EFFECT OF FISH OIL APPLICATION AND ITS COMBINED EFFECT WITH THERAPEUTIC ULTRASOUND ON TENDON HEALING: A RAT MODEL

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Effect of Fish Oil Application and Its Combined Effect with Therapeutic Ultrasound on Tendon Healing: A Rat Model

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CERTIFICATION 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 that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

All experiments in the present study were carried out solely by me except otherwise stated in the text. Any contribution made to the research by others, with whom I have worked at the Hong Kong Polytechnic University or elsewhere, is explicitly acknowledged in the thesis.

_____ (Signed)

__Karly Oi Wan CHAN_____(Name of student)

“One thing I know, that I know nothing.”

- Socrates

ABSTRACT

Full recovery of acute Achilles tendon rupture may not complete even at 2 years after injury irrespective of the type of treatment. This could be due to the deficiencies of current treatments, thus the need of exploring safe and effective long term treatment is implicated. Fish oil which contains high proportions of omega-3 polyunsaturated fatty acids (n-3 PUFAs) particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been shown to modulate inflammation, promote connective tissue healing, collagen synthesis and cutaneous wound healing in animal model. The lipophilicity and relative small molecular weight of n-3 PUFAs make them potential agents to penetrate the skin and hence serve as skin drug permeation enhancer. Therapeutic ultrasound has been used to treat soft tissue injuries for over half a century. It can also serve as transdermal enhancement modality. In light of this, the present series of studies were conducted to examine the biomechanical properties, histomorphological changes and collagen deposition of rupture tendons after 2-week and 4-week treatment of topical fish oil, therapeutic ultrasound and the combination of the two modalities.

A total of 121 female Sprague-Dawley rats were tested. All the rats

underwent the same surgical procedures that the right Achilles tendon was partially transected. The animals were randomly assigned into 4 groups, namely, control (CON), topical fish oil (FO), therapeutic ultrasound (US) and therapeutic ultrasound coupling with topical fish oil (FU). The animals received daily treatment at one day after surgery according to their group assignment for a period of either 2 weeks or 4 weeks. After the treatment period, all the rats were sacrificed and their Achilles tendons were harvested for analysis of biomechanical performance, histomorphological changes and collagen deposition. Results were analyzed using two-way analysis of variance (ANOVA).

The biomechanical analysis involved 85 rats and their Achilles tendons were tested for structural stiffness, ultimate tensile strength (UTS) and energy absorption capacity. At 2 weeks, only US group showed higher normalized UTS when compared with CON group ($p<0.05$). At 4 weeks, both US and FU groups demonstrated better UTS ($p< 0.05$), while both FO and FU groups had improved in structural stiffness ($p<0.05$). Four weeks of treatment with ultrasound using fish oil as coupling medium showed improvement in both structural stiffness and UTS ($p<0.05$).

The histomorphological and collagen analysis involved 36 rats. A histological examination was performed using hematoxylin and eosin, Masson's

trichrome and Picrosirius red stain. Immunohistological examination was used to distinguish type I and III collagen and to quantify immuno-positive-stained areas. Histological analysis suggested that FU group had more mature collagen fibre ($p<0.05$) at 4 weeks. The US group demonstrated significantly higher percentages of type I and type III collagen at 2 weeks ($p<0.05$). There were higher percentages of type I collagen at 2 weeks ($P<0.05$) and a higher proportion of mature collagen at 4 weeks in FO group ($p<0.05$). These findings demonstrated that therapeutic ultrasound expedited the healing response particularly in the early phase of healing while fish oil treatment had a more sustained effect on collagen deposition.

From the series of studies, it is concluded that therapeutic ultrasound coupled with topical fish oil could better restore UTS, stiffness, extracellular matrix organization and enhance mature collagen deposition.

PUBLICATIONS ARISING FROM THE THESIS

A. Articles published

Chan KOW, Tong HHY and Ng GYF (2016) Topical fish oil application coupling with therapeutic ultrasound improves tendon healing. Ultrasound in Medicine and Biology 42 (12):2983-2989.

Chan K, Tong H, Ng G (2015) Phonophoresis of fish oil improves tendon healing in a rat model. Physiotherapy 101:e209

Chan, K. O. W. and G. Y. F. Ng (2011). "A review on the effects of glucosamine for knee osteoarthritis based on human and animal studies." Hong Kong Physiotherapy Journal 29: 42-52.

B. Article to be submitted

Chan KOW, Tong HHY and Ng GYF. Therapeutic ultrasound and topical fish oil application enhanced the collagen production of tendon repair.

C. Article in preparation

Chan, K. O. W. and G. Y. F. Ng. A literature review on the effectiveness of omega-3 polyunsaturated fatty acids (n-3 PUFAs) on osteoarthritis

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Chan KOW, Tong HHY and Ng GYF (2016) The effect of topical fish oil and therapeutic ultrasound on tendon healing: a histological study in a rat model. Student Conference on Sports Science, Rehabilitation and Medicine, 26 November 2016, Hong Kong.

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DECLARATION OF INTEREST

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALA	Alpha linoleic acid
ANOVA	Analysis of variance
CON	Control group
COX	cyclooxygenase
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic acid
FO	Fish oil group
FU	Ultrasound coupling with fish oil group
GlcN	Glucosamine
H&E	Hematoxylin and eosin
HFS	High-frequency phonophoresis
IL	Interleukin
LIPUS	Low intensity pulsed ultrasound
LOX	Lipoxygenase
LTs	Leukotrienes

MMPs	Matrix metalloproteinases
n-3	Omega 3
n-6	Omega 6
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PGs	Prostaglandins
PSR	Picrosirius red
PUFAs	Polyunsaturated fatty acids
RCTs	Randomized controlled trials
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteinases
US	Ultrasound group
UTS	Ultimate tensile strength
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

CHAPTER 1 INTRODUCTION

This chapter aimed at presenting background information of tendon injuries from tendon biology, soft tissue injury repair mechanism to management of Achilles tendon rupture. The two possible treatment modalities namely therapeutic ultrasound and fish oil were introduced in this chapter.

1.1 Tendon

1.1.1 Structure and function

Tendon is a dense regular connective tissue consisting of a cellular component, collagen bundles and an extracellular matrix (Doroski et al. 2007). Water accounts for 70% of the total mass of tendon. Over 90% of the cellular elements of tendon are tenoblasts and tenocytes whereas the remaining 5-10% are chrondrocytes, synovial cells of the tendon sheath and vascular cells (Sharma & Maffulli 2005a). Tenoblasts are immature spindle-shaped tendon cells. They are metabolically active and have abundant cytoplasmic organelles. In contrast, tenocytes are mature elongated tendon cells with a lower nucleus-to-cytoplasm ratio, indicating that they have decreased metabolic activity (Sharma & Maffulli 2005b).

Seventy per cent of the dry weight of a tendon is collagen and 2% is elastin fibres. Type I collagen is predominant and the arrangement of fibre is exceptionally resistant to uniaxial tension and promotes elastic energy storage (Hulmes 2002). Type III collagen constitutes about 5% of the tendon collagen (Silver et al. 2006). Proteoglycans, glycosaminoglycans, glycoproteins and several other small molecules form the ground substance of the extracellular matrix (Longo et al. 2009). Proteoglycans are hydrophilic which enables rapid diffusion of water and soluble molecules and migration of cells

(Sharma & Maffulli 2006). This characteristic enables water to be bound to proteoglycans, which acts as a cushion that allows tendon to resist compressive loads. Water in the extracellular matrix resists the inward movement of the collagen fibres which takes up most of the loading during activities. This arrangement of collagen, proteoglycan and extracellular matrix provides the optimal mechanical strength and prevents the collagen from irreparable damage during normal loading conditions (Harley & Bergman 2008).

Tendon is responsible for transmitting forces from muscles to bones. It acts as a buffer by absorbing external forces to prevent muscle damage as well as enabling joint and limb movements (Sharma & Maffulli 2005a). To do this effectively, tendon has to act as a biological spring which is capable of resisting high tensile forces with limited elongation so as to minimize the energy loss and deformation (Maquirriain 2011). It also has the highest tensile strength among any other tissues in the body but yet is light and flexible (Harley & Bergman 2008). Tendon is non-contractile viscoelastic structure that exhibits both viscous and elastic characteristics when undergoing deformation. The collagen fibres and other matrix components such as water, proteoglycans and glycoproteins determine the viscoelastic behavior. For example, more hydrated tissues exhibiting greater stress relaxation characteristic and reduction in proteoglycan content would lower the tissue's viscoelasticity (Screen 2008). The elastic characteristic of tendon is observed within 4% strain which is time-independent. Tendon stores energy by active stretching because of inertial, gravitational, and muscle force (Wang et al. 2012a).

With mechanical loading, a stress-strain curve demonstrates the viscoelastic behavior of tendon (Figure 1.1). The tendon is strained up to 2% in the toe region,

representing flattening of the collagen crimp. With further increase in stress, tendon deforms in a linear fashion and within 4% strain, the tendon can return to its original length without damage. However, with 8 to 10% strain, macroscopic failure occurs (Sharma & Maffulli 2005b) and the tendon is permanently deformed. The viscoelastic behavior of tendon makes the structure more deformable at low strain rates but less deformable at high strain rates (Wang et al. 2012b). Tendon elongation under a given force is determined by its stiffness which is an important quality for explosive performance particularly at high strain rates (Magnusson et al. 2008).

Tendon also exhibits time-dependent behaviors that when the tissue is held at a constant strain level, the stress in the tissue decreases over time, and this phenomenon is called 'stress relaxation'(Figure 1.2). On the other hand, when a tendon is held at a constant stress level, strain in the tissue increases, which is known as 'creep elongation' (Duenwald et al. 2009) (Figure 1.3). These behaviours allow tendon to transmit forces with minimal deformation or energy loss (Kirkendall & Garrett 1997) and help to prevent fatigue failure of tendon (Jung et al. 2009).

1.1.2 Terminology

Tendinopathy is a generic term that describes all pathologies of non-ruptured tendon arising from overuse, which is characterized by an absence of inflammatory cells and a poor healing response featured by a combination of pain, swelling and impaired performance (Khan et al. 1999; Lake et al. 2008; Maffulli 1998). Tendinosis is a condition that describes collagen degeneration without histological signs of intratendinous inflammation. This degenerative tendinosis condition sometimes may be mistakenly referred to as tendinitis or tendonitis in which inflammation is the

fundamental problem. Tenditinis denotes a clinical syndrome and not a specific histopathological entity (Khan et al. 1999). Some researchers have suggested this misnomer should be abandoned because the overuse tendon conditions have a non-inflammatory pathology (Khan et al. 2002; Maffulli 1998). However, a brief period of true "tendinitis" is possible even though most tendinopathies are chronic in nature. Kader et al. (2002) suggested that the term "tendinosis" and "tendinitis" should only be used after proper histopathological examination is conducted.

1.1.3 Achilles tendon

Achilles tendon is formed by the tendinous portion of the medial and lateral gastrocnemius and soleus muscles. Although Achilles tendon is the strongest amongst all tendons in the body, it is commonly injured and there is high incidence of spontaneous rupture of this tendon (Kannus & Józsa 1991; Rees et al. 2006). Tendons like the Achilles that sustain high loadings have slower turnover than those that bear lower loads (Magnusson et al. 2016). Achilles tendon bears the largest load in the body particularly during running and jumping as the load can be up to 12.5 times body weight when one jumps or runs (Doral et al. 2010). The blood supply to Achilles tendon is from the musculotendinous junction, vessels in adjacent connective tissue and the osteotendinous junction (Nandra et al. 2012).

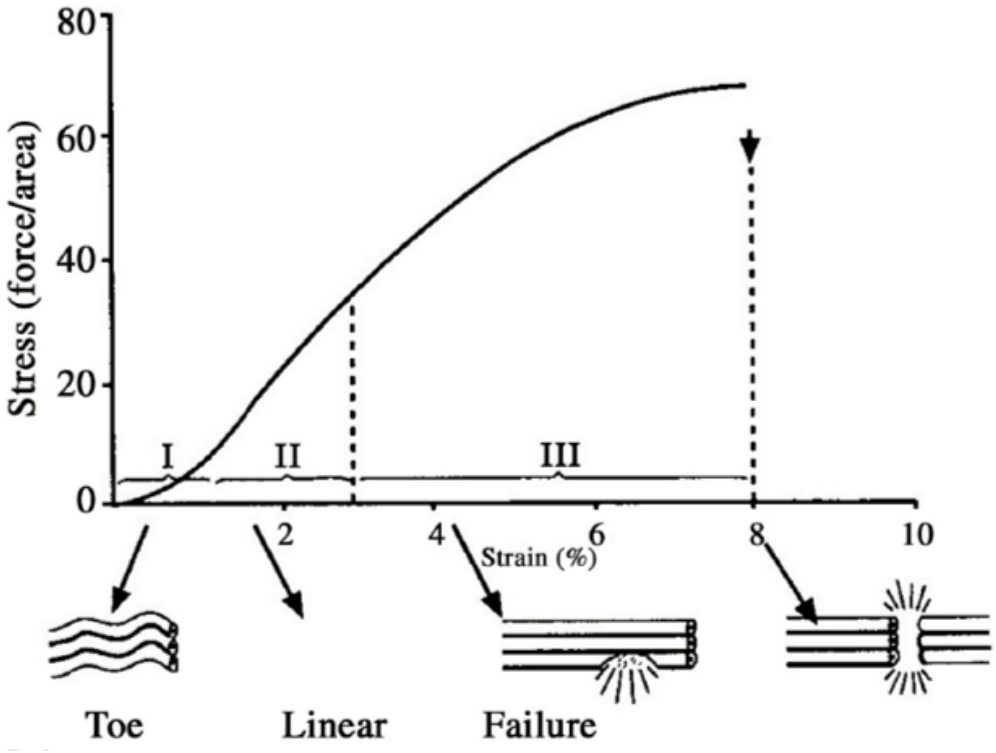


Figure 1.1 Stress strain curve of tendon (Adopted from Sharma and Maffulli (2005b))

Phase I is the toe region in which the collagen crimp is flattened. In phase II, tendon deforms in a linear fashion with increase in stress. In phase III, with 8-10% strain applied, the tendon is permanently deformed.

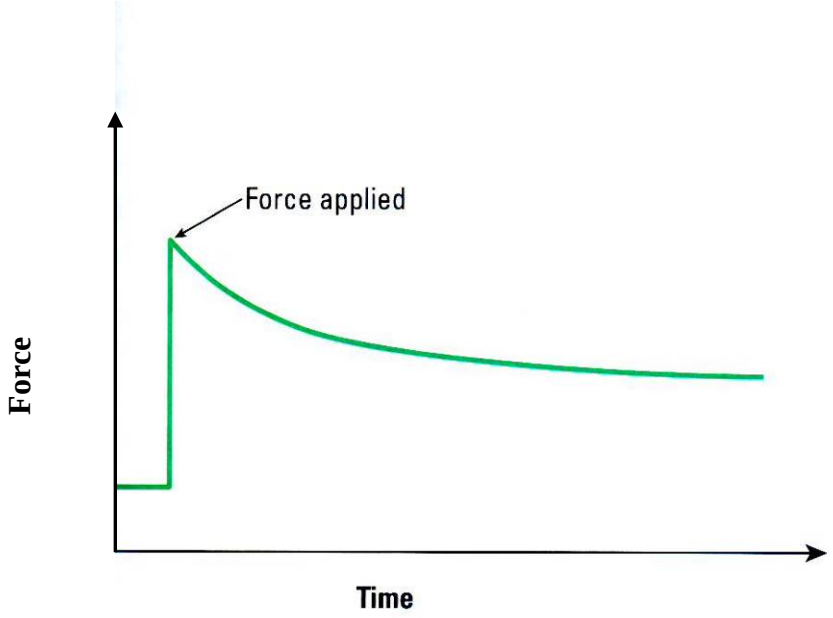


Figure 1.2 Force relaxation for connective tissue (Adopted from Ng (2003))

Under a constant strain level or in an isotonic contraction, the force in the tissue decreases over time.

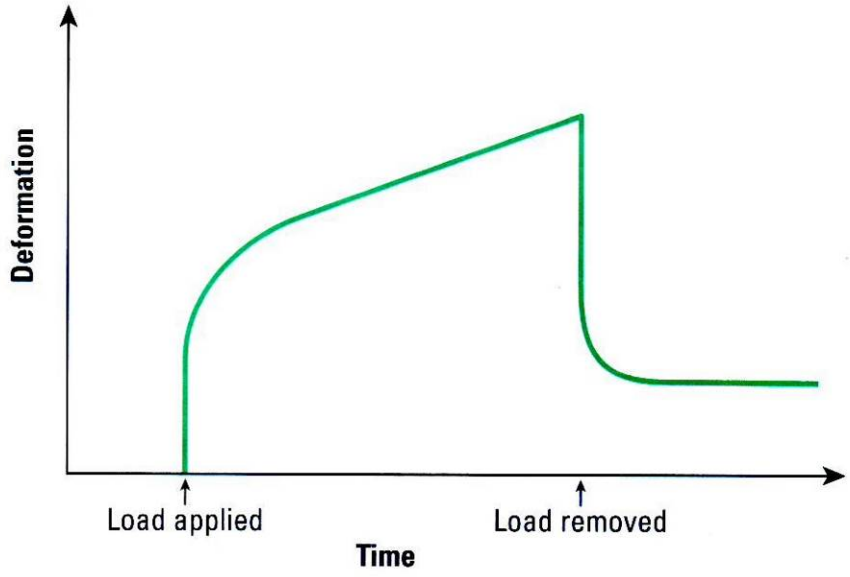


Figure 1.3 Creep-elongation curve for connective tissue (Adopted from Ng (2003))

When a tendon is held at a constant stress level or in an isometric contraction, the tendon will lengthen slightly and strain in the tissue increases.

Achilles tendinopathies are frequent amongst athletes who run and jump a lot (Maffulli 1998), and almost all Achilles tendon ruptures occur during sport activities. Achilles tendinosis is a risk factor for acute tendon rupture which is common in high-level athletes who participate in sports that require forceful plantar-flexion (Nandra et al. 2012). Middle-aged people (30-55 years old) are also a vulnerable group as age dependent degenerative process induces structural, biochemical and biomechanical changes of the tendon (Cook et al. 2002; Kader et al. 2005; Nandra et al. 2012).

1.1.4 Tendon injury and healing

Primary disorder of tendon is common especially in Achilles tendon (Rees et al. 2006). Poor tendon vascularity, degeneration, age, sex, changes in training pattern, previous injuries as well as improper footwear are possible factors leading to Achilles tendon rupture (Longo et al. 2009). There is no consensus on the etiology of Achilles tendon rupture but it is commonly believed that degenerative changes and repeated mechanical loading which reduces the tensile strength of tendon are the causative factors of Achilles tendon rupture (Tallon et al. 2001). Increasing infiltration of blood vessels, decreasing matrix organization and change in cellularity are common pathological findings in painful tendon (Riley 2008). Lesions in the human Achilles tendon are often associated with a hypovascular region located 2-6cm above the tendon insertion to bone (Lesic & Bumbasirevic 2004). This region has been shown to have less blood supply which is susceptible to degeneration and a common site for acute tendon rupture (Abate et al. 2009; Khan-Farooqui & Anderson 2010).

Tendon healing occurs in three overlapping phases, namely, inflammation,

proliferative/reparative (scar formation) and remodeling (scar remodeling) in essentially the same way as other soft tissues (Figure 1.4). The key regulators of these processes consist of a myriad of cytokines and growth factors which are released by the cells in response to the injury (Frank et al. 1999). In the first few days post injury, the tendon is in a state of acute inflammation (Battery & Maffulli 2011). Neutrophils, activated monocytes and macrophages arrive at the injury site. These cells would initiate the inflammatory process to clear the wound site of foreign particles such as bacteria, which also stimulates proliferation of tenocytes, angiogenesis and type III collagen synthesis (Abate et al. 2009; Sharma & Maffulli 2005a).

In the first few days after injury, the soft tissue is in a state of acute inflammation which stimulates proliferation of tenocytes and type III collagen synthesis. Type I collagen and granulation tissue synthesis are promoted during the subsequent repair or proliferation phase. The last phase is maturation and remodeling. Tendon cellularity decreases and the extracellular matrix of tendon becomes more organized.

Inflammation is involved in the early phase of tendon injury. Based on several animal studies, Ree at al. (2006) deduced that inflammatory reaction is present in acute situation but a degenerative process soon supersedes. Inflammatory and degenerative changes very often coexist in adjacent areas of the samples of tendinopathic tissue (Abate et al. 2009).

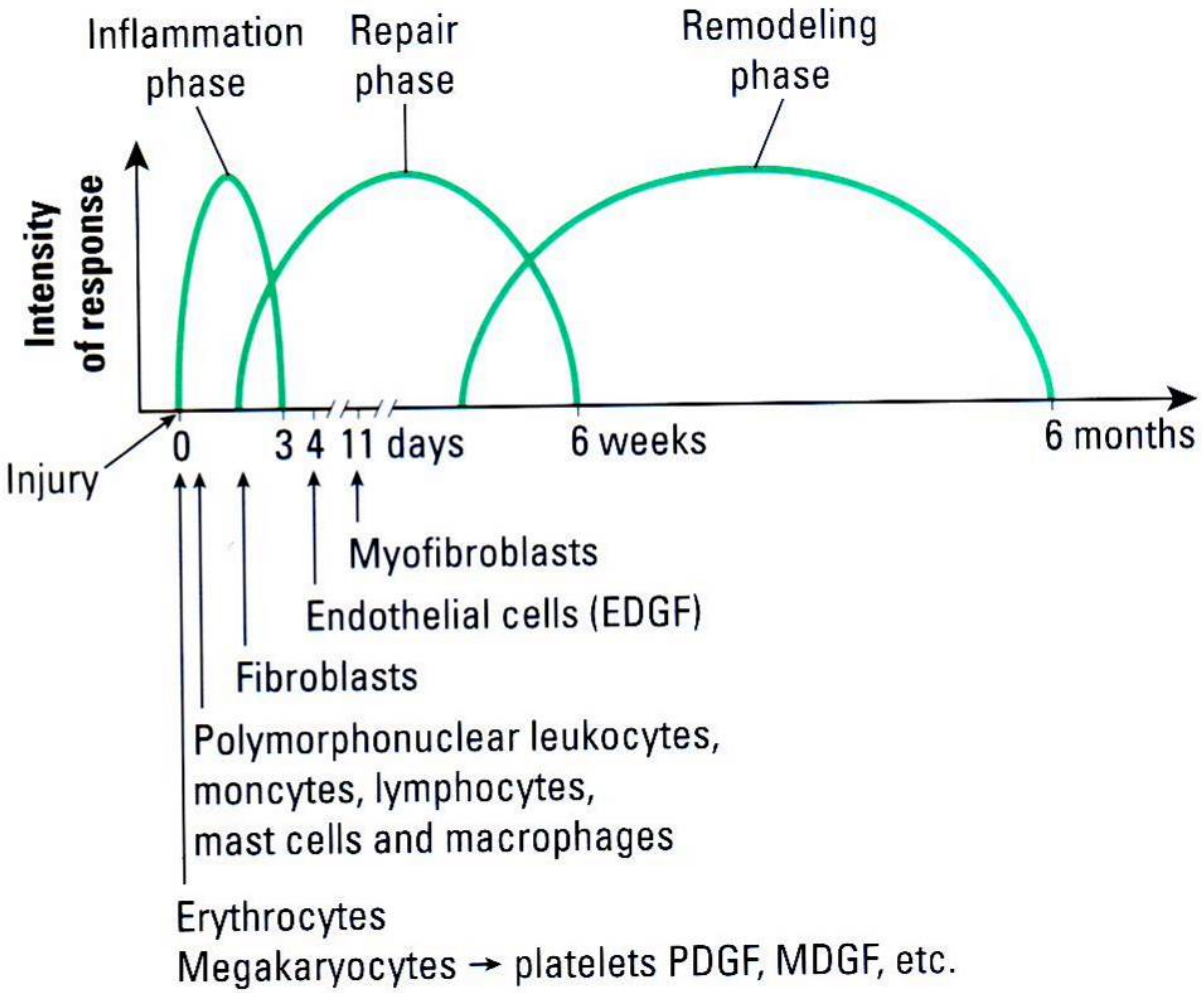


Figure 1.4 The major phases of soft tissue injury (Adopted from Ng (2003))

The proliferation phase lasts between 5 and 21 days, during which the wound is filled with macrophages and fibroblasts, numerous new blood vessels and a connective tissue matrix for producing collagen and granulation tissue (Watson & Young 2008). Early in the proliferation phase, cellular replication and migration are the major activities of fibroblasts (MacKay & Miller 2003). Later, fibroblasts synthesize type III collagen which will be replaced by type I collagen from day 12-14 with progressive increase in tensile strength and tendon stiffness (Kader et al. 2002). At approximately 4 weeks after injury, immature fibrous tissue which resembles granulation tissue will fill in the injured tendon (Dahlgren 2007).

The last phase is the maturation and remodeling which occurs concurrently with the proliferation phase and usually lasts for up to one year or longer (Abate et al. 2009; Enwemeka 1989b). During the remodeling phase, tendon cellularity decreases and the structure becomes less vascular. Adhesions formed after injury can anchor the tendon but not providing the strength for it to perform normal function. Treatment should favor intrinsic tendon healing so as to hasten the healing process and prevent abnormal adhesion formation (Greenwald et al. 1991).

Compared with normal Achilles tendon, the tenocytes from ruptured tendon produce greater quantities of type III collagen which has lower tensile strength (Longo et al. 2009). The type III collagen will later be transformed to type I collagen and hence type I collagen becomes dominant again in a well-healed tendon. Type III collagen increased to around 66% of the total collagen at 4 weeks after injury and type I collagen regained to over 90% by 8 weeks (Dahlgren 2007). The rate of the injured tendon to regain tensile strength is slow. Even after one year, spontaneously healed transected sheep Achilles tendons could only restore 56.7% of its normal strength (Bruns et al.

2000) and hence the authors suggested the injured tendon would be unlikely to ever regain its full strength.

Recovery of tendons after injury takes longer than muscles and bones as the metabolic rate of tendons is relatively limited with oxygen consumption 7.5 times lower than that of skeletal muscles (Sharma & Maffulli 2005b). Furthermore, the turnover time for tendon collagen varies from 50 to 100 days (Abate et al. 2009). It indicates a limited capacity to regenerate and hence natural healing of these injuries is insufficient (Doroski et al. 2007).

1.1.5 Management of acute Achilles tendon rupture

There is no consensus on the optimal protocol for the treatment of Achilles tendon injury. Management of acute Achilles tendon rupture can be widely classified into conservative and operative. For non-operative approach, a below-knee plaster cast or splint would be used to restrict the foot in plantar flexion with or without physiotherapy (Soroceanu et al. 2012). The operative management of acute Achilles tendon rupture includes open, minimally invasive and percutaneous repair of the tendon. This operative approach reduces the risk of re-ruptures compared with conservative approach (Weber et al. 2003) but it often associates with higher risk of delayed wound healing problems (Longo et al. 2009; Saxena et al. 2008). The prolonged immobilization of the conservative approach leads to elongation of the tendon thus compromising the strength of the calf muscles (Ding et al. 2013).

Percutaneous repair and minimally invasive repair minimizes skin healing problem but these management approaches have been reported to result in high incidence of sural nerve injuries and re-ruptures compared with open repair (Carmont et

al. 2011; Čretnik et al. 2005; Wong et al. 2002). However Gigante et al. (2008) and Hsu et al. (2015) found no significant difference in the risk of complications or clinical outcomes between open and percutaneous surgery. Both percutaneous Achilles tendon repair and open operative approach could help patients return to baseline activities by 5 months after surgery (Hsu et al. 2015).

The rate of tendon re-rupture has decreased after the introduction of functional brace in both conservative and surgical approaches. Functional bracing is better than traditional immobilization by avoiding the risks of immobilization, facilitating return to work and sport activities (Nandra et al. 2012). Early rehabilitation to improve the functional outcome may outweigh the choice of treatment (Holm et al. 2015). A more recent meta-analysis suggested that both surgical and nonsurgical treatment were comparable with regard to rerupture rate, range of motion, calf circumference and functional outcomes as long as early functional rehabilitation was included in the nonsurgical protocol (Soroceanu et al. 2012). Irrespective of the types of treatment, injured tendon may not return to its original strength even one year after injury. It implicates the need to explore new treatment that can supplement current practice and can be safe to use for a long period.

1.2 Omega 3 polyunsaturated fatty acids (n-3 PUFAs)

Fatty acid contains a hydrocarbon chain and a carboxyl terminal cluster. It is a structural component of biological membrane, source of energy and precursor of intracellular messengers. Fatty acids are divided into saturated, monounsaturated and polyunsaturated according to the number of double bond. The two principal families of polyunsaturated fatty acids (PUFAs) are the omega-6 (n-6) and omega-3 (n-3). Linoleic acid (n-6, LA) and α-linolenic acid (n-3, ALA) are essential fatty acids for humans

which cannot be synthesized by the body, thus it is necessary to obtain them from the diet. The two commonly known n-3 fatty acids are eicosapentaenoic acid (20:5 n-3; EPA) and docosahexaenoic acid (22:6 n-3; DHA) which are considered as conditionally essential fatty acids. These two fatty acids can be synthesized from ALA but the synthesis is extremely limited in humans and it is not sure if the endogenous conversion of EPA and DHA is enough for optimal health (Calder et al. 2010).

For a typical modern diet, there is a higher consumption of n-6 fatty acids than n-3 fatty acids (ratio of 15-20:1) because of decreasing fish consumption and increasing consumption of food items rich in n-6 fatty acids. N-6 fatty acids are commonly found in meat, soy, corn and sunflower oils, while n-3 fatty acids are found in green vegetables, fish, linseed (flaxseed oil) and canola oils (Simopoulos 1991 and 2008). It is postulated that most people in developed countries may not have sufficient intake of n-3 PUFAs. These fatty acids are essential for modulating inflammation and tissues healing processes in humans as they give rise to eicosanoids which are C20 oxylipids and consist of prostaglandins (PGs), thromboxanes , leukotrienes (LTs) and lipoxins (Yashodhara et al. 2009).

Oil from deep sea fish is a complex mixture of fatty acids containing high proportions of PUFAs of the omega-3 (n-3) series, typically containing 18% eicosapentaenoic acid (EPA) and 12% docosahexaenoic acid (DHA) (Cleland et al. 2006). Fish oil has been shown to have beneficial effects on a plethora of health conditions such as lowering the risk of cardiovascular disease mortality, improving blood lipid profile, chondroprotective, regulating inflammatory and renal diseases, maternal and child health, nervous system function, psychiatric disorders and several types of cancers (Tur et al. 2012). It has also applied in different musculoskeletal

problems and soft tissue healing such as rheumatoid arthritis (James et al. 2010), osteoarthritis (Hill et al. 2016) and cutaneous wound healing (Terkelsen et al. 2000). The potential effects of omega-3 PUFAs to mediate numerous eicosanoids and cytokines, suppress inflammation (Cleland et al. 2003), relieve pain (Tokuyama & Nakamoto 2011) and increase collagen formation (Otranto et al. 2010) are important qualities towards tendon healing but few studies have investigated in these areas.

1.3 Therapeutic ultrasound

US waves are longitudinal sound waves which transport mechanical energy through the local vibration of particles with frequency above that of the audible range of humans (above 20kHz) (Leighton 2007). The acoustic wave of frequency 1-12MHz transmits through body tissues by molecular vibrations and collisions (Khanna et al. 2009). To maximize the transmission of ultrasound energy to the targeted tissues, appropriate coupling media are applied between the sound head and the target tissues so as to prevent reflection of energy by the air gap. The common coupling media are gels with acoustic impedance similar to that of the skin in order to minimize reflection of the ultrasound wave. They should be in a sufficiently fluid state and relatively viscous (Watson & Young 2008). Based on the findings form Poltawski and Watson (2007), there was no clinically significant difference between the commonly available couplants such as physio-med ultrasonic transmission gel and Biofreeze topical analgesic gel. In general, tissues with higher protein content rather than water and fat would absorb ultrasound to a greater extent (Watson & Young 2008). Therefore, tendon which is high in collagen content should absorb significant amount of ultrasound energy.

The ultrasonic energy can be divided into high-intensity ultrasound with peak

intensities from 5000 to 15000W/cm^2, and low intensity ultrasound with peak intensities from 0.5 to 3000mW/cm^2 (Khanna et al. 2009). The description of intensity for pulsed exposures includes spatial peak intensity which is based on the maximum pressure measured in the field, and spatial average intensity which is based on a pressure averaged over a specified area. For energy delivery, the intensity quoted can be pulse average in which the intensity is averaged when the pulse is on, while temporal average is the average intensity over a period of pulse "on" and "off" time. Therefore, the intensity may be quoted as I_{SPTA} (spatial peak, temporal average intensity), I_{SPPA} (spatial peak, pulse average intensity), I_{SATA} (spatial average, temporal average intensity) and I_{SAPA} (spatial average, pulse average intensity) (ter Haar 2007). There is no quantitative clinical study to establish the optimal intensity for different conditions. In general, acute conditions required lower intensity such as 0.1-0.3W/cm^2, while chronic conditions can tolerate higher intensity such as 0.3-1.0W/cm^2 (Watson 2015). Most of the clinical studies and animal studies investigating the effectiveness of therapeutic ultrasound adopted low intensity (<3W/cm^2). Therefore the discussions in this thesis would mainly focus on low intensity US.

Therapeutic ultrasound therapy has been widely used in the clinical settings for more than half a century. It is commonly used to treat various musculoskeletal problems such as pain, collagen mobilization, management of inflammatory conditions (Granter 2006). Based on a survey on the usage of therapeutic ultrasound among sports physiotherapists in Australia from Warden and McMeeken (2002), only 1% among 171 respondents did not use US. A similar survey performed in England has also identified therapeutic ultrasound as the most frequently employed electrotherapeutic modality (Pope et al. 1995). Wong et al. (2007) found that at least two third of the physical

therapists interviewed would use ultrasound for soft tissue inflammation, tissue extensibility and scar tissue remodeling. About half of the respondents would use ultrasound to deliver medication via phonophoresis for soft tissue inflammation. Despite the popularity of this treatment, there were not many well-designed RCTs studies to support the use of therapeutic ultrasound (Gam & Johannsen 1995; Robertson & Baker 2001; Shanks et al. 2010).

While more favourable results were found in animal studies, its effects on collagen synthesis are not conclusive. Ultrasound treatment have been shown to improve strength (Enwemeka et al. 1990; Fu et al. 2008; Jackson et al. 1991; Ng et al. 2003; Yeung et al. 2006), enhance range of motion (Gan et al. 1995), increase collagen fibers synthesis (Jackson et al. 1991), hasten collagen fiber organization (da Cunha et al. 2001), and boost mean collagen fibril size (Ng & Fung 2007). However, both Carvalho et al. (2006) and Larsen et al. (2005) could not reveal any beneficial effect of therapeutic ultrasound on collagen content. More high quality studies are needed to explore the mechanisms of therapeutic ultrasound on soft tissues healing.

