**HEAT SHOCK DOES NOT ATTENUATE
LOW FREQUENCY FATIGUE**

by

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Abstract

Whole-body hyperthermia or heat shock confers protection to myocardial contractility against reperfusion-induced injury. The purpose of this study was to determine whether heat shock could provide similar protection for skeletal muscle against low frequency fatigue. Male Sprague-Dawley rats (6 rats/group) were heat-shocked at 41.5 degrees Celsius for 15 minutes either 24 hours or four days prior to fatiguing stimulation to compare the contractile responses of the plantaris to those of a non-heated group. Both 24 hours and four days after heat shock, the 72-kDa heat shock protein (hsp72) was elevated above control levels ($p < 0.05$). Heat shock elevation of hsp72 provided no benefit of improved resistance to fatigue or post fatigue force recovery above control responses, and in fact delayed the recovery of force. This study does not support the use of heat shock therapy to improve skeletal muscle performance.

Keywords

heat shock proteins; rat; skeletal muscle; low frequency fatigue; contractile properties; plantaris muscle

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1. Introduction

The topics covered in the following review of literature include the characteristics of the heat shock proteins and their proposed function in cellular protection, the theory of muscle contraction, the description and proposed etiologies of low frequency muscle fatigue, and the potential role of the heat shock proteins in minimizing low frequency fatigue.

1.1. The Heat Shock Proteins

1.1.1. Classification Of The Heat Shock Proteins

In response to a variety of stressors, organisms manufacture a set of proteins called heat shock proteins (hsps) (for review see Locke, 1997; Tomasovic, 1989; Welch, 1992). The hsps are characteristically elevated in response to increases in temperature or heat shock, and have been classified by molecular mass after isolation by denaturing polyacrylamide gel electrophoresis. They include the 28-kD, 58-kD, 72-kD, 73-kD, 90-kD, and 110-kD hsps (Welch and Suhan, 1986; Mizzen et al., 1989). The most common heat shock proteins are those having a molecular weight of approximately 70,000 Daltons, and are, thus, said to belong to the hsp70 family. The two most prominent isoforms are the hsp73 member, which is constitutively expressed (Sorger and Pelham, 1987), and the hsp72 form which is strongly inducible and considered to be an indicator of physiological stress (Welch and Suhan, 1986). The identification and characterization of the heat shock proteins have revealed that this class of proteins is highly conserved among various types of organisms, both prokaryotic and eukaryotic. Extensive homology exists between well defined clusters of amino acids of human hsp70 and those of *Drosophila*, *Xenopus*, yeast,

and dnaK from *Escherichia coli*, implicating an evolutionary conservation of functional domains in this protein (Mues et al., 1986).

1.1.2. The Heat Shock Response

The heat shock response was first observed in *Drosophila*. It was noted that elevations in temperature altered the puffing patterns of salivary gland chromosomes (Ritossa, 1962), which indicated variations in chromosome activity and increased transcription of particular genes. Heat shock will result in significant accumulation of heat shock proteins only after a threshold temperature is reached or exceeded (between 41 and 42 degrees Celsius in rats) (Hutter et al., 1994; Locke and Tanguay, 1996).

The heat shock response is now more commonly referred to as the “stress response” since it can be induced by many other conditions. Ischemia (Marber et al., 1993), ATP depletion (Van Why et al., 1994), denatured proteins (Ananthan et al., 1986), oxidative stress (Salo et al., 1991), chronic stimulation (Ornatsky et al., 1995), adenoviral infection (Nevins, 1982), and exercise (Locke et al., 1990) are all capable of activating the stress response.

The activation of the stress response begins with oligomerization, nuclear translocation and DNA-binding of heat shock transcription factor(s) or HSF(s) (Sarge et al., 1993; Baler et al., 1993). DNA sequence which is bound by the activated HSFs, the heat shock element or HSE, consists of inverted repeats of a 5 base pair recognition unit, NGAAN, and is located in the promoter region of the heat shock gene (Pelham, 1982; Perisic et al., 1989). Two members of the HSF family, HSF1 and HSF2, have been identified. HSF1 is activated by heat shock, whereas HSF2 is activated by hemin and may play a role in differentiation and development (Sistonen et al., 1994). HSF activation

is determined in a laboratory setting by HSF bound to HSE. HSF activation after heat shock appears to be immediate, by one hour after heat shock HSF activation is considerably reduced, and by two hours undetectable (Locke and Tanguay, 1996, Locke et al., 1995a).

