EFFECT OF DIETARY CHITOSAN SUPPLEMENT ON MUSCLE INFLAMMATION AND RECOVERY AFTER CARDIOTOXIN-INDUCED INJURY

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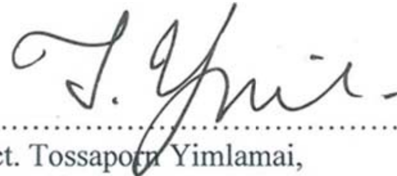
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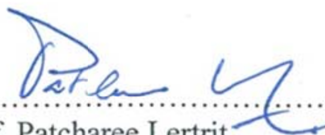
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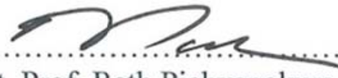
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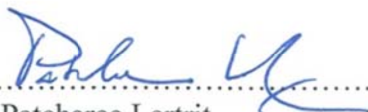
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EFFECT OF DIETARY CHITOSAN SUPPLEMENT ON MUSCLE INFLAMMATION AND RECOVERY AFTER CARDIOTOXIN-INDUCED INJURY

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ABSTRACT

Chitosan is a natural and non-toxic polymer that has a wide range of pharmaceutical application, including drug carrier, wound dressing and neuroprotective agents. This study aimed to evaluate the effect of chitosan on the inflammatory response and muscle recovery after acute injury. Adult male Wistar rats were randomly divided into 2 major groups: control intact and an injury group. The injury group was further divided into vehicle and chitosan treatment. After 1 week of treatment, injury was induced in tibialis anterior (TA) by cardiotoxin injection. At day 1 and day 3 post-injury, rats were sacrificed and contractile functions of TA muscles were measured. After 1 day post-injury, there was a significant reduction in force production in both the vehicle-treated (~42%) and chitosan-treated (~54%) groups compared to the control. These reduction in force, however, were recovered to control value in both injury groups by day 3 post injury, with chitosan-treated group (~14%) showed better improvement than that in the vehicle-treated group (~23%). This effect was associated with increases damage area and expressions of tumor nuclear factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) mRNA level in both injury groups at day 1 post-injury. On day 3 post-injury, however, the level of TNF-α and TGF-β mRNA were further increased only in the chitosan-treated group, while IL-6 and IL-10 mRNA expressions were unaffected in both injury groups. Taken together, these results suggest that chitosan treatment could enhance the inflammatory response but had a small effect on muscle recovery following cardiotoxin-induced injury.

KEY WORDS: CHITOSAN / INFLAMMATION / SKELETAL MUSCLE / INJURY

58 pages

ผลของอาหารเสริมไคโตซานต่อการอักเสบและการฟื้นฟูของกล้ามเนื้อหนูหลังจากได้รับบาดเจ็บจากพิษงู cardiotoxin

EFFECT OF DIETARY CHITOSAN SUPPLEMENT ON MUSCLE INFLAMMATION AND RECOVERY AFTER CARDIOTOXIN-INDUCED INJURY

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บทคัดย่อ

ไคโตซาน (Chitosan) เป็นสารพอลิเมอร์จากธรรมชาติที่ไม่มีพิษ จึงถูกนำมาใช้กันอย่างแพร่หลายในด้านเภสัชศาสตร์ อาทิเช่น ใช้เป็นพาหนะนำส่งยา, ใช้ในการทำแผล, และเป็นสารที่ใช้ป้องกันเซลล์ในสมองตาย การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของไคโตซานต่อการการบาดเจ็บและการฟื้นฟูสภาพของกล้ามเนื้อลาย ในช่วงการอักเสบ ในหนูแรทเพศผู้สายพันธุ์ wistarอายุ 8 สัปดาห์ โดยถูกสุ่มแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มควบคุม และกลุ่มที่ได้รับบาดเจ็บ ซึ่งจะถูกแบ่งออกเป็น 2 กลุ่มย่อย คือ กลุ่มที่ได้รับสาร vehicle และกลุ่มที่ได้รับสารสกัดไคโตซาน หลังจากได้รับสารดังกล่าวเป็นระยะเวลา 1 สัปดาห์ หนูกลุ่มที่ได้รับการบาดเจ็บจะถูกฉีดสารพิษ cardiotoxin เข้าสู่กล้ามเนื้อ tibialis anterior (TA) โดยในวันที่ 1 และวันที่ 3 หลังจากได้รับการบาดเจ็บ หนูจะถูกทำให้สลบโดยการฉีดยาสลบเข้าทางช่องท้อง หลังจากนั้นจะทดสอบแรงหดตัวของกล้ามเนื้อ TA โดยการกระตุ้นด้วยไฟฟ้า การทดลองพบว่า หลังจากได้รับบาดเจ็บ 1 วัน แรงหดตัวของกล้ามเนื้อลดลงร้อยละ 42 ในกลุ่มได้รับบาดเจ็บที่ได้รับสาร vehicle และร้อยละ 54 ในกลุ่มที่ได้รับสารสกัดไคโตซาน อย่างไรก็ตามแรงหดตัวของกล้ามเนื้อสามารถกลับสู่ระดับปกติได้ภายในวันที่ 3 หลังจากได้รับบาดเจ็บในทั้งสองกลุ่ม โดยกลุ่มที่ได้รับสารสกัดไคโตซานสามารถฟื้นตัวได้เร็วกว่ากลุ่มที่ได้รับสาร vehicle ข้อมูลนี้สัมพันธุ์กับจำนวนกล้ามเนื้อที่ได้รับความเสียหาย และ ระดับ mRNA ของ tumor nuclear factor-α (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), และ transforming growth factor-β (TGF-β) ในวันที่ 1 หลังจากได้รับบาดเจ็บในทั้งสองกลุ่ม อย่างไรก็ตามพบว่าในวันที่ 3 หลังจากได้รับบาดเจ็บ ระดับของ TNF-α และ TGF-β จะเพิ่มมากยิ่งขึ้นในกลุ่มที่ได้รับสารสกัดไคโตซาน ในขณะที่ระดับของ IL-6 และ IL-10 จะไม่เปลี่ยนแปลงในทั้งสองกลุ่ม จากข้อมูลสรุปได้ว่าสารสกัดไคโตซานสามารถเพิ่มระดับของสาร cytokine ในระยะต่อของการอักเสบ แต่มีผลกระทบน้อยมากในการป้องกันและฟื้นฟูสภาพของกล้ามเนื้อหลังได้รับบาดเจ็บจากสารพิษ cardiotoxin

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LIST OF ABBREVIATIONS

Akt	protein kinase B
ANOVA	analysis of variance
ATP	Adenosine triphosphate
CK	creatine kinase
cm	centimeter
COX	cyclooxygenase
CSA	cross-sectional area
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EF_{50}	Effective frequency 50
ERK	Extracellular signal-regulated kinases
g	gram
GAPDH	glyceraldehyde-3-phosphatedehydrogenese
HDL	high-density lipoprotein
HGF	hepatocyte growth factor
HPDLC	human periodontal ligament cell
Hz	hertz
H&E	Haematoxylin and Eosin
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IL-10	interleukin-10
IL-1β	interleukin-1beta
IL-6	interleukin-6
kDa	kilo Dalton
kg	kilogram
LDL	low-density lipoprotein

LIST OF ABBREVIATIONS (cont.)

LPS	lipopolysaccharide
L_o	optimal muscle length
m	meter
MAPK	Mitogen-activated protein kinase
mg	milligram
Min	minute
miRNA	micro ribonucleic acid
miR-133a	microRNA-133a
ml	milliliter
MPO	myeloperoxidase
MRF	myogenic regulatory factors
Mrf4	Myogenic regulatory factor 4
mRNA	messenger RNA
ms	millisecond
MyoD	Myogenic Differentiation
Myf5	myogenic factor 5
NAC	N-acetylcysteine
nAChR	Nicotinic acetylcholine receptors
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NSAID	non-steroid anti-inflammatory drug
NT2	NTera2
N/cm^2	Newton per centimeter square
ORAC	oxygen radical absorbance capacity
Pax7	paired box protein 7
PBS	Phosphate-buffered saline

LIST OF ABBREVIATIONS (cont.)

PI3K	phosphatidylinositol 3-kinase
PMN	polymorphonuclear neutrophilic leukocytes
P_o	isometric tetanic force
P_t	maximum isometric twitch
ROS	reactive oxygen species
SEM	standard error of the mean
sPo	Specific tension
SR	sarcoplasmic reticulum
TA	tibialis anterior
TGF-β	transforming growth factors-beta
TNF-β1	tumor necrotic factor-beta 1
UK	United Kingdom
USA	United States of America
V	volt
VEGF	vascular endothelial growth factor
^{0}C	degree Celsius
%	percent

CHAPTER I
INTRODUCTION

Chitosan is a cationic linear polysaccharide created by deacetylation of chitin from shells of crustaceans (Casettari et al, 2012). It is composed of beta-1,4 linked glucosamine with N-acetylated glucosamine residues. Chitosan has a wide range of pharmaceutical applications such as drug carrier, wound dressing (Yang et al, 2007), as well as other beneficial properties including antilipidemic, antioxidant, membrane-stabilizing, anti-fungal, anti-bacterial, anti-inflammatory properties and enhancing immune system (Anandan et al, 2012; Khodagholi, 2009; Anruku, 2008).

So far, the effects of dietary chitosan supplement are being actively investigated in many physiological systems as shown in a number of experiments. For instance, dietary chitosan supplement have been shown to exhibit hypolipididemic activities (Zhang et al, 2012), renoprotective activity (Anraku et al, 2012), in myocardium (Anandan et al, 2012) and hepatic system (Jeon et al, 2002). Moreover, its antiaging effect has recently been reported in both juvenile and old rats (Anandan et al, 2012). The mechanisms behind these benefits are believed to predominantly derive from antioxidative effects of chitosan. In humans, an experiment with water-soluble chitosan also demonstrated a significant reduction in blood glucose, atherogenic index and oxidized albumin ratio while there is an increase in high-density lipoprotein (HDL) concentration and total plasma antioxidant capacity (Anraku et al, 2009). Chitosan also have potent effect on inflammatory response that may be beneficial in muscle regeneration after injury. Chitosan has been shown to inhibit reactive oxygen species (ROS)-induced nuclear factor-kappa B (NF-κB) activation in NTera 2 (NT2) neurons (Khodagholi et al, 2010). Another study in human primary monocyte culture also demonstrated that chitosan caused a significant increase in pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor nuclear factor-beta1 (TNF-β1) in the early stage of macrophage infiltration that gradually decrease over time, while increasing anti-inflammatory cytokines production including interleukin-10 (IL-10) in

later stage (Oliverira et al, 2012). Altogether, these results suggest that chitosan can aid in macrophage polarization from pro-inflammatory macrophages to anti-inflammatory macrophages.

Despite many studies have investigated the effect of chitosan on various tissues, there is limited information available regarding the effect of chitosan on muscular tissue. Specifically, the effect of chitosan on skeletal muscle healing after injury has never been explored. Skeletal muscle injuries are among the most common injuries suffered during participation in sports and daily activities (Turner & Badylak, 2012). Skeletal muscle healing can be divided into three distinct, but overlapping phases. The destruction phase is characterized by the rupture, the formation of the hematoma, and the inflammatory reaction (Yin et al, 2011). The repair phase is composed of proliferation and growth of new myocytes, and the remodeling phase consists mainly of formation of new muscle fibers. Thus, any disturbances occur during remodeling phase either from excessive injuries or improper healing could result in fibrosis accumulation and scar tissue (Mann et al, 2011).

Among these three phases, there is now become clear that the inflammatory response is essential for successful skeletal muscle regeneration (Turner & Badylak, 2012; Yin et al, 2013). During this phase, two different subpopulations of macrophage (i.e. M1 and M2 phenotype) infiltrate the site of injury and become the dominant population of inflammatory cells. Whereas the role of M1 phenotype macrophage is associated with pro-inflammatory cytokines secretion such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) and interleukin-6 (IL-6) and responsible for phagocytosis of cellular debris, the M2 phenotype macrophage is responsible for cell fusion and differentiation by secreting anti-inflammatory cytokines IL-10 and transforming growth factors-beta (TGF-β). Importantly, suppression of macrophage to the injury site results in incomplete skeletal muscle regeneration (Ten Broek et al, 2010). However, prolonged macrophage accumulation is accompanied with skeletal muscle fibrosis.

To date, there is no safe and effective method available for the treatment of muscle injury. Although non-steroid anti-inflammatory drug (NSAID) is commonly prescribed for the treatment of skeletal muscle injury and inflammation, it is well known to have negative side-effects including asthma exacerbation, gastrointestinal

and renal side effects as well as hypertension when used inappropriately (Urso, 2013). The use of NSAID may also interfere with the process of muscle regeneration, specifically decreasing macrophage functions, thus resulting in an impairment of muscle functions (Urso, 2013; Gharaibeh et al, 2012). Considering chitosan is proven to have anti-inflammatory effect, it has a potential to be an excellent candidate for treating skeletal muscle injury. Thus, the aim of this study was to evaluate the effect of chitosan on skeletal muscle injury. Specifically, we asked whether dietary supplement of chitosan could alleviate the inflammatory response, thus enabling enhanced skeletal muscle recovery after injury.

Hypothesis

Chitosan treatment in the form of dietary supplement could provide a protection against inflammation and facilitate muscle recovery after cardiotoxin iduced-injury.

Objective

To determine the effect and mechanism of dietary chitosan supplement on the inflammatory response and functional recovery after cardiotoxin-induced injury.

Expected Results

Chitosan treatment could modulate the inflammatory response and facilitates muscle recovery after cardiotoxin-induced injury. If so, chitosan has a therapeutic potential as dietary supplement for treating skeletal muscle injury.

CHAPTER II
LITERATURE REVIEW

2.1 Chitosan

Chitosan is cationic linear polysaccharide created by deacetylation of chitin from shells of crustaceans (Casettari et al, 2012). It is composed of beta-1,4 linked glucosamine with N-acetylated glucosamine residues. Structurally, it has one primary amino and two free hydroxyl groups for each C6 building unit (Chen et al, 2011). This freely available amino group carries a positive charge which allows it to reacts with various negatively charged surfaces and polymers, as well as facilitating metal ions chelation (Fig. 2.1). Chitosan and their derivatives have a wide range of pharmaceutical applications such as drug carrier, wound dressing, scaffold material, intestinal absorption enhancer and drug carrier in cancer chemotherapy etc. (Yang et al, 2007; Kato et al, 2003). Other beneficial properties include antilipidemic, antioxidative, membrane-stabilizing, anti-fungal, anti-bacterial, anti-inflammatory properties and enhancing immune system (Anandan et al, 2012; Khodagholi, 2009; Anruku, 2008). A summary of biological activities of chitosan on various experimental models is shown in Table.1. In this review, however, the focus is on antioxidative and anti-inflammatory properties of chitosan.