CHAPTER 2 LITERATURE REVIEW

Injured tendon is unlikely to ever regain its full strength even after long time of repair. Treatments that can speed up healing, suppress excessive tissue scarring, promote formation of collagen and are safe to apply for long term are potentially suitable for treating tendon injury thus worth exploring. Possible treatment options for lower limb injuries including glucosamine, antioxidants, vitamin D, fish oil and therapeutic ultrasound were reviewed in this chapter.

2.1 Common nutraceuticals in the management of lower limb injury

Nutraceuticals are pharmaceutical forms (pills, capsules, powders etc) containing food bioactive compounds. They are diet supplements that deliver a more concentrated form of bioactive agent from a food with the purpose of enhancing healing (Espín et al. 2007). Nutraceuticals designed for musculoskeletal problems are popular as they provide the raw materials necessary for tissue synthesis or facilitate preservation of the structural integrity of the joint directly or indirectly (Vista & Lau 2011).

2.1.1 Glucosamine

Glucosamine (GlcN) is a naturally occurring amino monosaccharide composed of glucose with a bound amino group and present in the matrix of all connective tissues, including cartilage. It is one of the principal substrates used in the biosynthesis of Glycosaminoglycans, proteoglycans, aggrecans and hyaluronan, with all of which being fundamental components of articular cartilage (Wallace 2010). In addition, GlcN possesses anti-inflammatory and anti-catabolic properties via the cyclooxygenase-2 (COX-2) pathway which could further augment its treatment effectiveness

(Herrero-Beaumont et al. 2007b; Jang et al. 2007; Nagaoka et al. 2011), as well as enhancing collagen production in tendons and fibrous tissues (Lippiello 2007). The application of GlcN has been the focus of research for relieving symptoms of osteoarthritis (OA) as it is believed that abundant administration of GlcN would augment the endogenous production of GlcN as well as production of proteoglycan and hence maintain the normal turnover of the cartilage (Oegema Jr et al. 2002).

Animal studies have demonstrated the benefits of GlcN in decreasing cartilage lesion (Chen et al. 2010; Tiraloche et al. 2005; Wen et al. 2010), reducing osteoid volume (Wang et al. 2007), suppressing type II collagen degradation and enhancing type II collagen synthesis (Naito et al. 2010). However, in all of the studies (Chen et al. 2010; Lippiello 2003; Naito et al. 2010; Oegema Jr et al. 2002; Silva et al. 2009; Tiraloche et al. 2005; Wang et al. 2007; Wen et al. 2010), OA was induced by either surgery or enzyme injections which can produce the OA conditions quickly but may not truly simulate the natural progress of OA in humans.

Evidence in the literature suggest that GlcN was effective for managing OA or regular knee pain in improving the symptoms (Braham et al. 2003; Clegg et al. 2006; Herrero-Beaumont et al. 2007a; Pavelka et al. 2002; Reginster et al. 2001; Usha & Naidu 2004), preventing joint structural change (Pavelka et al. 2002; Reginster et al. 2001) and altering cartilage turnover in patients with osteoarthritis (Petersen et al. 2010) when compared with placebo treatment in human clinical studies (Table 2.1) (Chan & Ng 2011). In the literature review conducted by (Chan & Ng 2011), only half of the human studies reviewed have reported positive findings of GlcN towards knee OA. The reasons for the conflicting results may be the low sensitivity of outcome measures such as width of joint space, poor control of confounding variables such as baseline

characteristics of participants, rescue medications used before and during the experimental period, duration of treatment as well as the quality of the GlcN preparation. Recent studies advocated the combination of GlcN and chondroitin sulfate and long-term use of these supplements to treat knee OA (Fransen et al. 2015; Raynauld et al. 2016). Owing to the popularity of this supplement and the potential favourable effects towards cartilage metabolism and healing, further studies should explore the effective formulation, dosage, duration of treatment as well as the feasibility of treating other soft tissues injuries.

2.1.2 Antioxidants

Human body produces reactive oxygen species (ROS) continuously during cellular metabolism which is important for cell signaling and apoptosis (Scott & Nordin 2016). However, excessive and uncontrolled oxidative load may be associated with tendon pathology (Lewis & Sandford 2009) and abnormal adhesion formation (Binda et al. 2003). The results of oxidative stress can damage the constituents of the extracellular matrix such as proteoglycans and collagens (Hitchon & El-Gabalawy 2004). Scientists are interested in whether the use of antioxidants such as vitamin C, E, β-carotene and selenium can reverse the tissue damages and enhance tissue healing.

Vitamin C is a water soluble antioxidant acting as a reducing agent directly participating in the proline and lysine hydroxylation pathway to form procollagen which is essential for the later conversion to collagen (Kipp & Schwarz 1990). It also enhanced neutrophil function during inflammation (MacKay & Miller 2003), enhanced healing and collagen synthesis in bone, cartilage and tendon (Kipp et al. 1996). Other than the effects proposed, vitamin C may also enhance tendon healing by ensuring

tendon protein synthesis, cell permeability (Russell & Manske 1991), reducing extent of adhesion in injured tendon in a chicken model (Hung et al. 2013), stimulating angiogenesis as well as promoting type I collagen production in rats (Omeroğlu et al. 2009). Other antioxidants such as taurine, curcumin have also been found to enhance tendon healing (Akdemir et al. 2015; Jiang et al. 2016).

Apart from tendon injury, the pathogenesis of rheumatoid arthritis (RA) is associated with either increased oxidative stress or deficiency in antioxidant status or both (Canter et al. 2007; Mahajan & Tandon 2004). A low serum antioxidant level (vitamin E, β-carotene, and selenium) is therefore a risk factor of rheumatoid arthritis (Heliövaara et al. 1994).

However, controversy exists regarding the use of antioxidants towards musculoskeletal conditions. McAlindon et al. (1996b) found that antioxidants, particularly vitamin C, may slow down the disease progression in people with OA but not able to reduce the incidence of knee OA among participants without the disease. Most randomized controlled trials (RCTs) of antioxidants in the treatment of arthritis were generally of not high quality in which the descriptions of randomization and double-blinding were inadequate (Canter et al. 2007) and hence there was no convincing evidence to support the use of antioxidants (selenium, vitamin A, C or the combination product) for the treatment of any type of arthritis.

Table 2.1 Effect of GlcN on OA in human studies (Adopted from Chan and Ng (2011))

Study	Effectiveness[a]	WOMAC pain, stiffness and function	Lequesne	OMERACT/OARSI	Joint Space narrowing	Others
Braham et al.(2003)	Yes	N/A	N/A	N/A	N/A	Knee Pain Scale, $p<0.05$ Knee related quality of life, $p<0.05$
Cibere et al. (2004)	No	NS	N/A	N/A	N/A	EQ-5D questionnaire and disease flare, NS
Clegg et al. (2006)	No	NS	N/A	NS	N/A	Use of rescues medications and joint swelling etc, NS
Herrero-Beaumont (2007a)	Yes	$P<0.05$	$P<0.05$	N/A	N/A	Use of rescues medications, $p<0.05$
Hughes & Carr (2002)	No	NS	N/A	N/A	N/A	Global pain, McGill sensory etc, NS
McAlindon, T. (2004)	No	NS	N/A	N/A	N/A	Use of rescues medications, NS
Pavelka et al (2002)	Yes	$P<0.05$	$P<0.05$	N/A	$P<0.05$	Use of rescues medications, NS
Petersen et al. (2010)	Yes	N/A	N/A	N/A	N/A	Serum COMP and Urinary CTX-II, $p<0.05$
Reginster et al (2001)	Yes	$P<0.05$	N/A	N/A	$P<0.05$	Use of rescues medications, NS
Rindone et al (2000)	No	N/A	N/A	N/A	N/A	Visual analogue scale of pain at rest and during walking, NS
Sawitzke et al. (2010)	No	NS	N/A	NS	N/A	
Usha & Naidu (2004)	Yes	N/A	$P<0.05$	N/A	N/A	Pain index, swelling index, walking time and join mobility index, $p < 0.05$

[a] significant difference between GlcN group and control group

N/A: not applicable; NS: no significant difference between GlcN and control group; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; OMERACT/OARSI : Outcome measure in Rheumatology clinical Trials/ Osteoarthritis Research Society International; COMP: serum cartilage oligomeric matrix protein; CTX-II: c-telopeptide of Type II collagen, marker of type II collagen catabolism

McAlindon et al. (1996b) reported that high dose of antioxidants may be helpful only for those with a deficiency and may be useful for those who had the disease. Physiologic stress such as trauma and sudden injury may prompt the requirement of vitamins for collagen synthesis, tissue repair and fighting against oxidative stress (MacKay & Miller 2003). There is no consensus about the recommended intake of antioxidants. Taking antioxidants from fruits, vegetables, and flavonoid-rich foods is a more practical advice to the general public (Melton 2006).

2.1.3 Vitamin D

Vitamin D can be classified into two forms, namely, vitamin D3 (cholecalciferol) and D2 (ergocalciferol). Vitamin D3 is formed in the skin after exposure to ultraviolet B radiation and can also be obtained from animal sources such as salmon and cod liver oil, while vitamin D2 is obtained from plant sources such as Maitake mushrooms (Stechschulte et al. 2009). The major circulating form of vitamin D metabolized in the liver is 25-hyroxyvitamin D, 25(OH)D which is later converted into active form 1,25 $(OH)_2D$ in order to regulate up to 200 genes that facilitate cell growth and differentiation (Stechschulte et al. 2009). Blood level of 25(OH)D is an index to define deficiency. It is estimated over 1 billion people worldwide are either deficient or insufficient in vitamin D (Holick & Chen 2008) which would increase the medical costs on rickets in children, osteomalacia and osteoporosis in adults (Stechschulte et al. 2009).

Supplementation, sufficient intake or high serum level of vitamin D appear to reduce risk of bone fracture, rheumatoid arthritis, OA and enhance tendon healing and collagen production. Vitamin D metabolites promoted maturation and differentiation of

chondrocyte from cartilage by increasing collagen production (Schwartz et al. 1989) and inhibiting osteoblastic differentiation in mouse fibroblast which prevent mineralization of soft tissue (Chen et al. 2012). Three years of vitamin D3 and calcium supplementation would significantly reduce the risk of hip fracture by 43% and non-vertebral fracture by 32% among elderly women (Chapuy et al. 1992). Greater intake of either dietary or supplemental vitamin D may be associated with a lower risk of rheumatoid arthritis in older women (Merlino et al. 2004). On the other hand, low intake and low serum levels of vitamin D is associated with the loss of cartilage and osteophyte growth and may be associated with higher risk for progression of OA of the knee (Lane et al. 1999; McAlindon et al. 1996a). Vitamin D deficiency may be detrimental to tendon healing as less bone formation and less collagen fiber organization were observed in vitamin D-deficient rats (Angeline et al. 2014).

There is growing evidence for the use of nutraceuticals to manage musculoskeletal problems. However, most of the evidence is obtained from in vitro and animal studies particularly for tendon healing. It appears that supplementation is more applicable for those who have the diseases or deficiency in certain nutrients. Efforts should be put on ensuring more high quality RCTs to confirm the use of nutraceuticals.

2.2 Fish oil- Polyunsaturated fatty acids

2.2.1 Anti- Inflammation

Inflammation is the body's response to infection or injury which is manifested by clinical signs of redness, swelling, heat and pain. It is part of the normal, innate immune response but may lead to damage to the host tissues and diseases when uncontrolled (Calder 2006). The key regulators of these processes consist of a myriad of cytokines such as interleukin-1β (IL-1β) and IL-6 which are released by the cells in response to

the injury. Cytokines are regulatory glycoproteins produced by every nucleated cell types in the body and serve as local messenger proteins between cells. They can act positively or negatively and regulate all aspects of tissue remodeling (Border & Noble 1994). They are involved in such diverse processes as cell growth, cell differentiation, tissue repair and remodeling, and regulation of the immune response (de Jager & Rijkers 2006; Vilcek 2003). Therefore, control of various cytokines level may help prevent pathological inflammation and the subsequent excessive, irreparable damage to host tissues. Regulated, controlled inflammatory responses are essential for the body to remain healthy and maintain normal homeostasis (Calder 2009a).

Fish oil is believed to have anti-inflammatory effect as their components EPA and DHA competes with arachidonic acid (20:4 n-6; AA) for production of inflammatory eicosanoids. Both EPA and DHA are structurally similar to AA differing only in the presence of n-3 double bond (Figure 2.1). Both EPA and DHA are competitor substrates that inhibit oxidation of AA by the COX and lipoxygenase enzymes to form eicosanoids such as PGs and leukotriene (Cleland et al. 2003) (Figure 2.2). Fish oil ingestion increases the concentration of prostaglandins E3 (PGE_3) and leukotriene B5 (LTB_5) which have both anti-inflammatory and antimitogenic properties but reduces prostaglandin E_2 (PGE_2) and leukotriene B4 (LTB_4) (Bagga et al. 2003; Sköldstam et al. 1992). The PGE_3 induces down regulation to Tumour Necrosis Factor α (TNFα) and IL-1β synthesis by monocytes which would lessen its destructive effect to joint (Cleland et al. 2003) (Figure 2.3). Omega-3 fatty acids are hence transformed into less potent inflammatory eicosanoids (Simopoulos 2008). Other than the aforementioned mechanism, n-3 PUFAs are believed to alter the expression of genes encoding inflammatory mediator production such as IL-1β and IL-6 (Calder 2009b).

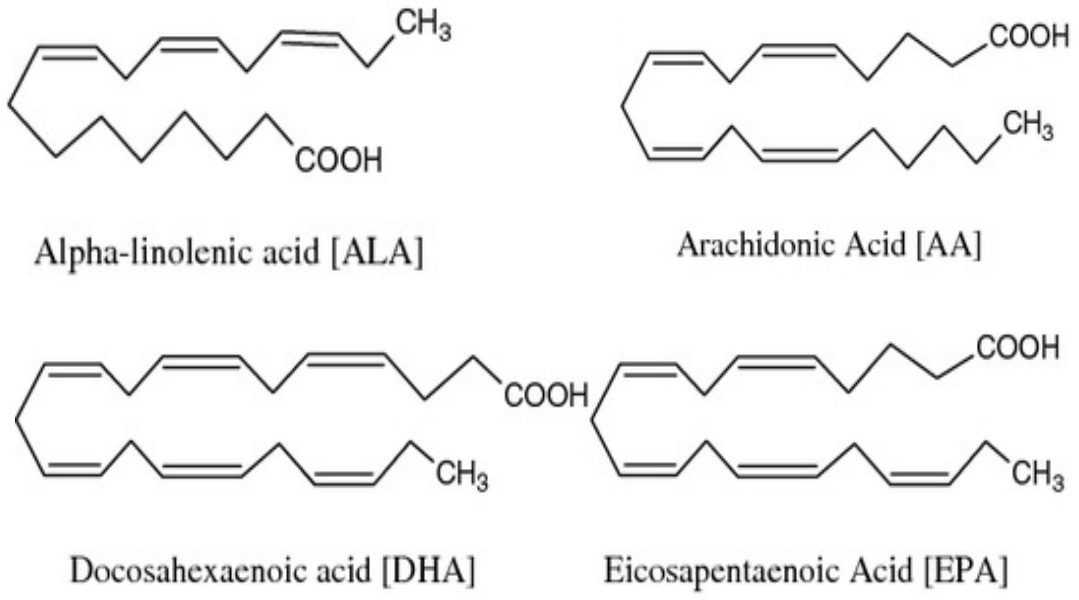


Figure 2.1 Structure of ALA, AA, DHA and EPA (Adopted from Asif (2011)).

ALA, DHA and EPA are omega-3 fatty acid while AA is an omega-6 fatty acid. Both EPA and DHA are structurally similar to AA differing only in the presence of n-3 double bond.

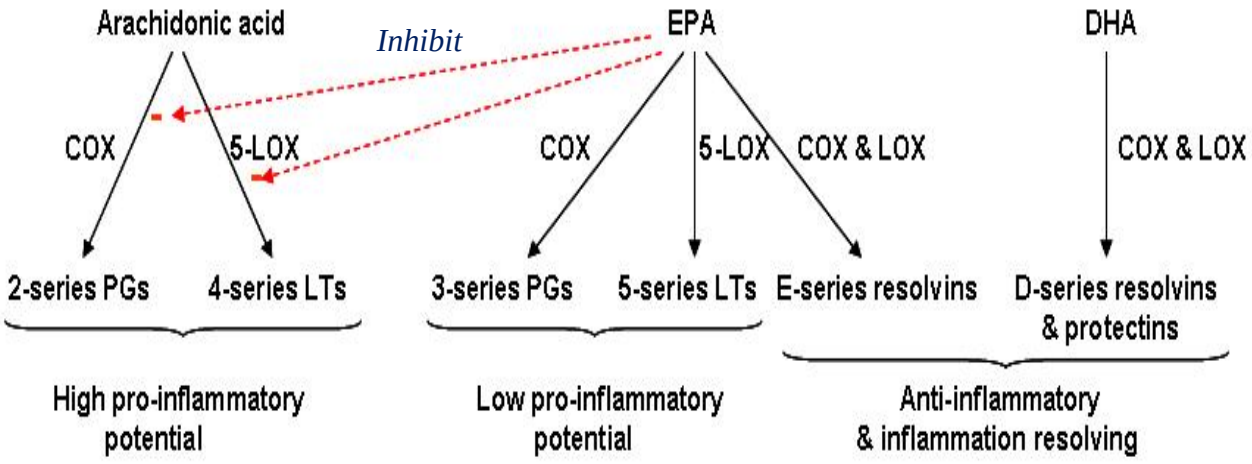


Figure 2.2 Overview of synthesis and action of lipid mediators (Adopted from Calder (2011)).

Both EPA and DHA are competitor substrates that inhibit oxidation of AA by the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes. Therefore, prostaglandins (PGs), leukotrienes (LTs), resolvins and protectins of low pro-inflammatory potential are produced.

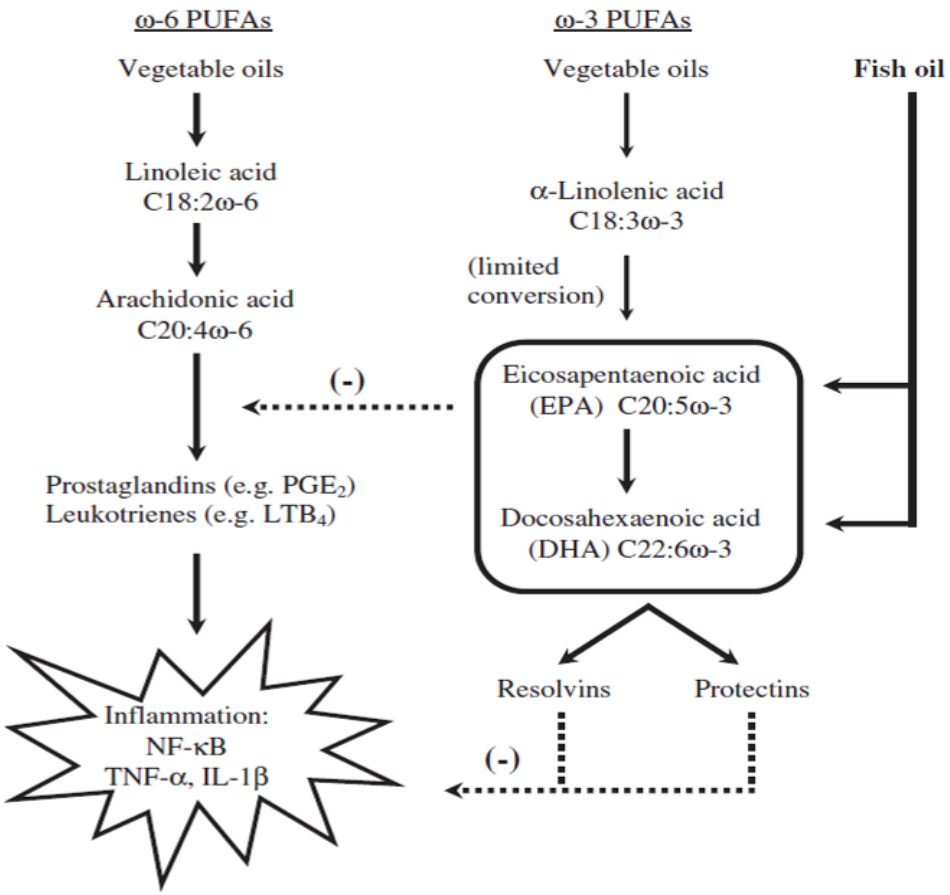


Figure 2.3 Metabolism of n-6 and n-3 fatty acids (Adopted from Goldberg and Katz (2007)).

EPA and DHA inhibit the formation of PGE_2 and LTB_4 and hence prevent the formation of nuclear factor kappa B (NF-κB), tumour necrosis factor (TNF) and interleukin-1β (IL-1β)

(-) = inhibition.

Recent studies have indicated that some anti-inflammatory lipids such as resolvins and protectins are produced by EPA and DHA (Goldberg & Katz 2007). Both Resolvins and protectins are anti-nociceptive (Tokuyama & Nakamoto 2011) and exert potent suppressive effects on neutrophils, macrophages, T cells and TNFα (Serhan et al. 2000; Serhan et al. 2008; Sommer & Birklein 2011). Omega 3 polyunsaturated fatty acids (n-3 PUFAs) was shown to suppress a lipid mediator- lysophosphatidic acid, which is strongly associated with the development of neuropathic pain (Miyazawa et al. 2003). Suppression of the synthesis of inflammatory cytokines and eicosanoids by n-3 PUFAs is also believed to reduce pain. Thus, n-3 fatty acids dampen inflammation and modulate pain but without inhibiting the healing process. Although there are numerous studies showing the production and activity of various components of the immune system can be modified by unsaturated fatty acids, the exact underlying mechanism of that has not been confirmed yet.

To summarize, the anti-inflammatory potential of n-3 PUFAs are due to

- reduced production of PGE_2 and LTB_4 metabolites which are pro-inflammatory,
- increased level in PGE_3 and LTB_5 which are weak inducers of inflammation,
- enhanced production of E-series resolvins, D-series resolvins and protectins which are anti-inflammatory and anti-nociceptive.

2.2.2 Omega 6 to Omega 3 ratio

PUFAs are incorporated in the cell membrane particularly phospholipids of practically all cells. N-6 cannot be converted to n-3 in mammalian cells and hence mammals depend on external sources to maintain the n-3 level within the body. The same system of enzyme processes all the families of essential fatty acids but with a

preference in the order of n-3, n-6 and n-9 (Claassen et al. 1995). As discussed in section 1.3.2, ingestion of EPA and DHA can replace arachidonic acid (AA) in membrane phospholipids (Benatti et al. 2004) and hence EPA and DHA are competitor substrates that inhibit oxidation of AA by the cyclooxygenase (COX) and lipoxygenase enzymes to form pro-inflammatory eicosanoids such as PGE_2 and LTB_4 (Cleland et al. 2003).

There are at least two isoforms of COX, namely COX-1 and COX-2. COX-1 is expressed in most normal tissues in the body to produce PGs to maintain cellular homeostasis, while COX-2 is mainly induced by pro-inflammatory mediators at the injured sites (Radi & Khan 2005). Zainal et al. (2009) supported that n-3 PUFAs including EPA, DHA and ALA reduce expression of COX-2 but not COX-1. EPA is particularly potent when compared with DHA and ALA. Suppression of COX-1 is associated with gastrointestinal ulcer formation (Cryer & Feldman 1998) and hence n-3 PUFAs are superior to NSAIDS which reduced both COX-1 and COX-2 (Wallace et al. 2000). Effectiveness of COX-2 inhibitors depends on the COX-2 expression and the supply of AA (Benatti et al. 2004). Therefore, some researchers proposed the concept of decreasing the ratio of n-6:n-3 in the diet so that more n-3 can compete with n-6 to produce less pro-inflammatory eicosanoids. According to this theory of substrate competition between n-6 and n-3 during eicosanoid metabolism, it is suggested that the ratios of n-6/n-3 rather than the absolute amount is more practical (Baker et al. 2012; Simopoulos 2008).

On the other hand, de Deckere (2001) and Zainal et al. (2009) disagreed with the above notion and advocated that the absolute amount of ALA, n-3 PUFAs and LA should be recommended. Since increasing the ratio alone by decreasing the dietary n-6

PUFAs level cannot reproduce the results as increasing n-3 PUFAs level. As humans need both n-6 and n-3, the ratio of n-6 to n-3 is not a useful and complete metric of health effects (Calder et al. 2010).

2.2.3 Plant and marine n-3 PUFAs

The 3 main n-3 PUFAs include ALA which comes from plant source, DHA and EPA which are mainly coming from fish. EPA and DHA are incorporated more easily into membranes than ALA, therefore the effects of fish oil is expected to be faster (Otranto et al. 2010). Supplementation of ALA could not raise blood DHA level, nor be converted to EPA and DHA completely (Brenna et al. 2009; Paschos et al. 2007). ALA from rapeseed oil (50g/ day) is metabolized to EPA in humans which resembled the effect of a weekly portion (50-100 g) of fatty fish (Valsta et al. 1996) and hence around 20% was metabolized to EPA (Tur et al. 2012). Consequently, supplementation of ALA could not fully mimic the effects of EPA and DHA because ALA, in particular, has the highest rate of oxidation among all PUFAs (Nettleton 1991). Due to the modest conversion of ALA to EPA, ALA may decrease eicosanoids and cytokines synthesis slightly. Therefore, it appears that marine n-3 PUFAs are more preferable if considered their effects on cytokines and eicosanoids.

2.2.4 Safety of omega-3 fatty acids

There are three potential problems of n-3 PUFAs therapy, namely, bleeding risk, exposure to environmental toxins and excessive intake of some fat soluble vitamins (Bays 2007). N-3 PUFAs control a wide range of functions in our body including blood pressure and blood clotting. They potentially contribute to a decrease in clinical

atherothrombosis which may increase the risk of bleeding particularly for patients who take anticlotting medications such as warfarin. In general, fish oil consumption of up to 6g per day (~1g n-3 PUFAs) for 4 weeks did not create a significant effect on the anticoagulation status of patients receiving chronic warfarin therapy (Bender et al. 1998). Fenton et al. (2013) reviewed the current evidence on the potential immunomodulatory effects and adverse effects of n-3 PUFAs supplementation and recommended up to 5g/day of combined DHA and EPA appear safe and not increase the risk of spontaneous bleeding or lipid peroxidation. On the other hand, high n-3 PUFAs consumption in animal may detrimentally affect the immune response to microbes in the gut, infection induced inflammation and cancer. It has been reported high dose of n-3 PUFAs (>10g/d) and low levels of AA was associated with hemorrhagic stroke in humans (Hamazaki et al. 2014). However, the result needs to be interpreted with cautions as it was only based on few cases of Greenland Eskimos.

Environmental toxins would be of particular concern for pregnant women, infants and young children. Pregnant women would benefit from fish intake (~2-3 servings per week) in ensuring the level of intelligence and visual recognition memory of the infant if a few species of fishes are avoided (Mozaffarian & Rimm 2006). Fish oil in dietary supplement, in general, is produced under stringent purification processes and quality control to minimize the risk of exposure to environmental toxins such as mercury and polychlorinated biphenyls (PCBs) and hence fish oil supplement may contain less toxins than fish (Bays 2007; Foran et al. 2003). Taking supplement from reliable manufacturers or avoid fishes high in heavy metals according to the suggestions from US Food & Drug Administration (2014) would be a possible way to get the n-3 PUFAs safely.

Another concern of fish oil supplement is the high intake of vitamin A and D. The physical form of fat soluble vitamin A determines the likelihood of hypervitaminosis in which oil based preparations is 10 times less toxic than water based (Myhre et al. 2003). Therefore, the vitamin A in fish oil is rarely reported to cause hypervitaminosis A if the consumption level of fish oil is in accordance with the recommended dosage. Fish oil, unlike cod liver oil, is manufactured from eviscerated fish and hence the level of vitamin A and D are generally present in very low levels (Phillipson et al. 1985).

Theoretically, topical administration of drugs could deliver more concentrated doses to the tissues below the site of application while maintaining low serum concentrations and hence reduced incidence of systemic adverse effects (Heyneman et al. 2000). Despite the fact that the safety of topical n-3 PUFAs has not been studied, topical administration of n-3 PUFAs should be well tolerated as fatty acids are components of the epidermis which rarely trigger allergic response on the skin (McCusker & Grant-Kels 2010). Henneicke-von Zepelin et al. (1993) confirmed that eight weeks of topical treatment of n-3 PUFAs was well tolerated by most patients with psoriasis.

Optimal n-3 PUFAs consumption is crucial to ensure the balance of risks and benefits. The general recommendations from American Heart association is that 2-3 servings of fish a week can provide 250-500mg of n-3 PUFAs per day (American Heart Association 2016). According to Australian Rheumatology Association, 2.7g of omega-3 (EPA and DHA) is required to reduce inflammation (Australian Rheumatology Association 2010). If higher dosage of n-3 PUFAs such as 5-6g/ day is required, dietary supplement from reliable manufacturers is more preferable than to taking fish.

2.2.5 Effect of fish oil on musculoskeletal problems and soft tissues healing

a) Rheumatoid arthritis

Rheumatoid arthritis is a chronic autoimmune inflammatory disorder affecting small joints of the hands and feet with eventual erosion of cartilage and bone causing severe pain, deformities and functional loss (Rosenbaum et al. 2010). A meta-analysis of randomized clinical trials on the n-3 PUFAs supplementation suggested that EPA/DHA supplementation could reduce joint pain intensity, morning stiffness and non-steroidal anti-inflammatory drug (NSAID) consumption in patients with rheumatoid arthritis (Goldberg & Katz 2007). Similar results were shown in another meta-analysis study (James et al. 2010) but that latter study focused more on fish oil supplementation rather than individual n-3 PUFAs. Daily fish oil consumption for 3 months was found to associate with decreased number of tender joints, use of NSAIDs and duration of morning stiffness in patients with rheumatoid arthritis of 10–11 years' duration. The anti-inflammatory and analgesic properties of n-3 fatty acids may account for the NSAID-sparing effect. The application of n-3 PUFAs in rheumatoid arthritis may be helpful in relieving symptoms.

b) Osteoarthritis

Osteoarthritis (OA) is a form of degenerative joint disease and the most common type of arthritis. It is believed to be caused by "wear and tear" of joints over time that leads to chondrocyte activation and cartilage breakdown and may affect the underlying bone and synovium (Vista & Lau 2011).

Animal and human studies with the key words "omega-3", "cartilage" and "osteoarthritis ", from the years 1999 were search from google scholar to screen all

citations that investigated the treatment effect of omega -3 fatty acids. Eleven animal studies and 6 human studies were identified.

N-3 PUFAs treatment improved signs and symptoms of OA in animals which was assessed by owners' assessed questionnaire on severity of OA (Corbee et al. 2013; Fritsch et al. 2010b; Roush et al. 2010b), investigators' assessed clinical signs (Fritsch et al. 2010a) and investigators' assessed reduction in NSAIDs usage (Fritsch et al. 2010b) (Table 2.2). Serum PUFAs concentration such as EPA, DHA and AA were significantly different between the groups after N-3 PUFAs treatment in three studies (Fritsch et al. 2010a; Roush et al. 2010b; Woodward et al. 2007) which proved the bioavailability of n-3 PUFAs. Three studies demonstrated lower level of pathologic markers in blood, synovial fluid and cartilage (Hansen et al. 2008; Knott et al. 2011; Manhart et al. 2009). Those markers included lower pro-matrix metalloproteinases-2 and 9 (proMMP) expression, lower urokinase plasminogen activator (uPA) activity, increased tissue inhibitors of metalloproteinases (TIMP) in synovial fluid (Hansen et al. 2008), lower denatured type II collagen (Knott et al. 2011), which were indicators of reduction in tissue degradation. Knott et al. (2011) also showed that Glycosaminoglycans content was higher in the n-3 group. The anti-inflammatory response was observed in n-3 group with decrease in synovial fluid white blood cell concentration and plasma PGE_2 (Manhart et al. 2009). Therefore, most animal studies have revealed the favourable effects of n-3 PUFAs treatment towards OA.

