Approval issued by the Animal Subjects Ethics Sub-committee of the Hong Kong Polytechnic University

THE HONG KONG
POLYTECHNIC UNIVERSITY
香港理工大學

MEMO

To : Dr Guo Xia, Department of Rehabilitation Sciences

From : Dr Maureen Boost, Chairman, Animal Subjects Ethics Sub-committee

Ref. : _____ Your Ref. : _____

Tel. No. : Ext. 6391 Date : _____

Ethical approval granted for teaching / research projects using animals
[Effect of acoustic pressure waves on callus innervation and fracture healing]
(ASESC No. 01/12)

I am pleased to inform you that approval has been given to extend the approval validity period for the above project up to 13 December 2005. You will be invited to advise on the status of your project by the end of the approval validity period.

You are required to inform the Animal Subjects Ethics Sub-committee if at any time the conditions under which the animals are kept and cared for no longer fully meet the requirements of the Procedures for the Care of Laboratory Animals. If you are keeping animals in the University's animal holding room, you should state the full title of the approved project and the ASESC no. on the cage cards of the cages holding the animals. The members of the Sub-committee may visit the animal holding room unannounced at any reasonable time.

I would like to draw your attention to the University requirement that holders of licences under Cap. 340 must provide the Animal Subjects Ethics Sub-committee with a copy of their licences and a copy of their annual returns to the Licensing Authority. These must be kept up to date for the duration of the above work. In this connection, you are requested to provide to Sub-committee with the updated license for the project when available.

Dr Maureen Boost
Chairman
Animal Subjects Ethics Sub-committee

c.c. Chairman, DRC (RS)

Appendix II a. License to Conduct Experiments

衞 生 署
九 龍 區 辦 事 處
九龍亞皆老街 147B 號
醫院管理局大樓一字樓

DEPARTMENT OF HEALTH
REGIONAL OFFICE (KOWLOON)
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HOSPITAL AUTHORITY BUILDING
147B ARGYLE STREET
KOWLOON

本署檔號 Our Ref.: (32) in DH/KRO/P07/01/2

電 話 Tel.: 2199 9100

傳 真 Fax: 2311 7537 (General Office)
2375 8451 (Health Office)

24 June, 2003

Mr. YEUNG Chi-keung
Department of Rehabilitation Sciene
Room ST114, Hong Kong Polytechnic University
Hung Hom
Kowloon

Dear Sir,

Animals (Control of Experiments) Ordinance
Chapter 340

I refer to your application dated 11.4.2003 and forward herewith the following licence and teaching permit issued under the captioned Ordinance :-

Form 2 : Licence to Conduct Experiments

Your attention is drawn to regulations 4 and 5 of the Animals (Control of Experiments) Regulations, copy of these regulations together with copies of Forms 6 and 7 are enclosed for your convenience. Failure to comply with either regulation 4 or regulation 5 is an offence, each offence punishable by a fine of HK$500 and to imprisonment for 3 months. Conviction of an offence against either regulation 4 or regulation 5 or failure to comply with either regulation may result in your licence being cancelled.

Please also be reminded that if you wish to continue your experiments after the specified periods as stated on the above licence, you should renew them at least one-moth before the end-dates. On the other hand, if you have completed or stopped your experiments before the specified periods, you should inform this Office immediately.

Yours faithfully,

(Ms. Sharon POON)
for Community Physician (Kowloon)
Department of Health

SP/mm

We are committed to providing quality client-oriented service

Appendix II b. License to Conduct Experiments

Form 2

Licence to Conduct Experiments

Name : Mr. YEUNG Chi-keung

Address : Department of Rehabilitation Science,
Room ST114
The Hong Kong Polytechnic University

By virtue of section 7 of the Animals (Control of Experiments) Ordinance, Chapter 340, the above-named is hereby licensed to conduct the type of experiment(s), at the place(s) and upon the conditions, hereinafter mentioned.

Type of experiment(s)

The aim of the experiment is to study the effectiveness of ultrasound on tendon healing and sensory nerve regeneration and the possible mechanism underneath. Under anaesthesia, Achilles tendon will be incised on all rats while neurotomy will be induced for the neurotomy group. Different modalities of ultrasound will be applied daily for four different groups. Painkillers will be given during the first week of the operation. At the end of the experiment, animals will be sacrificed by overdose of anaesthesia. The tendon harvested will be used for histological and biochemical analysis.

Place(s) where experiment(s) may be conducted

The Hong Kong Polytechnic University

Conditions

1. Such experiments may be conducted only for research investigation.

2. The validity of this licence is from 24.6.2003 to 23.6.2005.

Dated 24 June 2003

(Dr. S.Y. LEE)
for Director of Health
Licensing Authority

[mm]

Appendix III. Meta-analysis on therapeutic ultrasound on soft tissue healing (Abstract from the third post-graduate student seminar, Department of Rehabilitation Sciences, The Hong Kong Polytechnic University, 2006)

Therapeutic ultrasound on soft tissue healing: A meta-analysis

[1]Yeung CK, [1]Guo X, [1]Fung DTC, [1]Ng GYF

[1]Department of Rehabilitation Sciences, The Hong Kong Polytechnic University, Hong Kong

Aim: Many studies have been published concerning the effect of therapeutic ultrasound on soft tissue lesions. However, the results of these studies are inconclusive. We conducted a systematic review of the effectiveness of therapeutic ultrasound in the treatment of pain due to soft tissue lesions.

Methods: Randomized trials evaluating the effect of ultrasound on soft tissue healing published between 1966 and April 2004 were searched in the electronic database MEDLINE. We selected trials which met the inclusion criteria, namely, published studies on therapeutic ultrasound conducted on human subjects with soft tissue lesions, which included a comparison with a placebo treatment group and adopted a random allocation of treatments. Case studies of therapeutic ultrasound used in the healing of wounds and the treatment of ulcers, burn injuries, arthritis and non-specific shoulder pain syndrome and ultrasound therapy combined with iontophoresis were excluded. Two reviewers independently scored the selected articles for methodological quality using a 5-point scale. A mean effect size was then calculated from all selected trials that had a 95% confidence interval.

Results: We identified 218 English publications on that topic since 1966 and 11 of which met our inclusion criteria. There was a good agreement on the quality assessment score for these trials (Intraclass correlation coefficient 0.78, p=0.001). On further review of the 11 papers, 5 were excluded because they were either considered to have low quality or they contained insufficient data for us to calculate the effect size. The weighted average effect size of the remaining 6 studies showed that therapeutic ultrasound did not have a more significant effect in relieving pain associated with soft tissue lesions than that of the placebo treatment group. ($\overline{ES}$ =0.098, 95% CI= -0.034 to 0.230).

Discussion and Conclusions: There is insufficient evidence to show that therapeutic ultrasound is more effective than placebo intervention in the treatment of pain due to soft tissue lesions. There is also doubt that ultrasound can improve the function of joints affected by soft tissue lesions. Further clinical trials with a larger sample size and better design on outcome measure are warranted to justify the clinical efficacies of therapeutic ultrasound.

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