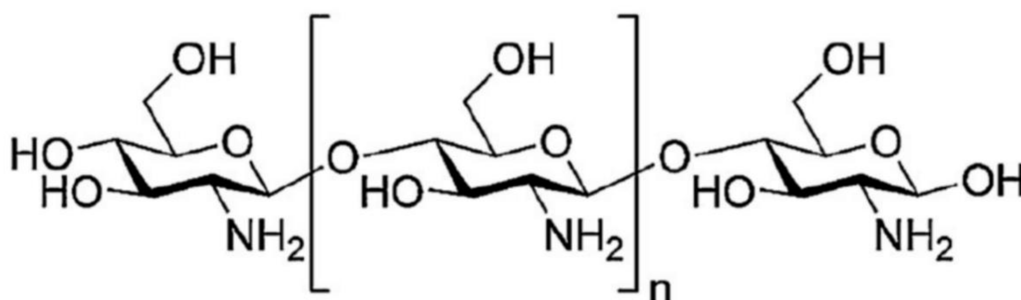

Figure 2.1 Structure of chitosan (Anandan et al, 2012).

Table 2.1 Summary of bioactivity of chitosan and its derivatives.

Studies	Animal Models	Experiment/ Treatment/Application	Results
Klokkevold et al., 1991	Rabbits	Lingual haemostasis model	Enhanced haemostasis due to decreased bleeding time
Rao and Sharma, 1997	Various	Blood	Haemagglutination and clot formation
Stone et al., 2002	Human	Healing at split skin graft donor sites	Enhanced wound healing
Ishihara et al., 2002	Mice	Tail bleeding and full thickness skin incision	Rapid cessation of bleeding and accelerated wound healing
Pusateri et al., 2003	Swine	Severe hepatic injury model	Reduced blood loss and increased haemostasis and survival
Kojima et al., 2004	Rat	Implantation of fabric impregnated with chitosan into skin wounds	Early wound healing process
Zhang et al., 2006	Rat	Artificially induced peritoneal adhesion	Reduced adhesion
Kozen et al., 2008	Pig	Haemorrhagic groin injury	Reduced bleeding and increased survival
Sugano et al., 1980; Vahouny et al., 1983; Jennings et al., 1998; Fukada et al., 1991	Rat, low/high cholesterol diet	Dietary administration	Reduced blood and liver cholesterol, lowered cholesterol and oleic acid absorption and altered intestinal bile acid metabolism
Gallaher et al., 2000	Rat with high cholesterol	Dietary feeding	Reduced liver cholesterol and cholesterol absorption and increased fecal fat and bile acid excretion
Miura et al., 1995	Mice	Dietary feeding	Reduced blood glucose, cholesterol and triglyceride
Hirano and Akiyama, 1995	Rabbit, high cholesterol diet	Dietary feeding	A hypocholesterolaemin action in the intestine, increased fecal bile acid and sterol levels

2.2 Antioxidatve Effects of Chitosan

Antioxidative properties of chitosan are determined by two physical characteristics namely molecular weight and deacetylation degree of chitosan (Yang et al, 2007). A comprehensive study of antioxidative properties of chitosan at various ranges of molecular weight and degree of deacetylation showed that chitosan with low molecular weight (4-5 and 22-30 kDa) and high degree of deacetylation is the most effective (Jung and Zhao, 2012). 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability, reducing ability, chelating ability and hydroxyl radical scavenging ability are tested in the experiment and the results showed that low molecular weight chitosan (4-5 and 22-30 kDa) scores higher than high molecular weight chitosan (61-79 and 280-300 kDa). High molecular weight chitosan (100 kDa), on the other hand, has been shown to be effective in reducing total cholesterol level and atherogenic index in human after 8 weeks of dietary supplement (Anraku et al, 2011). High molecular weight chitosan, unlike its low molecular weight variant, cannot be absorbed efficiently in the gastrointestinal tract; therefore LDL can be removed from the system when it binds to chitosan. Additionally, chitosan derivatives have even higher antioxidative capacity in oxygen radical absorbance capacity (ORAC) test than unmodified chitosan (Casettari et al, 2012). For example, N-acetylcysteine (NAC)-g-chitosan and Gallic acid-g-chitosan score 27 and 19 times higher than normal chitosan in ORAC test. Chitosan also has a potent effect on inflammatory response that may be beneficial in muscle regeneration after injury. Moreover, chitosan has been reported to inhibit ROS-induced NF-κB activation in NT2 neurons (Khodagholi et al, 2010). Up-to-now, there are very few studies examining the effect of chitosan on skeletal muscle system. In one study, chitosan oligosaccharide can induce mitochondrial biogenesis and enhance exercise endurance in rats (Jeong et al, 2012).

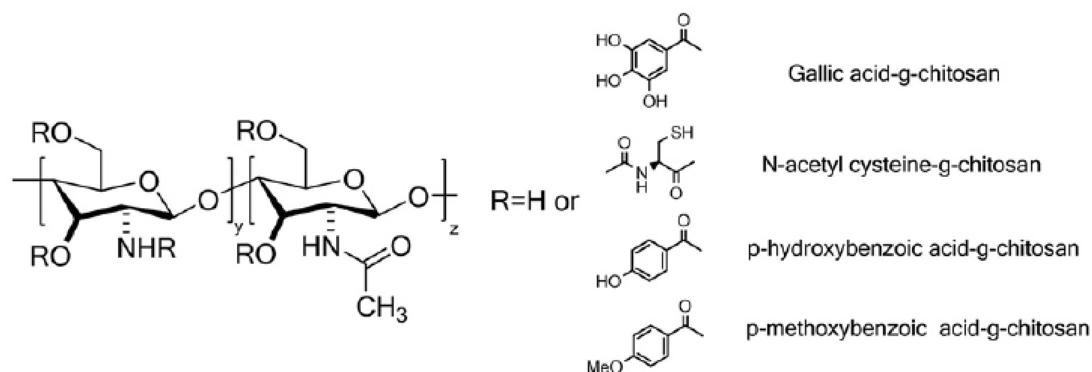

Figure 2.2 Chemical structures of chitosan and its derivatives (Casettari et al, 2012).

2.3 Anti-inflammatory Effect of Chitosan

Previous studies in human primary monocyte culture demonstrated a significant increase in pro-inflammatory cytokines such as IL-6 and TNF-β1 in the early stage of macrophage infiltration that gradually decreased over time, while increasing anti-inflammatory cytokines production including IL-10 in later stage (Oliverira et al, 2012). This result indicates that chitosan may help in macrophage polarization from pro-inflammatory macrophages to anti-inflammatory macrophages. In a study of mite allergen-induced airway inflammation in mice, water-soluble chitosan has been shown to reduce production of inflammatory cytokines IL-6 and TNF-α, thus modulating lung inflammation via the reduction of inflammatory cell infiltration (Chen et al, 2008). This process eventually results in the resolution of inflammatory process. Another experiment was carried out to examine the effect of chitosan on lipopolysaccharide (LPS)-stimulated human periodontal ligament cells (HPDLCs) (Ji et al, 2012). Consistent with previous reports, treatment with chitosan effectively inhibited pro-inflammatory cytokine production, specifically IL-1β and TNF-α up to 72 hours. Chitosan oligosaccharide, a chitosan derivative, has also been reported to decrease levels of key pro-inflammatory cytokines TNF-α and IL-1β in blood when challenged by LPS in vivo (Qiao, Bai & Du, 2011). Moreover, oral administration of chitosan oligosaccharide could protect against intestinal inflammation in mice with induced acute and chronic colitis (Yousef et al, 2012). The levels of nuclear factor kappa B (NF-κB), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were evidently decreased after chitosan oligosaccharide treatment.

2.4 Other Effects

In addition to its antioxidative and anti-inflammatory effect, dietary chitosan supplements have been demonstrated to reduce hepatic injury from chronic carbon tetrachloride (Jeon et al, 2002), exhibit hypolipididemic activities, especially a low molecular weight chitosan (Zhang et al, 2012) and renoprotective activity in nephrectomized rat (Anraku et al, 2012) as well as its antiaging effect via glutathione-dependent antioxidant system in both juvenile and old rats (Anandan et al, 2012). Although the exact mechanism (s) behind these benefits is not well understood, its antioxidative effects of chitosan have been suggested. In humans, an experiment with water-soluble chitosan demonstrated a significant reduction in blood glucose, atherogenic index and oxidized albumin ratio, with an increase in HDL concentration and total plasma antioxidant capacity (Anraku et al, 2009). Treatment with chitooligosaccharide was also reported to have stimulatory effects on mitochondria biogenesis in rat skeletal muscle and improved exercise endurance (Jeong et al, 2012). In cultured myocytes, chitooligosaccharide showed an increased in expression level of major regulators of mitochondrial biogenesis and key components of mitochondrial electron transfer chain, including silent information regulator two ortholong (Sirt 1) and AMP-activated protein kinase (AMPK). In other study involving primary myoblast cells and embryonic fibroblasts cells co-culture, culture plates coated with chitosan were compared with culture plate with no chitosan coating (Iyer et al, 2014). The results showed that at 5 hours after seeding, chitosan-coated culture plates had higher number of primary myoblasts with greater expression of integrin β_3, while primary fibroblasts had reduced F-actin fraction. The ratio of myoblasts to fibroblasts were increased even further at 48 and 72 hours after seeding in chitosan-coated culture plates, resulting in up to two-fold increase in ratio compared to uncoated culture plates. Other difference included higher organization of actin and integrin β_3 network around myoblast nucleus in culture plate with chitosan coating, while fibroblasts showed actin and integrin β_1 network reorganization.

2.5 Skeletal Muscle Structure and Function

In a human body, there are approximately 640 skeletal muscles which accounts for an estimated 40% of total body mass (Relaix & Zammit, 2012). The primary function of the skeletal muscle is force generation via contraction of its functional unit called sarcomere. Structurally, the force-generating functional unit consists of actin and myosin filaments contained within the sarcomeres. Thousands of sarcomeres are bound together to form myofibrils which make up the myofiber. The skeletal muscle's ability to generate contractile force enables the body to move, facilitate respiration and maintain postures.

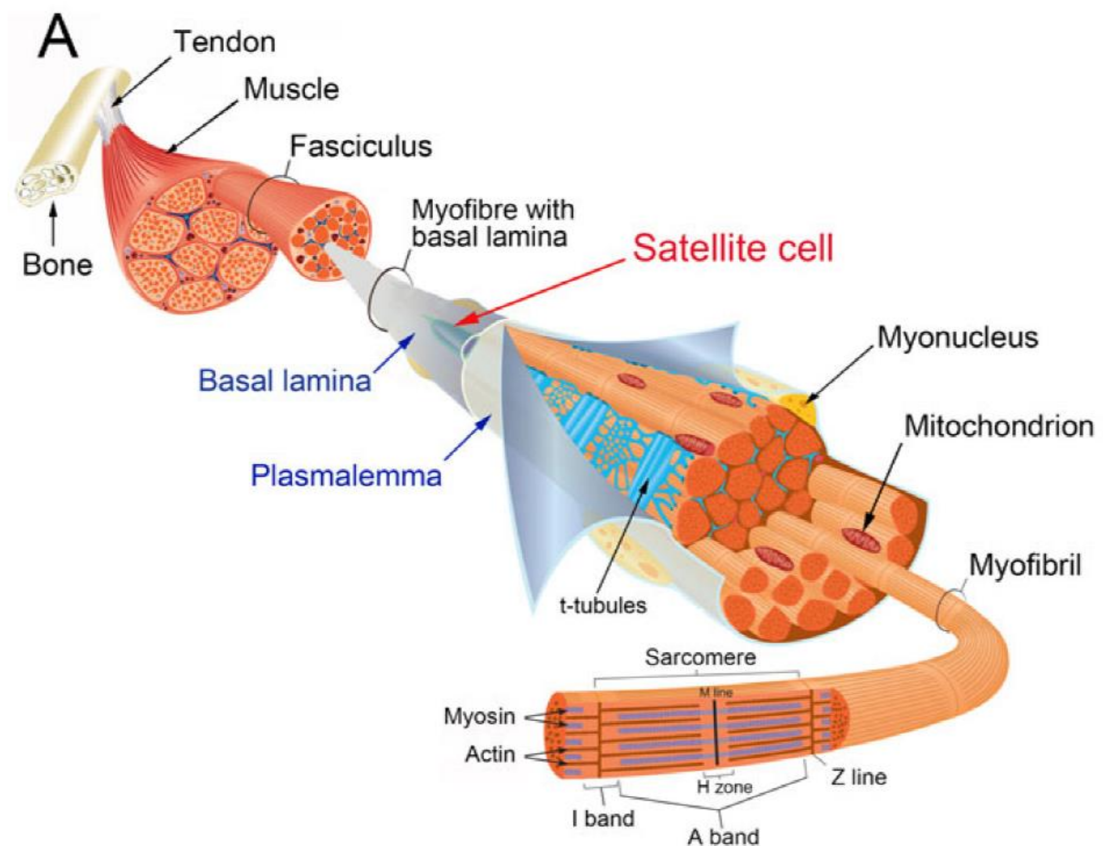

Figure 2.3 Structure of skeletal muscle (Relaix & Zammit, 2012).

2.6 Skeletal Muscle Injury

Tears, lacerations and contusions represent the most common forms of skeletal muscle injury encountered by an average individual with either contusions or

strains being responsible for more than 90% of sport injuries (Turner & Badylak, 2012). This type of injury can be categorized as physical trauma without volumetric loss of muscle mass. Skeletal muscle has innate ability to fully recover from such injury. Upon injury, the skeletal muscle will undergo three phases of regeneration, namely the destruction or inflammatory phase, the repair phase and remodeling phase (Ten Broek et al, 2010). The destruction or inflammatory phase consists of necrosis of damaged cells, formation of hematoma and inflammatory cells infiltration. In the repair phase, activated satellite cells initiate myofiber regeneration. Finally, in the remodeling phase, regenerated myofiber matures and contractile function is restored. More detailed mechanisms of each phase of regeneration are described below.

2.6.1 Muscle Regeneration – Inflammation Phase

Upon injury, the damaged skeletal muscle undergoes necrosis (Yin et al, 2011). First, dissolution of myofiber sarcolemma takes place, leading to increase in myofibers permeability. As a result, there will be an increase in plasma level of muscle proteins and micro ribonucleic acid (miRNA) such as creatine kinase and microRNA-133a (miR-133a). In addition, damaged muscle renders calcium influx or calcium release from sarcoplasmic reticulum (SR), thereby activating calcium-dependent proteolysis which stimulates tissue degeneration. An important endoproteinase is a calcium-activated protease, also known as calpain, which cleaves myofibrillar and other forms of cytoskeletal proteins. This stage of muscle regeneration also activates complement cascade and induces inflammatory response.