Table 2.2 Effect of n-3 PUFAs on OA in animal studies

Study	n	Study design	Animal model	Outcome measures- signs and symptoms	Outcome measures- others	Significantly favourable changes for n-3 group
Corbee et al. (2013)	21	Randomized, double-blinded, placebo-controlled, crossover design	Spontaneous OA of cats (client owned)	Owners' assessed questionnaire		Yes, most items
Fritsch et al. (2010a)	177	Randomized, double blinded and controlled	Spontaneous OA of dogs (client owned)	Clinical assessment by investigators, Owners' assessed questionnaire	Serum PUFAs concentration	Yes, in both clinical and owners' assessment; serum ALA, EPA, DHA, n-6 and AA concentration.
Fritsch et al. (2010b)	131	Randomized, double blinded and controlled	Spontaneous OA of dogs (client owned)	Clinical assessment by investigators, Owners' assessed questionnaire, NSAIDs usage determined by investigator		Yes, NSAIDs usage
Hansen et al. (2008)	24	Randomized, double-blinded and controlled	spontaneous cranial cruciate ligament CCL injury of dogs with surgical treatment and dietary treatment (client owned)		proMMP-2, uPa, TIMP-2, MMP,	Yes, on proMMP-2, uPa and TIIMP-2 in synovial fluid of nonsurgical knee
Knott et al. (2011)	40	Randomized, double blinded and controlled	OA –prone Dunkin-Hartley Guinea pigs (DH)		Cartilage and subchondral bone biochemistry Histological analysis	Yes, cartilage structure, OARSI criteria, Glycosaminoglycans content, type II collagen and MMP-2
Manhart et al. (2009)	16	Randomized group	Spontaneous arthritis of mature horses from the same university horse centre		Synovial fluid white blood cells, plasma PGE_2, fibrogen	Yes, white blood cell concentration and plasma PGE_2
Mehler et al. (2016)	78	Randomized, double blinded and placebo controlled	Spontaneous OA of dogs (client owned)	Lameness/Discomfort VAS assessed by the same investigator, Individual joint score (effusion, pain on palpation or range of motion, and crepitus)	iFATs: inflammatory fatty acid target score, ratio of ARA/(EPA+DHA)	Yes, in lameness/discomfort VAS, total joint severity score, iFats
Moreau et al. (2013)	30	Randomized, double-blinded and controlled l	Spontaneous OA of dogs (client owned)	Case-specific outcome measures (owner assessed)	Vertically oriented ground reaction force	No
Roush et al. (2010a)	38	Randomized, double blinded and controlled	Spontaneous OA of dogs (client owned)	Clinical assessment by investigators, Owners' assessed questionnaire	Peak vertically force	No
Roush et al. (2010b)	127	Randomized, double blinded and controlled	Spontaneous OA of dogs (client owned)	Owners' assessed questionnaire, Investigator assessments of clinical signs of OA	Serum PUFAs concentrations	Yes, Owners' assessed questionnaire, serum total n-3 and AA concentrations
Woodward et al. (2007)	12	Randomized groups	Spontaneous OA of horse from the same horse centre	Stride length, lameness scores	Plasma PUFAs concentrations	Yes, plasma DHA, AA concentrations

MMP: matrix metalloproteinases; proMMP-2: promatrix metalloproteinases-2; TIMP-2: tissue inhibitors of MMP-2; uPa: urokinase plasminogen activator activity, is a key agent in the proteolytic activation of MMP in the articular cartilage of arthritic joints; PGE_2: prostaglandin E_2; VAS: visual analogue scale

Table 2.3 Effect of n-3 PUFAs on OA in human studies

Study	n	Study design	Subject	Treatment	Significantly favourable changes for n-3 group
Caturla et al. (2011)	45	Randomized Double blind, placebo control	45 subjects with joint discomfort or pain for more than 3 months	• Fish oil and lemon verbena extract supplement • Placebo For 9 weeks	Yes Significant improvement on WOMAC pain, stiffness, function, Lequesne's functional index, starting from the 3rd or 4th week till the end of the study
Gruenwald et al. (2009)	164	Randomized, double blind (No placeb control)	182 patients with moderate-to-severe hip or knee OA	• Glucosamine sulfate, EPA, DHA • Glucosamine sulfate For 26 weeks	●No difference for WOMAC score ●Significant difference on the percentage of patients with ≥80% reduction (P=0.044) of WOMAC score ●If the criteria set at ≥90% reduction, superiority was achieved for the combination product (P=0.015)
Hill et al. (2016)	202	Randomized, Double blind trial, multicenter (No placebo control)	202 patients with knee OA and regular knee pain	• High dose fish oil (H): 4.5g n-3 • Low dose fish oil (L): 0.45g n-3 For 2 years	Yes , the L group ● lower pain scores at 18 and 24 months, ● better functional limitation scores at 24 months No difference in total cartilage volume, bone marrow lesion , use of analgesics and NSAIDS
Jacquet et al. (2009)	81	Randomized, double-blind	81 patients with OA of the knee or hip	• Phytalgic: consists of fish oils rich in omega-3 and omega-6 fatty acids, Urtica dioica, zinc and vitamin E; • Identical placebo For 3 months	Yes ●Significantly lower the mean use of analgesics and NSAIDS in treatment group ●Significantly decreased in the mean WOMAC scores for pain, stiffness and function, WOMAC global score
Peanpadungrat (2015)	75	Did not mention	75 patients with mild to moderate stage of knee OA	• 1000mg fish oil (EPA400mg, DHA 200mg) • 2000mg fish oil • Behavior modification only For 12 weeks	Yes, the two fish oil groups ● In WOMAC function subscale, 100m walking time, However, significant difference was observed at baseline in walking time and pain score.
Stammers et al. (1992)	86	Double blind, placebo controlled trial	86 patients with clinical diagnosis of OA	• 10 ml cod liver oil • 10 ml olive oil For 24 weeks	●No difference between the groups at baseline ●No difference between the group in pain, disability

Three human clinical trials have revealed that n-3 PUFAs supplementation could improve WOMAC function (Caturla et al. 2011; Jacquet et al. 2009; Peanpadungrat 2015) (Table 2.3). Significantly lower consumption of analgesics and NSAIDS was found in the fish oil group (Jacquet et al. 2009). Recently, Hill et al. (2016) compared high and low doses of fish oil and reported that improvements in WOMAC pain and functional scores were also observed in the lower dose group at 2 years. However, this study did not have a control group, thus one needs to be cautious in interpreting the results. No difference was found for WOMAC score in the report of Gruenwald et al. (2009) but significant difference was found when the cut-off difference level was set at ≥80% reduction of the score. It indicated that patients with more severe symptoms tended to benefit from the supplementation more.

The experimental design of those human studies was not satisfactory. For example, randomization and blinding were not clearly explained and the baseline walking time and pain score were different among groups (Peanpadungrat 2015). The treatment supplementation contained ingredients other than fish oil such as glucosamine sulfate, lemon verbena extract, vitamin E and zinc (Caturla et al. 2011; Gruenwald et al. 2009; Jacquet et al. 2009). Due to the extra ingredients, the actual effect of fish oil alone may be masked.

c) Cutaneous wound healing

Skin is the largest organ in mammals and has a vital role for maintaining electrolyte balance, immune function and thermoregulation. Cutaneous wound is the damage of the epithelial tissue and may affect the dermis, subcutaneous fat, fascia, muscle or even bone (Enoch & Price 2004). Wound healing is a complex biological

process depending on effective synthesis of healthy collagen to provide strength but not excessive scarring. Numerous factors can cause impaired wound healing such as increasing age, androgens in aged individuals, obesity as well as cytokines level (Guo & DiPietro 2010). The main foci on wound management are to accelerate healing by stimulating collagen synthesis, angiogenesis and pain relief (Singer & Dagum 2008).

Two in-vitro studies analyzing the effect of n-3 on fibroblasts (Hankenson et al. 2000; Jia & Turek 2004) have demonstrated higher connective tissue fibre deposition in cutaneous wounds in animal model with n-3 PUFAs administrated orally (Otranto et al. 2010) and topically (Cardoso et al. 2004) but delayed wound closure was observed in both studies (Table 2.4). The higher level of collagen deposition could be useful for conditions with deficiency of collagen synthesis. Scardino et al. (1999) found that n-3 fatty acid enriched diets would induce significantly less edema in sutured wounds even though there was no difference in epithelialization.

Apart from oral intake of n-3 PUFAs, few studies had tested the topical form of n-3 PUFAs on wound healing. Topical Cod liver oil rich in EPA, DHA, vitamin A and D, has been found to accelerate wound epithelialization and neovascularization in hairless mouse ear wound model (Terkelsen et al. 2000) but it is not sure which components are responsible for the effect. Topical administration of n-3 PUFAs solid emulsion gel in a pig wound model has been reported to have promoted wound repair by affecting cell division, induction of vascular endothelial growth factor (VEGF) expression and stimulating local angiogenesis (Shingel et al. 2008).

Table 2.4 Effect of n-3 PUFAs on skin wound in animal studies

Study	Animal model	Treatment	Control	Duration of treatment	Route of administration	Significant results in n-3 group
Cardoso et al. (2004)	Dorsal wound of male BALB/c mice	• ALA, n-3 • LA, n-6 • Oleic, n-9 (amount: 30 uM)	Vehicle	20 days (complete wound closure)	Topical	N-3 group had significantly larger wound than the n-9 group at days 5 & 10 N-3 delayed wound closure but significantly increased in connective tissue fibre deposition.
Gercek et al. (2007)	Full thickness dorsal wound of SD rats	• Dexamethasone & fish oil (DO) (1 ml/kg Commercial supplement Omegaven) • Dexamethasone (D)	Saline	14 days	Intraperitoneal injection	Fish oil did not aggravate the dexamethasone effects nor had adverse effects on wound healing
Otranto, Do Nascimento, and Monte-Alto-Costa (2010)	Full thickness dorsal wound of male Wistar rats	• Sunflower oil (n-6) • Linseed oil (vegetal source of N-3) • Fish oil (DHA, EPA) (amount: 1.5 ml/kg BM/day)	Water	14 days after wounding (30 days before wounding)	Oral gavage	The two n-3 groups delayed in wound closure but increased in collagen deposition
Scardino et al. (1999)	Full thickness dorsal wound of Beagle dogs	• Diet supplemented with Menhaden fish oil (n-6 :n-3: 0.3)	Diet supplemented with soya oil	20 days	Oral	Fish oil significantly lowered edema in sutured wound. No differences in epithelialization of the open wounds day 10 and beyond but less epithelization in n-3 group at day 5.
Shingel, Faure, Azoulay, Roberge, and Deckelbaum (2008)	Full thickness dorsal wound of pigs	• fish oil (EPA: 47.62 mg/g; DHA: 18.45 mg/g) • olive oil • Hydrogel wound dressing BioAquacare	Dry gauze	~30 days (complete wound closure)	Topical with solid emulsion gels	Fish oil induced greater reduction in wound area, stimulation of local angiogenesis and synthesis of vascular endothelial growth factor..
Terkelsen, Eskild-Jensen, Kjeldsen, Barker, and Hjortdal (2000)	Full thickness ears wounds of male hairless mice (HRS/J)	• Cod liver oil (EPA:8%; DHA: 9%) • Vehicle (yellow Vaseline)	Saline	~35 days (complete wound closure)	Topical	Cod liver oil ointment showed significantly faster epithelialization and neovascularization.

There were conflicting results regarding n-3 PUFAs treatment towards wound healing. One of the possible explanations for the difference is the duration of treatment. In the two studies showing positive potential of n-3 PUFAs, the treatment was applied to the wound till complete wound closure for at least 30 days (Shingel et al. 2008; Terkelsen et al. 2000). Since N-3 PUFAs is a COX-2 inhibitor and inflammation is essential for wound healing, it is possible that the anti-inflammatory effects of n-3 PUFAs can interfere with the wound healng process. Early administration of COX-2 inhibitors such as parecoxib would negatively affect tendon healing but applying for a longer period could improve healing afterward (Forslund et al. 2003; Virchenko et al. 2004). As tendon and skin are both rich in collagen and they have the same biological sequel towards injury, it is possible that treatment proved to be effective for skin wound may also work for tendon. Therefore, it is possible the timing and duration of n-3 PUFAs treatment may be critical for the healing of connective tissue.

d) Tendon injury

Martins et al. (2011) compared the effect of plain application of topical ovis aries gel, therapeutic ultrasound with topical ovis aries gel as coupling medium, oil-free lotion with message, therapeutic ultrasound with oil- free lotion as coupling medium, and plain application of oil-free lotion as control in wistar rats tendinitis model. They found combination of pulsed ultrasound and topical ovis aries resulted in significant less inflammatory cells number than the control group but no difference was found between other groups with seven days of treatment. Nevertheless, all treatment groups were significantly different from the control group after 14 days of treatment. Contrary to the belief of balancing the ratio of n-3 and 6 to suppress inflammation, this study

used fat from ovis aries which is abundant in oleic (n-9) and LA (n-6). However, the rationale for using ovis aries was not clearly explained in the report.

In a study using human subjects, Mavrogenis et al. (2004) used a randomized, double-blind, placebo-controlled design to analyze the effect of essential fatty acids (EPA, DHA and gamma-linolenic acid) and antioxidants consumption combined with ultrasound treatment on 40 recreational athletes suffering from chronic tendon disorders. All subjects received ultrasound treatment and consumed essential fatty acids daily whereas the control group consumed placebo tablet. The pain score in the treatment group has significantly decreased and the performance in sport-specific activity was increased. However, since the treatment group had also received US, the treatment effect of essential fatty acids alone was uncertain.

2.2.6 Transdermal absorption

The skin is the largest organ of the human body covering a surface area of approximately $2m^2$ and receives about one third of the body's blood circulation (Byl 1995). The skin is composed of epidermis, dermis and the subcutaneous tissue in which the skin appendages were interwoven within the two layers. The outermost layer of epidermis is the stratum corneum which is responsible for the barrier function of the skin and considered as the rate-limiting factor for transcutaneous drug delivery (Contri et al. 2011). The stratum corneum contains corneocytes and extracellular lipid molecules which are arranged in 15-20 layers (Nino et al. 2010). The most important predictors for permeation of topical drugs are lipophilicity and molecular weight of the medications (Russell & Guy 2009). As most substance penetrates the skin via intercellular pathway and pass through the extracellular lipid matrix, cutaneous

penetration of hydrophilic substances is limited, while lipophilic solutes usually have high permeability (Nino et al. 2010). The amount of cross-linking in collagen, extent of the skin appendages, age of the skin, hydration status as well as the presence of fatty acids would also determine the selective permeability of the skin (Byl 1995).

PUFAs have a high epithelial penetration ability and serve as a topical drug permeation enhancer such as ketoprofen (Heard et al. 2003; Puglia et al. 2005; Thomas & Heard 2005) and *p*-aminobenzoic acid (PABA) (Tanojo et al. 1997). Similar result was observed in fish oil. Zulfakar et al. (2010) found that fish oil would enhance corticosteroid delivery across full thickness skin of the porcine ears. PUFAs are one of the endogenous compounds in human skin lipids, which present in stratum corneum and play a key role on the properties of biological membranes (Tanojo et al. 1997).

It is believed that lipophilic substances readily enter the stratum corneum layer of the skin without partition out to the more hydrophilic dermis and blood circulation (Banga 2010a). However, Thomas et al. (2007) refuted this notion and suggested PUFAs could traverse full thickness skin of porcine ears and exert its effect on modulating inflammation.

Other than lipophilicity, molecular weight is also a critical factor for skin penetration. Lipophilic compounds of molecular weight more than 400g·mol^{-1} were poorly absorbed by rat skin in vivo. The molecular weight of DHA is 328.49g·mol^{-1} and that of EPA is 302.42g·mol^{-1}, their relatively light molecular weight suggests that both fatty acids should be easily absorbed by rat skin. In addition, they are much smaller than insulin (5808g/mol) which is a drug with high molecular weight and has reportedly been transported through the skin in rats with ultrasound phonophoresis (Mitragotri & Kost 2004).

The main adverse effects of oral consumption of fish oil is mild dyspepsia and belching (Yashodhara et al. 2009). As stated in section 1.3.5, environmental contaminants is a concern when some species which are top in the food chain such as sharks and swordfish are eaten for n-3 PUFAs. Moreover, it has been speculated high dose of n-3 PUFAs (>10 g /d) and low levels of AA was associated with hemorrhagic stroke (Hamazaki et al. 2014) and may exert immunosuppressive effect. However, these are speculations yet to be shown with sufficient clinical data to support the arguments (Eritsland 2000). The advantages of percutaneous administration of drugs include suppression of first hepatic pass effect and absence of degradation by the digestive tract and hence ensure more concentrated dosage transported to the affected area (Banga 2010b; Machet & Boucaud 2002).

As a summary, omega-3 PUFAs have diverse roles in soft tissue healing including suppression of inflammation, increasing collagen formation, pain relief as well as mediating various eicosanoids and cytokines. More evidence have derived in support of the application of n-3 PUFAs on inflammatory conditions such as rheumatoid arthritis and OA. However, there was controversy regarding the use of n-3 PUFAs on cutaneous wound and tendon injury. Only one human study has examined their effect on tendon healing but the treatment group included fish oil together with antioxidants and therapeutic ultrasound and hence the treatment effect of n-3 PUFAs alone was uncertain. Therefore, more studies are required to prove the effect of n-3 PUFAs on tendon healing and collagen synthesis.

2.3 Therapeutic ultrasound

2.3.1 Biophysical Effects

The therapeutic effects of ultrasound are due to its thermal and non-thermal or mechanical effects. The thermal effects include analgesic on nerves, increasing blood flow and collagen extensibility (Baker et al. 2001) but this effect is small for pulsed ultrasound (Khanna et al. 2009). The mechanical effects are usually associated with formation of bubbles and collapse of gaseous cavities in the tissues or simply named as "cavitation" (Baker et al. 2001). The physical forces of the sound wave cause gas bubbles in the tissue fluid to contract and expand. It can change the cell membrane's permeability by creating transient pores and hence the transportation of ions or membrane messengers such as calcium ions across the cell membrane, which is essential for tissue repair (Mortimer & Dyson 1988). Another mechanical effect is called "acoustic streaming" which is the physical forces of the sound waves that can displace ions and small molecules so as to stimulate cellular activities and metabolic processes such as protein synthesis, production of growth factors and fibroblast mobility changes at the boundary of the cell membrane and the surrounding fluid (Watson & Young 2008). The combined mechanical effects lead to growth retardation and a cellular recovery response that is characterized by an increase in protein production (Johns 2002). One more mechanical effect proposed by Johns (2002) is the frequency resonance hypothesis. It is the absorption of ultrasound energy by proteins and protein complexes and the subsequent change in signaling mechanisms within the cell. Thus, these changes may lead to enhance or decrease enzymatic activities or possible gene regulation. These cellular and molecular changes within cells are centrally involved in the inflammatory and healing processes and hence non-thermal effects are more critical for soft tissue healing (ter Haar 2007).

2.3.2 Effect on tendon healing- animal studies

Animal with the key words “tendon”, “ultrasound” and “animal ”, from the years 1990 were search from google scholar to screen all citations that investigated the treatment effect of therapeutic ultrasound alone on tendon healing. Eleven animal studies were identified summarized in table 2.5. Among the 11 studies, all except two showed favourable results towards ultrasound treatment in improving strength (Enwemeka et al. 1990; Fu et al. 2008; Jackson et al. 1991; Ng et al. 2003; Yeung et al. 2006), enhancing range of motion (Gan et al. 1995), increasing collagen fibers synthesis (Jackson et al. 1991), hastening collagen fiber organization (da Cunha et al. 2001), and boosting mean collagen fibril size (Ng & Fung 2007). However, both Carvalho et al. (2006) and Larsen et al. (2005) could not reveal any beneficial effect of therapeutic ultrasound on collagen content.

In the study of Larsen et al. (2005), the authors treated the tenotomized, sutured and immobilized rabbits with different intensities of ultrasound ranging from 0.05 to 2W/cm^2. A gradual decline in stiffness and collagen content was observed with increasing ultrasound intensities and there was no difference between the control and treated group. The authors did not explain the discrepancy between their study and other studies but argued that the important treatment qualities of ultrasound, namely, cavitation and acoustic microstreaming were still debatable in vivo.

Standardized histologic grading system has not been available for tendon. The histological analyses were commonly qualitative and semi-quantitative in which the reproducibility and validity has been questioned. Some of the studies adopted semi-quantitative approach to quantify the histolomorphology without explicit explanation on the grading system and running statistical analyses subsequently to

establish conclusion (Carvalho et al. 2006; Gan et al. 1995; Saini et al. 2002). Despite Carvalho et al. (2006) stating therapeutic ultrasound promoted fibrosis and collagen synthesis as illustrated histologically, the initial ANOVA analysis could not reveal a significant difference between the groups. The grading for semi-quantitative analysis of fibrosis and collagen synthesis was not described clearly in the article. The number of assessors involved and whether the assessors were blind to the group assignment were not explained thoroughly. Therefore, higher quality studies with more objective and stringent histological analyses are required to review the effect of therapeutic ultrasound on tendon healing.

Table 2.5 Effect of therapeutic ultrasound on tendon healing in animal studies

	Model	Group	US parameters-frequency, intensity, duration, duty cycle	Total treatment sessions	Outcome measures	Favourable results towards therapeutic ultrasound
Carvalho et al. (2006)	Crushing injury in the Achilles tendons of Malnourished albino Wistar rats	a) CON-Malnourished injured b) Malnourished injured with US c) Normal injured with US	3 MHz, 0.5 W/cm^2, 6 min, 20% duty cycle for both malnourished injured and normal injured rats	3 or 7 or 14	a) vessel neoformation, b) fibrosis c) collagen d) leukocytes count	No, with initiate ANOVA analysis between groups
Enwemeka et al. (1990)	Tenotomized, repaired and immobilized right Achilles tendons in rabbits	a) CON b) Treatment	1 MHz, 0.5 W/cm^2 (spatial average), 5 min, continuous	9	a) tensile strength b) tensile stress c) energy absorption capacity	Yes, for all outcomes
da Cunha et al. (2001)	Tenotomized Achilles tendon in Wistar rats	a) CON-tenotomized, mock-sonicated b) CON-normal tendon c) Treatment- pulsed US d) Treatment- continuous US	c) 1 MHz, 0.5 W/cm^2, 5 min, continuous d) 1 MHz, 0.5 W/cm^2, 5 min, 20% duty cycle	12	a) Organization of collagen fibers through birefringence	Yes, for pulsed mode but not the continuous mode
Fu et al. (2008)	Patellar tendon donor site injury of both legs of SD rats	a) CON b) Treatment-initiated at day1after injury c) Treatment- initiated at day 15 after injury d) Treatment- initiated at day 29 after injury	1 MHz, 30 mW/cm^2, 20 min, continuous	10, 20, 30	a) ultimate mechanical strength b) tensile modulus c) histological analysis (qualitative)	Yes, for the group treated for 2 weeks of LIPUS applied from day 1 post-lesions in ultimate mechanical strength
Gan et al. (1995)	Tenotomy of Zone 2 flexor tendons in leghorn chicken	a) CON b) Treatment- initiated at day 7 after injury c) Treatment- initiated at day 42 after injury	3 MHz, 0.2 W/cm^2, 3 min, 25% duty cycle	10	a) range of motion b) tensile strength c) scar maturation (qualitative and semi-quantitative)	Yes, for early administration group rather than the late administration group in range of motion. The two ultrasound groups showed a marked decrease in inflammatory infiltrate and a more regular pattern of scar formation (qualitative).
Jackson et al. (1991)	Partial rupture (puncture) of both Achilles tendon in Holtzman rats	a) CON b) Treatment	1.5 W/cm^2 , 4 min, continuous Unknown frequency	2,5,8,11, 14	a) breaking strength b) collagen synthesis (total hydroxyproline)	Yes , in breaking strength from day 5 -21 post-injury and collagen synthesis day 5 post-injury
Larsen et al. (2005)	Tenotomized sutured and immobilized Achilles tendons of rabbits	a) CON b) 7 different treatment groups with different in power output (0-1.2 2 W/cm^2)	3 MHz, 0, 0.05, 0.1, 0.2, 0.5, 0.75, 1, 2 W/cm^2, 5 min, pulsed but unknown duty cycle	10	a) breaking load b) stiffness c) collagen content (hydroxyproline)	No, slight decline in stiffness and collagen content was observed with increasing intensity of ultrasound treatment.
Ng et al. (2003)	Hemitenotomy of right medial Achilles tendons in SD rats	a) CON b) Treatment-1 W/cm^2 c) Treatment-2 W/cm^2	b) 1 MHz, 1 W/cm^2 , 4 min, continuous c) 1 MHz, 2 W/cm^2 , 4 min, continuous	22	a) Ultimate tensile strength b) Stiffness c) Load-relaxation d) Achilles functional index	Yes, for both ultrasound groups in UTS
Ng and Fung (2007)	Hemitenotomy of right medial Achilles tendons in SD rats	a) CON b) Treatment-0.5 W/cm^2 c) Treatment-1.2 W/cm^2 d) Treatment-2 W/cm^2	b) 1 MHz, 0.5 W/cm^2 , 4 min, continuous c) 1 MHz, 1.2 W/cm^2 , 4 min, continuous d) 1 MHz, 2 W/cm^2 , 4 min, continuous	22	a) Mean collagen fibril size	Yes, for all ultrasound groups. A trend towards lower intensity with increasing collagen fibril size was observed
Saini et al. (2002)	Tenotomy close to the point of insertion of left Achilles tendons in dogs	a) CON b) Treatment	0.5 W/cm^2 , 10 min, unknown frequency and duty cycle starting from day 3 after tenotomy	10	a) Clinical observations- weight bearing, swelling b) ultrasonography c) histomorphology	Yes, but all outcomes are based on qualitative and semi-quantitative analyses without statistical analyses to support the conclusion.
Yeung et al. (2006)	Hemitenotomy of right medial Achilles tendons and patella tenotomy in SD rats	a) CON b) Treatment	1 MHz, 0.5 W/cm^2 (SATA), 5 min, 20% duty cycle	6, 12	a) UTS b) stiffness c) load-relaxation d) histological analysis (qualitative and semi-quantitative)	Yes, UTS and stiffness at 2 & 4 weeks

CON: control group ; UTS: ultimate tensile strength; LIPUS: low-intensity pulsed ultrasound

2.3.3 Effect on lower limb injury

Human studies with the key words “ultrasound”, “human”, “musculoskeletal disorders”, “knee”, “ankle” and “tendon ”, from the years 1990 were search from google scholar to screen all randomized or quasi-randomized clinical trials that investigated the treatment effect of ultrasound on lower limb injury. Ten studies were identified and were summarized in Table 2.6. Among the 10 studies reviewed, half of them reported beneficial healing effects with therapeutic ultrasound but half of them did not. The reasons are multifaceted. First, the trials covered diverse range of conditions. Three of them focused on acute ankle sprains (Bradnock et al. 1996; Zammit & Herrington 2005), two on tendinopathy (Chester et al. 2008; Warden et al. 2008), four on OA of the knee (Falconer et al. 1992; Loyola-Sánchez et al. 2012; Özgönenel et al. 2009; Tascioglu et al. 2010) and one on plantar heel pain (Crawford & Snaith 1996). The conditions of the patients ranged from acute to chronic lasting for 4 months to 3 years. With such a wide range of problems, the pathologies behind individual conditions are different, it is not easy to make conclusion and comparisons (Table 2.7).

Second, there are no standardized ultrasound parameters for humans or for any given musculoskeletal conditions. The frequency adopted ranged from 45kHz to 3MHz, which could be pulsed or continuous. The power output ranged from 100mW/cm^2 to 2W/cm^2. The duration of treatment lasted from 2 to 20 minutes per session and the number of sessions ranged from 1 to 12.

Table 2.6 Effect of therapeutic ultrasound on different lower limb injuries in human studies- study design and outcomes

Study	Study design (all were randomized controlled trial)	Control of Pain medication	Additional exercise/other remedies	Outcomes measures	Favourable results towards US
Bradnock et al. (1996)	Single blinded	Not mentioned	Nil	Length of stride, symmetry of swing phase duration, cadence and walking velocity	Yes in low frequency group
Chester et al. (2008)	Single blinded	No additional treatments during and 6 weeks after the treatment	Nil	Pain (VAS), functional index, EuroQol generalized health questionnaire	Yes No dfference between heavy eccentric loading and ultrasound group
Crawford and Snaith (1996)	Double blinded	No pain controlling methods before and during treatment	Nil	Pain score (VAS)	No
Falconer et al. (1992)	Double blinded	Not mentioned	12 sessions following the US/sham treatment	Pain (VAS), Range of motion,	No
Loyola-Sánchez et al. (2012)	Double blinded	Not mentioned	Nil	medial tibia cartilage volume and thickness, subchondral cyst formation, bone marrow lesions, WOMAC scores	Yes in medial tibia cartilage thickness
Nyanzi et al. (1999)	Double blinded	Paracetamol was prescribed for those in need.	Tubigrip were given to all patients.	Pain (VAS), swelling, range of movement and weight bearing	No
Özgönenel et al. (2009)	Double blinded	No analgesics 10 days prior and during the study	Nil	Pain (VAS), WOMAC scores, 50 meters walking time	Yes for all outcomes
Tascioglu et al. (2010)	Single blinded	No analgesics 10 days prior and during the study	Nil	Pain (VAS), WOMAC scores	Yes for PUS group
Warden et al. (2008)	Double blinded	Not mentioned	Daily standardized eccentric exercise	Pain (VAS-usual and worst)	No
Zammit and Herrington (2005)	Single blinded	Not mentioned	Ice packs, tubigrip and exercises	Pain (VAS), swelling, range of motion	No

WOMAC : Western Ontario and McMaster Universities Osteoarthritis Index

Table 2.7 Effect of therapeutic ultrasound on different lower limb injuries in human studies - subjects conditions and ultrasound parameters

Study	Injury/condition	Subjects (no. of group)	Mean age	US parameters-power	duration
Bradnock et al. (1996)	Acute lateral Ankle sprains	47 (3)	N.A.	i) 3MHz,0.4W/cm^2 , 33% PUS ii) 45kHz, 0.19 W/cm^2 ,CUS	5 min, one off treatment , reviewed immediately after and 3 days later
Chester et al. (2008)	Achilles tendon pain	16 (2)	59±10 vs 48±12	3MHz, 0.5 W/cm^2 , 20% PUS	2min/cm^2 , twice a weeks for 6 weeks (12 sessions)
Crawford and Snaith (1996)	Plantar heal pain	19 (2)	N.A.	3MHz, 0.5 W/cm^2 , 20% PUS	8 min, twice weekly for 4 weeks (8 sessions)
Falconer et al. (1992)	Knee OA	69 (2)	65.7±12.8 vs 69.4±13.1	1 MHz, Intensity was progressed from 0.0 W/cm^2 to a maximum tolerable dosage >2.5 W/cm^2	12 min. 2-3 times per weeks over 4-6 weeks (12 sessions)
Loyola-Sánchez et al. (2012)	Mild to moderate Knee OA	27 (2)	61.15±11.5 vs 62.57±9.5	1MHz, 0.2 W/cm^2 , 20% PUS	9.5 minutes per session, 24 sessions (3 per week)
Nyanzi et al. (1999)	Acute Ankle sprains	58 (2)	50	3MHz, 0.25 W/cm^2, 20% PUS	10 minutes per session, for 3 consecutive days
Özgönenel et al. (2009)	Knee OA	67 (2)	56.2±8 vs 53.6± 6.9	1MHz, 1 W/cm^2,CUS	5 minutes/ session for total 10 sessions
Tascioglu et al. (2010)	Knee OA	90 (3)	60.04±2.83, 59.70±2.63, 61.64±3.74	i) 1MHz, , 2 W/cm^2 , CUS ii) 1MHz, 2 W/cm^2 , 20% pulsed,	5 minutes/ session, 5 days a week for 2 weeks (10 sessions in total)
Warden et al. (2008)	Patellar tendinopathy	37 (2)	27±7, 27±7	1MHz, 100 mW/ cm^2 , 2-ms burst of PUS	20 minutes, 7 days/weeks for 12 weeks (self-administrated)
Zammit and Herrington (2005)	Acute Ankle sprains	34 (3)	30±11, 33±12, 29±11	3MHz, 20% PUS, 0.25 W/cm^2 (for first 3 sessions); 3MHz, 33% PUS, 0.5 W/cm^2 (for the remaining sessions)	10 minutes (for 1st -3rd sessions), 6minutes (for the remaining sessions) alternate days for 2 weeks (total 6 sessions)

*PUS: pulsed ultrasound CUS: continuous ultrasound OA: osteoarthritis

Third, Baxter and Basford (2016) suggested that the human studies of therapeutic ultrasound are poorly controlled. The insufficient explanation of the study design has affected the methodological quality and casted doubt on the results. Seven studies out of the ten reviewed claimed themselves as "double blinded", but only 4 of them have clearly explained how the patients and the therapists were concealed from the sound and heat generated from the ultrasound machine (Falconer et al. 1992; Loyola-Sánchez et al. 2012; Nyanzi et al. 1999; Warden et al. 2008). In addition, pain is a common outcome measure for musculoskeletal conditions. However, four of the studies did not mention how the use of analgesics was controlled or recorded among patients before and during the treatment period (Falconer et al. 1992; Loyola-Sánchez et al. 2012; Warden et al. 2008; Zammit & Herrington 2005). The baseline level of pain was not discussed in most studies. Since pain is a subjective outcome measure for symptom relief and there might be gender difference in the perception of pain as women tended to report more pain and physical disability (Keefe et al. 2000), the gender distributions in the studies might also be a factor to consider.

Fourth, additional treatment such as standard exercise and ice packs for all patients may blur the effect of US. This is especially true when the outcome measures are not responsive enough to determine the changes based on a single type of treatment. Shanks et al. (2010) suggested that the effectiveness of therapeutic ultrasound was uncertain as there is a lack of high quality evidence to support its use. Considering there are many animal studies that supported the use of low intensity ultrasound on soft tissue healing, further studies have to focus on how to translate the results from animal studies to high quality human clinical trials.

2.3.4 Effect on Inflammation

Inflammation occurs in the acute phase of healing which is characterized by influx of monocytes and macrophages. These cells are responsible to remove damaged extracellular matrix, release vasoactive and chemotactic factors to initiate angiogenesis and stimulate cell proliferation (Dahlgren 2007). The acoustic streaming forces of therapeutic ultrasound have been shown to alter the mast cell permeability and vascular permeability which would lead to mast cell degranulation and release of histamine (Fyfe & Chahl 1984). The changes in membrane permeability stimulate the release of calcium ion which is an intracellular messenger to increase synthesis and secretion of various cytokines by the cells to mediate wound healing (Mortimer & Dyson 1988).

ElHag et al. (1985) advocated that therapeutic ultrasound has an anti-inflammatory effect leading to rapid resolution of edema after oral surgery. However, most evidence revealed the opposite. Therapeutic ultrasound of 2.3W/cm^2 stimulated inflammation with increased in PGE_2 and LTB_4 in the acute phase of injury (Leung et al. 2004) and the same group of researchers further demonstrated therapeutic ultrasound to up-regulate Transforming Growth Factor-β (TGF-β) at 10 days after injury in vivo (Leung et al. 2006). Li et al. (2002) also showed that ultrasound exposure augmented PGE_2 production in collagenase treated osteoblasts. The increased PGE_2 and LTB_4 could speed up the inflammatory process and TGF-β would direct monocyte migration and fibroblasts production. The pro-inflammatory effects are also signified by high peripheral leukocyte counts and cell density right after treatment with therapeutic ultrasound (Li et al. 2002; Lu et al. 2016). Watson (2006) suggested that therapeutic ultrasound is pro-inflammatory which acts as an inflammatory optimizer to speed up the process of inflammation and promote resolution of the inflammatory

events, hence therapeutic ultrasound accelerates the subsequent phases of healing. Therapeutic ultrasound tended to increase plasma extravasation (edema) but after a certain interval, the extravasation was found to be less in inflamed ankle treated with therapeutic ultrasound (Fyfe & Chahl 1985). Therefore, the injured tissues could be driven into proliferative phase sooner.

Similar finding was observed by Young and Dyson (1990a) that ultrasound treatment with low intensity of 0.1W/cm^2 with frequency either at 0.75MHz or 3MHz for 5 minutes daily induced fewer inflammatory cells and more extensive granulation tissue in the treatment groups at 5 days after full-thickness excised skin lesions. The results provide further support that therapeutic ultrasound accelerated inflammatory responses and early proliferative stages of repair as the peak of inflammation processes lasted for about 3 days or less.

2.3.5 Effect on collagen proliferative and remodeling phases

During collagen proliferative phase, the tissues become hypercellular and produce increasing amount of extracellular matrix (Dahlgren 2007). The wound will be filled with mainly macrophages and fibroblasts, numerous blood vessels and connective tissue matrix amid collagen deposition and granulation tissue formation (Watson & Young 2008). The injured tendon changes from cellular to acellular, avascular fibrous tissue and has an increase in type I collagen during remodeling phrase (Molloy et al. 2003).

Therapeutic ultrasound stimulated production of fibroblasts, endothelial cells and myofibroblasts (Doan et al. 1999; Ramirez et al. 1997; Watson & Young 2008), induced angiogenesis (Doan et al. 1999), and decreased expression of matrix metalloproteinase

(Curtis et al. 2002b) during proliferative phase.

During the remodeling phase, therapeutic ultrasound enhanced the orientation and aggregation of collagen fiber (da Cunha et al. 2001; Yeung et al. 2006), stimulated protein and collagen synthesis (Carvalho et al. 2006; Demir et al. 2004; Doan et al. 1999; Jackson et al. 1991; Ramirez et al. 1997). Therefore, the tensile strength would be better restored. The application of ultrasound tended to enhance the normal healing processes including inflammation, active repair and remodeling phases (Watson & Young 2008). However, the molecular mechanism of therapeutic ultrasound on collagen synthesis was tested in vitro only (Tsai et al. 2006). Whether therapeutic ultrasound could stimulate type I and III collagen still needs further investigation.

2.3.6 Ultrasound Dosage

Adjusting the ultrasound parameters viz. intensity, frequency, duty cycle, treatment time and period may produce different treatment effects to soft tissues. For example, the heating effect of ultrasound is more likely to occur at high frequency, high intensity and continuous ultrasound, while cavitation effect is more likely to happen with lower frequency (Watson & Young 2008). Pulsed model has on/off cycles and minimal heating effect when compared with continuous mode (Speed 2001) and evidence suggests pulsed mode to have better healing effect when compared with continuous mode particularly for acute injury (da Cunha et al. 2001; Tascioglu et al. 2010).