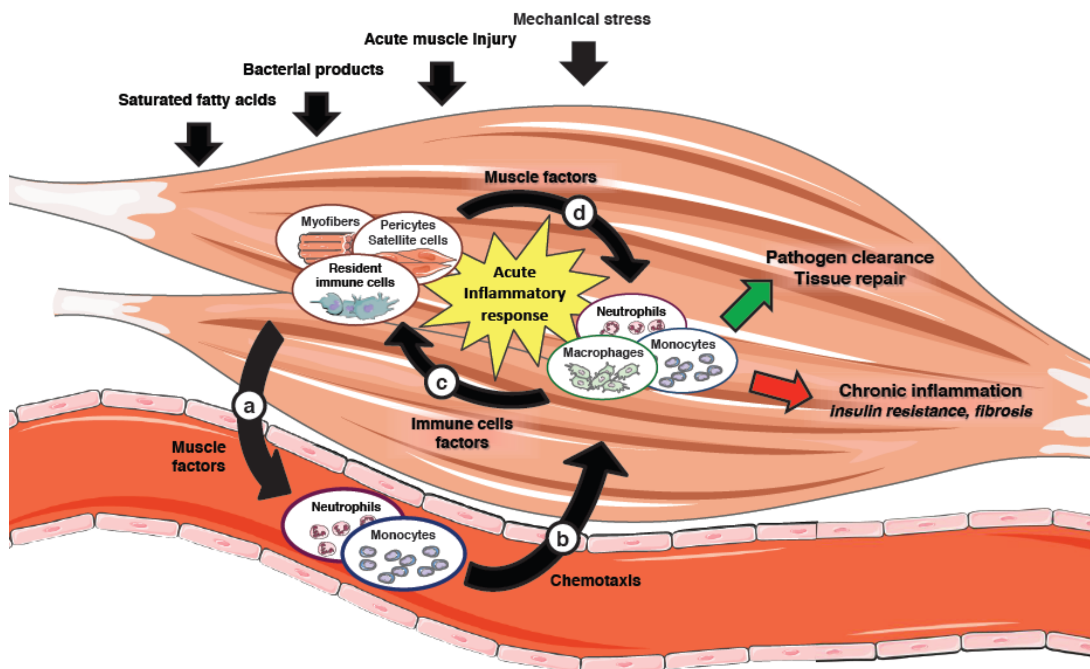

Figure 2.4 Inflammation of skeletal muscle (Pillon et al, 2012).

Inflammatory stage of muscle regeneration is characterized by chemotactic recruitment of systemic leukocytes to the site of injury (Yin et al, 2011). Among these, neutrophil is the first population of inflammatory cells to infiltrate damaged muscle during the first 1- 6 hours post injury and remain within the damaged tissue for up to 5 days (Tidball, 2005). The infiltrated neutrophils are known to be phagocytic as well as able to release proteases that facilitate in degrading cellular debris of the damaged muscle. It is very likely that the main role of neutrophils in the process of skeletal muscle regeneration is the oxidative of proteolytic modification of damaged muscle tissues, which enable phagocytosis of debris by neutrophils or macrophages during the inflammation stage.

Thereafter, 2 different subpopulations of macrophage infiltrate the site of injury and become the dominant population of inflammatory cells. The role of pro-inflammatory macrophage is to induce myogenic precursor cell proliferation and the role of anti-inflammatory macrophage is to induce differentiation and cell fusion. Indeed, suppression of macrophage to the injury site has been shown to cause incomplete skeletal muscle regeneration (Ten Broek et al, 2010). The first subpopulation of macrophages is pro-inflammatory macrophage or classically activated or M1 phenotype (Saclier et al, 2013). Upon muscle damage, this pro-

inflammatory macrophage population enters the damaged muscle fiber. Within the first 24 hours, it secretes pro-inflammatory cytokines such as TNF-α and interleukin IL-1β, which are responsible for phagocytosis of cellular debris. Then, IL-1β can then induce production of IL-6, resulting in myogenic proliferation of myogenic precursor cells, while reducing new myotube formation. Studies has shown that IL-6 null mice had lower number of monocyte/macrophage infiltration post-injury as well as impaired recovery, which resulted in decreased myofiber size during regeneration and increased tissue fibrosis (Pillon et al, 2012). In contrast to IL-1β, TNF-α can play different roles depending on the level of inhibition. Low inhibition of TNF-α results in stimulation of myogenic differentiation while high inhibition results in reduced myotube formation. Also, high level of TNF-α can stimulate myogenic cell proliferation. The overall result is increased myogenic cell proliferation and reduced fusion index of myogenic cells, which in turn inhibits large myotube (myotube with over 10 nuclei) formation (Saclier et al, 2013).

The other subpopulation of macrophage is the alternatively activated macrophage or M2 phenotype (Saclier et al, 2013). This subpopulation includes a subtype called M2c macrophage or the anti-inflammatory or deactivated macrophage. It is activated by anti-inflammatory compounds such as IL-10 and glucocorticoids and also secretes IL-10 as well as TGF-β. IL-10 is a key mediator in conversion of pro-inflammatory M1 macrophage to anti-inflammatory M2 macrophage which then promotes muscle growth and regeneration (Pillon et al, 2012). This anti-inflammatory cytokine stays in the site of injury until the end of inflammation (Broek et al, 2010). M2c macrophage also secretes TGF-β. This cytokine can inhibit cell myogenesis during myotube formation. The overall effect of M2c macrophage is the shift of focus from cell proliferation to myogenic expression and myotube formation (Saclier et al, 2013). The graphical representation of the timeline of inflammatory cells infiltration is shown in figure 2.5 (Tidball & Villalta, 2010).

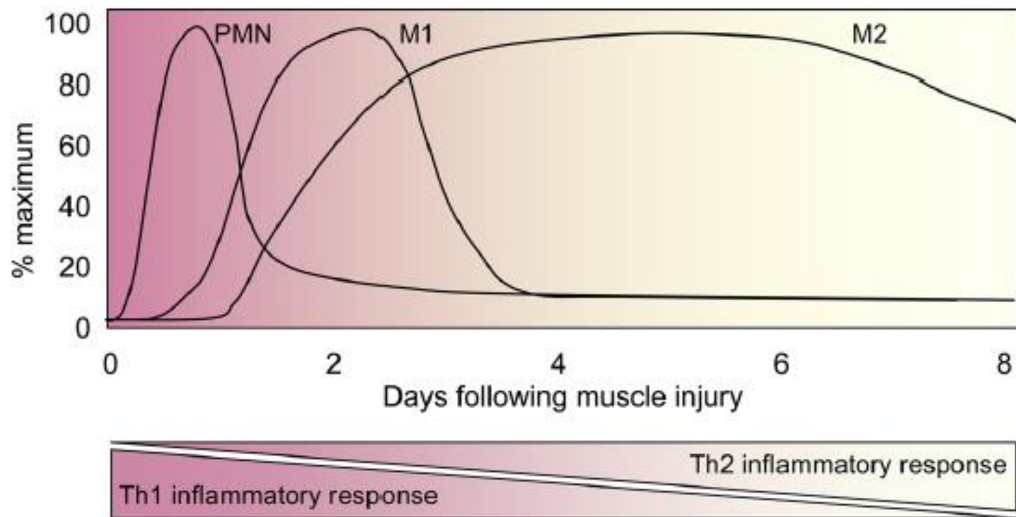

Figure 2.5 Timeline of inflammatory cells infiltration (adapted from Tidball & Villalta, 2010).

The process by which macrophage is switched from M1 phenotype to M2 phenotype is called macrophage polarization (Brown et al, 2012). This is possibly due to macrophage plasticity which allows macrophage to switch phenotype according to signals in the local microenvironment. Still the mechanism of macrophage polarization is not well understood. However, a key cytokine that facilitates macrophage polarization is IL-10. IL-10 is a general suppressive cytokine that inhibits pro-inflammatory responses including reducing production of pro-inflammatory cytokines and differentiation of M1 phenotype macrophage which results in resolution of inflammation phase (Ouyang et al, 2011; Hofmann et al, 2012).

Under normal physiological inflammatory response to injury, the site of damaged muscle tissue will undergo a transient infiltration of inflammatory cells (Fig. 2.6) (Mann et al, 2011). Under this stage of acute inflammation, the site can be expected to have transient collagen deposition before progressing to next stages of regeneration until reaching full recovery. However, under certain conditions including chronic injury and disease such as muscular dystrophies, persistent inflammatory infiltration is found at the site of injury instead of normal transient inflammatory infiltration. Under this chronic inflammation, collagen deposition is accumulated throughout all the stages of skeletal muscle regeneration which results in excessive fibrosis and scar tissue.

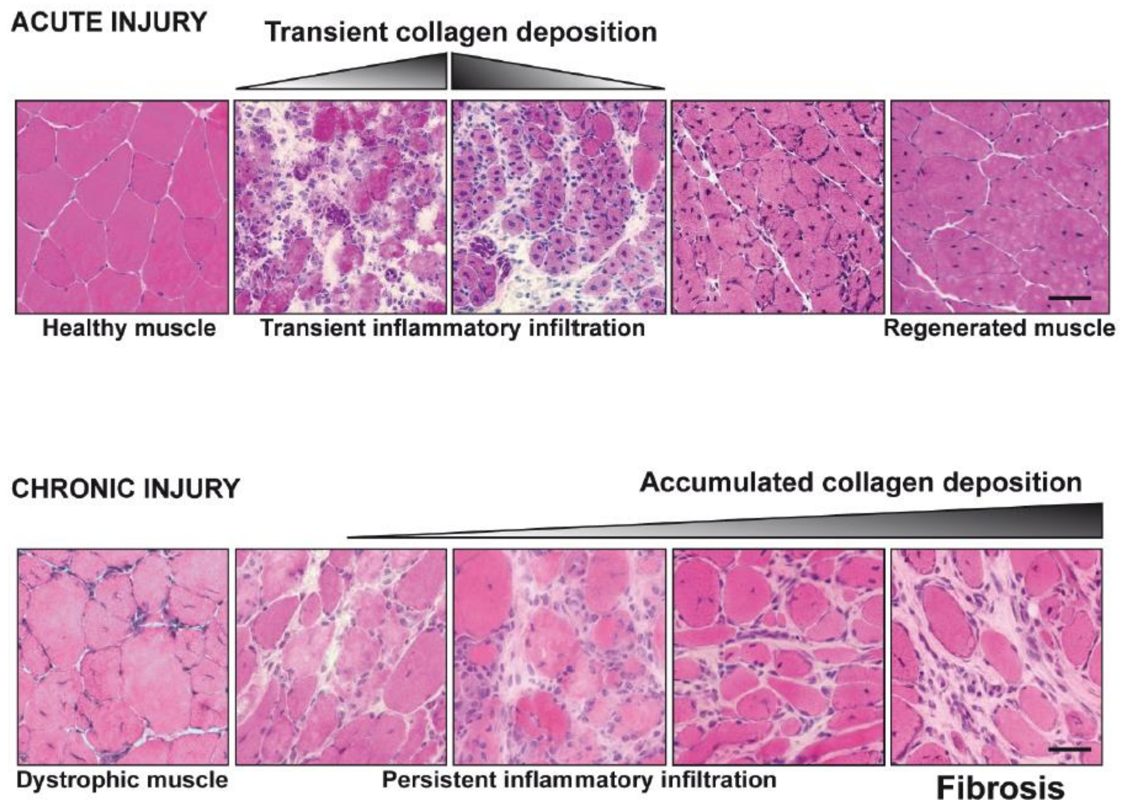

Figure 2.6 Histology of progression of acute and chronic muscle regeneration (Mann et al, 2011).

During inflammation phase of muscle regeneration, events associated with redox homeostasis can affect how damaged muscle fiber regenerated. Upon invasion and activation in the site of injury, neutrophils can release free radicals and proteases, causing a secondary injury to muscle fiber. Neutrophils are known to contain over 40 hydrolytic enzymes and toxic molecules inside the granules. These enzymes and molecules can produce oxidants such as superoxide anion, hydrogen peroxide and hypochlorus acid which target desmin and dystrophin, intermediate filament proteins essential in muscle contraction. Most of these neutrophils are polymorphonuclear neutrophilic (PMN) leukocytes (Pierce et al, 2007). Since PMN is considered to be a main factor the in early stage of inflammation, therefore its accumulation may even aggravate muscle damage. PMN releases myeloperoxidase (MPO), an important factor in PMN-mediated damage. A previous study showed that mutant MPO mice had lower level of muscle membrane injury than in wild-type mice (Pierce et al, 2007). One of MPO products is hypochlorus acid, which is a highly reactive oxidative agent. Once

generated, it reacts with cysteine, a protein in the binding site of creatine kinase (CK) and glyceraldehyde-3-phosphatedehydrogenese (GAPDH), enzymes critical to energy metabolism in cells. Cysteine is highly reactive and sensitive to oxidative stress, and is subjected to protein oxidation by hypochlorus acid, resulting in conformational changes in CK and GAPDH. In response, increased 20s proteasome activity and subsequently increased ubiquitinylated protein from cysteine oxidation is observed in the cells. As a result, enzyme activity of CK and GAPDH is reduced. PMN also attracts and activates macrophages to the site of injury. Macrophages eventually replace PMN population in the area and the main function is to ingest cellular debris. Both activated neutrophils and macrophages have reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex which can initiate 'respiratory burst', producing superoxide anion. These processes increase ROS production in the site of injury, causing secondary damage to the muscle.

2.6.2 Muscle Regeneration – Repair Phase

After phagocytosis of necrotic debris during destruction/inflammatory stage, myofibers regeneration can take place (Yin et al, 2011). The satellite cells are the major cell population responsible for muscle regeneration. When induced by injury, the quiescent satellite cells expressing paired box protein 7 (Pax7) migrate to the injured muscle (Ten Broek et al, 2010). Once there, the satellite cells up-regulate myogenic regulatory factors (MRF) including myogenic differentiation (MyoD) and myogenic factor 5 (Myf5). These factors enable quiescent satellite cells to become active and proliferate, allowing them to become myoblasts. When Pax7 level is down-regulated and myogenic regulatory factor 4 (Mrf4) and myogenin is up-regulated, the activated satellite cells will exit the proliferative stage and enter the differentiation stage. During this differentiation stage, differentiated myoblast will either form multinucleated myofiber (hyperplasia) or fuse with damaged myofiber (hypertrophy).

The process of myogenic proliferation and differentiation is regulated by various growth factors (Ten Broek et al, 2010). A major factor is insulin-like growth factor 1 (IGF-1), and subsequently IGF-2. IGF-1 regulates expression of myogenic regulatory factors, which stimulates proliferation and differentiation of myoblasts activated satellite cells. Many studies have established direct correlation between IGF-

1 and muscle development. Systemic administration of IGF-1 results in higher deoxyribonucleic acid (DNA) and protein content in muscle while direct injection can improve regeneration of muscle. Further, experiment has shown direct causal relationship between IGF-1 overexpression and muscle hypertrophy. The mechanism of IGF-1 in relation to muscle growth is regulated mainly by phosphatidylinositol 3-kinase (PI3K) leading to anti-apoptotic protein kinase B (Akt) activation. Other studies showed that pre-treatment with IGF-1 provided protection to skeletal muscle cells against oxidative stress through the action of PI3K/Akt as well as extracellular signal-regulated kinases (ERK) 1/2 mitogen-activated protein kinase (MAPK) pathway (Yang et al, 2010). Anti-inflammatory M2 macrophages can increase IGF-1 production, leading to myoblast proliferation (Pillon et al, 2012). For this reason, IGF-1 is considered to be the most important factor in myogenic proliferation and differentiation.

Other important factors are hepatocyte growth factor (HGF), which is the primary factor that induces satellite cells proliferation and help to migrate satellite cells to the site of muscle injury, and vascular endothelial growth factor (VEGF), which stimulates angiogenesis (Ten Broek et al, 2010).

2.6.3 Muscle Regeneration – Remodeling Phase

The last phase of muscle regeneration is the remodeling phase in which the regenerated myofibers mature and start to contract. Inhibitory growth factors are important factors in muscle remodeling processes. One of them is myostatin, which is expressed in satellite cells and myoblasts and its functions is down-regulates Pax3 and Myf5 expression and inhibits MyoD expression (Ten Broek et al, 2010). TGF-β1 is another factor secreted by anti-inflammatory M2c macrophage (Pillon et al, 2012). Its main role is inducing remodeling and repairing extracellular matrix and basal membrane through fibroblast stimulation. This action results in production of collagen and fibronectin which may form scar tissues. Indeed, inhibition of TGF-β1 by decorin showed less muscle fibrosis and more regeneration of skeletal muscle.

Reconstruction of sarcomeres takes place during the remodeling phase of muscle regeneration. This is the stage where contractile proteins, actin and myosin are organized into repeating units (Harris, 2003). This step is controlled by a group of

intermediate filament proteins such as vimentin, desmin, and nestin. When the organization of contractile protein is completed into mature myofibers, all three intermediate filament proteins are co-expressed at z-disc.

2.7 Cardiotoxin-induced Muscle injury

Intramuscular injection of cardiotoxin is one of most acceptable and reproducible model of muscle injury (Shiau et al, 1976). Cardiotoxin is also known as cytotoxin with direct lytic factors and membrane active polypeptide (Harris, 2003). It is single chain small polypeptides similar to nicotinic acetylcholine receptors (nAChR)-binding alpha-neurotoxins. It acts as a pore-forming agent, which causes plasma membrane of skeletal muscle to depolarize and degrade.

Upon direct contact with cardiotoxin, the muscle fibers will undergo rapid fragmentation and appearance of basal lamina sheaths of myofibers become continuous tubes (Couteaux et al, 1988). A few hours post-injection, neutrophilic leukocytes begin to infiltrate between necrotic fibers. One day after injection, there is an increase in leukocytes and macrophages number infiltrating the injured area, resulting in enhanced phagocytosis of old myofiber debris as well as intense edema. Neutrophils are the main inflammatory cell population during this period. By second day, spindle-shaped mononucleated cells, originated from satellite cells, first appear and mix with leukocytes, macrophages and necrotic debris. On third day, formation of new myotubes start and will continue to progressively transform myotubes into myofibers. At this stage, mononuclear cells become the dominant inflammatory cell population and are closely associated with necrotic myocytes. By day 7, the injured muscle will be largely repaired while some inflammatory cells remain on site (Pierce et al, 2007). On the 14th day, most inflammatory cells that infiltrated the injured myofiber will be resolved and muscle regeneration is completed. In regenerated muscle fiber, nuclei migrate from fiber axis to the periphery in a later stage of maturation than in normal developmental stage. In some cases the migration is incomplete in regenerated mature muscle fiber, resulting in higher number of central

myonuclei. Cardiotoxin does not damage basal lamina of muscle fiber, which is the location where muscle fiber regenerates (Harris et al, 2003).

2.8 Skeletal Muscle Injury Treatment

2.8.1 Antioxidants

Myburgh and colleges (2012) studied the effect of plant-derived polyphenol and its anti-oxidative property on rat skeletal muscle recovery. They showed that polyphenol facilitates in improving muscle recovery through stimulating satellite cells and macrophage activity, decreasing neutrophil infiltration and improving free radical quenching activity both in plasma and muscle tissue. They also found changes in different phases of muscle repair and inflammation both in histological and molecular aspects.

Comparing the histology of placebo group and polyphenol-treated group, at 4 hours post-contusion, both groups displayed extensive fiber and vascular damage as well as accumulation of red blood cell in interstitial space. There was no significant difference between the two groups. Significant difference in level of inflammatory cell infiltration between the two groups could be seen at day 1 and 3 post injury with the treated group having lower level of infiltration. Five days after injury, inflammation in the treated group was mostly resolved and structural re-organization began. By day 7, treated group showed better fiber regeneration while the placebo group still has centrally-located nuclei in the fiber and infiltration by immune cells, making basal lamina adhesion impossible between adjacent fibers. By day 14, the treated group had completed recovery, however this is not the case for the placebo group.

Changes in infiltration cell count and molecular cytokines was also observed in the study. In placebo group, the macrophage count was significantly elevated on day 3 and 5 before returning to baseline level on day 7. Neutrophil count increased as soon as 4 hours post-injury and peaks at day 1 and returns to baseline by day 3 in placebo group. In treated group, there was no significant increase in neutrophil count. In treated group, macrophage count is highest on day 1 before

gradually declining to baseline level on day 5. Pro-inflammatory cytokines TNF-α level in placebo group increased upon injury and was observed to be highest on day 3 in placebo group before returning to normal at day 5. When treated there was a significant decrease in day 3 elevation of TNF-α level compared to untreated. In the border zone, area around the damaged fiber, TNF-α level was significantly higher at all time points, peaking at day 1 when not treated. In the treated group TNF-α level elevation was significantly lower from day 1 till day 14. Similarly IL-6 level increases immediately upon contusion and peaks at day 3 before gradually dropping and returning to normal on day 14 in both treated and untreated groups with no significant difference between the groups. In the border zone, IL-6 level peaked on day 1 in both groups with the treated group having lower IL-6 level on day 3. Anti-inflammatory cytokine IL-10 level did not change at all time points in placebo group. In treated group, there is a significant increase on day 3. To test the free radical quenching capacity, both plasma and muscle oxygen radical absorbance capacity (ORAC) were observed. These results showed significant increase on day 3, with plasma ORAC being five times higher than muscle ORAC.

2.8.2 Inflammatory Drugs

A common treatment option for inflammation is to use non-steroid anti-inflammatory drugs (NSAIDs). NSAID modulates inflammation by inhibiting cyclooxygenase (COX) enzyme which is released at the onset of injury, producing prostaglandin which promotes inflammation (Urso, 2013). Examples of well-known and widely available NSAIDs are ibuprofen and aspirin. When used in appropriate dosage, NSAIDs can prevent excessive inflammation, otherwise can lead to edema and eventually anoxia and cell death (Baoge et al, 2012). However, its use for long period of time is not recommended. Chronic use can cause negative side-effects which include asthma exacerbation, gastrointestinal & renal side effects, and hypertension among others.

Other complication that comes with the use of NSAIDs for treatment of skeletal muscle injury is its effect on skeletal muscle healing. Prolonged use of NSAIDs can interfere with macrophage activity which limits phagocytic function and inhibiting the production for growth factors as well as inactivating proliferation and

differentiation of satellite cells, eventually leading to delayed muscle regeneration and reduced tensile strength of injured muscle (Baoge et al, 2012). COX-2 pathway is important in skeletal muscle healing and inhibition of COX-2 has been shown to slow down proliferation and maturation of differentiated myogenic precursor cells (Gharaibeh et al, 2012). The use of ibuprofen, which is a non-specific inhibitor of COX-1 and COX-2, has no effect on pain perception and soreness while loss of strength and serum creatine kinase is expected (Urso, 2013). NSAIDs that target COX-1 may inhibit COX-1-mediated protein synthesis. NSAIDs can increase expression level of TGF-β1 in lacerated muscle, resulting in a decrease in neutrophil and macrophage infiltration which ultimately lead to a delay in muscle healing. The use of NSAIDs also has detrimental effects on mitochondria. Some of the effects are increases in proton leak and decreases in rate of adenosine triphosphate (ATP) synthesis, uncoupled oxidative phosphorylation and inhibit mitochondrial respiration.

CHAPTER III
MATERIALS AND METHODS

3.1 Experimental protocol

Adult male Wistar rats (300-350 g) were used in this study. All procedures were approved by the Animal Care and Use Committee of the Faculty of Science, Mahidol University (protocol #268). Rats were randomly assigned into one of two groups: control (intact) and injury groups. The control group received no treatment whereas the injury group was given a cardiotoxin injection to induce muscle injury. Injury group was further divided into 2 subgroups. One group was given chitosan treatment via oral gavage. Another group was given vehicle in a form of 1% acetic acid solution. Animals received their respective treatment for 1 week prior to injury and treatment continued until the end of the experiment. After 1 and 3 days post-injury, the muscle function was measured and muscle samples removed.

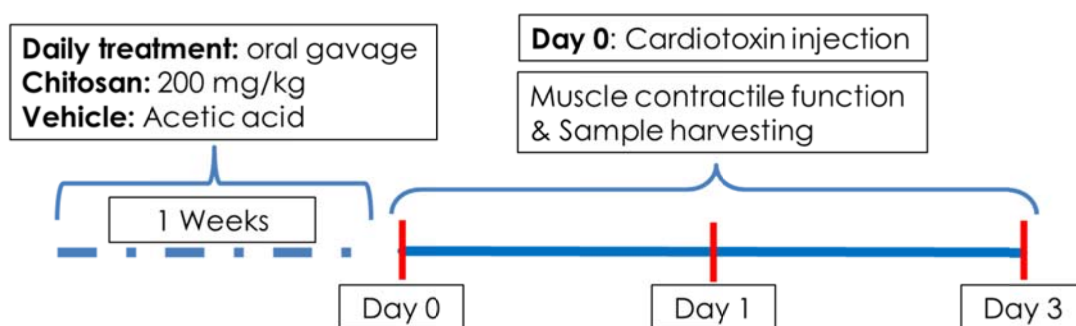

Figure 3.1 Schematic illustrating the experimental design

3.2 Induction of muscle injury

Rats were anesthetized by intra-peritoneal injection of cocktail of 25 mg/kg of Zoletil and 8 mg/kg of Xylavet. To induce muscle injury, a single dose of Cardiotoxin from cobra *Naja mossambica mossambica* (Sigma-Aldrich) dissolved in

phosphate-buffered saline (PBS) (0.03 ml of 0.5 mg/ml) was injected unilaterally into the left tibialis anterior (TA) muscles longitudinally (Clever et al, 2010).

3.3 Chitosan preparation and administration

Chitosan was obtained from Sigma-Aldrich, (Sigma-Aldrich, cat. # 448869). According to the company's product specification, the molecular weight is 50,000 to 190,000 daltons and deacetylation level is over 75%. Chitosan solution was prepared by dissolving chitosan in 1% acetic acid solution, then heat to 70^{0}C and stir overnight. Chitosan was administered by oral gavage daily at 200 mg/kg body weight in a 1 ml dose (Jeon et al, 2003; Kohda et al, 2012).

3.4 In situ measurement of muscle function

Isometric contractile properties of tibialis anterior muscles were determined at specified time points (day 1 and 3) using the method of Shin et al (Shin et al, 2008) with some modifications. Animals were anesthetized using a cocktail of 25 mg/kg of Zoletil and 8 mg/kg of Xylavet. Once a surgical plane of anesthesia was reached, as determined by the lack of withdraw from the pain reflex test, the injured tibialis anterior muscle was exposed to determine muscle contractile properties. The tendon of each muscle is securely tied with a surgical silk thread (3-0, Pearsalls Sutures, Somerset, UK) and connected to the force transducer (FT-03, Grass, Rhode Island, USA). Contractile properties were determined directly by stimulating peroneal branch of the sciatic nerve with supramaximal square wave pulse (3V, 0.2 ms duration). Force was recorded and analyzed using the PowerLab system (PowerLab 45/P, AD Instrument, USA). The optimal muscle length (L_o) was determined by stimulating the muscle to produce an isometric twitch response and adjusting muscle length to produce a maximum isometric twitch (P_t). The force-frequency relationship of the muscle was determined by stimulating at frequencies of 10, 20, 40, 60, 80, 100, 120, and 150 Hz, with a 2-min rest period between each stimulus to prevent fatigue. The animal's body temperature was maintained at 37°C using heating lamp throughout the experiment. Immediately after the functional measurements, the muscles were

carefully removed, trimmed free of connective tissues, blotted on filter paper, weighed and horizontally cut into halves. The lower half was frozen in liquid nitrogen and stored at -80°C for further analysis. The upper half was embedded with optimal cutting temperature (OCT) in Tissue-Tek compound (Sakura, Finetek, USA) and snap-frozen in isopentane pre-chilled in liquid nitrogen and stored at -80°C until used for histological analysis.