Even though it is impossible to identify a relationship between dosage and treatment responses (Robertson 2002), the effective treatment dosage of therapeutic ultrasound usually ranged from 0.5-2.0 W/cm^2 (SATA) (Tsai et al. 2006). Higher

intensity tended to induce more pro-inflammatory responses (Leung et al. 2004) and larger amount of heat produced (Baxter & Basford 2016).

Frequency of 0.8 to 1.1 MHz with 5 to 10 minutes per site are the most commonly used ultrasound parameters in physical therapy treatment (Tsai et al. 2006). Frequency of ultrasound is inversely correlated with the depth of penetration (ter Haar 2007). The energy of 1MHz is absorbed primarily by tissues at a depth of 3-5cm, while that of 3MHz is absorbed by tissues at a depth of 1-2cm (Larsen et al. 2005). No study has compared the biological effects of different sonication time on tendons but longer sonication time was favourable for bone regeneration after fracture (Chan et al. 2006).

Lu et al. (2016) compared the timing of low-intensity pulsed ultrasound (LIPUS) on tendon-bone healing in a rabbit model. The treatment started at either immediately after operation, 7 days or 14 days after operation. All treatment groups demonstrated significantly better biomechanical and histological parameters but the 7-day delayed group was more effective among all the treatment regimens in that study. Fu et al. (2008) and Gan et al. (1995) also advocated ultrasound could improve tendon healing if the treatment was initiated at early but not in the late tendon healing phase.

2.3.7 Phonophoresis

Ultrasound is well known for its effect on delivering drugs through the skin and this process is referred to as sonophoresis or phonophoresis. High-frequency phonophoresis (HFS, frequencies $\geq$ 0.7 MHZ) can enhance skin penetrations by 1- 10 fold and has been used for topical or regional drug delivery (Polat et al. 2011). The ultrasound treatment head is in contact with the skin for HFS with the ultrasound waves delivered in pulsed mode which helps to decrease the thermal effects associated with

the treatment. With a water-soluble gel as the coupling medium, ultrasound at the frequency of 1MHz has been reported to penetrate 2 to 4cm into the subcutaneous tissue (Byl 1995). The frequency determines the depth of penetration of ultrasound with lower frequency penetrating deeper. Therefore, HFS should be used to deliver drugs to more superficial layers.

Phonophoresis can help to enhance penetration of drugs by thermal and non-thermal effects. The associated increase in temperature during sonication raises the kinetic energy of the molecules in the drug and the cell membrane, which may also enhance substance penetration (Nino et al. 2010). The frequency, intensity and duration of sonication affects the thermal effect (Merino et al. 2003). With sonication over 30 minutes at 1MHz frequency and 1W/cm^2 intensity, the local temperature would increase by 11^{o}C (Brucks et al. 1989). Some harmful side effects may appear after sustained exposure to high temperatures such as burns, epidermal detachment and necrosis of the viable epidermis (Boucaud et al. 2004).

Acoustic cavitation which alters the skin barrier function by creating gas bubbles in a liquid is believed to be the major mechanism of phonophoresis. The oscillation of the sound waves would resonate the cells at high speed and change the resting potential of the cell membrane or even disrupt the cell membrane (Byl 1995). Bommannan et al. (1992) confirmed that 20 minutes of sonication at 10 or 16 MHz would alter the structure of stratum granulosum and stratum basale cells. The increase in membrane permeability is due to defects and channels created within the lipid bilayers. The barrier properties are not lost permanently, which will be recovered several hours after cessation of sonication (Meidan & Michniak 2004). For HFS, cavitation mainly occurs in skin appendages or at locations near the kerotinocytes of the stratum corneum (Polat

et al. 2011). HFS can deliver drugs with low molecular weight of <1000Da (g/mol) for local and regional ailments (Polat et al. 2011).

Boucaud et al. (2002) took phonophoresis of insulin as an example to indicate that sonophoretic enhancement is dependent on the energy level. With 10 W/cm^2 elicited greater effect on blood glucose level than 5 and 2.5 W/cm^2. For the duration, 18 minutes of sonication elicited greater effect than 6 and 12 minutes which is consistent with the cavitation-based mechanism. Smith et al. (2003) performed similar experiment to compare the effects of ultrasound exposure time on insulin delivery and found that 20 minutes of exposure time showed similar results as the 60 minutes exposure group. It is possible that the transdermal delivery of insulin could be maximized by increasing the duration of phonophoresis to around 20 minutes.

2.3.8 Safety of ultrasound application

The safety concerns of therapeutic ultrasound mainly lie in its heating effects (Baxter & Basford 2016). Ultrasound treatment particularly at high intensity may induce uneven heating effect which leads to significant temperature changes. People with metal implants and sensitive structures such as eyes, pregnant uterus, brain, are more vulnerable to uneven heating (Gersten 1958). When the modality is correctly applied, the risk of burn appears to be low. No protective gears are needed for ultrasound therapy as the ultrasound waves are dispersed and poorly transmitted in air (Miller et al. 2012).

2.4 Conclusion

Connective tissues such as tendon, ligament, cartilage, bone and skin are composed of cells and extracellular matrix including fibers, proteoglycans and

glycoproteins (Liu et al. 1995). All soft connective tissues when injured are believed to heal via a similar mechanism but the repaired tissue would unlikely to return to a perfectly normal condition (Adzick & Longaker 1992). Tissue healing occurs in three overlapping phases, namely, inflammation, proliferation (scar formation) and remodeling (scar remodeling) (Frank et al. 1999). Cytokines, growth factors, the degradative MMPs and the TIMPs are important to maintain the balance between synthesis and degradation of connective tissues. The cyclooxygenase pathways are the major focus of soft tissue metabolism (Culav et al. 1999).

` There is no consensus on the etiology nor the best treatment for Achilles tendon rupture. Considering the poor vascularity and low metabolic activity of tendon, it has limited healing capacity. Injured tendon is unlikely to ever regain its full strength even after long time of repair. Treatments that can speed up healing, suppress excessive tissue scarring, promote formation of collagen and are safe to apply for long term are potentially suitable for treating tendon injury thus worth exploring. Topical administration of drugs is particularly useful for delivering drugs to the tissue area in the proximity of the skin like Achilles tendon. It can bypass the hepatic metabolism, prevent the drugs from degradation in the gastrointestinal tract and possible interaction with food.

N-3 PUFAs have been shown to suppress inflammation, promote collagen formation, relieve pain and mediate numerous eicosanoids and cytokines in various soft tissues including skin, cartilage and ligament. N-3 is an appealing treatment for soft tissue injury for it is safe and easily available. Topical n-3 PUFAs promoted wound healing by accelerating wound epithelialization, neovascularization and stimulating synthesis of vascular endothelial growth factor. In light of the high epithelial

penetration ability of n-3 PUFAs, it is possible that n-3 PUFAs can traverse intact skin and exert its effect towards the nearby tissues. This has prompted the question that whether topical n-3 PUFAs could improve tendon healing.

Therapeutic ultrasound has been used to treat soft tissue injuries for several decades. It appears that early administration of therapeutic ultrasound, in particular the pulsed mode, during acute and sub-acute conditions with frequency of less than 3 W/cm^2 would be preferable for soft tissues healing. It can improve tendon healing by enhancing the biomechanical strength as reported in animal studies. Apart from its direct effect on soft tissue injuries, it can facilitate drug delivery across the skin. Yet, inconclusive results were observed in its molecular mechanism on collagen synthesis and human studies. While the biomechanical properties can be assessed with well-established methods, the histomorphological analysis still lack objectivity to draw proper conclusions on the effect of US.

Tendon is a kind of connective tissue which has similar origin as the dermis of skin, bone and cartilage. The therapeutic potential of n-3 PUFAs observed in other soft tissues may be replicated in tendon, but the current evidence on n-3 PUFAs and tendon healing is scarce. Achilles tendon is chosen for the current studies because it is located just beneath the skin which makes topical application of n-3 PUFAs more effective. Phonophoresis is a conceivable strategy to enhance n-3 PUFAs delivery to tendon. In clinical practice, interventions are usually used in combination. Therefore, topical fish oil coupled with therapeutic ultrasound is a potential treatment modality for tendon injury.

2.5 Hypotheses to be tested

The hypotheses for the study are as follow:

1. Topical fish oil treatment improves biomechanical properties of injured tendon.
2. Topical fish oil modifies the histomorphologic properties of injured tendon.
3. Topical fish oil treatment enhances total collagen, type I and III collagen synthesis in injured tendon.
4. Two weeks of fish oil treatment shows significant improvement in tendon healing.
5. Four weeks of fish oil treatment demonstrates significant better healing effects than two weeks of treatment.
6. Therapeutic ultrasound speeds up healing responses.
7. There is improvement in the histomorphologic properties of tendon tissue after treatment of US.
8. Therapeutic ultrasound stimulates total collagen, type I and III collagen synthesis in injured tendon.
9. Therapeutic ultrasound coupled with fish oil is superior to either treatment alone on improving Achilles tendon healing

CHAPTER 3 BIOMECHANICAL ANALYSIS

3.1 Introduction

When a tendon is injured, an indicator of functional recovery is whether the tissue has regained its mechanical properties including ultimate tensile strength (UTS), structural stiffness and energy absorption capacity. The mechanical properties of tissues were determined by the distribution of different collagen fibril and their specific organization patterns (Doroski et al. 2007). Hence, tensile mechanical behavior is a gold standard for the evaluation of the Effectiveness of any treatments or modalities (Uysal et al. 2012). Considering the slow healing rate of tendon and the prolonged immobilization after injury, the Effectiveness of any physical agents and modalities which can speed up healing and lessen complications has been the focus of recent studies (Huang et al. 2013; Ng 2011; Ng & Fung 2009).

3.2 Aims

This study aimed to (i) examine the effect of topical fish oil application on the biomechanical performance of healing rat Achilles tendon at 2 weeks and 4 weeks post-injury, (ii) compare the effect of topical fish oil application with normal therapeutic ultrasound, and (iii) test if there is synergistic effect on therapeutic ultrasound coupled with fish oil.

3.3 Methodology

3.3.1 Animal Model

Eighty-five female, 3-month old Sprague-Dawley rats were used in the study. The animals had mean body weight of 243 ± 26g (range 325-209g). All the animals were

obtained from the Laboratory Animal Services Centre of the Chinese University of Hong Kong and the Centralized Animal Facilities (CAF) at the Hong Kong Polytechnic University (PolyU). The animals were kept at the animal house in the CAF at PolyU. Each animal was randomly assigned with a number and allocated into the designated group as detailed in table 3.1. All the surgical operations and treatments were performed in the operation room of the CAF. The biomechanical tests were performed in the Orthopaedic and Microscopy Laboratory, Department of Rehabilitation Sciences at PolyU from Sep 2012 to Nov 2014.

3.3.2 Ethical consideration

This study was approved by the Animal Subjects Ethics Sub-committee of PolyU. The approval form is attached in Appendix 1. A license to conduct animal experiments was also endorsed by the Department of Health of the Hong Kong Government. A copy of the license is attached in Appendix 2.

3.3.3 Surgical procedures

The surgical procedures were based on a previous report by Ng and Fung (2009). The skin at the incision site was shaved before surgery. The procedures were conducted under general anesthesia induced by continuous administration of gas isoflurane. The vaporizer was turned to a concentration of 2-3%. The rat inhaled the vaporized anesthetic through a nose cone during the surgery (Figure 3.1). A longitudinal incision was made over the skin anterior and lateral to the Achilles tendon in order to prevent the topical ointment from diffusing through the skin wound to the tendon. The medial and lateral portions of the Achilles tendon were identified and separated by a blunt probe.

The medial Achilles tendon was then transected at the mid-point with the lateral Achilles tendon left intact in order to create a partial tenotomy of the Achilles tendon (Figure 3.2). The skin on the calf was closed by suturing. Partial rupture was adopted to prevent retraction of the cut ends and promote spontaneous healing (Ng et al. 1996).

The animals were housed in the laboratory with a 12-hour light-dark cycle and the temperature was maintained at about 20°C. Water and food were given ad libitum during the study. All treatments were given on a daily basis starting from the first day after surgery.

3.3.4 Experimental design

The animals were randomly allocated into 4 groups, namely, control (CON), fish oil (FO), therapeutic ultrasound (US) and fish oil plus ultrasound combination group (FU) as shown in table 3.1. The right Achilles tendon of all rats was hemi-transected to mimic a partial tendon rupture. The rats were treated for either 2 or 4 weeks after surgery.

All the animals received daily treatment according to their group assignment. Vaseline plain petroleum jelly was applied over the skin of the Achilles tendon and secured further with adhesive bandage in the CON group (Figure 3.3). A cone collar was put on the neck of each rat to prevent the animals from biting off the dressing which would dry off the next day (Figure 3.4). The vaseline with total weight of 0.8-1g was changed every day. The treatment for FO group was similar to the CON except that fish oil (F8020, Sigma-Aldrich Co., Poole, United Kingdom) mixed with vaseline in a ratio of 1:1.5 to form a fish oil ointment was applied over the skin of the Achilles tendon. The fish oil was standard refined Menhaden oil and comprised 10-15% EPA and 8-15%

DHA. This ointment was changed daily for each animal.

The ultrasound treatment was applied by using an ultrasound machine (150 plus, Dynatron, Salt Lake City, USA) with a 2.0cm^2 treatment head (Figure 3.5). The machine was calibrated with an ultrasound wattmeter (UW4 Ultrasound Wattmeter, Fluke Biomedical, Carson City, USA). The animals in the US group received pulsed ultrasound of 50% duty cycle at 1MHz, at the intensity of 0.5W/cm^2 for 4 minutes (Spatial average-temporal average, SATA: 0.25W/cm^2). Vaseline was used as the coupling agent. The combination group was similar to US group except that fish oil ointment acted as coupling agent (Figure 3.6).

Figure 3.1 Isoflurane vaporized anesthetic system.

The anesthetic system provided gas isoflurane continuously. Rats was anesthetized through a nose cone during the surgery.

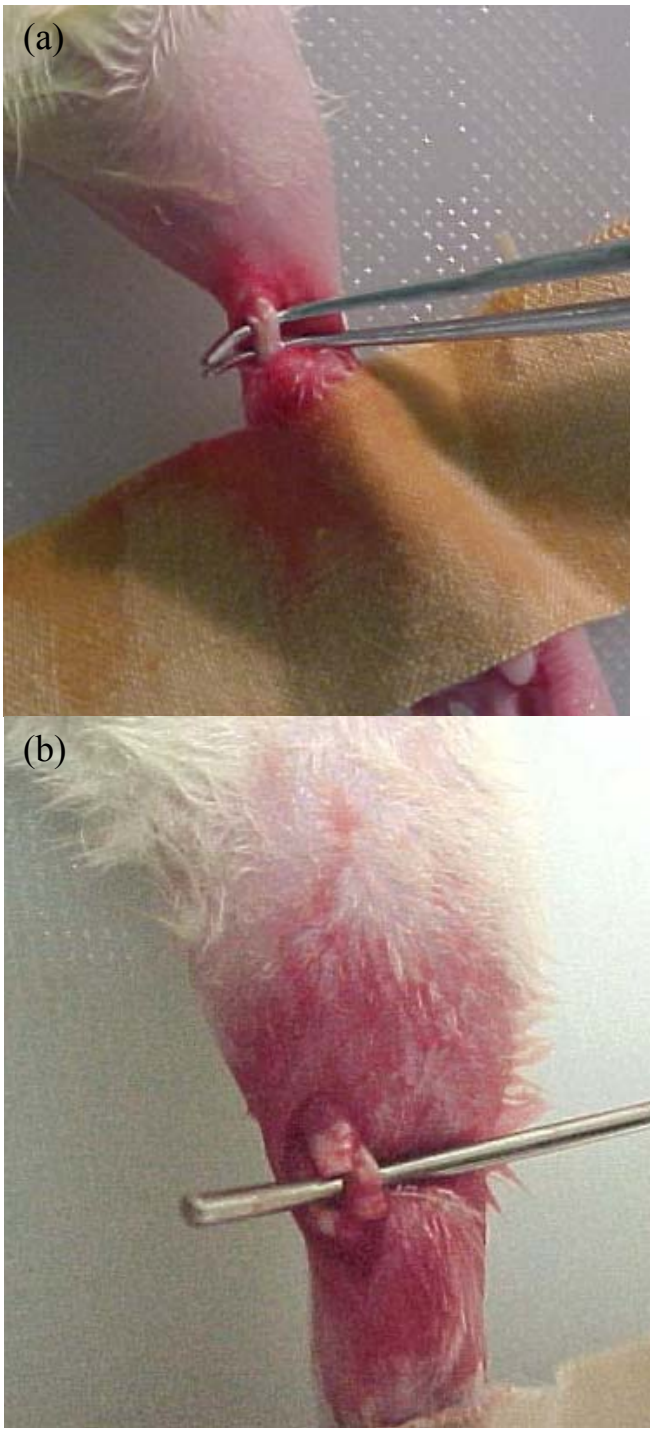


Figure 3.2 Partial tenotomy of the Achilles tendon (Adopted from Yeung (2007)).

(a) The Achilles tendon was identified. (b) The medial Achilles tendon was transected at the mid-point with the lateral Achilles tendon left intact.

Group	Treatment duration	Details of the ointment/ coupling agent	Ultrasound parameters
CON at 2 weeks	2 weeks	Topical Vaseline plain petroleum jelly	NA
FO at 2 weeks	2 weeks	Topical fish oil ointment which consisted of 40% fish oil and 60% Vaseline	NA
US at 2 weeks	2 weeks	Vaseline plain petroleum jelly	50 % duty cycle, 1 MHz at the intensity of 0.5 W/cm^2 for 4 minutes
FU at 2 weeks	2 weeks	Same as fish oil group	same as US group
CON at 4 weeks	4 weeks	Topical Vaseline plain petroleum jelly	NA
FO at 4 weeks	4 weeks	Topical fish oil ointment which consisted of 40% fish oil and 60% Vaseline	NA
US at 4 weeks	4 weeks	Vaseline plain petroleum jelly	50 % duty cycle, 1 MHz at the intensity of 0.5 W/cm^2 for 4 minutes
FU at 4 weeks	4 weeks	Same as fish oil group	same as US group

Table 3.1 Details of the treatment groups and control group

Figure 3.3 Adhesive bandage secured the treatment ointment.

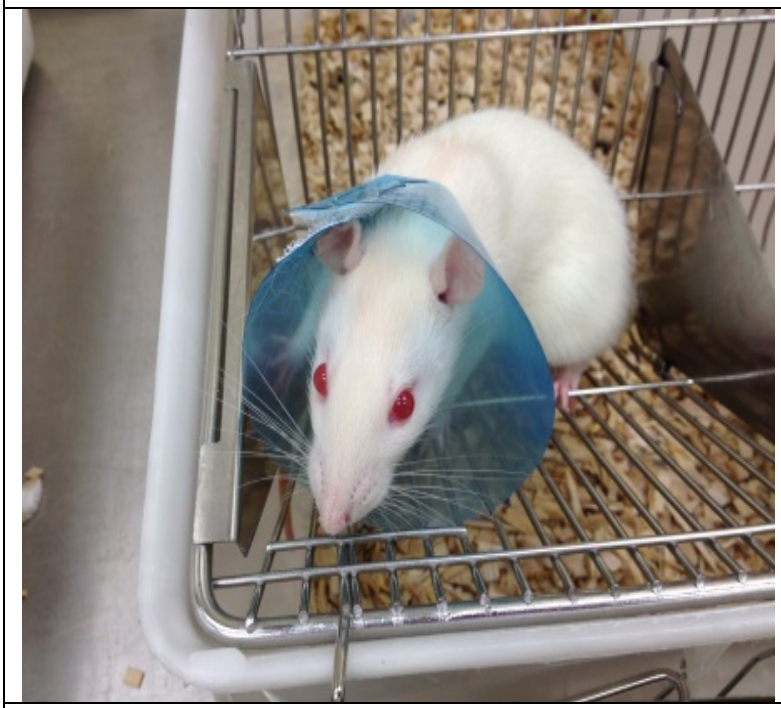


Figure 3.4 A cone collar on the rat to prevent the animal from licking the legs

.

Figure 3.5 Ultrasound treatment unit and ultrasound wattmeter.

Figure 3.6 Treatment for the FU group.

The rats was having ultrasound treatment with fish oil ointment as coupling agent.

3.3.5 Biomechanical testing

The biomechanical tests were done based on the established procedures of our laboratory (Ng 2011; Ng & Fung 2009). Half of the rats were euthanized at day 15 and the remaining half at day 29 after surgery by carbon dioxide inhalation. The lower limbs were harvested by hip joint disarticulation and the leg specimens were kept in a plastic re-sealable bag and stored in a freezer at -40^{o}C. At least six hours before testing, the specimens were retrieved from the freezer and allowed to thaw inside the plastic bag at room temperature. The specimens were dissected to remove all soft tissues, leaving the intramuscular tendinous fibres, Achilles tendon, and calcaneus intact. The intramuscular tendinous fibres were then secured between two strips of fine grade sandpaper which could provide extra anchorage for gripping the tendinous tissue. The specimens were kept moist with normal saline during the whole testing procedure. The intramuscular tendinous fibers and the calcaneus were then mounted on the cross heads of a material testing machine (MTS Synergie 200, MTS Systems Corporation, Eden Prairie, Minnesota, USA). An extensometer (Dynamic model 634.12 F-24, MTS Systems Corporation, Eden Prairie, Minnesota, USA) was attached to the margin of the cross heads for measuring the local strain in the tendon (Figure 3.7). The room temperature was controlled at 25˚C throughout testing.

A vernier caliper was used to measure the tendon length of the taped tendon-calcaneus complex for later calculation of the strain value for the preconditioning and load relaxation testing. Before mounting the specimen, both the load cell and the extensometer of the MTS machine were calibrated by running a built-in protocol.

a) Preconditioning

In order to minimize the effect of deep freezing on the tissue, each specimen after mounted on the material testing machine was preconditioned with 10 oscillation cycles of 2.5% strain at a rate of 10mm per minute (Woo et al. 1986)

b) Load relaxation testing

After preconditioning, the specimen was elongated to 2.5% strain and maintained for 10 minutes (Ng & Fung 2008). The loads were recorded throughout the test at a sampling rate of 5Hz. The difference between the initial and final load was expressed as a percentage of the initial load to represent the load-relaxation.

c) Ultimate tensile strength and structural stiffness

After load relaxation testing, the specimen was unloaded and left for 5 minutes on the machine to allow the tendon to restore to its original length. During the unloading phase, the tendon was kept moist with normal saline spray. Afterwards, specimen was elongated at a loading rate of 500mm per minute until breakage. Load and displacement data were recorded at a sampling rate of 50Hz throughout the test. The maximum load from the load-displacement curve represents the ultimate tensile strength (UTS), and the gradient in the linear portion immediately after the toe region of the curve represents the structural stiffness (Figure 3.8).

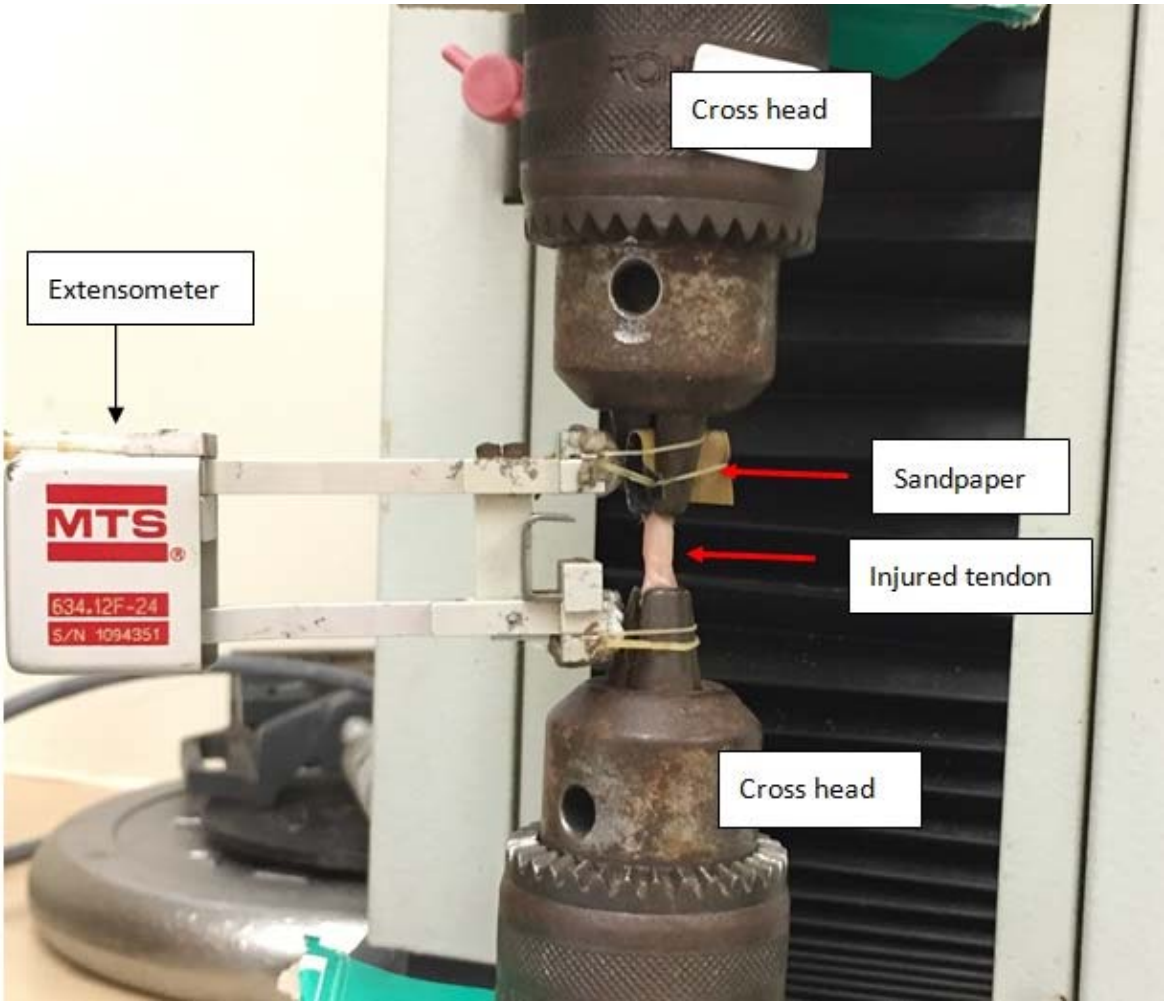


Figure 3.7 The specimen mounted on the cross heads of a material testing machine.

The intramuscular tendinous fibres were secured between two strips of find grade sandpaper. The extensometer was attached to the margin of the cross heads for measuring the local strain in the tendon. The sandpaper and the calcaneus were then mounted on the cross heads of a material testing material.

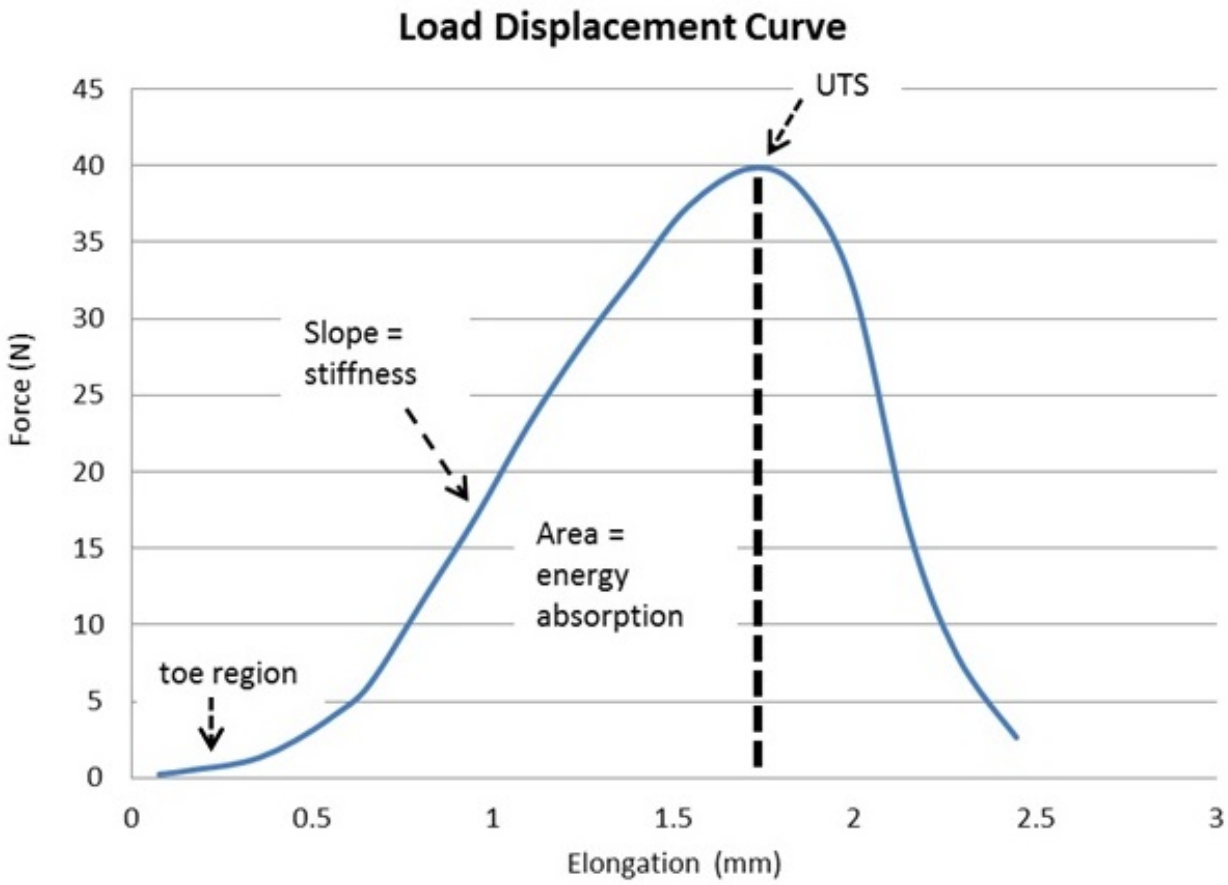


Figure 3.8 The load displacement curve of tendon

The maximum load of the curve represents the ultimate tensile strength (UTS). The gradient in the linear portion after the toe region of the curve represents the structural stiffness. The area under the graph from zero deformation to the maximum load represents energy absorbed by the tendon. (The graph was plotted based on actual data of a sample)

d) Energy absorption capacity

In the load-displacement curve of each specimen, a vertical line was drawn from the failure point to the abscissa. The energy absorption capacity of each tendon was derived by measuring the area beneath each curve (Enwemeka 1992). The area under the load-displacement graph represents energy absorbed by the tendon. Higher the amount of energy absorbed indicates lower efficiency of the structure (de Oliveira et al. 2011).

3.4 Statistical analysis

The values of structural stiffness, UTS and energy absorption capacity of the medial portion of the right Achilles tendon were normalized against the left Achilles tendon of each animal so as to minimize individual variations (Ng & Fung 2008; Yeung et al. 2006). These normalized values were then compared between groups and treatment duration using two-way analysis of variance (ANOVA). Homogeneity of variance was checked by Cochran C. (Cochran 1941) and Harley F-max (Hartley 1950) tests. Fisher's least significant difference post-hoc comparisons were conducted for significant ANOVA results. An α level of 0.05 was set for all statistical comparisons. The Statistica for Microsoft Windows version 10.0 (StatSoft, Inc. Tulsa, USA) was used in the data analysis.

3.5 Results

On gross examination, the right TA in all groups showed signs of repair but the CON group generally had a thicker fibrous scar formation than the other groups (Figure 3.9).

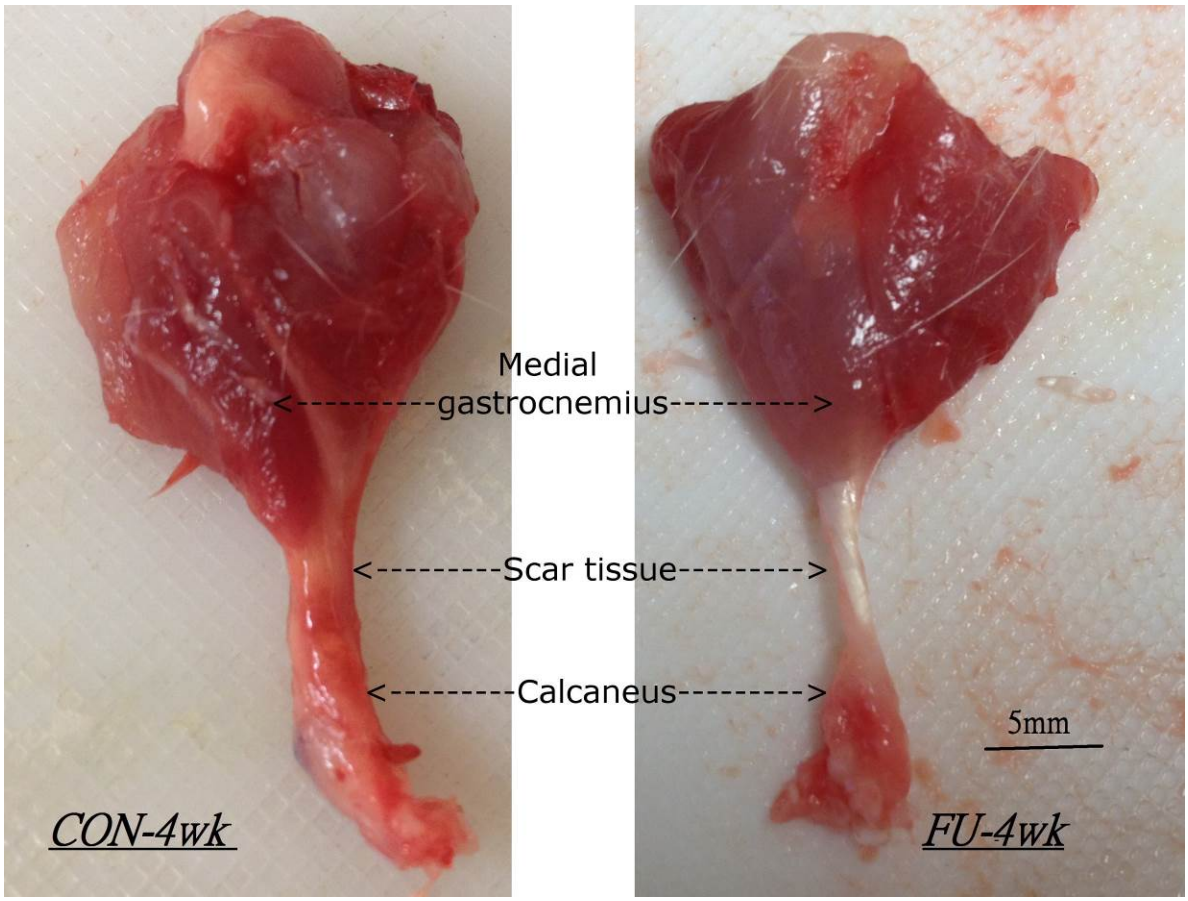


Figure 3.9 Gross examination of Achilles tendon.