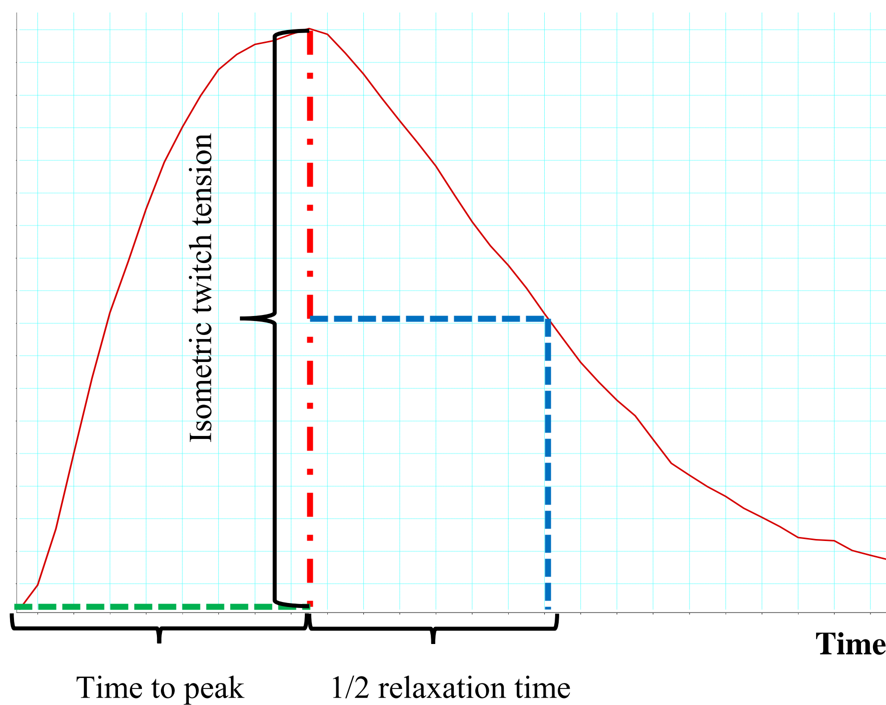

Figure 3.2 Isometric twitch tension. Time to peak is the time from the onset of contraction to the peak of isometric twitch. Half-relaxation time is the time for decay of tension from peak of isometric twitch to one half of peak tension. Isometric twitch tension is the peak tension generated by a single stimulus.

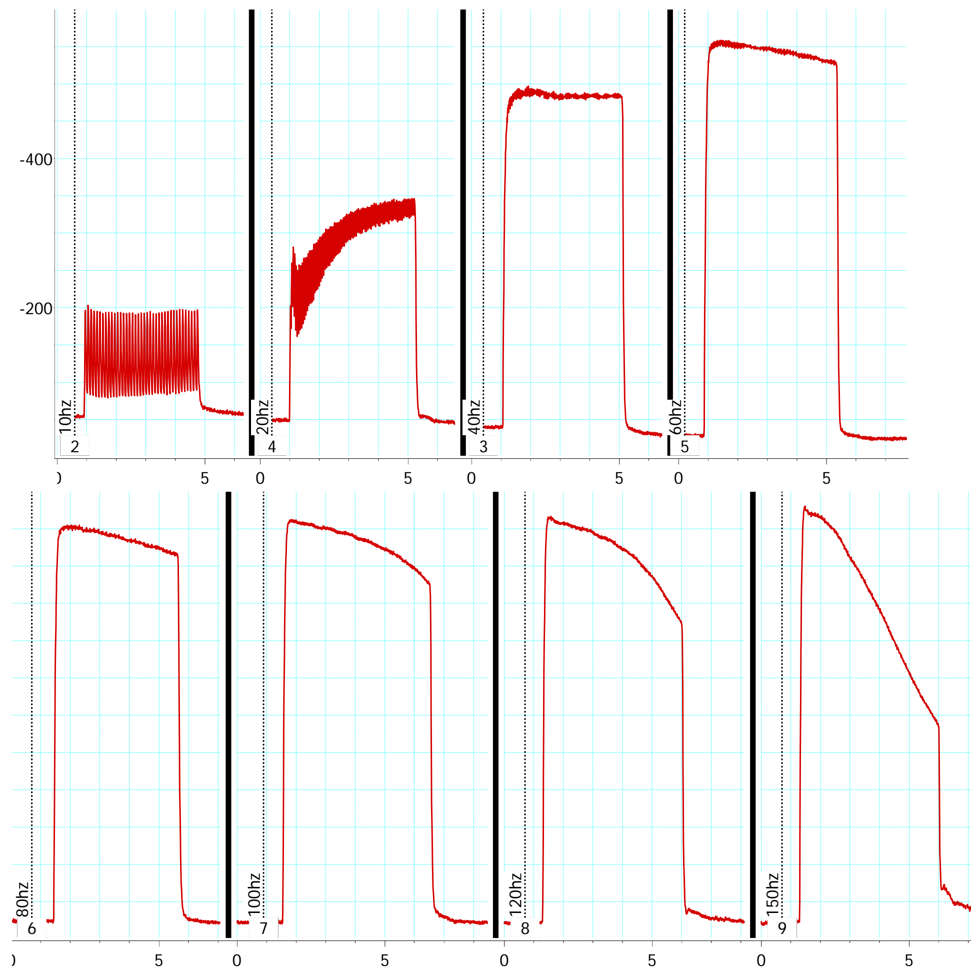

Figure 3.3 A tracing of isometric tetanic force

The maximal isometric tetanic force (P_o) was determined from the plateau of the frequency-force relationship. Muscle cross-sectional area (CSA) was calculated as muscle mass (g)/muscle length (cm) /muscle density 1.06 g/m^3. Specific tension (sP_o) was expressed as the maximum force per CSA of the muscle (N/cm^2). Effective frequency (EF_{50}), or stimulus frequency which produces 50% of peak tetanic force, was calculated using nonlinear curve fit analysis (McDonald et al, 2015). It is a measure of skeletal muscle's sensitivity to electrical stimulus.

3.5 Histological studies

Embedded TA muscles were cut into 10 μm sections. The section was stained with Hematoxylin and Eosin (H&E) for descriptive and histological analysis. Five to six random fields per each sample were captured by using BX53 microscope[c] (Olympus) with 40x magnification for cross-sectional area analysis and with a 200x magnification as representative sample. The area of damage was evaluated using the ImageJ v.1.49U software (NIH, Bethesda, MD, USA).

3.6 Dry and wet muscle weight ratio

Approximately 100 mg of TA muscle were weighted and individually allocated in 2-ml plastic tubes. The samples were freeze-dried at -50°C for 24 hours and the dry samples were then weighted. Dry weight to wet weight ratio was determined as a marker of muscle inflammation and edema.

3.7 Determination of markers of inflammatory response

RNA expression levels of TNF-α and IL-6 were used as markers of pro-inflammatory response whereas IL-10 and TGF-β were used as markers of anti-inflammatory response. RNA expression levels were determined by real time-PCR. Total RNA was extracted from TA muscle by pulverization and incubated in Trizol (Invitrogen) at 4°C overnight. The samples were then grinded and centrifuged to separate RNA from other protein and DNA component. The procedure for RT-PCR was based on Lisi et al, 2012. Briefly, the sample RNA was isolated and purified through a series of centrifugation and treatment with DNase I and RNase-free solution. Then the RNA was converted to cDNA with reverse transcriptase and oligo (dT) primer. SYBR Green was used to as fluorescent dye for real-time amplification. Quantitative changes in mRNA levels were estimated by real-time PCR using the following cycling conditions: 35 cycles of denaturation at 95°C for 20s; annealing at 59°C for 30s; and extension at 72°C for 30s. Relative mRNA concentrations were calculated from the take-off point of reactions (threshold cycle, C_t) using the

comparative quantitation method. The results were expressed as fold change relative to GAPDH mRNA expression. List of primer sequences for all inflammatory cytokines used were in the table 3.1.

Table 3.1 Primer sequence

Cytokine	Primer sequence	Reference
TNF-α	Forward: 5'- CCACCAAGCGGAGGAGCAGC-3' Reverse: 5'-TCGGCTGACGGTGTGGGTGA-3'	Lisi et al, 2012
IL-10	Forward: 5'-CAGCTGCGACGCTGTCATCGA-3' Reverse: 5'- GCAGTCCAGTAGATGCCGGGTG-3'	Lisi et al, 2012
IL-6	Forwards: 5'-TCCTACCCCAACTTCCAATGCTC-3' Reverse: 5'-TTGGATGGTCTTGGTCCTTAGCC-3'	Ibrahim et al, 2013
TGF-β	Forward: 5'- TCCGGGCTGCGGCTGCAGC-3' Reverse: 5'-TCGCGGGTGCTGTTGTACA-3'	dos Santos et al, 2014

3.7 Data analysis

All data were expressed as means and standard error of the mean (means ± SEM). Shapiro-Wilk test was performed to determine normal distribution of data set. The Levene's test was used to verify the equality of variance. One way analysis of variance (ANOVA) was used to compare variables that met the homogeneity of variances assumption, followed by Tukey's post-hoc test. Krusal-Wallis test was used to compare differences between groups for non-parametric data, followed by pairwise group comparison as post-hoc test. The p-values less than 0.05 ($p<0.05$) was set for the significant difference between groups.

CHAPTER IV
RESULTS

In this study, **the effect of chitosan treatment was being investigated on the inflammatory response and functional recovery after cardiotoxin-induced injury.**

4.1 Effect of chitosan on body weight and TA muscle weight.

The animals' characteristics were reported in Table 4.1. The body weight was not significantly different among groups at both day 1 and day 3 post-injury. Similarly, there was no significant difference in TA wet weight of both injury groups at day 1 post-injury compared with the control group. At day 3 post-injury, however, TA mass was slightly decreased, although did not reached statistically significant, as compared to the control. The ratio of TA wet weight and body weight also showed no significant difference among groups at both day 1 and 3 post-injury.

Table 4.1 Effect of chitosan treatment on animal's body weight and muscle mass in the control, vehicle-treated and chitosan-treated groups 1 day and 3 days post injury. Values were presented as mean ± SEM. (n=4-5/group for each time point).

	1 Day Post-injury			**3 Days Post-injury**		
	Control	**Vehicle**	**Chitosan**	**Control**	**Vehicle**	**Chitosan**
Body mass, g	365.6 ± 1.6	378.4 ± 4.7	364.1 ± 10.3	371.2 ± 11.1	366 ± 9.5	362.8 ± 15.4
TA mass, mg	719.1 ± 14.5	773.6 ± 20.2	775.9 ± 49.9	775.3 ± 44.2	633.7 ± 73.2	698.0 ± 39.2
TA/Body mass, mg/g	2 ± 0.03	2.0 ± 0.06	2.1 ± 0.08	2.1 ± 0.1	1.7 ± 0.19	1.9 ± 0.05

4.2 Skeletal muscle contractile function

To evaluate the efficacy of chitosan treatment on muscle function following injury, series of contractile function tests were carried out.

4.2.1 Twitch kinetic

A twitch kinetic including isometric twitch tension, time to peak and half relaxation time was measured to see the effect of chitosan treatment on skeletal muscle contractile function.

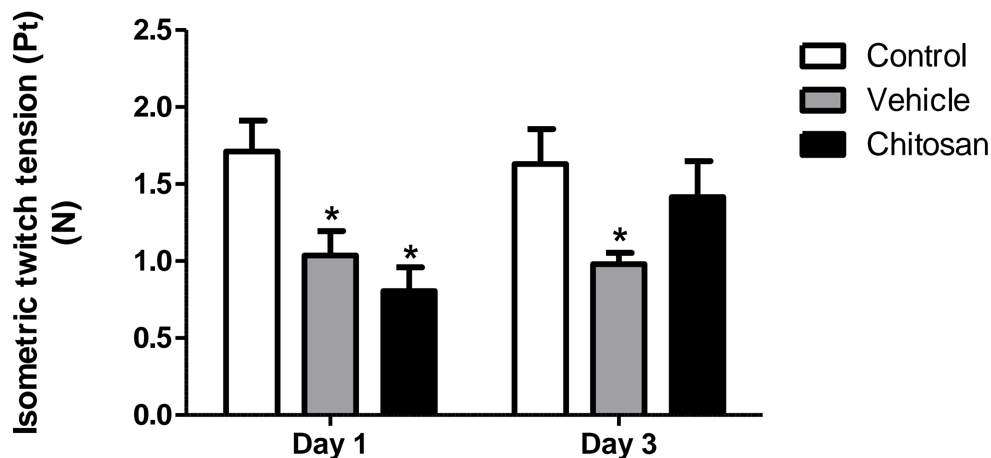

Figure 4.1 Effect of chitosan treatment on twitch force of TA muscle at day 1 and day 3 post-injury. * $p < 0.05$, significant difference from control.

Figure 4.1 showed changes in isometric twitch force in vehicle-treated and chitosan- treated group as well as that of the control at two times pointed. At day 1 post-injury, isometric twitch tension (P_t) of TA muscles from both vehicle-treated (1.03 ± 0.16, $p<0.05$) and chitosan-treated groups (0.81 ± 0.15, $p<0.05$) were significantly lower than that in the control group (1.77 ± 0.16). By day 3 post-injury, force production was recovered and comparable between the control (1.63 ± 0.23) and chitosan-treated (1.42 ± 0.23) while vehicle-treated (0.98 ± 0.07, $p<0.05$) group remained significantly lower than the control.

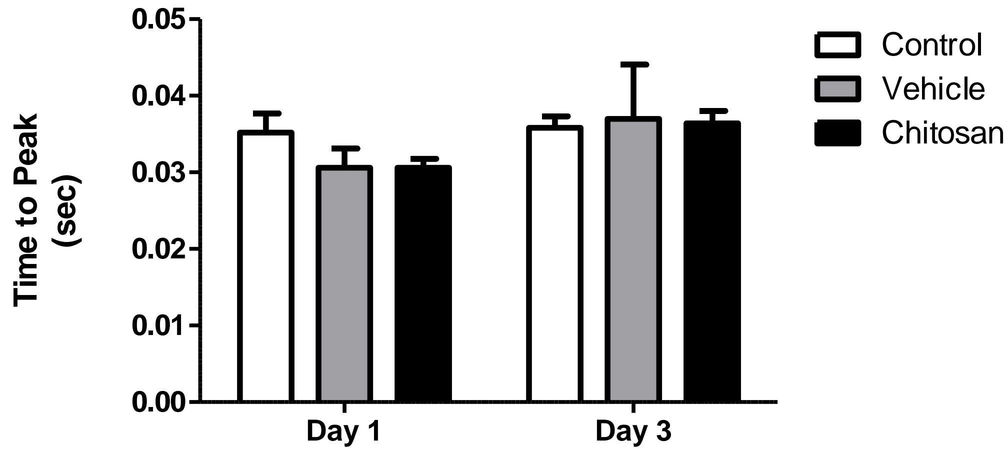

Figure 4.2 Effect of chitosan treatment on time to peak of TA muscle at day 1 and day 3 post-injury. * $p < 0.05$, significant difference from control.

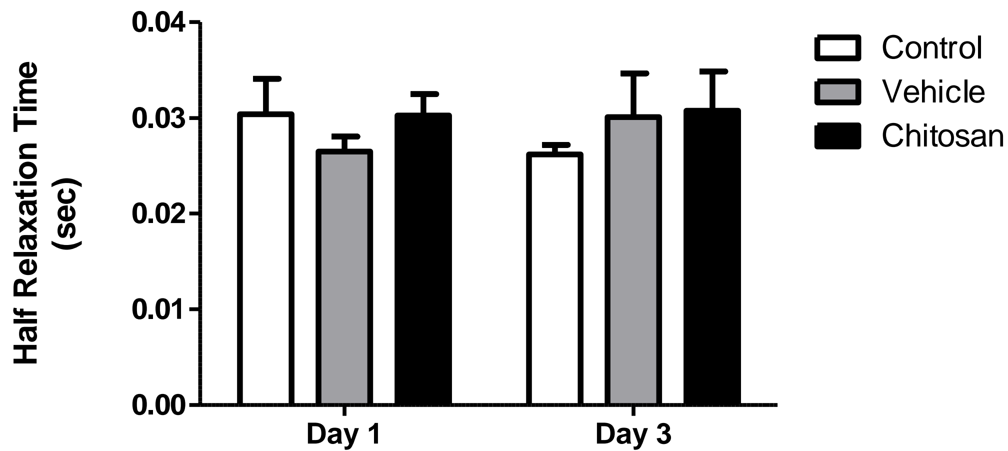

Figure 4.3 Effect of chitosan treatment on half relaxation time of TA muscle at day 1 and day 3 post-injury. * $p < 0.05$, significant difference from control.

Neither time to peak value nor half-relaxation time were significant differences among groups at both day 1 and day 3 post-injury.

4.2.2 Tetanus

Isometric tetanic contraction was performed on the TA muscle to determine the maximal force generation capacity after injury and the effect of chitosan treatment.

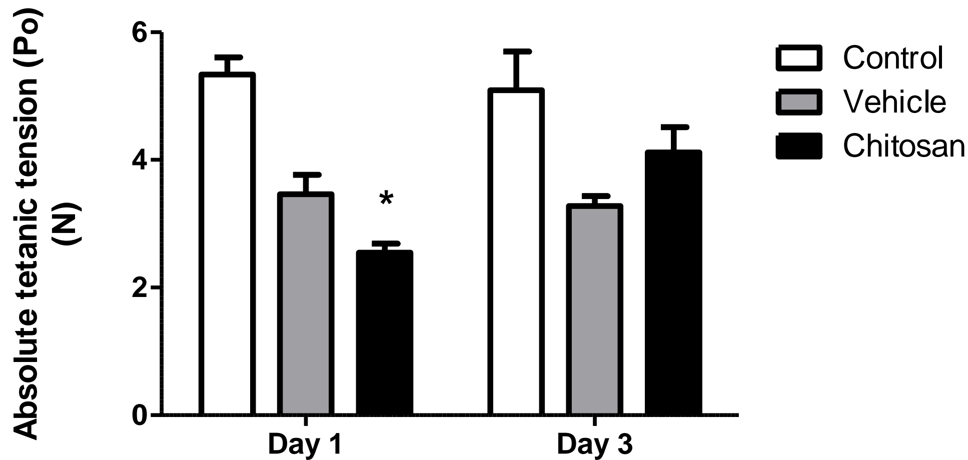

Figure 4.4 Effect of chitosan treatment on isometric tetanic force of TA muscle at day 1 and day 3 post-injury. * $p < 0.05$, significant difference from control.

There was a significant decrease in maximum absolute isometric tetanic force (P_o) in vehicle-treated group (3.46 ± 0.3) but chitosan-treated group decreased significantly (2.55 ± 0.14; $p<0.05$) compared with the control (5.34 ± 0.27) at day 1 post-injury. At day 3 post-injury, however, the loss of force began to recover in the chitosan-treated group and was no longer significantly different compared to the control. In the vehicle-treated group, on the other hand, this deficit in force remained unchanged on day 3 post-injury as compared to day 1 post-injury. There was no significant difference in P_o between vehicle-treated and chitosan-treated groups at both time points.

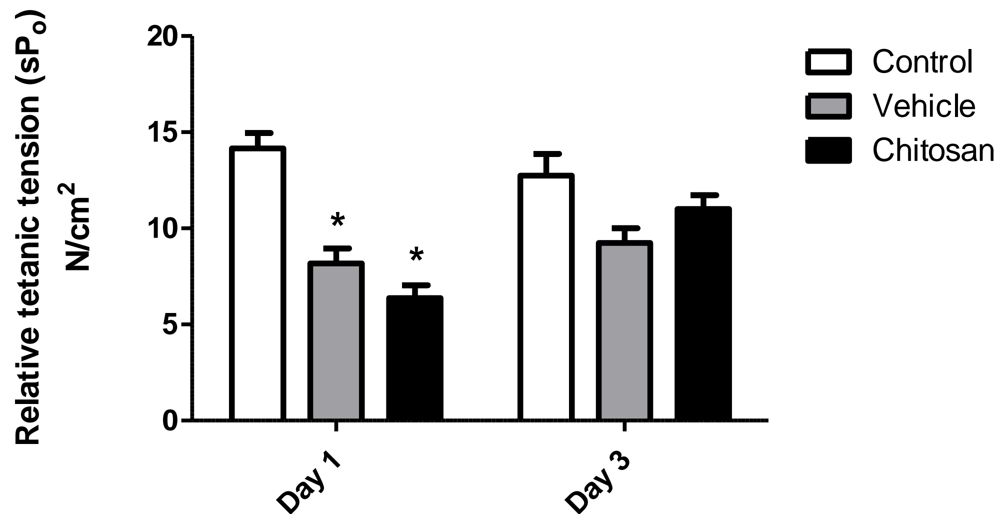

Figure 4.5 Effect of chitosan treatment on the relative tetanic force of TA muscle at day 1 and day 3 post-injury. * $p < 0.05$, significant difference from control.

Similar results were obtained for the relative tetanic force. At day 1 post-injury, the relative tetanic force of both vehicle-treated (8.18 ± 0.79; $p<0.05$) and chitosan-treated (6.34 ± 0.66; $p<0.05$) groups were significantly lower than that of the control (14.16 ± 0.79). However, these decreases in relative force of both vehicle-treated (9.25 ± 0.75) and chitosan-treated (11.01 ± 0.72) groups were recovered and no longer significantly different compared with the control group (12.75 ± 1.14) by day 3 post-injury. No significant difference in the relative tetanic force was observed between the vehicle-treated and chitosan-treated groups at both time points studied.

4.2.3 Force-Frequency Relationship

The force-frequency relationship were analyzed to determine the effect of chitosan treatment on contractile performance at different stimulus frequencies.

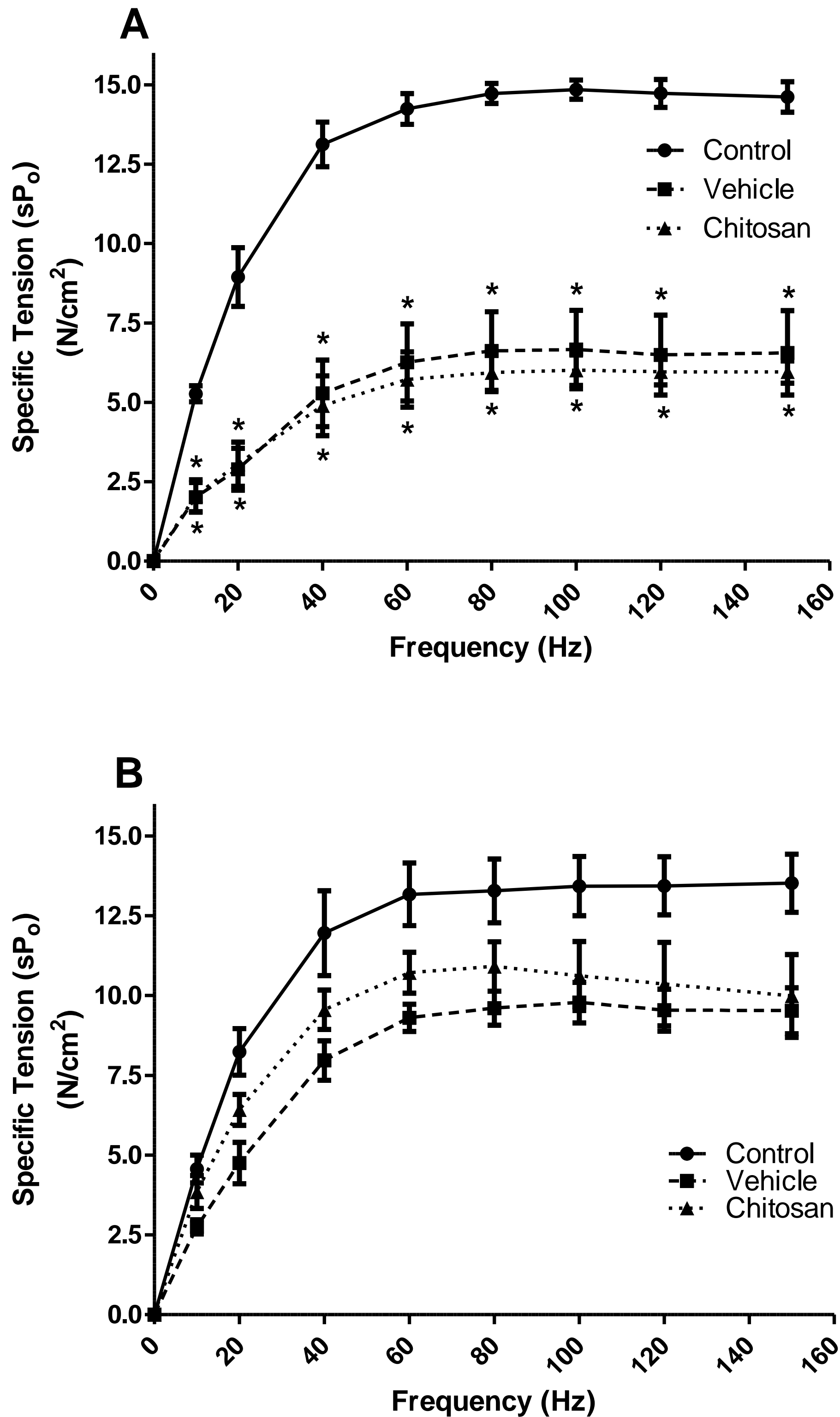

Figure 4.6 Effect of chitosan treatment on force frequency curve at day 1 post-injury (A) and day 3 post-injury (B). * $p < 0.05$, significant difference from control.

The force-frequency relationship of the vehicle-treated and chitosan-treated muscles at day 1 and 3 post-injury were shown in Figure 4.6. As expected, there was a significant reduction in force production ($p<0.05$) across all stimulation frequencies at day 1 post-injury in both injury groups compared to the control. However, no such difference was observed between vehicle-treated and chitosan-treated groups over the entire frequency.

By day 3 post-injury, only the vehicle-treated group exhibited a significant decrease in the recovery force as compared with the control. There was no significant difference in muscle function observed between vehicle-treated and chitosan-treated groups across all stimulation frequencies.

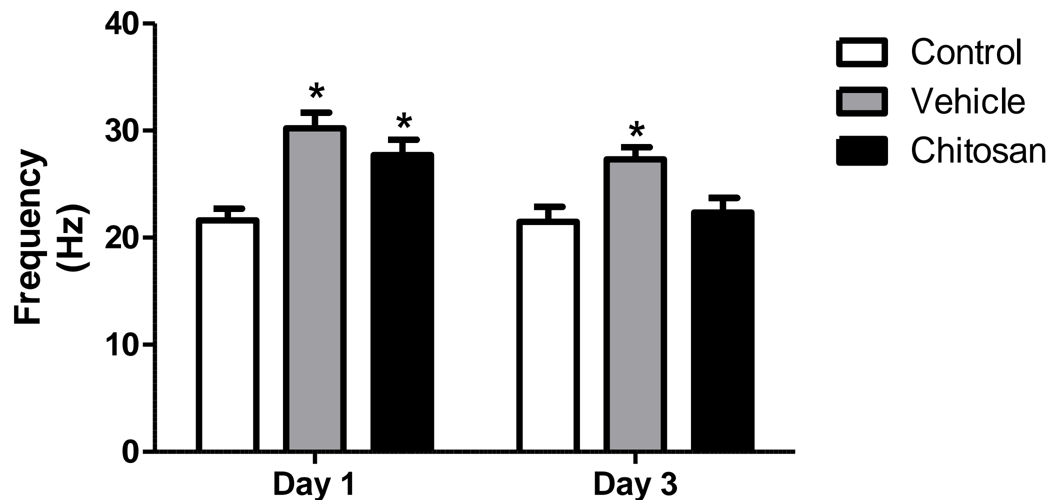

Figure 4.7 Effect of chitosan treatment on effective frequency 50 of TA muscle at day 1 and day 3 post-injury. *, $p < 0.05$, significant difference from control.

To further determine whether chitosan treatment affected the sensitivity of injured muscle to stimuli, EF_{50}, which is the stimulus frequency eliciting 50% of peak force, was calculated. At day 1 post-injury, the EF_{50} significantly increased in both vehicle-treated (30.22 ± 1.46, $p<0.05$) and chitosan-treated groups (27.71 ± 1.45, $p<0.05$) compared with that of the control (21.61 ± 1.12). Interestingly, however, this increased EF_{50} value was returned close to control only in the chitosan-treated group (22.36 ± 1.36) but remained elevated in the vehicle-treated group (27.33 ± 1.12, $p<0.05$) at day 3 post-injury.

4.3 Histological Studies

Histological studies were performed to compare the morphology of injured myofibers and the level of inflammation as well as the effect of chitosan on these parameters.

Figure 4.8 (A-F) showed the H&E staining of TA muscles from both the vehicle-treated and chitosan-treated groups as well as those of the control groups. At day 1 post-injury, there was an increase in the number of infiltrating inflammatory cells and necrotic fibers in the damaged area. By day 3 post-injury, this influx was progressively increased in muscles from both injury groups. There was no visible difference in the degree of inflammation between the vehicle-treated and chitosan-treated groups at both time points.

To quantify the protective effect of chitosan treatment on skeletal muscle damage after injury, the area of damaged tissues was analyzed. At both day 1 and day 3 post-injury, both vehicle-treated and chitosan-treated groups showed no significant difference in percentage of damaged area compared to control group (Fig. 4.9). No significant difference in damaged area between vehicle-treated and chitosan-treated group was observed at both time points. The average percent of damage area in vehicle-treated and chitosan-treated groups were 15.41 ± 1.70 and 16.41 ± 2.42 %, respectively, at day 1 post-injury, and were 33.98 ± 6.00 and 29.53 ± 2.06 %, respectively, at day 3 post-injury.