On gross examination, the CON group displayed a thicker fibrous scar than the FU group.

a) Load relaxation testing

Load relaxation was not significantly different among groups and across time (Figure 3.10).

b) Ultimate tensile strength

For the 2-week group, significantly higher normalized UTS was observed in the US group when compared with CON ($p<0.05$). For the 4-week group, however, both US and FU had higher UTS than the CON ($p<0.05$) (Figure 3.11).

c) Structural stiffness

There was no difference in structural stiffness among treatment groups at 2 weeks. Both FO and FU at 4 weeks showed greater improvement in structural stiffness than CON at the same time point ($p<0.05$). (Figure 3.12)

d) Energy absorption capacity

Significantly higher energy absorption capacity was found in FO at 2 weeks when compared with FU at 2 weeks ($p<0.05$) (Figure 3.13)

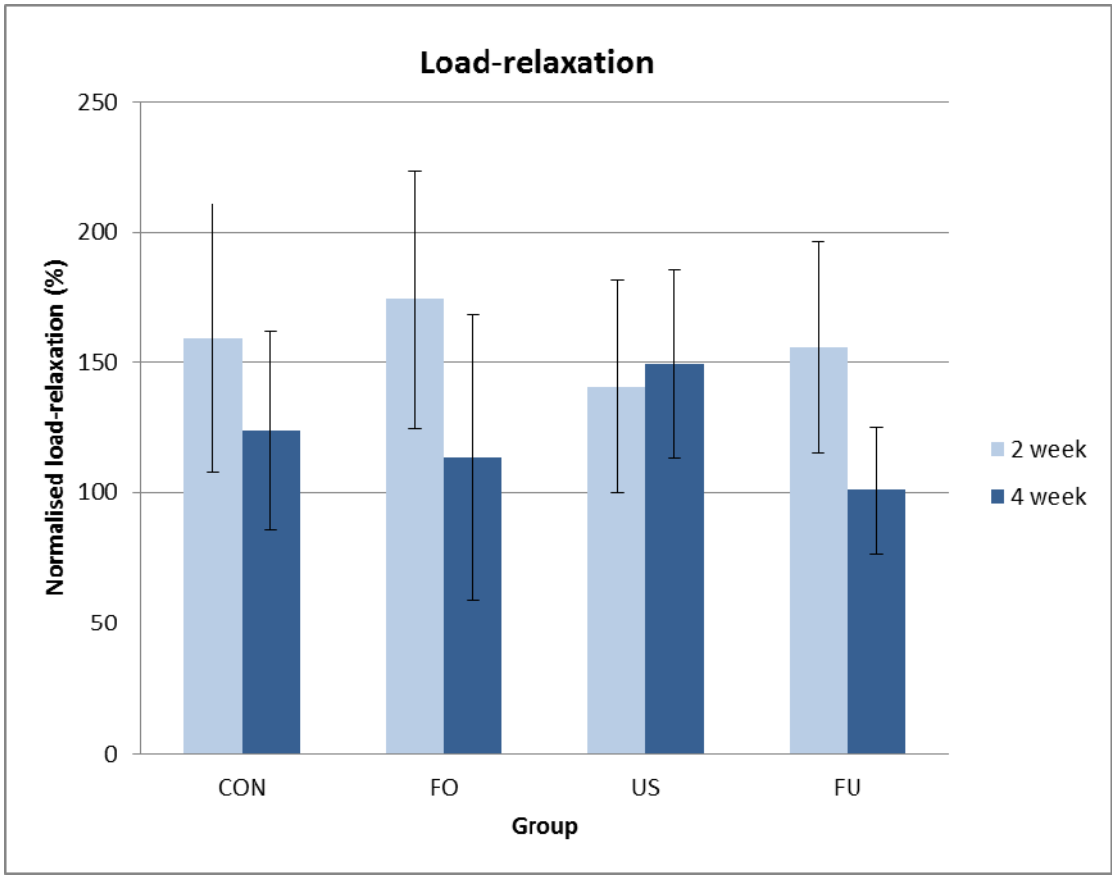


Figure 3.10 Results of normalized load relaxation.

No significant differences were found among groups. Data are expressed as mean ± standard error of the mean values.

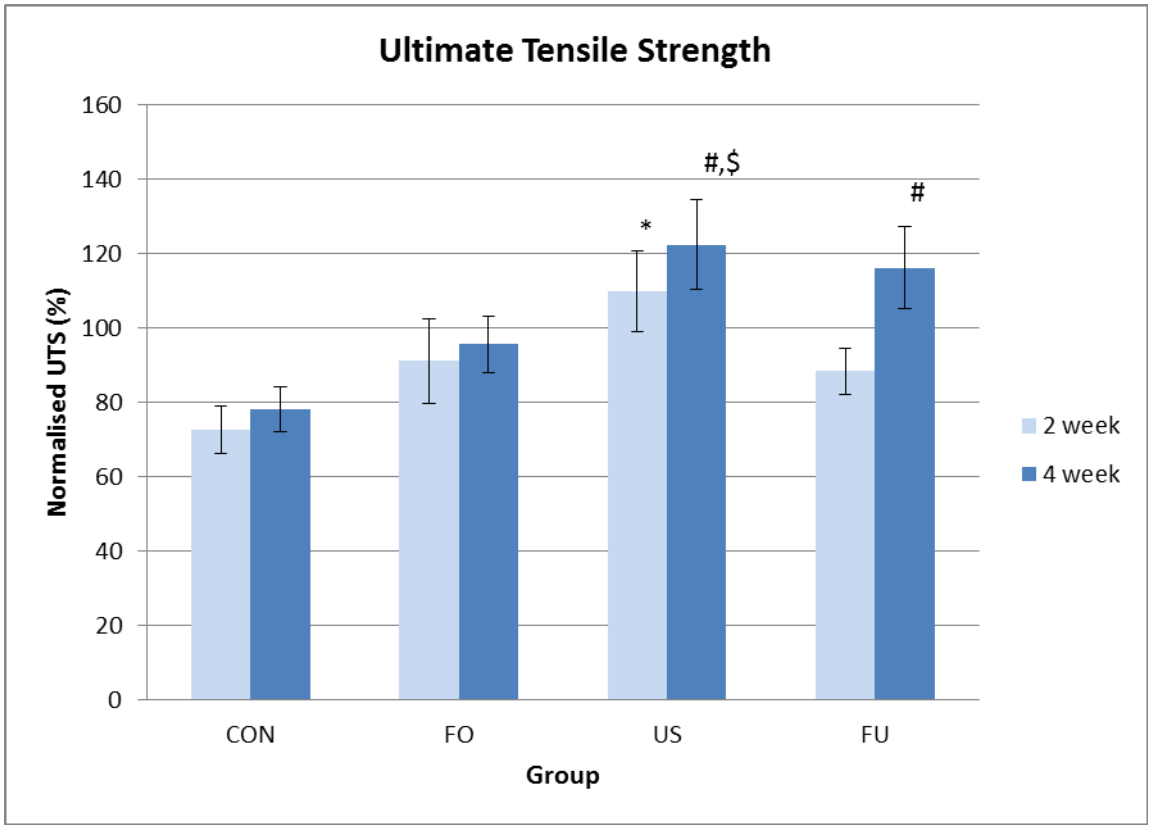


Figure 3.11 Results of normalized UTS.

The UTS of US at 2 weeks was significantly higher than CON at 2 weeks. Both US at 4 weeks and FU at 4 weeks have significantly higher UTS than CON at 4 weeks. US at 4 weeks had significantly higher UTS compared with FO at 4 weeks. Data are expressed as mean ± standard error of the mean values.

* compared with CON at 2 weeks, $p<0.05$; # compared with CON at 4 weeks, $p<0.05$; $ compared with FO at 4 weeks, $p<0.05$.

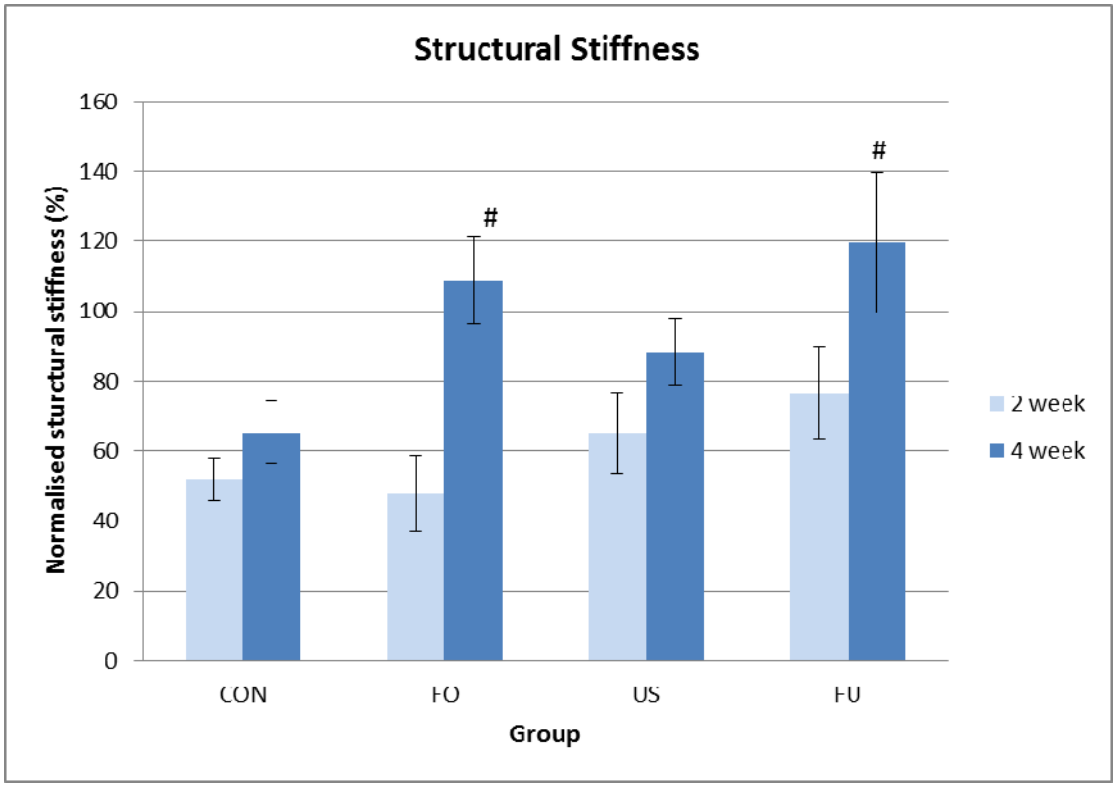


Figure 3.12 Results of normalized stiffness.

Both FU at 4 weeks and FO at 4 weeks showed significantly higher structural stiffness than CON at 4 weeks. Data are expressed as mean ± standard error of the mean values. # compared with CON at 4 weeks, $p<0.05$;

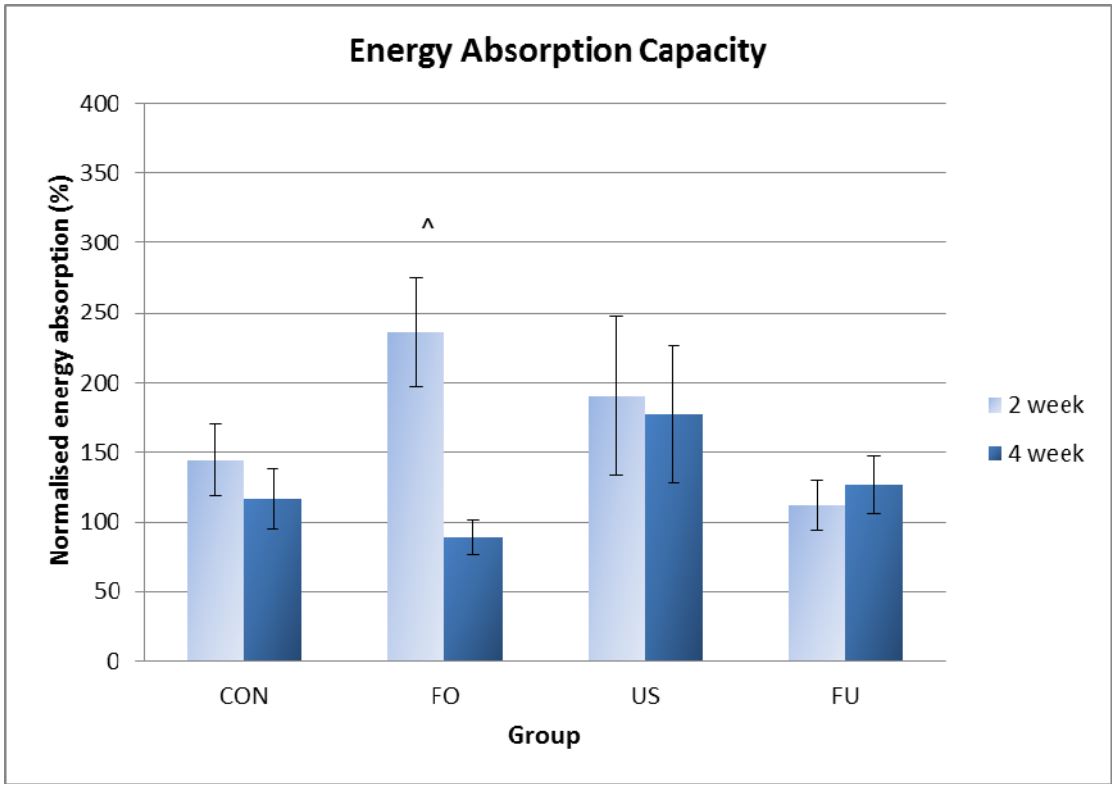


Figure 3.13 Results of energy absorption capacity.

Significantly higher energy absorption capacity was found in FO at 2 weeks compared with FU at 2 weeks. Data are expressed as mean ± standard error of the mean values.

^compared with FU at 2 weeks, $p<0.05$.

3.6 Discussion

The present findings revealed therapeutic ultrasound has hastened the tendon recovery as manifested by an increase in mechanical performance of UTS for the injured Achilles tendon. Furthermore, combined treatment of therapeutic ultrasound coupled with fish oil resulted in an overall improvement of UTS and structural stiffness, particularly at 4 weeks after injury.

The Effectiveness of a topically applied treatment depends on the pharmacological effect and effective skin permeation ability of the active components of the medication. The outermost layer of epidermis is the stratum corneum which acts as a barrier for the skin and is regarded as the rate-limiting factor for transcutaneous drug delivery (Contri et al. 2011). The most important predictors of permeation of topical drugs are lipophilicity and molecular weight (Russell & Guy 2009; van Ravenzwaay & Leibold 2004). Since most substance would penetrate the skin via intercellular pathway and pass through the extracellular lipid matrix, cutaneous penetration of hydrophilic substances is limited, while lipophilic solutes have high permeability (Nino et al. 2010). Fish oil has high lipophilicity and hence good epithelial penetration potential. This is supported by Zulfakar et al. (2010) who found fish oil could enhance corticosteroid delivery across full thickness skin. This refuted the idea that small molecules with both hydrophobic and hydrophilic properties are most readily absorbed by the skin and pass through the hydrophobic stratum corneum and hydrophilic dermis easily (ECETOC 1993). This was supported by van Ravenzwaay and Leibold (2004) who found molecular weight was a more critical factor. An inverse relationship between molecular weight and the rate of dermal absorption was observed in studies *in vivo*, irrespective of the water and fat solubility of the compounds. Lipophilic compounds of molecular weight more than $400 g \cdot mol^{-1}$ would be poorly absorbed by rat skin *in vivo*. The

molecular weight of DHA is 328.49g·mol^{-1} and that of EPA is 302.42g·mol^{-1}, their relatively light molecular weights suggests that both fatty acids should readily pass through the rat skin. The previous report from Thomas et al. (2007) that fish oil could permeate full thickness porcine skin and exert an anti-inflammatory effect is in line with the present findings in rats..

Fish oil was mixed with Vaseline plain petroleum jelly to form the ointment in FO and FU groups. Vaseline acts as an optimum ointment base and is compatible with various medicaments, providing emollient effect and occlusive properties (Ammar et al. 2007). The occlusive properties of Vaseline refers to its serving as impermeable films outside the skin, which increases penetration of lipid-soluble, non-polar molecules (Zhai & Maibach 2002). Since the Achilles tendon is located just beneath the skin, it is possible that fish oil exerted its effect on the tendon after permeating the full thickness of skin.

Ultrasound is well known for its effect on delivering drugs through the skin, which is referred to as sonophoresis or phonophoresis (Polat et al. 2011). It increases the percutaneous absorption of drugs by acoustic cavitation which alters the skin barrier function (Machet & Boucaud 2002). The increase in membrane permeability is due to defects and channels created within the lipid bilayers (Meidan & Michniak 2004). Molecules can then penetrate through the microchannels to the subdermal tissue (Ting et al. 2004). Theoretically, such skin permeation enhancement effect should also be applicable to other substances with reasonable molecular size such as fish oil.

The ultrasound parameters used in the current study with 4 minutes of pulsed ultrasound (1MHz) at the intensity of 0.5W/cm^2 was based on a few studies that demonstrated favorable results towards tendon healing and relief of symptoms in

arthritic rats by coupling ultrasound with different substances including diclofenac, aloe vera gel, hydrocortisone and Panax Notoginseng (Hsieh 2006; Koeke et al. 2005; Maia Filho et al. 2010; Ng & Wong 2008). This ultrasound frequency (1 MHz) is categorized as therapeutic ultrasound or medium frequency which restricts the action of ultrasound to more superficial layers and hence optimizes its effect on the skin (Merino et al. 2003).

Insulin is a drug with high molecular weight of 5808g/mol and has reportedly been effectively transported through the rat skin with ultrasound phonophoresis (Mitragotri & Kost 2004). It is possible that the penetration of EPA and DHA, which have much smaller molecular weights than insulin, can be enhanced by ultrasound sonication. However, whether phonophoresis had actually taken place in the FU group with enhancement of fish oil penetration into the repairing tendons would need to be confirmed by biochemical analysis of the tendon specimens. This warrants to be explored in future studies.

When a tendon is injured, an indicator of functional recovery is whether the tissue has regained its mechanical properties of UTS, structural stiffness and energy absorption capacity. The distribution of different collagen fibril sizes and their specific organization patterns determine the mechanical properties of tissues (Doroski et al. 2007). Apart from that, composition of non-collagenous components such as proteoglycans and glycosaminoglycans present in the extracellular matrix are also crucial factors (Parry 1988). Similar to the reports of previous studies (Ng et al. 2003; Yeung et al. 2006), the present results showed no difference in load relaxation between groups at both testing timelines of 2 weeks and 4 weeks of treatment. Very few studies have compared the elements that influence the strength and stiffness of

tendons separately but both properties were found to be influenced by collagen density (Roeder et al. 2002). Both fish oil and ultrasound have been reported to promote collagen synthesis (Hankenson et al. 2000; Jackson et al. 1991), which may explain the improved mechanical properties in the three treatment groups. Similar to the results from Majewski et al. (2009), structural stiffness did not differ significantly from the control until week 4, as it takes time for collagen to form cross-links during remodeling of the repair site. Significantly improved structural stiffness was observed in FO and FU groups at 4 weeks. The question on why fish oil groups rather than US group showed greater improvement in stiffness needs further investigation.

Inflammation is a part of the normal, innate immune response to injury and this process is particularly important in the first 48 to 72 hours so as to prepare the injured tissue for repair. However, if there is excessive inflammation, it may result in pain and over-scarring or damage to the host tissues during remodeling (Calder 2006). Fish oil is believed to have an anti-inflammatory effect as both EPA and DHA are competitor substrates that inhibit oxidation of arachidonic acid (20:4 n-6; AA) via the cyclooxygenase (COX) pathway to form eicosanoids such as PGE_2 and LTs (Cleland et al. 2003). The non-significant effect of the fish oil groups at 2 weeks could be because the fish oil had inhibited the COX-2 pathway as it has been demonstrated that early administration of COX-2 inhibitors such as parecoxib would negatively affect tendon healing. However, when the COX-2 inhibitors were applied for a longer period, it would improve healing (Forslund et al. 2003; Virchenko et al. 2004). Our results coincided with the above findings that significant improvement was observed in the fish oil groups only for longer treatment period. Therefore, it is suggestive that the timing for application of fish oil is critical for the healing process.

Energy absorption capacity is the area under the load-displacement curve from an estimated zero deformation to the maximum load. Tendon is a kind of non-contractile viscoelastic structure that exhibits both viscous and elastic behaviors when deformed. Tendon becomes less viscous but more effective in transmitting heavy loads if it absorbs less energy. This scenario occurs during fast loading conditions (Maquirriain 2011). The higher energy absorption capacity of FO group at 2 weeks with relatively low UTS and structural stiffness indicated a lower efficiency of the tendon. In this situation, the muscle fiber may contract slower over a longer period of time and decrease the required average muscle fiber power output (Prilutsky et al. 1996). This finding also implied an unfavorable effect of fish oil at the early healing period. However, the data should be interpreted with caution as the standard deviations of energy absorption capacity were large particularly for the FO and US at 2 weeks.

The present findings revealed topical fish oil alone has improved structural stiffness of the repairing tendon and the combined treatment effect of therapeutic ultrasound coupled with fish oil showed improvement in both stiffness and strength at 4 weeks. Apart from acting as a COX inhibitor, several studies have shown that omega-3 fatty acids would stimulate collagen synthesis (Cardoso et al. 2004; Hankenson et al. 2000; Jia & Turek 2004; Otranto et al. 2010), up-regulate growth factors such as vascular endothelial growth factor (Shingel et al. 2008), and reduce the loss of Glycosaminoglycans (Curtis et al. 2002a), which are crucial for tendon healing. It has been reported that the anti-inflammatory properties of fish oil would modulate pain but not inhibit the later healing process (Goldberg & Katz 2007).

The FU group had significantly higher UTS and structural stiffness than the

CON group at 4 weeks. This might be the results of the summated therapeutic effects of ultrasound and fish oil on soft tissues and also the possibility of the penetration enhancement effect of ultrasound that has facilitated the fish oil transport to the injured tissue. Considering the factors of safety, easy availability, low cost and versatility of fish oil, the use of it to treat soft tissue injury has a promising future. Further work is warranted to include other outcome measures to confirm the findings and investigate whether the healing involves the COX pathway as well as the optimum time window for topical fish oil application.

3.7 Conclusion

Topical fish oil application, therapeutic ultrasound and combined ultrasound with fish oil improved the biomechanical properties of injured Achilles tendon. Only the combined ultrasound and fish oil treatment group has shown improvement in both UTS and structural stiffness of injured Achilles tendon at 4 weeks.

CHAPTER 4 HISTOMORPHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

4.1 Introduction

Collagen is the major component of soft connective tissues such as tendon. It constitutes up to 70% of the dry weight of the structure and its arrangement determines the tissue's strength and function (Battaglia et al. 2003). In mature tendons, type I collagen is the dominant type which provides mechanical strength and stiffness to the tendons (Amiel et al. 1984) while Type III collagen constitutes a few per cent of the tendon collagen and its production is most active after injury. This type of collagen usually aggregated into small fibril without crosslinking so as to give flexibility to the newly formed repairing tissue (Silver et al. 2006).

The hierarchical organization of collagen fibers are the major determining factor of the tendon's mechanical strength (Battaglia et al. 2003). The fibril diameter is also an important factor as large fibrils could withstand higher tensile forces (Parry 1988). Therefore, strategies that stimulate collagen synthesis without abnormal scarring and its reorganization into fibre bundles during the healing processes are of paramount importance.

4.2 Aims

Taking into account the importance of collagen deposition and its subsequent fibre alignment on tendon healing, the purposes of this experiment were to test the hypotheses that tendon healing was promoted by therapeutic ultrasound and fish oil treatment by modulating extracellular matrix organization as well as collagen synthesis, and compare the treatment effects of the two modalities on Achilles tendon healing.

4.3 Methodology

4.3.1 Animal model

Thirty-six female, 3-month old Sprague-Dawley rats were used in the study with mean body weight 206±14g (range 237- 183g). All the animals were obtained from the Laboratory Animal Services Centre of the Chinese University of Hong Kong and the Centralized Animal Facilities (CAF) at the Hong Kong Polytechnic University (PolyU). The animals were kept at the animal house in the CAF at PolyU. Each animal was randomly assigned with a number and allocated into the designated group. All the surgical operations and treatments were performed in the operation room of the CAF. The subsequent histological analysis was performed in the Orthopaedic and Microscopy Laboratory, Department of Rehabilitation Sciences at PolyU from Feb 2013 to Mar 2016.

4.3.2 Ethical consideration

This study was approved by the Animal Subjects Ethics Sub-committee of PolyU. The approval form is attached in Appendix 1. A license to conduct animal experiments was also endorsed by the Department of Health of the Hong Kong Government. A copy of the license is attached in Appendix 2.

4.3.3 Surgical procedures

The surgically induced partial tendon rupture model was the same as that described in chapter 2. In brief, skin on the anterior aspect of the right calf of the anesthetised rat was shaved, incised and retracted to expose the Achilles tendon. The tendon of the medial gastrocnemius was cut with a scalpel at its mid-point without suturing. The skin wound was closed by non-absorbable suture that was removed three

days after surgery. The animals were allowed to roam freely inside their cages. The rats were kept in an animal house with a 12-hour light-dark cycle and the temperature was maintained at approximately 20°C. Water and food were given *ad libitum* during the experiment. All treatments were given every day one day after surgery.

4.3.4 Experimental design

The animals were allocated randomly into eight groups, including four different treatment forms and two treatment periods (ie. 2 weeks and 4 weeks). All of the animals received daily treatment as described below (Table 4.1):

CON: the animals received topical Vaseline plain petroleum jelly over the skin of the right Achilles tendon, which was secured further with adhesive dressing to their calf.

FO: the animals were treated daily with a topical fish oil ointment over the skin of the right Achilles tendon, which was secured further with an adhesive dressing. The fish oil ointment was prepared by mixing 40% fish oil (F8020, Sigma-Aldrich CO., Poole, United Kingdom) with 60% Vaseline. The fish oil was standard refined Menhaden oil and comprised 10-15% EPA and 8-15% DHA.

US: the animals received pulsed ultrasound of 50% duty cycle at 1MHz, at an intensity of 0.5W/cm^2 for 4 minutes daily (spatial average temporal average of 0.25W/cm^2) (Ng et al. 2003; Ng & Wong 2008) with Vaseline acting as the coupling medium. The same ultrasound machine (150 plus, Dynatron, Salt Lake City, USA) with a 2.0cm^2 soundhead was used throughout the study.

FU: the animals received similar treatment as the US group except that the fish oil ointment acted as the coupling medium.

Group	Number of animals	Treatment period (days)	Treatment
CON at 2 weeks	3	14	Topical Vaseline
FO at 2 weeks	3	14	Topical fish oil ointment
US at 2 weeks	3	14	Ultrasound and Vaseline
FU at 2 weeks	3	14	Ultrasound and fish oil ointment
CON at 4 weeks	6	28	Topical Vaseline
FO at 4 weeks	6	28	Topical fish oil ointment
US at 4 weeks	6	28	Ultrasound and Vaseline
FU at 4 weeks	6	28	Ultrasound and fish oil ointment

Table 4.1 Experimental groups and the treatment applied to each group.

4.3.5 Tissue preparation

One third of the animals were euthanised on day 15 and the remaining were euthanised on day 29 after surgery. CO_2 was used to euthanise the animals. The harvested Achilles tendon was fixed in 4% paraformaldehyde for a day and then embedded in paraffin. Longitudinal sections were cut into 5μm thin from the middle of the repairing tissue. The sections were then mounted directly on SuperFrost/Plus glass slides.

Deparaffinised sections from each specimen were stained with hematoxylin and eosin (H&E), Masson's trichrome, and Picrosirius Red (PSR) respectively. Sections were analysed with a Nikon microscope Eclipse 80i (Nikon, Tokyo, Japan) and digitised with a SPOT insight digital camera using SPOT Advance software (SPOT imaging, Diagnostic Instruments, Sterling Heights, MI, US). Then, quantitative analysis was performed by two assessors with ImageJ software (National Institutes of Health, Bethesda, Maryland, US). All the sections being assessed were labelled with numbers, and the assessors were not informed of the treatment group of their sections in order to eliminate bias.

4.3.6 Hematoxylin-eosin staining

The slides were deparaffinized and rehydrated by immersing into xylene and different concentrations of ethanol ranged from 100% to 70%, progressively. The sections were then stained with Harries hematoxylin and eosin (H&E). The sections were finally dehydrated in grades by immersing into ethanol and xylene. The sections stained with H&E showed nuclei with a deep blue-purple color while the cytoplasm and extracellular matrix have varying degrees of pink staining (Fischer et al. 2008).

Quantitative analysis of H&E stained sections to determine fibroblast density was performed by three assessors and ImageJ software (NIH, Bethesda MD). Cell counts were performed at x600 magnification for 3 high-powered fields per specimen throughout the tendon scar (Kollitz et al. 2014). Two assessors counted the number of cell in each section and one used ImageJ software to measure cell density by adjusting the thresholds for red, green and blue (RGB value) which was expressed as per cent of the total field area.

4.3.7 Masson's trichrome

Collagen quantification was carried out at x40 magnification modified from the protocol of Bauman et al. (2014) and Moshaverinia et al. (2014). The stain was used to distinguish collagen fibers with nuclei and cytoplasm. The deparaffinized sections were stained overnight with Bouin's solution at room temperature. The sections were washed and stained with Weigert's Iron hematoxylin for 10 minutes. After rinsing in distilled water, Biebrich Scarlet-Acid Fuchsin was applied for 5 minutes and then treated with Phosphotungstic / Phosphomolybdic Acid for 10 minutes. Collagen was stained with Anline Blue solution for 5 minutes. Before dehydrating and mounting, the sections were rinsed in distilled water and 1% acetic acid. Blue colour indicated collagen fibers, while red colour indicated cytoplasm. Quantification was performed by ImageJ software. Image calculator plug-in was used to perform background subtraction. Blue colouration of each image was separated manually by setting the threshold of the hue (121-179), saturation (20-255) and brightness (10-255). The area of blue colouration was shown as a percentage of the total tissue area.

4.3.8 Picrosirius red staining (PSR)

The deparaffinized sections were stained with Weigert's haematoxylin for 10 minutes and PSR for an hour, respectively. After washing in two jars of acidified water, the sections were dehydrated in ethanol and xylene and mounted on slides. PSR was used to assess collagen maturity. Thick and mature fibers appear as orange or red while thin and immature fibers appear as yellow or green (Rich & Whittaker 2005). A polarising filter was put on the light source of the microscope to enhance collagen birefringence. The stained sections were viewed at x40 magnification. Background colouration was removed similarly to the Masson's trichrome stain. ImageJ macros for the quantification of individual colours of birefringence were developed using the following hue (H), saturation (S), and brightness (B) ranges: red (H 1-13, S 10-255, B 20-255), orange (H 14-25, S 10-255, B 20- 255), yellow (H 26-52, S 10-255, B 20-255), and green (H 53-110, S 10-255, B 20-255) (Bauman et al. 2014). The number of pixeled areas within each hue range was determined and expressed as a percentage of the total collagen pixels which were the sum of pixeled areas of all four colours.

4.3.9 Immunohistochemical staining

The sections were deparaffinised, washed with phosphate-buffer saline and incubated in 3% H_2O_2 and normal horse serum. The sections were then reacted with anti-type I collagen (Rabbit polyclonal to collagen I, 1:200, ab34710, Abcam, Cambridge, MA, US) or anti-type III collagen (Rabbit polyclonal to collagen III, 1:100, ab7778, Abcam, Cambridge, MA, US) overnight at 4^oC. The sections were subsequently reacted with amplifier antibodies and secondary antibodies respectively for 30 minutes at room temperature. Finally, a diaminobenzidene chromogen system

was added for coloration (ImmPRESS Excel amplified staining kit, Vector Laboratories, Inc, Burlingame, CA, US). Slides were analyzed blindly by the same assessor under x40 magnification with ImageJ according to Fuhrich et al. (2013) and subsequently cross-checked by another assessor. The images were separated into diaminobenzidine (DAB) only images by the plug-in “colour deconvolution”. A freehand-selection tool was used to remove the non-tendon tissues from the analysis. The colour image was then converted into a black-and-white image. The percentage of black areas relative to the total tissue area was calculated as immuno-positive stained areas (Kaemmer et al. 2010).

4.4 Statistical analysis

A comparison between the groups and the treatment duration was performed using a two-way analysis of variance (ANOVA). The homogeneity of variances was tested using Cochran C. (Cochran 1941) and Harley F-max (Hartley 1950) tests. Fisher's least significant difference (LSD) post-hoc comparisons were conducted for significant ANOVA findings. An α level of 0.05 was set for all statistical comparisons. The Statistica for Microsoft Windows version 10.0 (StatSoft, Inc. Tulsa, USA) was used in the data analysis.

4.5 Results

4.5.1 Hematoxylin-eosin staining

Histological analysis revealed that a normal tendon had densely packed, parallel collagen bundles oriented in only one direction with a few slender fibroblasts, whereas all the injured tendons exhibited densely distributed fibroblasts. Although the results were not significant, the cell count generally decreased with time. The 4-week groups

appeared to have fewer cells, more extracellular matrices and more regular tissue organisation patterns (Figure 4.1). However, the remodeling of the tendon extracellular matrix was not completed even on day 29 post injury. The densely distributed fibroblasts indicated the repair process was still under way in all the injured groups. There was a strong propensity towards fewer fibroblasts ($p=0.0616$) in FU at 4 weeks and less cell area in US at 4 weeks when compared with CON ($p=0.0831$) (Table 4.2).

4.5.2 Masson's trichrome staining

The normal tendons appeared uniformly red while the injured tendons were stained mainly blue (Figure 4.2). The injured tendons at 4 weeks displayed a mainly blue colouration which signified the active repair process was on-going (Table 4.3).

4.5.3 Picrosirius red staining

Red and orange colours represent thicker and mature collagen fibres while yellow and green colours represent newly formed immature collagen fibres. The normal tendons were dominantly red and orange, while slimmer, faint yellow and green fibres were detectable at the repair site in the injured tendons even at 4 weeks post injury (Figure 4.3). Based on the quantitative analysis, FO and FU at 4 weeks contained significantly more red and orange fibres (FO:60.69±17.95%; FU: 69.62±16.08%, $p<0.05$) and fewer yellow and green fibres than the CON at this time point (FO: 39.31±17.95%; FU: 30.38±16.08%, $p<0.05$) (Figure 4.4).