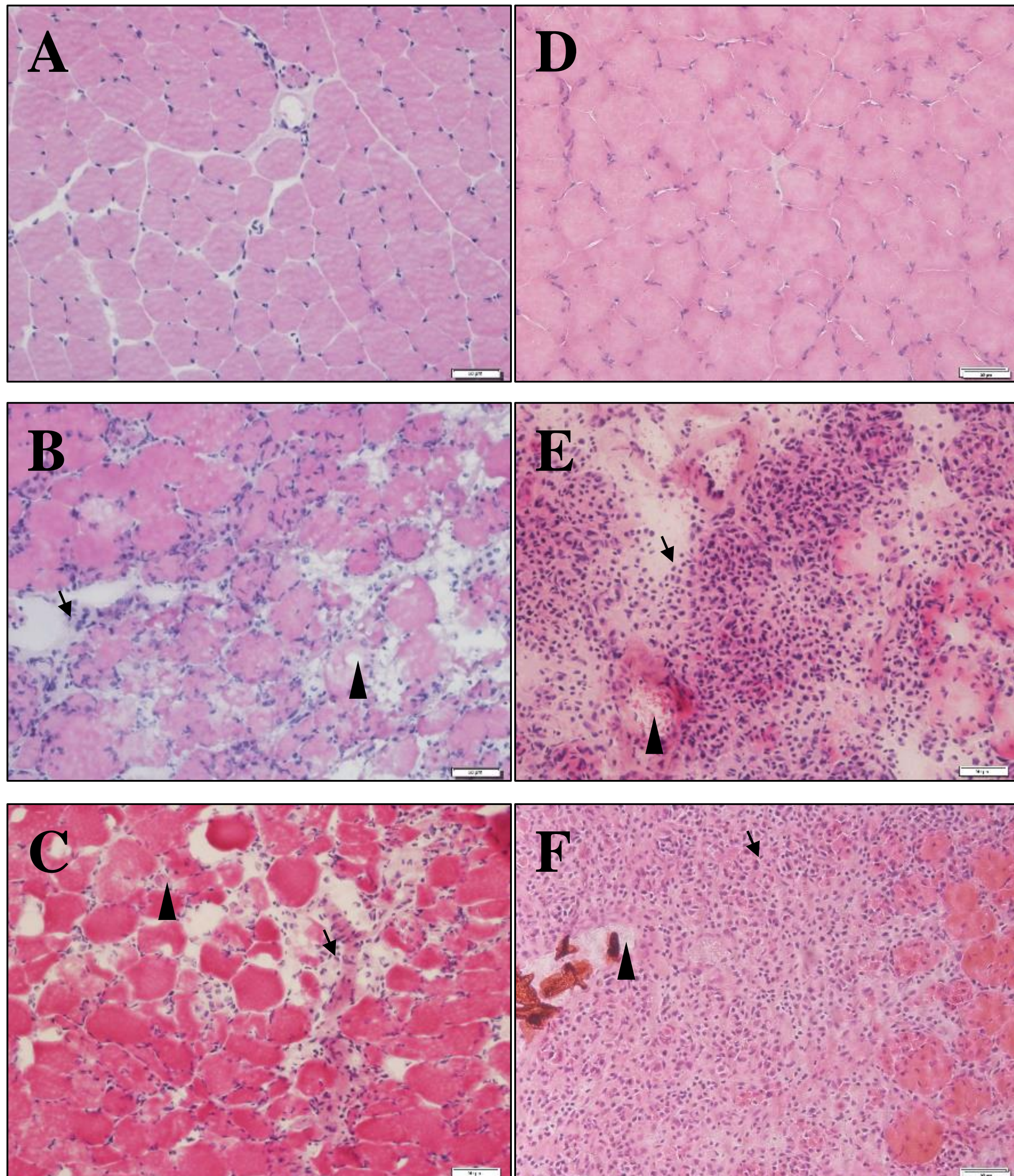

Figure 4.8 Representative of histology cross section of TA muscle stained with hemotoxylin and eosin (200x magnification). (A) Control day 1 post-injury. (B) Vehicle-treated day 1 post-injury. (C) Chitosan-treated day 1 post-injury. (D) Control day 3 post-injury. (E) Vehicle-treated day 3 post-injury. (F) Chitosan-treated day 3 post-injury. Arrow indicated infiltrating inflammatory cells. Triangle showed myofiber undergoing necrosis.

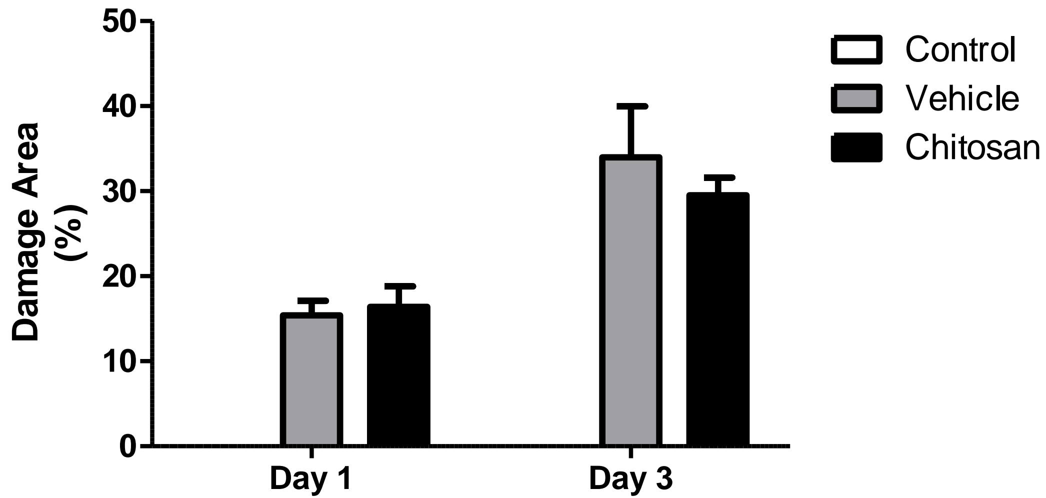

Figure 4.9 Effect of chitosan treatment on skeletal muscle damage area at day 1 and day 3 post-injury. Percentage damage area was calculated as the cross-sectional area of muscle damage over the total area of TA muscle.

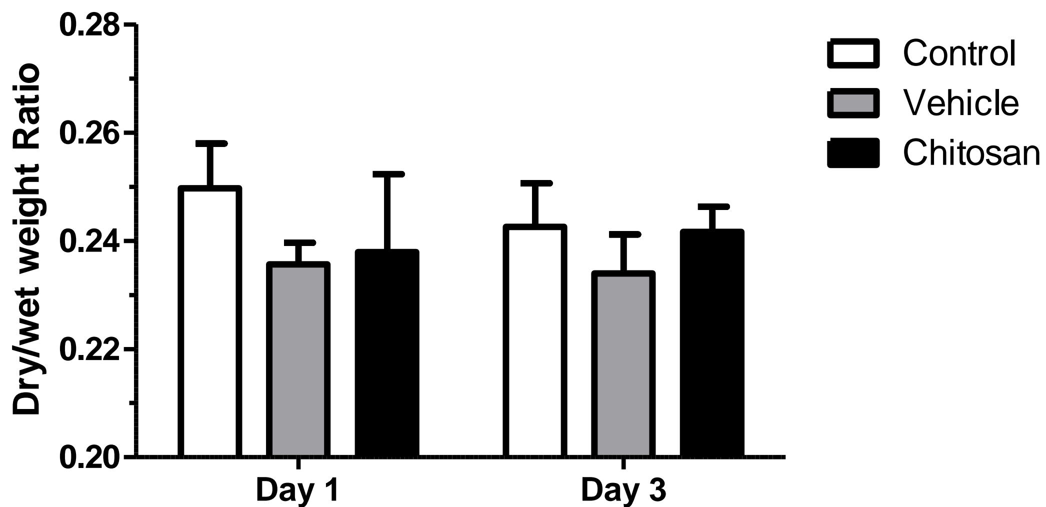

Figure 4.10 Effect of chitosan treatment on wet weight to dry weight ratio of TA muscle at day 1 and day 3 post-injury.

In addition, the muscle wet weight to dry weight ratio was measured as a gross marker of edema during the inflammatory response to injury. There was no statistical significance in muscle wet weight to dry weight ratio between the vehicle-treated and chitosan-treated groups at both day 1 and day 3 post-injury (Figure 4.10).

4.4 Inflammatory cytokine expression

The inflammatory response plays an essential role during skeletal muscle regeneration. To further determine the effect of chitosan treatment on the attenuation of the inflammatory response following injury, mRNA expression levels of both pro- and anti-inflammatory cytokines were evaluated at day 1 and day 3 post-injury.

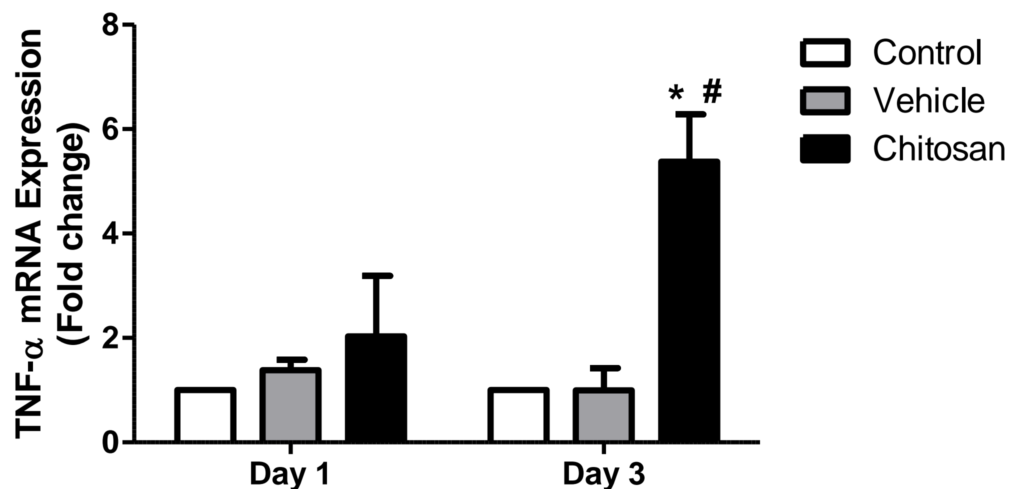

Figure 4.11 Effect of chitosan treatment on relative mRNA expression level of pro-inflammatory cytokines TNF-α of injured TA muscles at days 1 and 3 post-injury. * $p < 0.05$, significant difference from control group. # $p < 0.05$, significant difference from vehicle group.

The mRNA expression level of TNF-α and IL-10 was measured as pro-inflammatory markers of injured TA muscles. At day 1 post injury, the level of TNF-α mRNA was slightly elevated in both chitosan-treated (~2.0 fold) and vehicle-treated groups (~1.4 fold), although no significant difference was detected, as compared with the control group (Fig. 4.11). At day 3 post-injury, however, these values was further increased only in the chitosan-treated group ($p<0.05$) but remained unchanged in the vehicle-treated group as compared with the control.

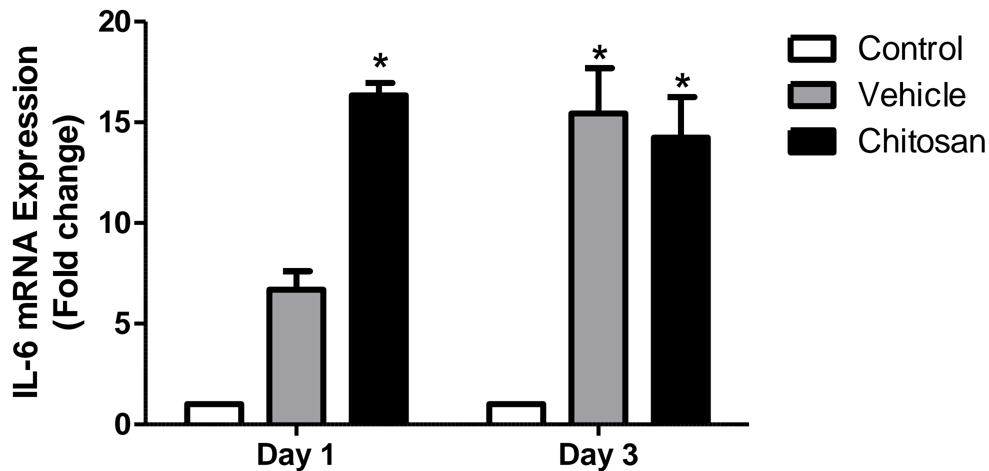

Figure 4.12 Effect of chitosan treatment on relative mRNA expression level of pro-inflammatory cytokines IL-6 of injured TA muscle at days 1 and 3 post-injury. * $p < 0.05$, significant difference from control group.

Figure 4.12 showed significant increases in the relative IL-6 mRNA expression level of injured TA muscles compared with the control. The expression level of IL-6 mRNA in vehicle-treated and chitosan-treated groups were increased by ~6.7 and ~16.3 fold, respectively, at day 1 post-injury. These values, however, remained elevated by day 3 post-injury, with the level in vehicle-treated group increased even further to ~15.4 fold while the chitosan-treated group remained unchanged.

The relative expression level of TGF-β and IL-10 were measured as anti-inflammatory markers in the present study. The relative mRNA expression of IL-10 was increased significantly in chitosan-treated (~35.7 fold; $p<0.05$) and vehicle-treated group (~19.5 fold; $p<0.05$) compared with the control at day 1 post-injury (Fig. 4.13). These values, however, remained elevated a day 3 post-injury.

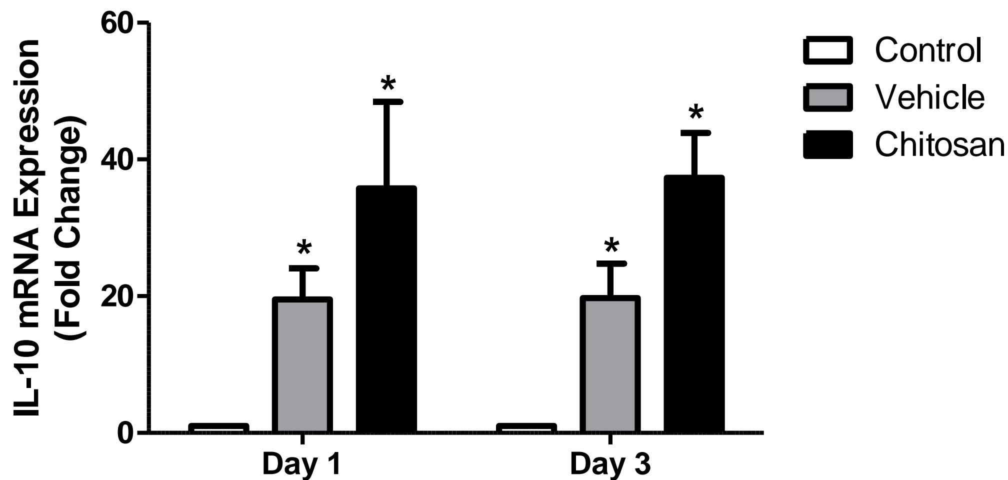

Figure 4.13 Effect of chitosan treatment on relative mRNA expression level of anti-inflammatory cytokines IL-10 of injured TA muscle at days 1 and 3 post-injury.* $p < 0.05$, significant difference from control group.

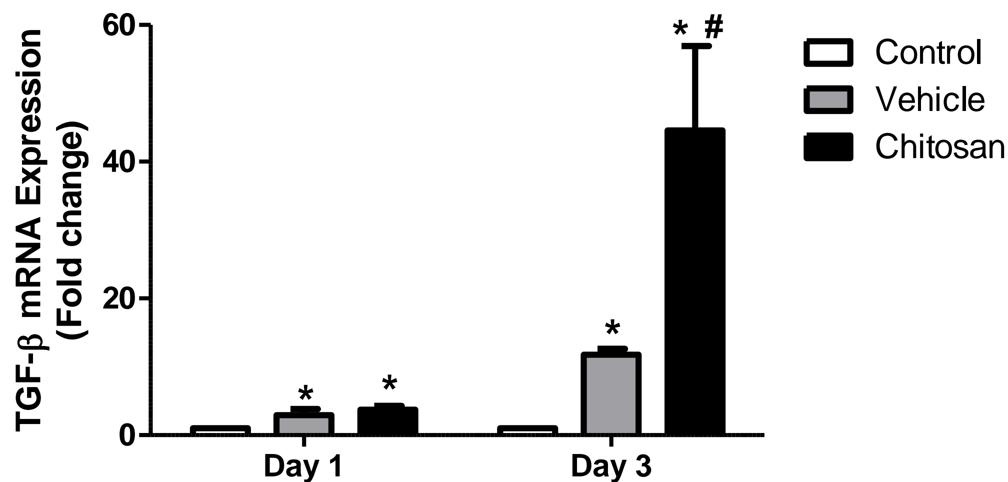

Figure 4.14 Effect of chitosan treatment on relative mRNA expression level of anti-inflammatory cytokines TGF-β of injured TA muscles at days 1 and 3 post-injury. * $p < 0.05$, significant difference from control group. # $p < 0.05$, significant difference from vehicle group.

At day 1 post-injury, the relative mRNA expression of TGF-β was significantly higher in both chitosan-treated (~3.7 fold; $p<0.05$) and vehicle-treated groups (~2.9 fold; $p<0.05$) than that of the control (Fig. 4.14). At day 3 post-injury,

however, these values was increased ($p<0.05$) further in both two groups, with higher ($p<0.05$) TGF-β mRNA expression was observed in the chitosan-treated group.

CHAPTER V
DISCUSSIONS

In this study, we investigated the effect of dietary chitosan supplement on the inflammatory response and muscle functional recovery after cardiotoxin-induced injury. Our data revealed that chitosan treatment could modulate the inflammatory response but had minimal effect on muscle contractile function after cardiotoxin-induced injury. A detailed discussion of these and other major findings follow.

5.1 Chitosan treatment had no effect on body and muscle weights after cardiotoxin-induced injury

Dietary chitosan supplement has been reported to help reduce body weight (Mhurchu et al, 2005). In this study, although the average body weight of animals in the chitosan-treated group was lower than in other groups at both day 1 and day 3 post-injury, the difference was not statistically significant. This finding was consistent with some previous reports (Mhurchu et al, 2005) but not the other (Zhang et al, 2012). This discrepancy in results between studies is possibly due to the differences in molecular weight of chitosan. High-molecular weight chitosan was reported to promote higher lipid excretion with fecal matter than low-molecular weight chitosan employed in this study.

In addition, we found that TA muscle weights were similar among groups. At day 1 post-injury, the TA weight of both vehicle-treated and chitosan-treated groups was higher, although not reached a significant level, than that of the control. By day 3 post-injury, however, the muscle weight of the vehicle-treated and chitosan-treated groups had slightly decreased below the level of control group. The reason for this reduction is not known but could be due to a removal of damaged cellular debris following injury as observed in our histological study.

5.2 Chitosan treatment had minimal effect on muscle contractile function after cardiotoxin-induced injury

To determine the effect of chitosan treatment on muscle contractile function, a single twitch and isometric tetanic tension were measured. As expected, our result showed that the isometric twitch tension in both the vehicle-treated and chitosan-treated groups was significantly lower than that of the control group at day 1 post-injury. This could be due to a direct effect of cardiotoxin injection since the TA weight was unaffected after injury. Interestingly, this decline in force was recovered to normal control only in the chitosan-treated group, but not the vehicle-treated group, by day 3 post-injury. This result suggests that chitosan treatment may help restore contractile function by day 3 post-injury. Since neither time to peak nor half-relaxation time was altered in both injury groups, thus the improvement of recovery force observed in this study was due to other factors instead of alterations in rates of calcium release and re-uptake by the sarcoplasmic reticulum.

Despite this, we also found that the relative tetanic force, a functional index of muscle injury, was significantly decreased in both injury groups at day 1 post-injury. In the chitosan-treated group, however, there was a trend for the contractile force to return close to normal by day 3 post-injury. This finding suggests that chitosan treatment may facilitate the restoration of contractile force following cardiotoxin-induced injury.

In corresponding with a reduction in tetanic force, there was a rightward shift of the force-frequency curve in both chitosan-treated and vehicle-treated groups as compared to the control at day 1 post-injury. This finding suggests the impairment of contractile function following cardiotixin-induced injury. Interestingly, however, the shift of the force frequency curve was better prevented, although not statistical difference, in the chitosan-treated group, by day 3 post-injury. This result suggests that chitosan treatment could have an impact on the force-generating capacity of damaged muscle fibers, suggesting a damaging myofibers.

To further delineate the possible mechanism responsible for this attenuation, EF_{50} was measured to reflect a change in sensitivity to stimulus. As expected, our result showed that the EF_{50} significantly increased in muscles from both the vehicle-treated and chitosan-treated groups at day 1 post-injury, suggesting a

decrease in sensitivity of skeletal muscle to stimulus. At day 3 post-injury, however, the increased EF_{50} was recovered only in the chitosan-treated group. Since the injured TA muscle did not atrophy in the present study; thus it is possible that chitosan could attenuate cardiotoxin-induced contractile dysfunction by modulating the sensitivity of myofibers to stimuli (e.g. the affinity of the thin regulatory proteins for Ca^{2+}, the actomyosin cross-bridge kinetics) which in turn directly or indirectly impact on contractile function.

During the inflammatory phase of skeletal muscle regeneration, neutrophils and macrophages infiltrating the injured muscle tissue can increase ROS production, resulting in additional damages to the skeletal muscle and negatively impact muscle functions (Pierce et al, 2007). Given that chitosan have both anti-inflammatory and anti-oxidative effects, it is likely that chitosan could prevent more damage to the muscle tissue by limiting ROS production during inflammation, thereby improving functional recovery in chitosan group. However, a further study is needed to confirm this possibility.

5.3 Chitosan treatment increased the pro-inflammatory and anti-inflammatory cytokines expression after injury

Histological studies revealed normal healthy myofibers with polygonal shape in the control group. In the injury groups, however, the muscle tissues were undergoing necrosis and inflammatory cells began to infiltrate the sites of injury starting at day 1 post-injury. At day 3 post-injury, this influx was progressively increased and accumulated surrounding all the damaging area. This finding was concomitant with a slightly increase of TA muscle wet weight, probably due to the presence of edema during the inflammatory response following injury.

There is increasing evidence that the inflammatory response is essential for efficient muscle regeneration after injury (Yin et al, 2013). Typically, a complete inflammatory response after injury requires two distinct subsets of inflammatory macrophage populations in order to illicit an acute inflammatory response that is needed for proper skeletal muscle regeneration. The first macrophage population is the pro-inflammatory macrophage or designated as M1 macrophage which secretes pro-

inflammatory cytokines such as TNF-α and IL-6. The second macrophage population is the anti-inflammatory macrophage or designated as M2 macrophage. This population of macrophage secretes anti-inflammatory cytokines including TGF-β and IL-10 which play a key role in resolution of inflammation.

One of the most important pro-inflammatory cytokines is TNF-α. The high level of TNF-α has been shown to stimulate myogenic proliferation (Saclier et al, 2013). In this study, we found that chitosan treatment significantly increased expression level of TNF-α in injured muscle at day 1 and day 3 post-injury, compared with that of both control and vehicle groups. The reason behind the up-regulation of TNF-α expression is currently unknown. Nevertheless, a previous study reported that chitosan could act as an immunomodulator (Oliveira et al, 2012). Hence, the presence of a high level of TNF-α after chitosan treatment in this study may facilitate the inflammatory reaction against injury. This in turn speeds up skeletal muscle recovery.

In this study, we also found that chitosan treatment displayed a significant increase in the mRNA expression of IL-6 in injured muscle at both day 1 and day 3 post-injury, with the chitosan-treated group showing a greater increase in the IL-6 mRNA expression than that in the vehicle-treated group. Since IL-6 is known to be responsible for facilitating macrophage infiltration to the site of injury (Saclier et al, 2013), it is very likely that the increased IL-6 expression could influence on later stages of inflammation and the subsequent skeletal muscle regeneration. Collectively, the increased relative mRNA expressions of both pro-inflammatory cytokines at day 1 post-injury in the chitosan-treated group over that of the vehicle-treated group may enhance the rate at which inflammatory cells infiltrate to injury site, thereby allowing for more rapid phagocytosis of injured tissues, which may affect muscle contractile performance.

Additionally, chitosan treatment significantly increased the relative mRNA expression of IL-10, an anti-inflammatory cytokine, which could inhibit the pro-inflammatory response including production of pro-inflammatory cytokines (Ouyang et al, 2011; Hofmann et al, 2012). Because one of the most important function of IL-10 is the promotion of fusion and maturation of myotubes (Brown et al, 2012); therefore, the up-regulation of IL-10 expression observed in our study may give rise to a small improvement in contractile performance at day 3 post-injury.

TGF-β is another anti-inflammatory cytokine released by M2 macrophage population. During inflammatory period, TGF-β is responsible for inhibiting action of pro-inflammatory cytokines, stopping myoblasts proliferation and promoting expressions of myogenic maturation and myotube formation (Saclier et al, 2013). It is also responsible for repairing extracellular matrix and basal membrane via fibroblast stimulation at a later stage of skeletal muscle remodeling. Our data showed that chitosan treatment had a strong effect on TGF-β mRNA expression on both day 1 and day 3 post-injury. Surprisingly, despite the overwhelming increase in TGF-β mRNA expression, there was no noticeable change in the contractile force. The reason for the lack of effect is not known, but there is a possibility that any stimulatory effect of TGF-β could be observed at later stages of muscle regeneration.

It should be noted, however, that the up-regulation of mRNA of both pro-inflammatory and anti-inflammatory cytokines does not always result in an increase in activity and protein expression; therefore this data should be interpreted with caution. Other quantitative measures, such as western blot analysis, are required to verify this actual effect.

Taken together, our result revealed that chitosan treatment increased expression of both pro-inflammatory and anti-inflammatory cytokines with in favor of the latter and this could affect the inflammatory response during skeletal muscle repair. However, this beneficial effect of chitosan might not directly translate to enhanced functional recovery after cardiotoxin-induced injury.

5.4 Limitation of the study

There are several limitations in the present study. Firstly, neither the level of chitosan nor the oxidative markers were measured in this study; therefore it is not known whether the dose of chitosan used in this study is optimal to provide a protection against oxidative injury induced by cardiotoxin injection. Nevertheless, previous studies have proven that chitosan at concentration of 200 mg/kg body weight, which were similar to this experiment, had detoxifying effects on chronic hepatic injury induced by carbon tetrachloride (Jeon et al, 2003). Secondly, the experiment focused on the inflammatory stage of skeletal regeneration, which occurred during the

first 3 days after injury. However, an extending of the experimental period beyond this time point to encompass the repair or remodeling phase at week 1 or week 2 post injury may provide a more meaningful and relevant data in term of muscle functional recovery. Thirdly, the damaged area of each sample was only 15% and 30% of total cross section area on day 1 and day 3 post-injury respectively. This could present a problem in selecting samples with appropriate injury level for further studies where whole muscle was not needed. It was possible that some samples selected may only contain area with partial or even no injury present, thus affecting the results. Lastly, an addition of a positive control group to the experimental model would help in making comparison of effectiveness of chitosan treatment during inflammation period with commercially available anti-inflammatory drugs or other treatments.

CHAPTER VI
CONCLUSIONS

Based on the results of this study, it could be concluded that

1. Cardiotoxin injection caused significant decreases in isometric muscle twitch and tetanic tension and a rightward shift of the force-frequency curve as compared to the control at both day 1 and day 3 post-injury.

2. These changes were associated with muscle inflammation and up-regulations of pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (TGF-β, IL-10) cytokines mRNA levels at the same time points.

3. One week of chitosan treatment at a dose of 200 mg/kg significantly increased TNF-α and TGF-β mRNA expressions but had a small effect in the attenuation of cardiotoxin-induced contractile dysfunction.

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APPENDIX

1. RT-PCR Fold Induction Calculation

1.1 Average C_t values for all gene replicated (TNF-α, TGF-β, IL-6, IL-10 and GAPDH) for each group

1.2 Calculate ΔC_T value: $\Delta C_T = C_{T\ target} - C_{T\ reference}$

$C_{T\ target}$ is target genes (e.g. TNF-α) and $C_{T\ reference}$ is housekeeping gene (e.g. GAPDH)

1.3 Calculate $\Delta\Delta C_T$ values: $\Delta\Delta C_T = \Delta C_{T\ experiment} - \Delta C_{T\ control}$

$\Delta C_{T\ experiment}$ is ΔC_T value from experiment group, $\Delta C_{T\ control}$ is ΔC_T value from control group

1.4 Calculate fold change: $2^{(-\Delta\Delta CT)}$

2. Muscle cross-sectional area and damaged area measurement

2.1 In ImageJ (version 1.49U), open the histological image of a sample

2.2 Use Handfree selection tool, outline the total cross-sectional area of the sample

2.3 Select Analyze, then Measure. Record the result under Area column

2.4 Use Handfree selection tool, outline the damaged cross-sectional area of the sample

2.5 Select Analyze, then Measure. Record the result under Area column

2.6 Calculate the percentage of damaged area: Damaged area/Total area x 100

2.7 Repeat step 2.1 – 2.6 with all other images from the same sample and aggregate the total percentage of damaged area.

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