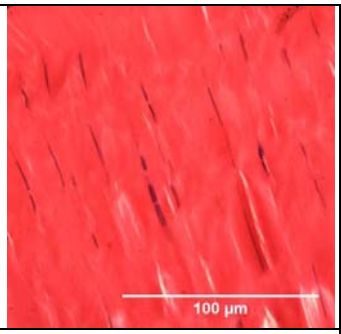
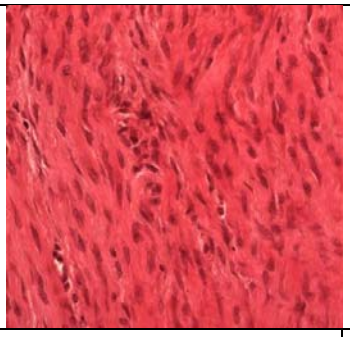
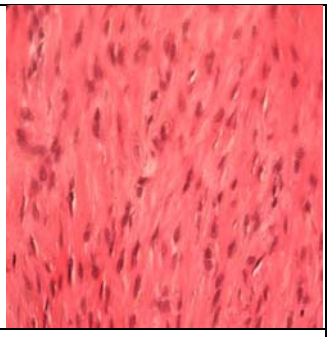
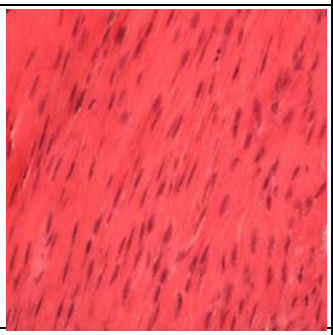
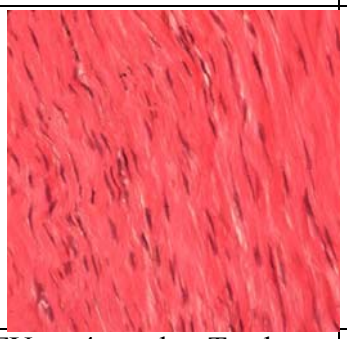
		
a) Normal tendon. It had parallel collagen bundles oriented in only one direction with few slender fibroblasts.	b) CON at 4 weeks. Fibroblasts abundant with plump nuclei, found in a random pattern. The tissue was less woven.	c) FO at 4 weeks. Most nuclei appeared in plump and the tissue was less woven.
		
d) US at 4 weeks. Tendon tissue appeared with less cellularity and showed greater tendency to be aligned in parallel, more structured and with a more developed crimp pattern. More nuclei appeared in slender shape.	e) FU at 4 weeks. Tendon tissue appeared with less cellularity and showed greater tendency to be aligned in parallel, more structured and with a more developed crimp pattern. More nuclei appeared in slender shape.	

Figure 4.1 Photomicrographs showing histological aspects of the tendons.

	H&E staining	
Group	Cell Count	% area of cell
CON at 2 weeks	146.33±47.86	16.30±5.28
FO at 2 weeks	144.67±19.34	12.16±3.39
US at 2 weeks	138.11±29.17	13.21±4.64
FU at 2 weeks	148.11±25.20	11.66±2.97
CON at 4 weeks	176.22±21.69	12.37±7.90
FO at 4 weeks	159.89±49.44	11.86±10.93
US at 4 weeks	138.56±57.45	5.48±4.05
FU at 4 weeks	129.75±41.11	7.72±4.58
normal	32.67	0.95

Table 4.2 Quantitative results of tendon sections with H&E staining.

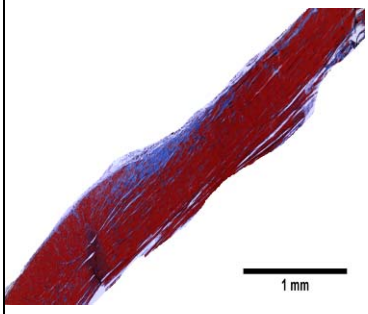
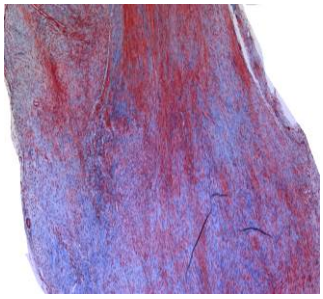
		
a) Normal tendon	b) CON at 4 weeks	c) FO at 4 weeks
		
d) US at 4 weeks	e) FU at 4 weeks	

Figure 4.2 Sections with Masson's trichrome staining.

The normal tendon appeared uniformly red, while the injured tendon stained nearly completely blue. The US and FU at 4 weeks showed deeper blue colour which indicated tendency to have higher collagen deposition.

Group	% blue area
CON at 2 weeks	43.54±18.85
FO at 2 weeks	62.77±22.27
US at 2 weeks	64.76±13.79
FU at 2 weeks	52.68±14.79
CON at 4 weeks	61.94±23.89
FO at 4 weeks	63.04±9.16
US at 4 weeks	68.50±23.88
FU at 4 weeks	75.04±19.62
normal	19.80

Table 4.3 Quantitative results of tendon sections with Masson's trichrome staining.

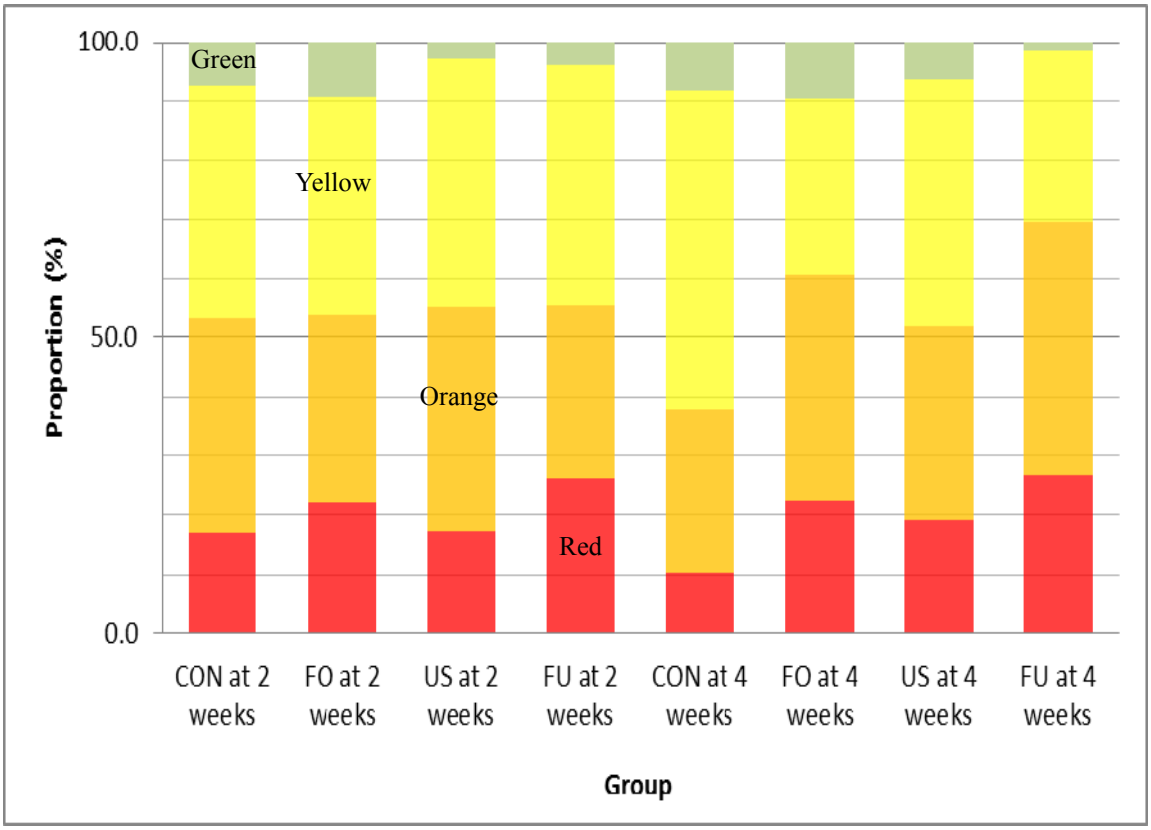


Figure 4.3 Picrosirius red birefringent colour proportions among groups.

Higher proportions of red and orange fibers was observed in FO and FU at 4 weeks ($p<0.05$)

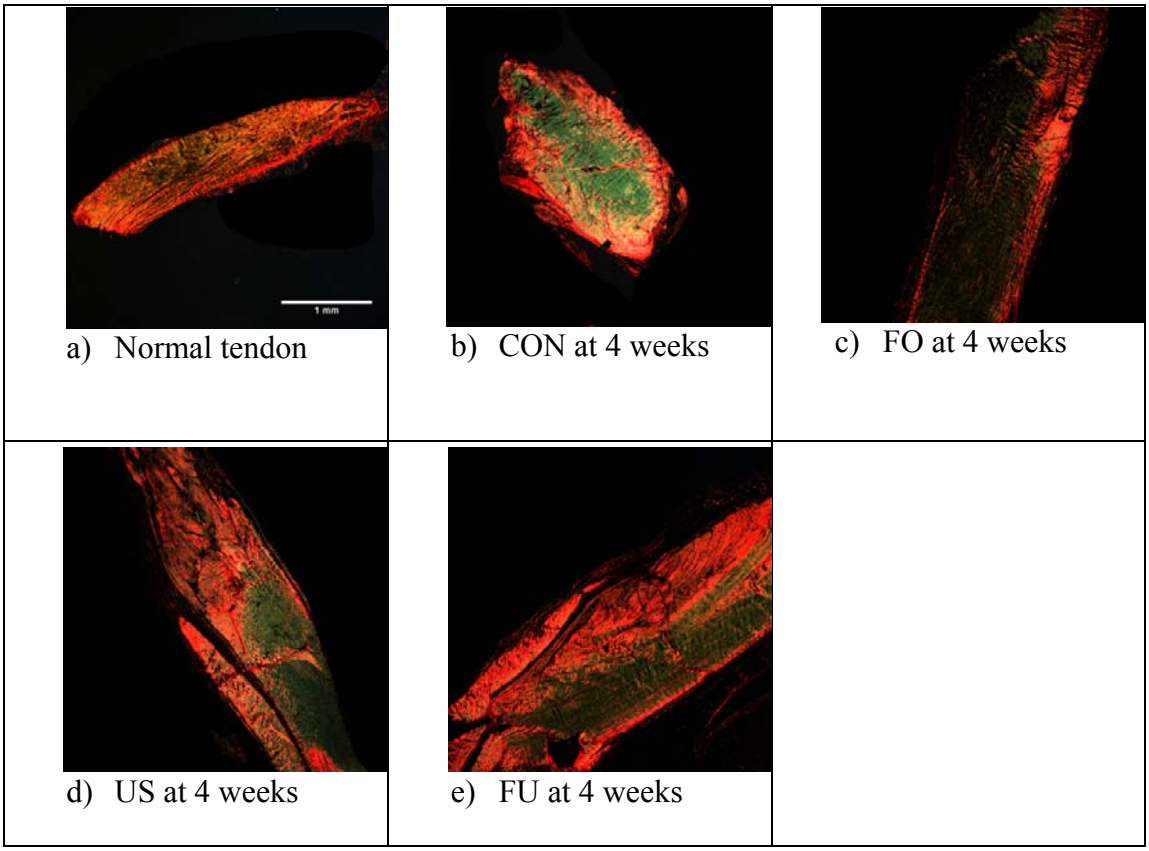


Figure 4.4 Tendon sections stained with PSR examined by polarized light microscopy. Large collagen fibers were stained red and orange, while small fibrils were stained yellow and green. Compared with normal tendon, injured tendons showed higher proportion of yellow and green fibers around the scar tissues.

4.5.4 Immunohistochemistry

Type I collagen was scattered randomly throughout the repair site with some forming slim, crimped, fibre-like wires. The FO and US groups at 2 weeks showed a higher percentage of type I collagen relative to tissue area when compared with CON at the same time point ($p<0.05$) (Table 4.4). The US group at 2 weeks demonstrated a higher percentage of type III collagen relative to the total tissue area when compared with CON at the same time point ($p<0.05$). There was no difference for the 4-week groups in both type I and III collagen.

Group	Type I- % immune-positive –stained area	Type III - % immune-positive –stained area
CON at 2 weeks	24.97±9.72	56.79±28.81
FO at 2 weeks	72.56±18.51*	73.14±33.30
US at 2 weeks	71.63±20.26*	92.21±3.30*
FU at 2 weeks	55.47±26.05	68.62±17.51
CON at 4 weeks	50.92±24.18	74.15±21.92
FO at 4 weeks	49.47±18.92	87.24±8.25
US at 4 weeks	46.90±20.09	74.44±13.27
FU at 4 weeks	50.99±22.68	82.94±11.75

Table 4.4 Quantitative analysis of immunostaining of type I and type III collagen. US at 2 weeks showed significantly higher % of area for type I and III collagen. FO at 2 weeks exhibited significantly higher % of area for type I collagen.

* compared with CON at 2 weeks, $p<0.05$

4.6 Discussion

The main findings of this study were that topical fish oil, therapeutic ultrasound and ultrasound coupled with fish oil favored tendon healing by regulating collagen synthesis. After an injury, the inflammatory phase lasts for the first two to three days. The repair phase starts on day 3. During this phase, the fibroblasts become highly proliferative, metabolically active and larger in size to synthesise collagenous fibres. The climax of this process is around the 5th day of injury (Júnior et al. 2014). The large number of newly produced collagen fibrils are scattered and randomly oriented in the extracellular matrix (Enwemeka 1989a). The reorganization of collagen fibres would start around the 14th day post-injury to align the collagen fibrils in the longitudinal axis of the tendon (Maia Filho et al. 2010). Tendon cell proliferation for synthesis of the matrix and collagen is vital in the healing process but the cellular content decreases during the remodeling phase (Molloy et al. 2003). The current study analysed the collagen content at 2 and 4 weeks post-injury. This was a reasonable time frame to observe the changes in cell count and collagen content. The findings of the FU and US groups coincided with the normal healing process and demonstrated a tendency towards faster healing particularly at 4 weeks.

A profound anabolic phase occurs seven days post-injury in which type III collagen is rapidly deposited with a parallel pattern and is characterised by small and thinner fibrils that have greater elasticity (Eriksen et al. 2002; Tsai et al. 2006). It can rapidly form cross-links to stabilise the repair site and facilitate scar formation (Wang et al. 2012b). The deposition of type I collagen becomes dominant later in the repair process to restore the strength of the tendon. Consistent to Hou et al. (2009), both type I and III collagen were found at the repair sites of all groups during the first 2 weeks with a predominance of type III collagen. Based on the proportion of type I and type III

collagen at 2 weeks, it is clear therapeutic ultrasound expedited the healing process. The possible mechanism is that therapeutic ultrasound regulates the inflammatory response and the early proliferative stage of repair by increasing the number of fibroblasts and macrophages (Young & Dyson 1990a). The regulatory processes involved an increase in PGE_2 and LTB_4 in the acute phase (Leung et al. 2004) and up-regulation of TGF-β at 10 days post-injury (Leung et al. 2006). TGF-β would then direct the monocyte migration and fibroblasts production for the active repair process. Watson (2006) reported that the pro-inflammatory action of ultrasound speeds up the inflammatory process and promotes resolution of the inflammatory events, thus enhancing the subsequent phases of healing. Unlike therapeutic ultrasound, n3 PUFAs are anti-inflammatory, this is in keeping with their role as a COX-2 inhibitor. It was found that early administration of COX-2 inhibitors negatively affected tendon healing (Forslund et al. 2003; Virchenko et al. 2004). That may explain why the US group demonstrated a faster healing effect as shown in the higher percentage of type I and III collagen at two weeks.

Masson's trichrome stain was mainly used to differentiate collagen fibres, nuclei, cytoplasm, and pathological status of a tendon (Martinello et al. 2015). The blue colour is dominant around 30 days after injury as formation of new extracellular matrix and collagen were most active (Martinello et al. 2015). Our results agreed with Martinello et al. (2015) that all the injured tendons at 4 weeks showed high proportion of blue colour particularly in the FU groups which demonstrated a higher proportion of collagen deposition when compared with CON at 2 weeks.

The thickness of the collagen fibres determines the polarisation colours observed in PSR staining (Dayan et al. 1989; Junqueira et al. 1982). Collagen network is

intensely birefringent and hence polarised light should be used to examine the structure (Wolman 1975). The intensity of birefringence is determined by the cross-links between fibrils (Junqueira et al. 1979). Tightly packed, thicker collagen fibres shift to the colour spectrum with longer wavelengths, ie. red. The stain can distinguish between procollagens, intermediate, pathologic and normal collagen. Therefore, PSR cannot quantify the absolute collagen content but it can reflect tissue rigidity, stability and ongoing remodeling (Kaemmer et al. 2010). This method cannot reflect the molecular components of the collagen and thus cannot identify the collagen types (Dayan et al. 1989; Piérard 1989). The results of PSR cannot be directly compared with IHC of type I and type III collagen. The findings of PSR staining were consistent with that of Masson's trichrome. FO and FU groups at 4 weeks appeared to have higher collagen deposition and demonstrated higher proportions of red-orange fibres which implied their collagen fibres were better arranged, more organized and tightly packed when compared with the CON group at this time point.

The effect of fish oil on collagen synthesis was associated with its ability to modulate cytokines. PGE_2, which inhibits type I and III collagen formation in fibroblasts (Varga et al. 1987), was reduced by n-3 PUFAs via the suppression of the pro-inflammatory COX-2 pathway (Bagga et al. 2003; Sköldstam et al. 1992). Moreover, n-3 PUFAs are believed to alter the expression of genes encoding inflammatory mediator production such as IL-6 (Calder 2009b). Several *in vitro* studies consistently demonstrated that collagen synthesis was enhanced by EPA, which was associated with the increased IL6 and reduced PGE_2 levels (Hankenson et al. 2000; Jia & Turek 2004 and 2005). The exact mechanisms on how n-3 PUFAs promote soft tissue healing and collagen synthesis have yet to be explored. Further studies are needed to

analyse the changes and interactions of various cytokines and eicosanoids in the injured tendons.

Both fish oil and therapeutic ultrasound are common treatment modalities for musculoskeletal problems and are considered safe. This study showed that all treatment groups produced modest effects on cellularity and collagen deposition. It is worth exploring the optimal treatment combinations to improve the current practice for tendon injury management.

4.7 Conclusions

The treatment groups including topical fish oil application, therapeutic ultrasound and therapeutic ultrasound coupled with fish oil improved the collagen synthesis of injured Achilles tendon to different extents. Therapeutic ultrasound elicited a faster healing effect whereas fish oil treatment elicited a prolonged benefit on collagen synthesis.

CHAPTER 5 GRAND DISCUSSIONS AND CONCLUSION

5.1 Summary of the key findings

The effects of topical fish oil, therapeutic ultrasound and combination of ultrasound and fish oil on tendon healing were examined biomechanically and histologically. The three treatment groups namely, FO, US & FU showed modest beneficial effects. Among them, FU tended to display better outcomes at 4 weeks which was signified by a significant improvement in UTS, structural stiffness and mature collagen fibres. therapeutic ultrasound appeared to trigger faster healing responses and hence significantly higher UTS, type I and III collagen were observed at 2 weeks and higher UTS at 4 weeks. FO also demonstrated enhanced structural stiffness and mature collagen deposition at 4 weeks (Table 5.1).

	Biomechanical analysis		Histolomorphological analysis			Immunohistochemical analysis	
	UTS (Figure 3.11)	Structural stiffness (Figure 3.12)	H&E-cell count (Table 4.2)	H&E- cell density (Table 4.2)	PSR- mature collagen fibres (Figure 4.3)	Type I collagen (Table 4.4)	Type III collagen (Table 4.4)
FO at 2 weeks						$p<0.05$	
US at 2 weeks	$p<0.05$					$p<0.05$	$p<0.05$
FU at 2 weeks							
FO at 4 weeks		$p<0.05$			$p<0.05$		
US at 4 weeks	$p<0.05$			$p=0.0831$			
FU at 4 weeks	$p<0.05$	$p<0.05$	$p=0.0616$		$p<0.05$		

p value compared with the corresponding CON group.

Table 5.1 Summary of the key findings.

5.2 Effectiveness of therapeutic ultrasound

Both biomechanical and immunohistological results indicated therapeutic ultrasound induced faster healing effect by improving the UTS, increasing type I and III collagen level at 2 weeks. Numerous studies have confirmed that therapeutic ultrasound could promote early healing in tendon and ligament injuries (Fu et al. 2008; Gan et al. 1995; Jackson et al. 1991; Warden et al. 2006). The improvement was associated with the time of administration of therapeutic ultrasound. Warden et al. (2006) applied ultrasound treatment at few hours after injury, Jackson et al. (1991) and Fu et al. (2008) started the treatment at one day after injury, while Gan et al. (1995) administrated the treatment 7 days after injury. All of the studies indicated favourable effects of therapeutic ultrasound. Lu et al. (2016) presented a clear picture as their study compared the healing effect of pulsed ultrasound initiated at immediate, 7, and 14 days after partial patellectomy. All groups showed significant increase in bone volume, bone mineral content, ultimate strength and stiffness at postoperative week 8 when compared with the control but the 7-day group was superior to other groups in stiffness, ultimate strength, bone volume and bone mineral content at the same time point. Therefore, the optimum time frames for administration of therapeutic ultrasound should be within the first 7 days after injury as most benefits with pulsed ultrasound were observed within the initial 4 weeks after injury rather than latter time points (Warden 2003). The current study agreed with the above notion that ultrasound treatment initiated 1 day after injury displayed beneficial effect at 2 weeks and 4 weeks.

There was still controversy regarding whether therapeutic ultrasound is pro- or anti-inflammatory. It may depend on how the “pro” and “anti” inflammatory are defined. Therapeutic ultrasound is a pro-inflammatory treatment as it has been

reported to increase the levels of PGE_2 and LTB_4 (Leung et al. 2004), stimulate macrophages to affect fibroblast mitogenic factors release (Young & Dyson 1990b) , induce angiogenesis in fibroblasts (Doan et al. 1999), elevate peripheral leukocyte counts and cell density in the acute phase of injury (Li et al. 2002; Lu et al. 2016). However, Gan et al. (1995) advocated the anti-inflammatory effect of therapeutic ultrasound as the amount of inflammatory infiltrate around the repair site was decreased at 4 weeks when therapeutic ultrasound was applied at 7 days after injury. However, there is a point to note in the report of Gan et al. (1995) that the conclusion was drawn based on the results at 4 weeks which was the period of repair and remodeling phases rather than inflammatory phase. Current study could not prove the inflammatory effect either but it has revealed the rapid resolution of inflammation and a tendency towards lower number of fibroblasts.

Tendon has abundant collagen content and demonstrates high absorption of acoustical energy (Michlovitz 2005). Thinner type III collagen will be deposited immediately after injury and thicker type I collagen will later replace type III collagen during the repair process. Higher type I collagen content is associated with higher tensile strength (Longo et al. 2009). Ultrasound treatment increased both type I and type III collagen formation *in vitro* (Tsai et al. 2006). The present results have also demonstrated an increase in both type I and III collagen at 2 weeks which can be explained by the higher UTS. On the other hand, a decrease in stiffness may also be correlated with an increase in immature collagen matrix (Matsumoto et al. 2003). The relatively lower proportions of mature collagen coincided with the lower stiffness of the US group at 4 weeks (Figure 3.12 & 4.3) of this study. These results reinforced the idea that most therapeutic effects of ultrasound would be observed at the early stage of

healing rather than later stages. The timing and duration of ultrasound treatment may be a key determinant for its treatment effect.

Current studies applied pulsed ultrasound to minimize the thermal effect and hence the non-thermal effects should be the major contributor to the positive findings. Based on the present understanding on the biophysical effects of therapeutic ultrasound, non-thermal effects would be more essential towards tendon healing. The non-thermal effects of therapeutic ultrasound increase membrane's permeability, displace ions and small molecules so as to stimulate cellular activities and metabolic processes such as protein synthesis, production of growth factors and mobilization of fibroblasts (Watson & Young 2008). The absorption of ultrasound energy by proteins in cells would lead to the change in signaling mechanisms. The enzymatic activities and possible gene regulation may be enhanced or suppressed subsequently (Johns 2002). These cellular and molecular changes within cells are crucial for the inflammatory and healing processes.

5.3 Effectiveness of fish oil

This is the first study to demonstrate that fish oil promoted tendon healing by enhancing the biomechanical properties and collagen synthesis. The hierarchical organization of collagen fibers are the major determining factor for the mechanical strength of tendons (Battaglia et al. 2003). The higher UTS and structural stiffness in the fish oil group could be explained by the enhanced mature collagen fibers. Deficiency of essential fatty acids was associated with loss of normal collagen synthesis in bone and in other organs (Kruger & Horrobin 1997). Both *In-vitro* and *in-vivo* studies have shed lights on the possible action of n-3 PUFAs on collagen synthesis (Cardoso et al. 2004; Hankenson et al. 2000; Jia & Turek 2004 and 2005).

Administration of EPA had led to an increase in collagen production in porcine medial collateral ligament fibroblasts (Hankenson et al. 2000). The possible effects were associated with increase in IL-6 production in which significant linear correlation between IL-6 level and collagen production was observed (Hankenson et al. 2000). Manipulation of n-6:n-3 ratio would regulate collagen production in murine 3T3-Swiss fibroblasts by mediating PGE_2 (Jia & Turek 2004), and the nuclear factor -κB (NF-κB) pathway (Jia & Turek 2005). NF-κB is a protein complex and a family of inducible transcription factors that controls the transcription of DNA related to collagen formation (Li & Verma 2002; Tak & Firestein 2001). N-3 PUFAs treatment has reportedly elevated the level of IL-1 in human subjects which would regulate fibroblast proliferation and collagen synthesis and activate the insulin-like growth factor (IGF)-I (McDaniel et al. 2008). They play an important role in collagen and proteoglycan synthesis (Di Battista et al. 1997; Efron & Moldawer 2004; McDaniel et al. 2008). Therefore, elevating IL-6, suppressing PGE_2, mediating NF- κB and increasing IL-1 could be the possible pathways for n-3 PUFAs to regulate collagen deposition. In addition, two animal studies have also demonstrated the effect of n-3 PUFAs on connective tissue fibre deposition in cutaneous wound model (Cardoso et al. 2004; Otranto et al. 2010). The use of PUFAs to control collagen formation is still in the preliminary stage which requires more studies to prove the actual mechanism and how they may be applied in different saturations.

The effect of FO and FU was more noticeable at 4 weeks. A reason for the slow healing effect of topical fish oil could be because of the rate of transport of active ingredients across the skin. It normally takes several hours or even days for the drug to permeate through the skin and migrate across the transdermal system and in order to

accumulate sufficient pharmacological dosage (Prausnitz et al. 2004). Another reason may be the potential inhibition effect of fish oil on the COX pathway which is the synthetic pathway of pro-inflammatory eicosanoids such as PGs and LTs. Those pro-inflammatory eicosanoids promote dilation of blood vessels and increasing local vascular permeability which is believed to be useful after acute injury (Warden et al. 2006). N-3 PUFAs similar to NSAIDS, are COX inhibitors that block the formation of pro-inflammatory eicosanoids (Zainal et al. 2009). Supplementation of COX inhibitor ie. parecoxib has been shown to have an adverse effect on early tendon repair (post-injury days 1-5), but improved tendon remodeling when given at later stages of repair (days 6–14) (Forslund et al. 2003). When applied throughout the healing phase for 14 days, another two COX inhibitors namely indomethacin and celecoxib have been demonstrated to reduce the cross sectional area without compromising the failure load and increase the tensile stress of transected tendons in rats (Forslund et al. 2003). Similar results were found in the study by Yuan et al. (2003) that treatment with flurbiprofen (non-selective NSAIDs) would significantly decrease cross-sectional area and increase tendon failure stress thus resulting in better collagen reorganization. The presence of COX inhibitors would decrease PGE_2 release and DNA synthesis during the proliferative phase of a healing but increasing protein synthesis in maturation and remodeling phase in-vitro (Almekinders et al. 1995). The beneficial effect of COX inhibitors in the later remodeling phase may dilute the negative effects in the early proliferation phase (Dimmen et al. 2009). Although EPA and DHA are COX inhibitors, whether they would exert similar effects as NSAIDs have not been proved. The fish oil group in the current study did not show any notable unfavourable effect during early healing, In addition, previous studies have suggested n-3 PUFAs are safer alternatives

to NSAIDS for treatment of non-surgical neck or back pain (Maroon & Bost 2006), rheumatoid arthritis (Belch et al. 1988) as well as OA (Hill et al. 2016) .

5.4 Comparison on the effect of fish oil and therapeutic ultrasound

Most evidence supported the beneficial effects of therapeutic ultrasound was observed at the early stage of healing while fish oil appeared to exert more influential effect in the later healing phases. If COX-2 is the major pathway to explain the effect of fish oil, pulsed ultrasound was not dependent on COX-2 pathway nor affected by the early detrimental effect of COX inhibitors (Li et al. 2007; Warden et al. 2006). Theoretically, both modalities would not antagonize one another. Although the effect of therapeutic ultrasound coupling with fish oil appeared to be superior to either treatment alone, it is not known whether the two modalities work separately or synergistically. Since the phonophoresis of fish oil and the transdermal movement of topical fish oil have not been analysed, the actual mechanism behind could not be identified in the present study.

5.5 Technical considerations

5.5.1 Animal model

The animal model adopted in this study was a partial hemi-transected tendon model which is a simple, quick and reproducible method to induce injury but may not simulate the natural development of the disease in humans. There is no consensus on the etiology of Achilles tendon rupture but it is commonly believed that degenerative changes and repeated mechanical loading which reduces tensile strength of the tendon are the causative factors of Achilles tendon rupture (Tallon et al. 2001). The abrupt cutting of the Achilles tendon adopted in this study is different from the natural Achilles tendons

rupture in patients as they may have long history of degeneration and the rupture is triggered by a sudden mechanical over-load. The present injury model cannot reproduce the gradual cellular responses and the changes in the extracellular matrix due to repetitive loading (Ng et al. 2011). Animal model of tendinosis such as the one developed by Ng et al. (2011) should be a better alternative in future studies.

5.5.2 Quantitative evaluation for histological analysis

Histological analysis commonly includes qualitative approach to describe the changes of the tissue sections such as collagen alignment and the colour of the stain (Martinello et al. 2015). Without conversion into a scoring system, the descriptions are just the subjective ratings from the assessors. Semi-quantitative evaluation such as Bonar and Movin scale are developed to grade the tissues in different aspects but these methods are heavily dependent on the experience and training of the assessors and the precise descriptions of the classification system. The slides have to be assessed several times to improve the reproducibility (Longo et al. 2008). The scale may not be linear regarding the changes in the injured tendons. A formal assessment of their validity and reliability would need to be performed.

On the other hand, the automated computerized system such as ImageJ, which is the software used in this study, can analyse the sections objectively and rapidly. For example, the different colours observed in the slides with PCR stain in this study was quantified by setting hue (H), saturation (S), and brightness (B) ranges of the captured image. The hue ranges selected for PSR analysis are empiric and hence it eliminates the trouble on describing the colours (Rich & Whittaker 2005). This method is time saving, efficient and requires little technical know-how. Once the thresholds setting is

confirmed, the analysis of all the slides can be automated and quantified by the same set of thresholds. One of the determining factors for a successful automated computerized analysis is that the tissue images have to have high uniformity (Corey et al. 1983).

Another example is immunohistochemical analysis. Diaminobenzidene (DAB) is commonly used in immunohistochemical analysis to identify the horseradish peroxidase (HRP)-labeled antigen. DAB and HRP complex give a brown coloration and hematoxylin stains the background tissue blue. The traditional assessment of immunohistochemical staining is highly dependent on the experience and training of the assessors to describe the intensity and area of stain. With the computerized method, the percentage of positive stain can be quantified. The results obtained from ImageJ were highly comparable to the experienced researchers. Intraobserver and interobserver variation of DAB immunohistochemical analysis using ImageJ was 0% (Fuhrich et al. 2013).

The qualitative descriptions of the tissues should be retained to highlight the special and unique features of the tissues, whereas quantitative approaches should be explored to offer more objective and unbiased comparisons.

5.6 Limitations

It is important to recognise the limitations of this study. First, the transdermal permeation efficiency of the fish oil ointment and the ultrasound phonophoretic effect of fish oil have not been examined. The high epithelial penetration potential of n-3 PUFAs suggested it is a promising topical agent, as discussed in session 2.6. However, caution should be taken for the difference between rat and human skin. Based on a literature review on skin penetration rate between rats and humans for a total 14 chemicals with molecular mass ranged from 231 to 466 $g \cdot mol^{-1}$, rat skin was, on

average, 10 times more permeable than human skin (van Ravenzwaay & Leibold 2004). In addition, there is considerable inter-individual variability regarding the transdermal absorption of topical drugs in humans which may be attributed to individual skin differences such as hydration status, permeability characteristics and subcutaneous vasculature (Heyneman et al. 2000). Therefore, it is not known whether the current formulation would be suitable for humans. Further research is required to find out the permeation enhancers for the topical formulation of n-3 PUFAs for humans.

Second, histological analyses are usually done qualitatively rather than quantitatively. The sample size commonly ranged between 3 and 6 (Aro et al. 2013; Majewski et al. 2008; Majewski et al. 2009; Moshaverinia et al. 2014; Yeung et al. 2006). Automated computerised analysis is a new trend in histological study and a few studies of tendon injury have adopted this approach (Kollitz et al. 2014; Moshaverinia et al. 2014). The small sample size in current study may increase the chance of a type II error, particularly as there were relatively large variations for quantitative assessment compared to the semi-quantitative assessment.

5.7 Future research

The promising findings in topical fish oil treatment implies a new direction in soft tissue management. Further studies with more outcome measures such as functional assessment are necessary to confirm the healing effects and explore the mechanism of topical fish oil on soft tissue injury. Functional assessment can be performed by gait analysis (Davidson et al. 1997). The gait of the animals will be video-recorded to measure the changes in stride length, stride frequency and ankle angular displacement.

It is believed that fish oil would exert its anti-inflammatory effect via the COX-2 pathway and early administration of COX-2 inhibitors would negatively affect tendon

healing (Virchenko et al. 2004). The inhibition of COX by fish oil and the subsequent effect on tendon injury should be verified. While delay application of fish oil is suggested (Forslund et al. 2003), early initiation of ultrasound treatment is proposed (Gan et al. 1995). Therefore, the importance of the timing for application of fish oil and therapeutic ultrasound should be confirmed by initiating the treatment at different days after injury.

Based on the results of biomechanical studies, the FU group appeared to be superior to either treatment alone. Whether phonophoresis has taken place would need to be confirmed by biochemical analysis of the FU group tendon specimens. The summated effects of ultrasound and fish oil needs to be examined too as it appears that ultrasound and fish oil exert conflicting effects on inflammation.

As discussed in section 2.6, it is likely that the omega 3 fatty acids could permeate skin as they are lipophilic and have light molecular weight. This assumption should be tested and the permeation ability of the formulation to human skin should further be investigated as rat skin is on average more permeable than human skin. Other permeation enhancement methods may need to be explored.

5.8 Conclusion

This is the first study to examine the effect of topical fish oil application on rupture tendons. Fish oil promoted tendon healing by faster resolution of inflammation, augmented mature collagen deposition and hence improved the UTS and stiffness of the injured tendons but the effect took time to develop. Therapeutic ultrasound appeared to support inflammation and shorten the inflammatory phase which expedited the subsequent repair and remodeling phase. There was no detrimental effects observed when topical fish oil and therapeutic ultrasound were applied together and the

therapeutic effect of the combination of the two modalities seems superior to either treatment alone.

APPENDICES

1. Ethical approval

Memo

To Prof. Gabriel Ng (RS)

From Prof. Samuel CL Lo, Chairman, Animal Subjects Ethics Sub-committee (ASESC)

Ref. _____ in _____ Your ref. _____ in _____

Tel no. Ext. 8669 Fax no. _____

Email samuel.chun-lap.lo@ Date 18 August 2014

Application for Ethical Review for the Use of Animals in Teaching or Research

Project Title	Effect of topical polyunsaturated fatty acids (PUFAs) and ultrasound phonophoresis on Achilles tendon healing in a rat model
ASESC Case #	13/48

Your application for ethics review for the use of animals in the above project has been approved for a period from the date of this memo up to 16 April 2016. Please note that in case the licence issued by the Department of Health is revoked, this animal ethics approval granted by the Animal Subjects Ethics Sub-committee (ASESC) will also be withdrawn.

The animal ethics approval is granted with regard to the ethical aspects of the protocol. Granting of animal ethics approval by the ASESC DOES NOT automatically give you the right of access to the service of the Centralised Animal Facilities (CAF). Although CAF operates on an equal opportunity principle and strives to serve each CAF user equally, CAF operates on limited budget and resources. Therefore, CAF may not have the space and manpower to support your experiments. CAF has the independent and ultimate authority to accept or reject the housing, provision of daily care and the performance of the proposed animal experiments within their premises. **You are personally responsible to liaise with CAF on the operational details of your experiments.**

You are required to inform the ASESC 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 CAF, 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 ASESC members may visit the CAF unannounced at any reasonable time to inspect if you have compile with the requirements of your licences.

I would like to draw your attention to the University requirement that holders of licences under Cap. 340 must provide the ASESC 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.

Samuel CL Lo (Prof.)
Chairman
Animal Subjects Ethics Sub-committee

c.c. Chairman, DRC(RS)

THE HONG KONG POLYTECHNIC UNIVERSITY
香港理工大學

Memo

To	Prof. Gabriel Ng (RS)		
From	Dr Mason Leung, Chairman, Animal Subjects Ethics Sub-committee (ASESC)		
Ref.	_____ in _____	Your ref.	_____ in _____
Tel no.	Ext. 4831	Fax no.	
Email	rsmcpleung	Date	19 July 2012

Application for Ethical Review for the Use of Animals in Teaching or Research

Project Title: *Effect of Topical Polyunsaturated Fatty Acids (PUFAs) and Ultrasound Phonophoresis on Achilles Tendon Healing in a Rat Model*
ASESC No.: 11/46

Your application for ethics review for the use of animals in the above project has been approved for a period of two years from the date of this memo.

Kindly note that the animal ethics approval is granted with regard to the ethical aspects of the protocol. Granting of animal ethics approval by the Animal Subjects Ethics Sub-committee (ASESC) DOES NOT automatically give you the right of access to the service of the Centralised Animal Facilities (CAF). Although CAF operates on an equal opportunity principle and strives to serve each CAF user equally, CAF operates on limited budget and resources. Therefore, CAF may not have the space and man-power to support your experiments. CAF has the independent and ultimate authority to accept or reject the housing, provision of daily care and the performance of the proposed animal experiments within their premises. **You are personally responsible to liaise with CAF on the operational details of your experiments.**

You are required to inform the ASESC 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 CAF, 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 ASESC members may visit the CAF unannounced at any reasonable time to inspect if you have compile with the requirements of your licences.

I would like to draw your attention to the University requirement that holders of licences under Cap. 340 must provide the ASESC 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.

Mason Leung (Dr)
Chairman
Animal Subjects Ethics Sub-committee

c.c. Chairman, DRC(RS)

2. License to conduct experiments

Form 2

Licence to Conduct Experiments

Name : CHAN Oi Wan [Ref No.: (14-41) in DH/HA&P/8/2/4 Pt.8]

Address : Department of Rehabilitation Sciences, 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)

Rats will be used in the experiment. Under general anaesthesia, a skin incision will be made in the anterior aspect of the right calf of the animals to expose the tendo-Achilles. The tendon of medial gastrocnemius will be identified and separated from the lateral tendon. The medial tendon will be cut at its midpoint and the cut ends will not be sutured. After surgery, the skin wound will be closed by suture. Treatments of topical polyunsaturated fatty acids to the Achilles tendons will be applied to the animals. Blood samples will be obtained from the tail vein at different time points for analysis. Conditions of the animals will be monitored. At the end of the experiment, the animals will be sacrificed by carbon dioxide asphyxiation. Tissues will be harvested for analysis.

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

Centralised Animal Facilities, The Hong Kong Polytechnic University

Conditions

1. Such experiment(s) may only be conducted for the following purposes-

   To examine the effects of topical polyunsaturated fatty acids on Achilles tendon healing in a rat model.

2. This licence is valid from 17 April 2014 to 16 April 2016

Dated 17 April 2014

Licensing Authority

Form 2

Licence to Conduct Experiments

Name : CHAN Oi Wan [Ref No.: Rev(12-32) in DH/HA&P/8/2/4 Pt.5]

Address : Department of Rehabilitation Sciences, 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)

Rats will be used in the experiment. Under general anaesthesia, a skin incision will be made in the anterior aspect of the right calf of the animals to expose the tendo-Achilles. The tendon of medial gastrocnemius will be identified and separated from the lateral tendon. The medial tendon will be cut at its midpoint and the cut ends will not be sutured. After surgery, the skin wound will be closed by suture. Treatments of topical polyunsaturated fatty acids to the Achilles tendons will be applied to the animals. Blood samples will be obtained from the tail vein at different time points for analysis. Conditions of the animals will be monitored. At the end of the experiment, the animals will be sacrificed by carbon dioxide asphyxiation. Tissues will be harvested for analysis.

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

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Conditions

1. Such experiment(s) may only be conducted for the following purposes-

   To examine the effects of topical polyunsaturated fatty acids on Achilles tendon healing in a rat model.

2. This licence is valid from 17 April 2012 to 16 April 2014

Dated 17 April 2012 (Amended on 4 January 2013)

DEPARTMENT OF HEALTH 衞生署 HONG KONG

Licensing Authority

3. Raw data

Biomechanical analysis

A. UTS

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	78.93	77.23	135.65	68.36	75.05	72.40	193.38	68.94
2	56.27	163.40	43.65	101.19	55.74	109.73	103.71	156.85
3	76.02	148.74	81.15	97.56	78.52	79.31	76.50	76.46
4	90.10	100.99	124.81	68.01	70.26	95.01	110.10	153.11
5	32.71	86.55	91.70	94.38	72.14	126.12	155.08	120.51
6	48.10	71.01	107.91	127.55	68.84	134.06	137.19	95.22
7	58.59	54.08	85.73	98.57	102.89	82.84	141.84	156.29
8	90.02	64.20	151.82	86.91	120.81	56.24	105.44	137.48
9	78.38	58.20	162.53	65.12	87.22	125.65	135.21	120.12
10	91.67	117.56	87.16	75.56	77.71	78.91	64.76	76.84
11	98.40	59.84	135.74		50.96	91.00		
Mean	72.65	91.07	109.81	88.32	78.19	95.57	122.32	116.18
SD	20.93	37.57	35.68	19.57	19.88	25.16	38.13	41.23

* FU at 2 week, US at 4 week and FU at 4 week had 10 samples only.

B. Structural stiffness

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	43.80	34.99	87.14	27.23	18.12	134.71	122.90	40.19
2	73.04	88.26	14.41	50.63	90.57	144.22	54.97	111.22
3	60.09	132.55	43.93	57.48	101.97	79.59	90.82	42.57
4	71.47	39.53	95.96	40.29	85.38	52.55	110.60	202.29
5	71.46	18.21	61.63	74.21	20.93	162.21	107.23	182.15
6	29.76	57.40	74.02	174.82	43.72	127.50	39.22	97.80
7	24.62	10.45	53.72	87.35	59.09	143.73	84.29	133.74
8	46.24	15.70	150.52	103.42	93.03	52.56	128.31	162.40
9	19.89	41.65	39.84	55.93	58.00	142.61	87.72	186.10
10	57.32	27.97	76.91	94.83	92.10	89.70	57.16	38.17
11	73.80	57.40	20.39		55.14	65.98		
Mean	51.95	47.65	65.31	76.62	65.28	108.67	88.32	119.66
SD	20.36	36.01	38.40	42.22	29.54	41.07	30.12	50.85

C. Energy absorption capacity

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	137.86	176.53	272.62	555.71	215.20	28.07	102.54	99.96
2	37.89	273.43	79.81	243.17	37.94	65.91	137.24	246.28
3	84.89	137.25	130.58	62.41	83.58	61.11	31.70	219.52
4	109.38	264.12	124.60	95.92	50.16	145.57	121.68	46.46
5	12.17	451.37	116.85	104.71	163.39	98.74	189.58	78.09
6	107.85	97.87	112.78	75.13	93.86	167.26	548.41	105.60
7	198.01	284.78	121.36	126.52	228.84	56.40	309.01	83.13
8	163.46	315.34	162.57	95.90	185.39	51.27	68.40	94.30
9	274.25	65.27	729.97	64.85	132.81	104.45	207.80	120.14
10	263.90	416.49	48.26	79.44	38.16	78.00	52.94	172.78
11	201.39	114.87	103.03		54.82	121.32		
Mean	144.64	236.12	182.04	150.38	116.74	88.92	176.93	126.63
SD	85.21	128.85	190.28	151.74	71.89	42.82	154.55	137.39

Histomorphological and immunohistochemical analysis

A. H&E staining

Cell count

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	201.00	133.33	144.33	130.67	164.33	149.00	209.33	169.00
2	112.00	133.67	163.67	136.67	163.00	230.33	47.67	188.00
3	126.00	167.00	106.33	177.00	211.33	152.67	136.00	114.33
4					170.33	94.50	149.00	91.67
5					154.67	129.50	105.67	128.50
6					193.67	203.33	183.67	87.00
Mean	146.33	144.67	138.11	148.11	176.22	159.89	138.56	129.75
SD	47.86	19.34	29.17	25.20	21.69	49.44	57.45	41.11

% Total area

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	16.17	15.83	17.45	14.13	13.39	33.33	9.56	10.42
2	11.09	11.51	8.25	12.49	11.93	11.29	0.81	13.72
3	21.64	9.14	13.92	8.36	26.07	4.90	11.30	11.06
4					4.14	7.84	4.12	2.72
5					5.05	3.54	3.21	4.81
6					13.66	10.28	3.86	3.58
Mean	16.30	12.16	13.21	11.66	12.37	11.86	5.48	7.72
SD	5.28	3.39	4.64	2.97	7.90	10.93	4.05	4.58

B. Masson's trichrome staining

%Blue area

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	22.20	73.11	61.41	39.25	73.68	60.05	82.57	86.08
2	57.93	78.00	79.92	50.26	82.16	55.67	27.59	107.03
3	50.49	37.21	52.96	68.53	61.21	55.64	80.16	57.28
4					51.84	60.31	53.13	53.88
5					19.90	66.77	75.38	70.96
6					82.82	79.77	92.16	75.04
Mean	43.54	62.77	64.76	52.68	61.94	63.04	68.50	75.04
SD	18.85	22.27	13.79	14.79	23.89	9.16	23.88	19.62

C. Picrosirius red staining

% Orange and red area

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	34.57	88.01	66.66	41.90	23.93	66.75	74.84	80.97
2	75.36	26.45	44.23	85.68	53.56	46.13	47.98	83.39
3	50.28	47.65	54.85	38.93	47.77	40.90	67.98	44.28
4					30.02	60.78	35.97	60.44
5					29.19	91.73	26.22	64.25
6					43.56	57.85	57.85	84.41
Mean	53.40	54.04	55.25	55.50	38.01	60.69	51.81	69.62
SD	20.58	31.27	11.22	26.18	11.90	17.95	18.70	16.08

% Green and yellow

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	65.49	11.99	33.34	58.10	76.07	33.25	25.16	19.03
2	24.64	73.55	55.77	14.32	46.44	53.87	52.02	16.61
3	49.72	52.35	45.15	61.07	52.23	59.10	32.02	55.72
4					69.98	39.22	64.03	39.56
5					70.81	8.27	73.78	35.75
6					56.44	42.15	42.15	15.59
Mean	46.60	45.96	44.75	44.50	61.99	39.31	48.19	30.38
SD	20.58	31.27	11.22	26.18	11.90	17.95	18.70	16.08

D. Immunohistochemical analysis

% immune-positive stained area of type I collagen

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	36.12	83.96	48.31	54.10	50.49	30.40	11.20	21.61
2	18.30	82.51	81.60	82.18	30.49	24.21	49.88	68.94
3	20.48	51.20	84.97	30.14	58.59	72.43	46.59	30.69
4					16.15	62.65	54.03	81.25
5					81.46	58.71	73.05	57.13
6					68.36	48.43	46.66	46.32
Mean	24.97	72.56	71.63	55.47	50.92	49.47	46.90	50.99
SD	9.72	18.51	20.26	26.05	24.18	18.92	20.09	22.68

% immune-positive stained area of type III collagen

	CON	FO	US	FU	CON	FO	US	FU
Treatment period	2 weeks				4 weeks			
1	50.97	94.73	89.67	60.49	84.94	79.55	87.36	69.06
2	31.34	89.90	91.02	88.72	89.64	81.60	54.86	72.64
3	88.07	34.78	95.93	56.66	48.70	94.76	72.84	97.58
4					44.77	78.22	72.25	77.11
5					96.44	93.87	68.09	87.08
6					80.41	95.46	91.26	94.19
Mean	56.79	73.14	92.21	68.62	74.15	87.24	74.44	82.94
SD	28.81	33.30	3.30	17.51	21.92	8.25	13.27	11.75

4. Publications derived from this study

a. Chan KOW, Tong HHY and Ng GYF (2016) Topical fish oil application coupling with therapeutic ultrasound improves tendon healing. Ultrasound in Medicine and Biology 42 (12):2983-2989.

Ultrasound in Med. & Biol., Vol. 42, No. 12, pp. 2983–2989, 2016

0301-5629/$ - see front matter

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● *Original Contribution*

TOPICAL FISH OIL APPLICATION COUPLING WITH THERAPEUTIC ULTRASOUND IMPROVES TENDON HEALING

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Abstract—Fish oil has been shown to promote collagen synthesis, and hence, connective tissue healing. Therapeutic ultrasound is commonly used to treat soft tissue injuries. This study aimed to investigate the therapeutic effect of topical fish oil on the management of Achilles tendon rupture, comparing normal therapeutic ultrasound with a combination of ultrasound and fish oil. Eighty-five Sprague-Dawley rats underwent surgical hemitenotomy of the right medial Achilles tendon. The rats received daily treatment of either topical placebo ointment (control group [CON]), topical fish oil (FO), therapeutic ultrasound (US) or ultrasound with fish oil as the coupling medium (FU). The treatment started on post-surgical day 2 over a 2-wk or 4-wk period. On days 15 and 29, the rats were sacrificed and their Achilles tendons were tested for structural stiffness, ultimate tensile strength (UTS) and energy absorption capacity. At 2 wk, only US showed higher normalized UTS compared with CON ($p < 0.05$). At 4 wk, both US and FU demonstrated better UTS ($p < 0.05$), while both FO and FU had improved in structural stiffness ($p < 0.05$). Four wk of treatment with ultrasound using fish oil as coupling medium showed improvement in both structural stiffness and UTS ($p < 0.05$). (E-mail: cckarly@hkcc-polyu.edu.hk) **© 2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Fish oil, Omega-3, Achilles tendon rupture, Therapeutic ultrasound, Repair.**

INTRODUCTION

The Achilles tendon (AT) is the largest and strongest tendon in the body, which bears up to 12.5 times of the body's weight during running and jumping (Doral et al. 2010). However, the incidence of spontaneous rupture of the AT is particularly high (Kannus and Józsa 1991; Rees et al. 2006), and it is believed that degenerative changes and repeated mechanical loading that reduce the tensile strength in this tendon are the causative factors of AT rupture (Tallon et al. 2001). Because of the relatively low metabolic rate and long turnover time for tendon collagen synthesis (Abate et al. 2009), natural healing is often very slow and the repaired tissue may never attain the normal pre-injury level of function (Doroski et al. 2007).

There is no consensus on the treatment of acute AT rupture (Nandra et al. 2012). The treatment options include two main approaches, namely operative and conservative. The operative approach is associated with higher risk of post-surgery infection (Bhandari et al. 2002), while the conservative approach may have higher risks of re-rupture (Khan et al. 2005; Weber et al. 2003). Both require 7–9 wk of immobilization, which may compromise the strength of the calf muscles (Ding et al. 2013). Additionally, it has been reported that Achilles tendons cannot fully recover even at 2 y after rupture, irrespective of the type of treatment (Olsson et al. 2011). Due to the deficiencies of current treatments, there is a need to explore potential treatment alternatives that can shed new light on current practices.

Oil from deep-sea fish contains high proportions of polyunsaturated fatty acids (PUFAs) of the omega-3 series that are particularly rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Cleland et al. 2006). Omega-3 fatty acids are believed to have anti-inflammatory properties essential for the healing of rheumatoid arthritis, osteoarthritis and chronic tendonitis (Berbert et al. 2005; Caturla et al. 2011; Mavrogenis et al. 2004). Furthermore, omega-3 PUFAs promote collagen synthesis in connective tissues such as in the

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porcine ligament fibroblasts (Hankenson et al. 2000), mouse fibroblasts (Jia and Turek 2004, 2005) and mouse and rat cutaneous wounds (Cardoso et al. 2004; Otranto et al. 2010). The omega-3 PUFAs are lipophilic and possess high epithelial penetration abilities, meaning they can even serve as a skin drug permeation enhancer for ketoprofen (Heard et al. 2003) and para-aminobenzoic acid (PABA) (Tanojo et al. 1997).

Therapeutic ultrasound has been used to treat soft tissue injuries for over six decades. Therapeutic ultrasound improves tendon healing by increasing biomechanical strength (Ng et al. 2003; Yeung et al. 2006), collagen synthesis (Jackson et al. 1991) and collagen fibril size (Ng and Fung 2007).

Taking into account the wide use of fish oil on different connective tissues and the popularity of ultrasound in treating tendon injuries, the aims of the present study were to (i) investigate the effects of topical fish oil application, (ii) compare the effect of topical fish oil with normal therapeutic ultrasound and (iii) examine if there is synergistic effect on therapeutic ultrasound coupled with fish oil for treatment of AT rupture.

METHODS

Experimental Design

Eighty-five female Sprague-Dawley rats (mean weight, 243.14 g ± 25.64 g; range, 325.00–208.76 g) aged 12 wk at the time of surgery were used. Ethics approval was obtained from the Animal Ethics Review Committee of the administrating institution before the study.

All rats underwent the same surgical procedures modified from a previous report by Ng and Fung (2008). The rats were anesthetized with isoflurane through a nose cone during the surgery. Skin on the anterior aspect of the right calf was shaved, incised and retracted to expose the AT. The tendon of the medial gastrocnemius was identified and separated from the lateral tendon with a blunt probe, and the medial tendon was cut with a scalpel at its mid-point without suturing. The lateral tendon was left intact to simulate a partial tendon rupture, and to prevent retraction of the cut ends. The skin wound was closed by non-absorbable suture that was removed 3 d after surgery. The limbs were not immobilized and the rats were allowed to have free activity inside their cages. The rats were kept in an animal house with a 12-h light-dark cycle, and temperature was maintained at about 20°C. Water and food were given *ad libitum* during the study. All treatments were given on the second post-surgical day.

Treatment

The animals were allocated randomly into eight groups. All animals received same amount of topical ointment daily, but with different compositions and treatment protocols according to their group assignment for either 2 wk or 4 wk (Table 1). Vaseline plain petroleum jelly acted as a control (CON) and was applied over the skin of the right AT and secured further with adhesive dressing. For the fish oil group (FO), fish oil ointment was applied over the skin of the right AT and secured further with adhesive dressing. A cone collar was put on the neck of each rat to prevent the animals from removing the dressing which would dry off the other day. The fish oil ointment was prepared by mixing 40% fish oil (F8020; Sigma-Aldrich, UK) with 60% Vaseline. The fish oil was standard refined Menhaden oil and comprised 10–15% EPA and 8–15% DHA. This ointment was changed daily for each animal. The animals in the ultrasound group (US) received pulsed ultrasound at a duty cycle of 50% at 1 MHz, at an intensity of 0.5 W/cm^2 for 4 min daily (spatial average temporal average of 0.25 W/cm^2) (Ng and Wong 2008; Ng et al. 2003), with Vaseline acting as the coupling medium. An ultrasound machine (Dynatron 150 Plus, Dynatronics, Salt Lake City, Utah, USA) with a 2.0 cm^2 soundhead was used throughout the study. The machine was calibrated with an ultrasound wattmeter (UW4 Ultrasound Wattmeter, Fluke Biomedical, Carson City, Nevada, USA) before the experiment. The combination group (FU) received similar treatment as the US group, except that fish oil

Table 1. Details of the three treatment groups and the control group

Group	Treatment	Details of the ointment/coupling agent	Ultrasound parameters
Control at 2 wk and Control at 4 wk	Topical Vaseline	Vaseline plain petroleum jelly	NA
Fish oil at 2 wk and Fish oil at 4 wk	Topical fish oil ointment	Fish oil ointment which consisted of 40% fish oil and 60% Vaseline	NA
Ultrasound at 2 wk and Ultrasound at 4 wk	Ultrasound and Vaseline	Vaseline plain petroleum jelly	50% duty cycle, 1 MHz at the intensity of 0.5 W/cm^2 for 4 min
Combination at 2 wk and Combination at 4 wk	Ultrasound and fish oil ointment	Same as fish oil group	Same as ultrasound

NA = not applicable.

ointment acted as the coupling medium. The coupling medium dried off by the motion of the ultrasound probe.

Half of the animals were euthanized at day 15 and the remaining at day 29 after surgery by CO_2 breathing. Both lower limbs were harvested by disarticulation at the hip joint, sealed in a plastic bag and stored in a freezer at −40°C for later testing.

Biomechanical testing procedures

The biomechanical tests were done following the previously established procedures (Ng et al. 2004). At least 6 h before testing, the specimens were retrieved from the freezer and thawed inside the plastic bag at room temperature. The specimens were dissected and the lateral portion of AT was removed at the musculotendinous junction, leaving the medial portion of the intra-muscular tendinous fibers, the medial AT and calcaneus intact. The intra-muscular tendinous fibers were secured between two strips of fine sandpaper. The specimens were kept moist with normal saline during the whole testing procedure. The sandpaper and the calcaneus were then mounted on the cross heads of a material testing machine (MTS Synergie 200; MTS Systems Corporation, Eden Prairie, Minnesota, USA). An extensometer (Dynamic model 634.12 F-24; MTS Systems Corporation, Eden Prairie, Minnesota, USA) was attached to the margin of the cross heads for measuring the local strain in the tendon (Fig. 1). The room temperature was controlled at 25°C during the test.

The specimen mounted on the material testing machine was preconditioned with 10 oscillation cycles of 2.5% strain at a rate of 10 mm/min, to minimize the effect of deep freezing on the tissue. After the above procedure, the specimen was subjected to a tensile failure test at a loading rate of 500 mm/min. The load-displacement curve was plotted and the maximum load recorded represented the ultimate tensile strength (UTS), whereas the gradient in the linear portion immediately after the toe region of the curve represented the structural stiffness. Energy absorption capacity was the area under the load-displacement curve, from estimated zero deformation to the maximum load (Fig. 2).

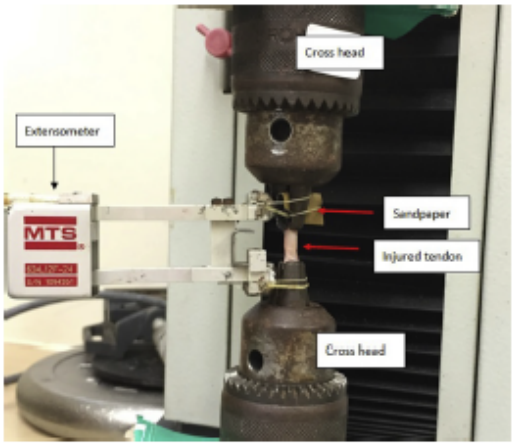

Fig. 1. The specimen was mounted on the cross heads of a material testing machine. The intra-muscular tendinous fibers of the tendon were secured between two strips of fine sandpaper. The sandpaper and the calcaneus were then mounted on the cross heads of a material testing machine.

Statistical analysis

The values of structural stiffness, UTS and energy absorption capacity of the medial portion of the right AT were normalized against those of the left AT of each animal so as to minimize individual variations (Ng and Fung 2008; Yeung et al. 2006). These normalized values were then compared between groups and treatment duration using two-way analysis of variance. Fisher's least significant difference post-hoc comparisons were conducted for significant analysis of variance. An α level of 0.05 was set for all statistical comparisons. The Statistica for Microsoft Windows version 10.0 was used in the data analysis.

RESULTS

On gross examination, the right AT in all groups showed signs of repair, but the CON group generally had a thicker fibrous scar formation than the other groups (Fig. 3).

UTS

At the 2-wk testing time frame, significantly higher normalized UTS was observed in the US group compared

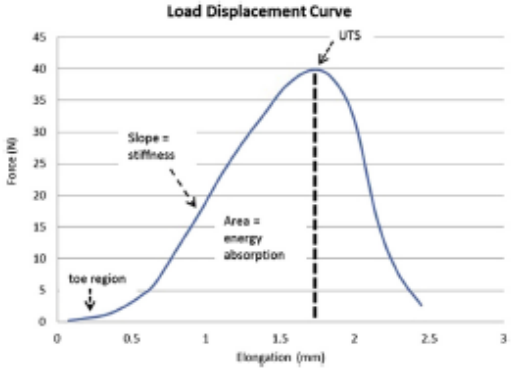

Fig. 2. The load displacement curve of tendon. The maximum load recorded represented the ultimate tensile strength (UTS). The gradient in the linear portion immediately after the toe region of the curve represented the structural stiffness. Energy absorption capacity was the area under the load-displacement curve, from estimated zero deformation to the maximum load.

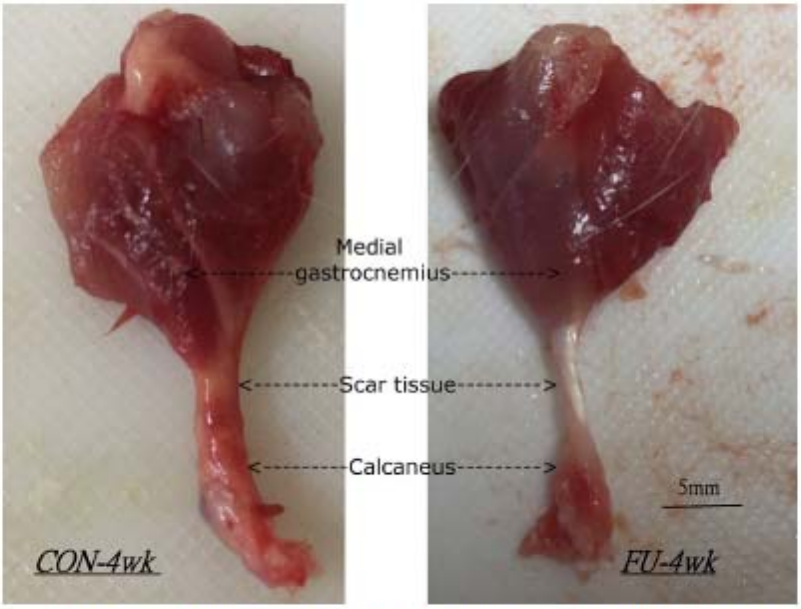

Fig. 3. Gross examination of Achilles tendon. The control group (CON) at 4 wk revealed thicker fibrous scar formation. (Scale bar = 5 mm).

with CON ($p < 0.05$). At the 4-wk testing time frame, however, both US and FU were superior to the CON ($p < 0.05$) (Fig. 4). When comparing the groups between the 2-wk and 4-wk testing time frames, UTS of the FU at 4 wk was significantly higher than the FU at 2 wk ($p < 0.05$).

Structural stiffness

There was no difference in structural stiffness among treatment groups at 2 wk. Both FO at 4 wk and FU at 4 wk showed greater improvement than CON at 4 wk ($p < 0.05$) and their corresponding groups at 2 wk *viz*. FO at 2 wk and FU at 2 wk ($p < 0.05$; Fig. 5).

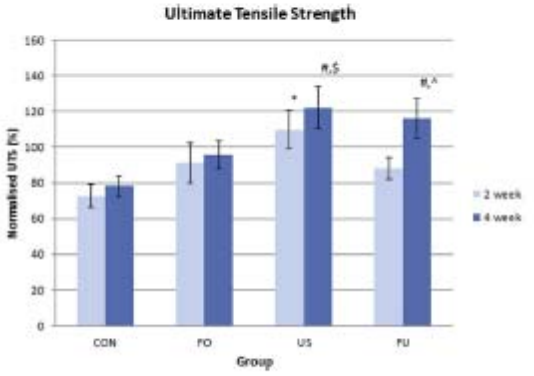

Fig. 4. Normalized ultimate tensile strength (UTS) of all groups. The UTS of US at 2 wk was significantly higher than CON at 2 wk. Both US at 4 wk and FU at 4 wk have significantly higher UTS than CON at 4 wk. FU at 4 wk demonstrated significant improvement compared with FU at 2 wk. Data are expressed as mean ± standard error of the mean values. *Compared with CON at 2 wk, $p < 0.05$; Compared with FU at 2 wk, $p < 0.05$; #Compared with CON at 4 wk, $p < 0.05$; $Compared with FO at 4 wk, $p < 0.05$.

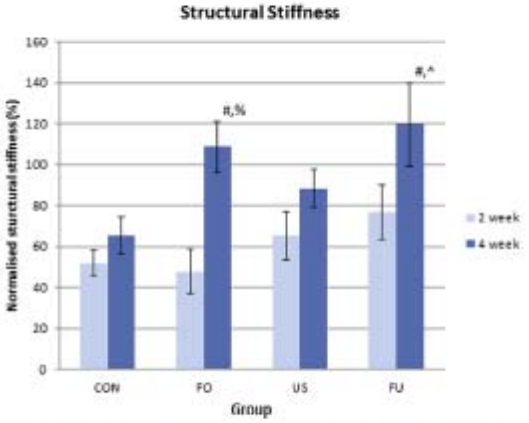

Fig. 5. Normalized structural stiffness of all groups. Both FU at 4 wk and FO at 4 wk showed significantly higher structural stiffness than CON at 4 wk and significant improvement compared with their corresponding 2-wk groups. Data are expressed as mean ± standard error of the mean values. #Compared with CON at 4 wk, $p < 0.05$; Compared with FU at 2 wk, $p < 0.05$; %Compared with FO at 2 wk, $p < 0.05$.

Energy absorption capacity

Significantly higher energy absorption capacity was found in FO at 2 wk compared with FU at 2 wk ($p < 0.05$). The FO at 4 wk was significantly lower than FO at 2 wk ($p < 0.05$; Fig. 6).

DISCUSSION

The present findings revealed ultrasound has hastened the recovery as manifested by an increase in mechanical performance of UTS for the injured Achilles tendon. Furthermore, combined treatment of US coupled with fish oil resulted in an overall improvement of UTS and structural stiffness, particularly at 4 wk after injury.

The efficacy of a treatment depends on the effective skin permeation ability of the active components. The outermost layer of epidermis is the stratum corneum, which acts as a barrier for the skin and is considered the rate-limiting factor for transcutaneous drug delivery (Contri et al. 2011). The most important predictors of permeation of topical drugs are lipophilicity and molecular weight (Russell and Guy 2009; van Ravenzwaay and Leibold 2004). Since most substances penetrate the skin *via* inter-cellular pathways and pass through the extracellular lipid matrix, the cutaneous penetration of hydrophilic substances is limited, while lipophilic solutes have high permeability (Nino et al. 2010). Fish oil has high lipophilicity and hence epithelial penetration potential, which is supported by Zulfakar et al. (2010), who found that fish oil could enhance corticosteroid delivery across full thickness skin. Other than lipophilicity, molecular weight is also a critical factor for skin penetration. Lipophilic compounds of molecular weights more than 400 g/mol would be poorly absorbed by rat skin *in vivo*. The molecular weight of DHA is 328.49 g/mol and that of EPA is 302.42 g/mol—their relatively light molecular weights suggest that both fatty acids should readily pass through the rat skin.

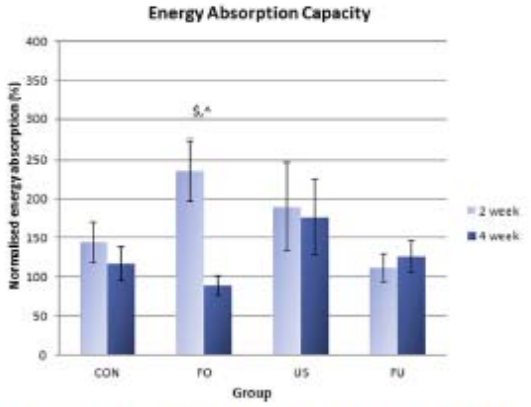

Fig. 6. Normalized energy absorption capacity of all groups. Significantly higher energy absorption capacity was found in FO at 2 wk compared with FU at 2 wk and FO at 2 wk. Data are expressed as mean ± standard error of the mean values. $Compared with FO at 4 wk, $p < 0.05$; ^Compared with FU at 2 wk, $p < 0.05$.

Fish oil was mixed with Vaseline plain petroleum jelly to form the ointment in the FO and FU groups. Vaseline acts as an optimum ointment base and is compatible with various medicaments, providing emollient effects and occlusive properties (Ammar et al. 2007). The occlusive property of Vaseline refers to its serving as impermeable films outside the skin, which increases penetration of lipid-soluble, non-polar molecules (Zhai and Maibach 2002). Since the Achilles tendon is located just beneath the skin, it is possible that fish oil exerted its effect on the tendon after permeating the full thickness of skin.

Ultrasound is well known for its effect on delivering drugs through the skin, which is referred to as sonophoresis or phonophoresis (Polat et al. 2011). It increases the percutaneous absorption of drugs by acoustic cavitation, which alters the skin barrier function (Machet and Boucaud 2002). The increase in membrane permeability is due to defects and channels created within the lipid bilayers (Meidan and Michniak 2004). Insulin (5808 g/mol) is a drug with a high molecular weight and it has reportedly been effectively transported through rat skin with ultrasound phonophoresis (Mitragotri and Kost 2004). It is possible that the penetration of EPA and DHA, which have much smaller molecular weights than insulin, can be enhanced by ultrasound phonophoresis. However, whether phonophoresis had actually taken place in the FU group with enhancement of fish oil penetration into the repairing tendons would need to be confirmed by biochemical analysis of the tendon specimens. This warrants being explored in future studies.

When a tendon is injured, an indicator of functional recovery is whether the tissue has regained its mechanical properties, including UTS, structural stiffness and energy absorption capacity. The distribution of different collagen fibril sizes and their specific organization patterns determine the mechanical properties of tissues (Doroski et al. 2007). Apart from that, composition of non-collagenous components such as proteoglycans and glycosaminoglycans present in the extracellular matrix are also crucial factors (Parry 1988). Limited studies compared the elements that influence the strength and stiffness of tendons separately, but both properties were found to be influenced by collagen density (Roeder et al. 2002). Both fish oil and ultrasound promoted collagen synthesis (Hankenson et al. 2000; Jackson et al. 1991), which may explain the improved mechanical properties in the three treatment groups. The question of why the fish oil groups showed greater improvement in stiffness needs further investigation.

Inflammation is part of the normal, innate immune response to injury, and this process is particularly important for the first 48 to 72 h. However, if there is excessive inflammation, it may result in pain and over-scarring or damage to the host tissues during remodeling (Calder 2006). Fish oil is believed to have an anti-inflammatory effect, as both EPA and DHA are competitor substrates that inhibit oxidation of arachidonic acid (20:4 n-6; AA) *via* the cyclooxygenase (COX) pathway to form eicosanoids such as prostaglandin E_2 (PGE_2) and leukotrienes (Cleland et al. 2003). The non-significant effect of fish oil groups at 2 wk could be because the fish oil had inhibited the COX-2 pathway as it has been demonstrated that early administration of COX-2 inhibitors such as parecoxib would negatively affect tendon healing. However, when the COX-2 inhibitors were applied for a longer period, it improved healing (Forslund et al. 2003; Virchenko et al. 2004). Our results coincided with the above findings that significant improvement was observed in the fish oil groups only for longer treatment periods. It is possible that the timing for the application of fish oil is critical for the healing process.

Energy absorption capacity is the area under the load-displacement curve, from estimated zero deformation to the maximum load. A tendon is a non-contractile viscoelastic structure that exhibits both viscous and elastic behaviors when deformed. Tendons become less viscous but more effective in transmitting heavy loads if they absorb less energy. This scenario occurs during fast loading conditions (Maquirriain 2011). The higher energy absorption capacity of FO at 2 wk with relatively low UTS and structural stiffness indicated a lower efficiency of the tendon. In this situation, the muscle fiber may contract slower over a longer period of time and decrease the required average muscle fiber power output (Prilutsky et al. 1996). This finding also implied an unfavorable effect of fish oil at early healing period. However, the data should be interpreted with caution as the standard deviations of energy absorption capacity were large particularly for the FO at 2 wk and US at 2 wk.

In this study, topical fish oil alone improved structural stiffness of the repairing tendon, and the combined treatment effect of ultrasound coupled with fish oil showed improvement in both stiffness and strength at 4 wk. Apart from acting as COX inhibitor, several studies have shown that omega-3 fatty acids stimulate collagen synthesis (Cardoso et al. 2004; Hankenson et al. 2000; Jia and Turek 2004; Otranto et al. 2010), up-regulate growth factors such as vascular endothelial growth factor (Shingel et al. 2008) and reduce the loss of glycosaminoglycans (Curtis et al. 2002), which are crucial for tendon healing. It has been reported that the anti-inflammatory properties of fish oil can modulate pain but not inhibit the later healing process (Goldberg and Katz 2007).

The FU group had significantly higher UTS and structural stiffness than the CON group at 4 wk. This might be the result of the summated therapeutic effects of ultrasound and fish oil toward soft tissues and also the possibility of the penetration enhancement effect of ultrasound that has facilitated the transportation of fish oil to the injured tissue. Considering the safety, availability, low cost and versatility of fish oil, using it to treat soft tissue injury has a promising future. Further work is needed to include other outcome measures to confirm the findings and investigate whether the healing involves the COX pathway, as well as the optimum time frame for topical fish oil application.

CONCLUSIONS

Topical fish oil application, therapeutic ultrasound and combined ultrasound with fish oil improved the biomechanical properties of injured AT. Only the combined ultrasound and fish oil treatment group manifested improvement in both UTS and structural stiffness of injured tendon at 4 wk.

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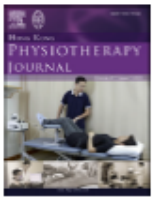

LITERATURE REVIEW

A review on the effects of glucosamine for knee osteoarthritis based on human and animal studies

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KEYWORDS
experimental osteoarthritis;
glucosamine;
osteoarthritis;
knee

Abstract Glucosamine (GlcN) is a popular nutritional supplement/prescription for relieving symptoms of osteoarthritis (OA), particularly for the knee joint. Although there are certain studies reporting the positive effects of GlcN for OA, its use remains controversial and the mechanism behind is unclear. This article critically reviewed published papers on the effects of GlcN in human clinical trials and animal studies. Twelve human clinical studies were reviewed and half of the studies reported positive effects of GlcN for OA or regular knee pain. Eight animal studies were reviewed and most of them had involved histological examination of cartilage, glycosaminoglycan content, subchondral bone, and synovium. Besides, nociceptive behaviour, biochemical markers, and immunohistochemistry of the joints were also examined. There is some evidence showing the beneficial effects of GlcN on joint structural repair in animals, but further research is needed to confirm the applicability of these models in human.

Introduction

Osteoarthritis

Osteoarthritis (OA) is the most common joint disorder affecting elderly people. According to the report from the World Health Organisation, OA is the 6th leading cause of nonfatal burden in the world in the new millennium. The most frequently affected joints are the hands, knees, and hips [1]. Most studies of OA have focused on the change of cartilage, but OA is not solely a disorder of the articular cartilage, other components of the joint such as the subchondral bone [2,3], synovial lining [4], ligaments, and periarticular muscles [5] are also affected. The symptoms are often associated with inflammation; which include pain, stiffness, and loss of mobility thus resulting in functional impairment [6]. The condition is not reversible and has few effective medical remedies [1].

Glucosamine

Glucosamine (GlcN) has been the focus of research for relieving symptoms of OA for the last two decades with most of the studies focussing on the knee joint. Glucosamine (2-amino-2-deoxyalpha-o-glucose) is a naturally occurring amino monosaccharide comprising a glucose

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molecule attached to an amino group and present in the matrix of all connective tissues. The human body can naturally synthesize GlcN by means of the hexosamine pathway by combining glutamine with fructose [7]. GlcN is one of the principal substrates used in the biosynthesis of hyaluronan, chondroitin, dermatan, keratin, glycosaminoglycans, and proteoglycans and all of which are fundamental components of the extracellular matrix of articular cartilage [7]. Therefore, the notion of GlcN in relieving the symptoms of OA is based on the assumptions that abundant administration of the precursors of extracellular matrix components would help chondrocytes to proliferate and replace the degenerated cartilage.

The half-lives of the metabolic turnover of cartilage proteoglycans were measured in days in younger animals and months in elder animals [8], thus cartilage is believed to be continually rebuilding itself [9,10]. Oral consumption of GlcN is thought to augment the endogenous production of GlcN as well as proteoglycan and hence maintain a normal turnover of the cartilage [11]. Furthermore, recent studies have proposed that the efficacy of GlcN may be because of its anti-inflammatory and anti-catabolic properties [12,13].

The Osteoarthritis Research Society International (OARSI) has recommended GlcN as a symptom-relieving and structure-modifying agent for knee OA [14]. Oral GlcN is widely used for modulating pain associated with OA. It can come in combination with other supplements such as chondroitin or by itself in the form of GlcN hydrochloride or GlcN sulphate. The recommended dosage is 1500 mg/d (20 mg/kg in a 75 kg subject) or 500 mg three times a day, which can ensure plasma concentrations of 10μM of GlcN [15], but without recognizable pattern of adverse effects [16–19].

Although GlcN is a popular nutritional supplement/prescription around the world, its efficacy is uncertain. There are three meta-analyses critiquing the efficacy of GlcN and advocating the benefits of GlcN on OA [20–22]. However, cautions should be paid on the interpretation of the results as some studies had suffered from methodological problems such as inadequate allocation concealment and absence of intent-to-treat approaches, which might have overestimated the actual benefits of GlcN [20]. Towheed et al [22] showed that GlcN prepared by Rotta Pharmaceuticals (Eatontown, NJ, USA) was superior to placebo in the treatment of pain and functional impairment resulting from OA. Contrary to those reports, a recent meta-analysis has concluded that GlcN could neither reduce joint pain nor increase joint space [23]. The use of GlcN to treat OA remains controversial and the mechanism behind is unclear. Therefore, this article aimed to critically review articles of GlcN on OA, with focus in the knee joint, in both human clinical trials and animal studies.

Human clinical studies

A Medline search was performed in February 2011 for the years 2000–2010 using the key words "glucosamine", "human clinical trials", "OA", and "knee" to screen all citations that involved GlcN in the management of knee OA or knee pain. Studies that compared GlcN-only preparations with placebo and double-blind, placebo controlled randomized clinical trials were deemed appropriate. With the above criteria, 12 studies were identified to fit in the criteria and were included for this review (Tables 1–4).

Baseline characteristics of participants

To have meaningful comparisons between treatment groups, the patient characteristics should be similar at baseline. Nevertheless, the baseline characteristics of patients were significantly different between the groups in two of the reports [24,25]. In McAlindon's [25] study, the placebo group had more female participants, used more nonsteroidal anti-inflammatory drug, and had higher body mass index than the GlcN group at baseline. Higher body mass index has been reported to associate with increase in physician- and patient-assessed levels of pain [26], which might affect the outcome measure. In Cibere's [24] study, more female participants and less severity in OA were found in the placebo group.

Gender difference among the participants

In most of the studies, women had accounted for more than 50% of the subjects ranging from 56.5% to 87.4%. The exceptions were Braham's [10] and Rindon's [27] studies in which the proportion of women was 28.3% and 5.1% correspondingly (Table 1). Women, particularly, those aged 55 years or above have a higher risk of developing knee OA, and more functional disabilities than men [28]. The gender difference of coping with knee OA should also be considered. Men and women adopt different gait strategies to reduce pain and to cope with the loads acting on the affected joints [29]. Women having OA reported more severe pain and physical disability [30]. Because the common clinical outcome measures of OA are self-reported pain and functional scales, it is possible that gender difference in perception of pain may affect the scores in the outcome measures.

Medication used during the experimental period

Because nonsteroidal anti-inflammatory drugs and analgesics would provide significantly greater pain relief than placebo [31], continuous use of these mediations during the experimental period may mask the efficacy of GlcN. Two of the studies reviewed did not encourage the use of rescue medication [10,32] and hence less than 9% of participants in these studies had consumed rescue medication (Table 2). For the study of Braham et al [10], it also set a washout period of 1 week before the assessment date.

In the study of McAlindon et al [25], the amount of rescue medication use was a secondary outcome measure, but this outcome measure is questionable because significant difference between groups was found at baseline measurement. In the study of Herrero-Beaumont et al [33], there were significantly less subjects completing the study using rescue medication in the GlcN group. Other studies which demonstrated GlcN to be more effective over placebo had set washout periods ranging from 1 week to 2

Table 1 Baseline characteristics of participants

Study	No. of subjects (groups)	% of female participants	Mean age	Mean BMI	History of OA/knee pain (yr)	Severity of OA/knee pain	Duration of treatment
Braham et al [10]	46 (2)	28	43	Not shown	median >10	Pain section of KOOS score 55	12 wk
Sawitzke et al [19]	662 (5)	68	57	~50% of subjects had BMI >30	10	KL score 2–3	24 mo
Cibere et al [24]	137 (2)	57 (placebo *vs.* GlcN, $p < 0.05$)	64	27.5	3	KL score 2–4 (placebo *vs.* GlcN, $p < 0.05$)	6 mo
McAlindon, T [25]	186 (2)	64 (placebo *vs.* GlcN, $p = 0.04$)	45–95, median 55–64	31 in GLcN, 34.1 in placebo, $p = 0.01$	Not shown	84% of participants with severe OA (total joint space loss)	12 wk
Rindone et al [27]	98 (2)	5	64	Not shown	13	KL score 1–4	2 mo
Petersen et al [32]	36 (3)	60	62.4	27.8	Not shown	KL score 1–4	12 wk
Herrero-Beaumont [33]	318 (3)	88	64	27.7	7	KL score 2–3	6 mo
Reginster et al [34]	212 (2)	76	66	27.4	7.9	KL score 2–3	3 yr
Usha & Naidu [35]	118 (4)	64	51	Not shown	3	Lequesne index 8–18	12 wk
Clegg et al [36]	1583 (5)	64	59	31.7	9.5–10.4	KL score 2–3	24 wk
Hughes & Carr [37]	80 (2)	68	62	Not shown	7.62	KL score 1–4	6 mo
Pavelka et al [38]	202 (2)	78	62.4	25.7	10.5	KL score 2–3	3 yr

KOOS, consists of 5 sections namely pain, symptoms, activities of daily living, sport/recreation, and knee related quality of life. It ranges from 0 to 80 with higher scores representing lower pain levels KL, ranges from 0 to 4, with 0 being normal and 4 being severe OA Leguesne index: OA specific questionnaire addressing pain, function limitation and ability to walk. It ranges from 0 to 24 with higher score indicating more severe of OA.
BMI = body mass index; GlcN = Glucosamine; KL = Kellgren and Lawrence radiographic grading scale; KOOS = Knee injury and osteoarthritis outcome score; OA = osteoarthritis.

Table 2 Medication used

Study	% of subjects taking rescue medications at baseline	% of subjects taking medication during the study period	Mean quantity of medication used	Washout period before assessment	Difference between groups	
					At baseline	During treatment
Braham et al [10]	Not shown	8.7% (6.5% in placebo, 2.2% in GlcN)	Not shown	1 wk	Not shown	
Sawitzke et al [19]	Washout period before baseline	Not shown	570 mg of paracetamol daily	24 hr	No	Not shown
Cibere et al [24]	68% in GlcN, 73%in placebo (acetaminophen and NSAID)	72% in GlcN, 76% in placebo	Not shown	No	No	No
McAlindon, T [25]	74% in GlcN, 87% in placebo, $p = 0.03$ (NSAID)	Not shown	Not shown	Not described	Significantly less subjects used NSAID in GlcN	No
Rindone et al [27]	28% (NSAID, acetaminophen, hydrocodone)	Not shown	Not shown	Not described	No	Not shown
Petersen et al [32]	33.3% (NSAID)	8.6% (5.7% in placebo, 2.9% in ibuprofen)	50 mg of tramadol for less than 5 d	Not described	Not shown	
Herrero-Beaumont [33]	Washout period before baseline	91% in placebo, 78% in GlcN	0.2–0.26 tablets of 400 mg ibuprofen daily	1 wk	No	Significantly less subjects used ibuprofen in GlcN
Reginster et al [34]	49% (NSAID, analgesics, corticosteroids)	50%	Less than 1 dose of rescue drug every 6 d	5 half-lives	No	No
Usha & Naidu [35]	Washout period before baseline	Not shown	30–95 tablets of 500 mg paracetamol	2 wk	No	Significantly less subjects used paracetamol in combination of GlcN and MSM groups
Clegg et al [36]	Not shown	Not shown	1.6–1.9 tablets of 400 mg acetaminophen daily	24 hr	No	No
Hughes & Carr [37]	22.5% analgesics, 46% NSAIDs	Not shown	43 tablets of paracetamol in GlcN, 45 in placebo	Not described	No	No
Pavelka et al [38]	Not shown	30–40% in both groups	500 mg Acetaminophen every 3 d	Not described	No	No

GlcN = Glucosamine; MSM = Methylsulfonylmethane; NSAID = nonsteroidal anti-inflammatory drug.

Table 3 Form of glucosamine

Study	Dosage (mg/d)	Form of GlcN	Supplier of GlcN	Selection criteria of GlcN use at baseline
Braham et al [10]	2000	HCl	Not shown	Not shown
Sawitzke et al [19]	1500	HCl	Not shown	Not shown
Cibere et al [24]	Equivalent to the dosage of GlcN intake before, max: 1500	SO_4	Vita health (dietary supplement)	Present daily-user for >1 mo
McAlindon, [25]	1500	SO_4	Physiologics (initial supplier) Rotta Pharmaceuticals (prescription drug) (Subsequent supplier)	Present users excluded
Rindone et al [27]	1500	SO_4	Applehart Laboratories	Past and present users excluded
Petersen et al [32]	1500	SO_4	Ferrosan	Washout period: 1 mo
Herrero-Beaumont [33]	1500	SO_4	Rotta Pharmaceuticals (prescription drug)	Washout period: 6 mo
Reginster et al [34]	1500	SO_4	Rotta Pharmaceuticals (prescription drug)	Not shown
Usha & Naidu [35]	1500	SO_4	Healers Limited	Not shown
Clegg et al [36]	1500	HCl	Ferro Pfanstiehl Laboratories	Not shown
Hughes & Carr [37]	1500	SO_4	Health Perception (dietary supplement)	Not shown
Pavelka et al [38]	1500	SO_4	Rotta Pharmaceuticals (prescription drug)	Not shown

weeks or 5 half-lives of the drug before symptom assessment [10,33–35].

History of glucosamine used

Among the articles reviewed, seven did not mention whether the participants had used GlcN before baseline measurement [10,19,34–38] (Table 3). Two had excluded present users of GlcN [25,27] and two others had set washout period for subjects with histories of GlcN consumption [32,33]. Based on the data from a long-term study [38], the change of Lequesne index after GlcN consumption was greater in the first year. Because GlcN has become popular and easily available, it is difficult to recruit subjects who have never consumed this substance before. Furthermore, there may also be carryover effect with GlcN consumption [39], which is not well reported and it should be further investigated.

Quality control of glucosamine preparation

The classification of GlcN is different among countries. Although it is a prescription in all European Union countries, it is classified as a dietary supplement in the USA [40]. Substances in the class of dietary supplement are usually under less stringent control when compared with prescriptions [41]. Among the 12 studies, 4 had used registered drug [25,32–34,38], 2 had used dietary supplement [24,37] and the others had used unknown sources of GlcN (Table 3). Most of the studies did not describe the quality control on the composition of GlcN. Based on a study assessing the content of active ingredient in over-the-counter GlcN sulphate preparations, it has revealed that the amount of active ingredient varied from 41% to 108% when compared with the content stated on the label [42]. The large discrepancy in the claimed compositions and actual content of the GlcN preparations would affect the analysis of the efficacy of GlcN.

Towheed et al [22] and Vlad et al [43] suggested that GlcN prepared by Rotta Pharmaceuticals was superior to placebo in the treatment of pain and functional impairment resulting from symptomatic OA. The effect size for trials using the Rotta pharmaceuticals preparation was much higher than that of other preparations (ES: 0.55 vs. 0.11) [43]. Although the GlcN manufactured by Rotta Pharmaceuticals is under the category of pharmaceuticals/prescription drug, this might explain the superiority of this GlcN preparation to the placebo group [33,34,38].

Forms of glucosamine

The common form of GlcN includes GlcN sulphate and GlcN hydrochloride. Only three of the reviewed studies had used the hydrochloride form (Table 3). One of them showed pain relief and improvement in knee related quality of life [10], whereas another showed decreased in OA-related pain level [Western Ontario and McMaster Universities (WOMAC) OA pain score 301–400] when GlcN was consumed with chondroitin sulphate [36]. Early hypothesis suggested that a component of the activity of GlcN sulphate was related to the sulphate residues in the compound as sulphur is an essential nutrient for stabilizing the extracellular matrices of connective tissue [7]. Vlad et al [43] also concluded that GlcN hydrochloride had no effect on pain as the effect size of GlcN hydrochloride was much smaller than that of GlcN sulphate. However, Verbruggen [44] found that the preparation used in many studies were two single molecules of GlcN and sulphate instead of a GlcN sulphate ester, therefore the active ingredient should be GlcN. Block et al [15] also rejected the hypothesis that increasing sulfate anion supply could boost the synthesis of the tissue matrix, because the concentration of serum sulfate after consumption of

Table 4 Outcome measures of human trials

Study	Efficacy	WOMAC pain, stiffness and function	Lequesne index	OMERACT/ OARSI	Joint space narrowing	Others
Braham et al [10]	Yes	N/A	N/A	N/A	N/A	(Knee Pain Scale, $p = 0.004$; knee related quality of life, $p = 0.038$)[a]
Sawitzke et al [19]	No	NS (pain, $p = 0.97$, function, $p = 0.56$)	N/A	NS ($p > 0.05$)	N/A	
Cibere et al [24]	No	NS (total score, $p = 0.96$)	N/A	N/A	N/A	NS (EQ-5D questionnaire and disease flare, $p > 0.05$)
McAlindon, T [25]	No	NS (total score, $p = 0.81$)	N/A	N/A	N/A	NS (use of rescue medications, $p = 0.12$)
Rindone et al [27]	No	N/A	N/A	N/A	N/A	NS (Visual analogue scale of pain at rest and during walking, $p = 0.66$–0.90, NS)
Petersen et al [32]	Yes	N/A	N/A	N/A	N/A	(Serum COMP, $p = 0.0378$), NS (Urinary CTX-II, $p = 0.1$)[a]
Herrero-Beaumont [33]	Yes	($p = 0.018$)[a]	($p = 0.01$)[a]	N/A	N/A	(Use of rescue medications, $p = 0.027$)[a]
Reginster et al [34]	Yes	($p = 0.016$)[a]	N/A	N/A	($p = 0.038$)[a]	NS (use of rescue medications, $p > 0.05$)
Usha & Naidu [35]	Yes	N/A	($p < 0.001$)[a]	N/A	N/A	(Pain index, $p < 0.001$; swelling index, walking time and join mobility index, $p < 0.05$)[a]
Clegg et al [36]	No	NS (Pain, $p = 0.73$; stiffness, $p = 0.68$)	N/A	NS ($p = 0.35$)	N/A	NS (use of rescue medications, joint swelling etc, $p > 0.05$
Hughes & Carr [37]	No	NS ($p = 0.54$ –0.77)	N/A	N/A	N/A	NS (Global pain, McGill sensory etc, $p > 0.05$)
Pavelka et al [38]	Yes	($p = 0.01$)[a]	($p = 0.002$)[a]	N/A	($p = 0.01$)[a]	NS (use of rescue medications, $p > 0.05$)

[a] significant difference between GlcN group and control group.
Efficacy: treatment of GlcN was significantly superior to the placebo in improving the outcome measure(s) stated.
COMP = serum cartilage oligomeric matrix protein, marker of aggrecan catabolism; CTX-II = c-telopeptide of Type II collagen, marker of type II collagen catabolism; EQ-5D = European Quality of Life Questionnaire; GlcN = Glucosamine; N/A = not applicable; NS = no significant difference between GlcN group and control group; OMERACT/OARSI = Outcome Measure in Rheumatology Clinical Trials/ Osteoarthritis Research Society International; WOMAC = Western Ontario and McMaster Universities Osteoarthritis.

glucosamine sulfate is far below the concentration required for effective uptake of the cell.

Efficacy of glucosamine on OA

The outcome measures of OA have generally been focused on symptomatic, structural, and biochemical changes. GlcN is said to be "efficacious" towards OA if GlcN treatment was significantly superior to placebo in improving the outcome measure(s) (Table 4). Among the articles reviewed, 6 showed that GlcN was effective towards OA or regular knee pain which included improving the symptoms [10,33–35,38], preventing joint structural change, [34,38] and altering cartilage turnover in the subjects [32] when compared with placebo treatment. The durations of treatment ranged from 6 weeks to 3 years. For those reporting positive effects of GlcN on OA, the treatment would last for more than 12 weeks. Nonetheless, the action of GlcN on OA-related symptoms could be detected as early as 2 weeks [21]. It may imply that the present outcome measures may not be stable and sensitive enough to detect the changes.

Most of the studies have adopted the WOMAC OA index as the primary or secondary outcome measures (Table 4). The WOMAC scale is a questionnaire that measures dysfunction and pain associated with OA of the lower extremities and it consists of 3 subscales on pain, stiffness, and physical functioning [45]. This instrument has been well studied and found to be reliable and valid [46,47]. However, as patients with OA often take analgesics, the effects of analgesics may cloud the action of other treatment preparation. Therefore, it is not sufficient to only measure pain without some objective measures.

Table 5 Outcome measures of animal studies

Study	Induced OA model	Mankin score/OOCHAS	Macroscopic cartilage assessment	GAG content	Others
Omegma et al [11]	Injection of protease	(More lesion in protease/GlcN, $p < 0.05$)[a]	N/A	(Higher GAG content in protease/GlcN, $p < 0.01$)[a]	(Higher expression of biglycan in protease/low GlcN, $p < 0.05$)[a]
Lippiello et al [53]	ACLT	(Less lesion in ACLT/combination treatment trial of CS, GlcN, & manganese ascorbate; $p < 0.05$)[a]	N/A	NS	N/A
Tiraloche et al [54]	ACLT	(Less lesion in ACLT/GlcN, $p = 0.049$)[a]	(Lower rate of disease in the ACLT/GlcN trial at LTP, $p = 0.046$)[a]	(Higher GAG content in ACLT/GlcN, $p < 0.05$)[a]	N/A
Wang et al [55]	ACLT	N/A	N/A	N/A	(Higher osteoid volume in ACLT/GlcN, $p = 0.041$)[a] (Higher trabecular bone volume in ACLT/GlcN, $p = 0.033$)[a]
Chen et al [56]	ACLT	(Less lesion in ACLT/GlcN, $p < 0.05$)[a]	N/A	N/A	(Higher expression of TGF-β in ACLT/GlcN, $p < 0.05$)[a] (Lower expression of IL-1-β in ACLT/GlcN, $p < 0.05$)[a]
Naito et al [57]	ACLT	NS	N/A	N/A	(Lower CTX-II level in ACLT/GLcN, $p < 0.001$)[a] (Increased CPII level in ACLT/GlcN, $p < 0.001$)[a]
Silva et al [58]	ACLT	(Less cartilage damage in combination of ACLT/combination treatment of GlcN and CS, $p < 0.05$)[a]	N/A	N/A	(Lower pain value in ACLT/combination of GlcN and CS, $p < 0.05$)[a]
Wen et al [59]	ACLT	(Less lesion in ACLT/GlcN, $p < 0.001$)[a]	(Lower macroscopic score in ACLT/GlcN, $p = 0.005$)[a]	N/A	(Lower synovitis score in ACLT/GlcN, $p < 0.001$)[a] (Improved nociceptive behavior in ACLT/GlcN, $p < 0.001$)[a] (Lower p38 kinase in ACLT/GlcN, $p = 0.002$)[a] (Lower JNK in ACLT/GlcN, $p = 0.006$)[a]

[a] significant difference compared with ACLT or protease/control.

ACLT = anterior cruciate ligament transaction; CP-II = carboxy propeptide of Type II collagen, marker of Type II collagen synthesis; CTX-II = Type II collagen C-telopeptide, marker of Type II collagen catabolism; GAG = Glycosaminoglycan; GlcN = Glucosamine; IL-1-β = interleukin-1- beta; JNK = c-Jun N-terminal kinase, activated in response to inflammation cytokines; LTP = Lateral tibial plateau; N/A = not applicable; NS = no significant difference compared with ACLT or enzyme induced/control; OA = osteoarthritis; OOCHAS = Osteoarthritis Research Society International (OARSI) Cartilage Histopathology Assessment System; p38 kinase = belongs to the family of mitogen-activated protein kinase. It is activated in response to inflammation cytokines; TGF-β = transforming growth factor beta.

Presently, measurement for the width of joint space is commonly used as an indirect measure of cartilage repair. However, the measurement is not sensitive enough as it often takes 1—2 years before there is reliable information on the preventive effect of the treatment preparation [48] and it also has large precision errors [49]. Biochemical markers could be a more specific outcome measure for improvement of joint disease. There was a study using biochemical markers, namely, serum cartilage oligomeric matrix protein and urine c-telopeptide of Type II collagen (CTX-II) as indicators of catabolism of aggrecan and Type II collagen, respectively, and found significant changes in the serum cartilage oligomeric matrix protein level [32]. Nevertheless, cautions should be paid when interpreting the data because it is not sure whether the markers level in blood/urine truly represents the change in joint structure [48]. Further research is needed to test the reliability of specific markers and develop more sensitive indicators for monitoring the disease.

Animal studies

Owing to the slow progress and unclear pathogenesis of OA [50], human clinical trials have mainly focused on symptomatic relief. The poor sensitivity of the diagnostic tools and the difficult access to disease tissues also hinder the research of OA in human [51]. Animal models can therefore provide an alternative for studying potential antiarthritis agents.

A Medline search was performed for the years 2000—2010 using the key words "glucosamine", "*in vivo*", "OA", and "experimental OA" to screen all citations that involved oral consumption of GlcN in the management of experimental OA. Studies that compared GlcN-only preparations with placebo (induced OA control) were included. There were 8 studies meeting the criteria above (Tables 5 and 6).

Models adopted

The design of animal models on prediction of the effectiveness of a drug should be based on the track record of predictability of drug induced modification of the disease progression. Although there are no such agents proven to modify disease progression of OA [52], most of the models adopted in testing GlcN were based on the histopathological similarities to human disease [50]. For example, anterior cruciate ligament transaction (ACLT) of rabbits [53—56] and rats [57—59] and enzyme-induced model [11] are commonly used to study OA. The advantages of these models include rapid development and reproducible damage relevant to the traumatic forms of OA [51]. On the other hand, spontaneous models are better in simulating the slow progress of the human disease but it usually takes a long time before observable changes can be recorded [52]. As yet, there is no consensus on which model and species are the most relevant for human OA.

Efficacy of glucosamine on change of joint structures

Histologic/Histochemical Grading System (HHGS, Mankin score) and the OARSI Cartilage Histopathology Assessment System are two common methods for analyzing the progression of cartilage lesions. Both the HHGS and OARSI Cartilage Histopathology Assessment System have excellent

Table 6 Glucosamine treatment in animal studies

Study	Model	Form of GlcN	Dosage of GlcN	Determination of dosage
Omegma et al [11]	Injection of CP to the middle of the patellar tendon	GlcN HCl	20 mg/kg/d for low GlcN diet, 100 mg/kg/d for high GlcN diet	Based on the recommended dosage for human (1.5 g/70 kg/d = 20 mg/kg/d approx.)
Lippiello et al [53]	ACLT, PCLT and medial meniscus removed NZ white rabbits	GlcN HCl	2% by weight of 500 mg GlcN daily	Not shown
Tiraloche et al [54]	ACLT, NZ white rabbits	GlcN HCl	100 mg/d	Based on the recommended dosage for human (1.5 g/70 kg/d = 20 mg/kg/d approx.)
Wang et al [55]	ACLT NZ white rabbits	GlcN HCl	100 mg/d	Not shown
Chen et al [56]	ACLT, NZ white rabbits	GlcN HCl	150 mg/kg/d	Not shown
Naito et al [57]	ACLT, Sprague-Dawley rats	GlcN HCl	1000 mg/kg/d	Based on a pharmacokinetics study of rat [64]
Silva et al [58]	ACLT male Wistar rats	GlcN SO_4	500 mg/kg/d	Based on preliminary studies
Wen et al [59]	ACLT, Wistar rats	GlcN SO_4	250 mg/kg/d	Not shown

ACLT = anterior cruciate ligament transaction; CP = protease-chymopapain; GlcN = Glucosamine; NZ = New Zealand; PCLT = posterior cruciate ligament transaction.

intra- and inter-observer reproducibility. The correlation between the scores is good [60]. (Table 5).

A number of studies have reported that the positive treatment effect of GlcN would decrease the ACLT induced cartilage lesion as compared with the ACLT control [54,56,59]. On the other hand, two of the studies reviewed showed that combination of GlcN and CS was superior to GlcN-only treatment in reducing cartilage damage [53,58].

In the study by Tiraloche et al [54], the GlcN group had a significantly lower rate of disease in the lateral tibial plateau compartment compared with that of the placebo group. The less severe cartilage lesion and erosion in tibial plateau compartment but not the other compartments implied the site-specificity of GlcN. As suggested by Handley [61], chondrocytes are sensitive to their biomechanical environment and therefore the metabolic characteristics are different for weight-bearing and non-weight bearing regions of articular cartilage.

Although the HHGS was based on study of specimens with very advanced OA [62], it is valid for normal and severe OA cartilage, but it does not show a linear relationship with the change of articular cartilage in mild and moderate OA [63]. Therefore, caution should be paid on Oegema's [11] study, as it used a slightly lower dose of enzyme to induce a mild OA condition. Contrary to the studies discussed above [54,56,59], the Mankin score was significantly higher in GlcN group compared with control, which indicated more severe damage in the GlcN group.

In studies that measured the cartilage glycosaminoglycan (GAG) content, controversies were reported. Some researchers found that the GlcN-treated group had significantly preserved the GAG content when compared with the placebo group [11,54], whereas Lippiello et al [53] could not find differences in the GAG content between the ACLT/GlcN and the ACLT/placebo group.

Apart from cartilage, a study had reported significant reduction in the osteoid volume of the ACLT/GlcN group compared with that of the ACLT/placebo group [55]. This finding implied that GlcN treatment has reduced the subchondral bone changes. Furthermore, based on the histological assessment, synovial inflammation was less severe in the ACLT/GlcN group compared with that of ACLT/placebo group [59].

Efficacy of glucosamine on pain relief

Pain is a parameter commonly measured in clinical studies but not with animal studies because of the question of validity of measuring pain in animals [51]. In the study by Silva et al [58], pain was assessed using an articular incapacitation method of the paw elevation time when the sole was placed on a rotating cylinder to induce painful stimulation to the paw. They found that a combination of GlcN and CS had significantly reduced the paw elevation time, but not with the GlcN treatment alone. Wen et al [59] investigated the nociceptive behaviour of OA rats with mechanical allodynia and weight-bearing distribution test. The force required for paw withdrawal in the mechanical allodynia and the amount of weight shifted to the non-injured limb were indicators of nociceptive behaviour. They found that GlcN treatment did reduce pain in the rats.

Effect of glucosamine on changes of biochemical markers

There is substantial interest on the use of biochemical markers to assess the progress of diseases. It is important to use biochemical markers in the clinical monitoring for patients with OA because these markers usually response rapidly to treatment [48]. Articular cartilage is rich in Type II collagen and according to the study of Naito et al [57] the treatment of GlcN would suppress Type II collagen degradation and enhance Type II collagen synthesis in rats with the ACLT.

Dosage of glucosamine

Most *in vivo* studies did not determine the Cmax (peak concentration of a drug observed after its administration) of GlcN, which may affect the therapeutic relevance of these studies for human OA [15]. Some studies determined the dosage based on the recommendation for human of 1.5 g/70 kg BW/d i.e., about 20 mg/kg/d [11,54] (Table 6). Although GlcN is consumed orally, the absorption efficiency and metabolism of GlcN may be different among species.

The prediction method used in animal studies could be used to estimate the effective dosage for a particular animal model, but the result may not be extrapolated to human OA. Naito et al [57] had used the dosage of oral GlcN that achieved comparable serum level with human according to a pharmacokinetics study of rat. This determination method was close to the suggestion from Block et al [15] and it showed better relevance for Human OA.

Conclusion

Based on the results of human clinical trials, half of the studies reported beneficial effects of GlcN for OA or regular knee pain. The conflicting results may be because of the different study design and control of confounding variables.

Treatment of GlcN for experimental OA has been found to facilitate cartilage healing, increase cartilage GAG content, reduce subchondral structural changes, relieve nociceptive behaviour, and synovitis. However, because there are large variations in the animal models, GlcN administration, and outcome measures, the efficacy of GlcN on different animal models is hard to compare. Further research is needed to study the validity of these animal models and the applicability of these models for human.

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