There are three factors which account for the rapid and preferential translation of the heat shock proteins. First, the HSP70 gene is transcribed as an uninterrupted mRNA and therefore, splicing of the HSP mRNA is not required (Wu et al., 1985). Secondly, since mRNA processing is impeded during heat shock due to faulty ribonucleoprotein assembly (Mayrand and Pederson, 1983), rapid accumulation of the heat shock proteins following stress is favoured over other cellular proteins which require further processing. Finally, during HSP70 mRNA translation, pre-existing control mRNAs are found to be highly undertranslated (Ballinger and Pardue, 1983), which enhances the selective translation of the heat shock proteins. After heat shock, the HSP70 mRNA can be detected immediately, remains elevated for at least two hours, and returns to control levels approximately six to eight hours post heat shock (Locke et al., 1995a; Kiang et al., 1994). While the heat shock proteins are not synthesized during heat treatment, they are produced during recovery from hyperthermia (Fujio et al., 1987). The inducible hsp70 protein has been detected two to three hours post heat shock (Currie and White, 1983; Li and Werb, 1982; Kiang et al., 1994), and can remain above baseline levels after four days (Kiang et al., 1994, Karmazyn et al., 1990).

Heat shock gene expression appears to be autoregulated by the hsp70, itself. Under denaturing conditions, hsp70 remains bound to proteins that have been rendered insoluble and a normal stress response is initiated (Beckmann et al., 1992). Baler and colleagues (1992) also found that when cells were pretreated with a protein inhibitor prior

to heat shock, resulting in reduced amounts of newly synthesized proteins to which hsp70 could bind, HSP70 gene expression was suppressed. Given these findings, Beckmann and colleagues (1992) and Baler and colleagues (1992) proposed a model of heat shock gene regulation. It is known that hsp70 is able to bind HSF (Baler et al., 1992). During times of metabolic stress or protein synthesis, hsp70 binds to any unfolded proteins produced. The “free” levels of hsp70 available to bind with HSF would be lower, permitting HSF to oligomerize and bind to HSE in the activation of the stress response. In the absence of stress, when there are lower levels of unfolded proteins to which hsp70 will bind, the so-called “free” levels of hsp70 will rise and hsp70 will bind to HSF preventing its activation. Therefore, the extent to which a stress response is initiated is controlled by a feedback mechanism that responds to the “free” levels of hsp that are available to bind HSF.

1.1.3. The Function Of Hsps

The exact function of stress proteins is not currently known. However, their roles as cellular chaperones for protein synthesis have been implicated (for review see Gething and Sambrook, 1992). The primary stress proteins in *E. coli*, homologous to those in mammals, have been shown to interact with one another in a sequential-type action to chaperone a folding polypeptide (Langer et al., 1992). Members of the hsp70 family are able to bind nascent polypeptide chains (Beckmann et al., 1990) and mature proteins that have become unfolded (Palleros et al., 1991). Not only do the 70-kDa hsps bind polypeptides, but they have also been shown to assist polypeptide trans-location into target organelles for further processing (Chirico et al., 1988; Deshaies et al., 1988;

Murakami et al., 1988). These observations indicate a role for the heat shock proteins as chaperones in the course of maturation of cellular proteins.

As noted above, the presence of abnormal or misfolded proteins in the cell actually triggers the accumulation of heat shock proteins (Goff and Goldberg, 1985). Sherman and Goldberg (1992) have shown that hsps act as essential components in the breakdown of denatured proteins possibly by binding to and marking those proteins for degradation. It is thought that in response to stress-induced denaturation of proteins, hsps will bind to the unfolded proteins, thereby acting to repair or remove abnormal protein structures and prevent inappropriate aggregations with key cellular proteins (Tomasovic, 1989; Li et al., 1995).

Hsp72 and hsp73 colocalize within the nucleus and nucleolus in the initial periods of recovery after heat shock, and during the later periods relocate to the cytoplasm (Welch and Feramisco, 1984; Welch and Suhan, 1985; Welch and Suhan, 1986; Brown et al., 1993). Given that Mayrand and Pederson (1983) found reduced ribonucleoprotein assembly upon heat shock, it is possible that the hsps could be involved in the inhibition of the nucleolar activity noted during heat shock or in its recovery from some heat-induced damage (Welch and Suhan, 1985). When the hsps relocate to the cytoplasm later in recovery, they have been found associated with the ribosomes (Welch and Suhan, 1986). Since these structures may be very sensitive to denaturing agents, the hsps may be responsible for ensuring that the cell returns to normal levels of protein translation post heat shock (Welch and Suhan, 1986).

Through all of the above processes, it may be that hsps are responsible for the restoration of normal protein balance in the stressed cell (Welch and Suhan, 1986).

1.1.4. The Protective Role Of The Stress Proteins

Given the findings that members of the hsp70 family may help to preserve cell function in the stressed cell, investigators have further studied their “protective” role. The exposure of cell lines or whole organisms to heat shock has been shown to confer resistance to subsequent treatment with lethal temperatures (Dean and Atkinson, 1983; Weshler et al., 1984; Gerner and Schneider, 1975). This so-called “thermotolerance” is probably acquired through the functions of hsp72, itself, since cells expressing elevated levels of the inducible hsp72 protein show protection against high temperatures (Heads et al., 1994; Angelidis et al., 1991; Johnston and Kucey, 1988; Riabowol et al., 1988).

Hsp-related protection is further supported by the phenomenon of “cross-tolerance”, whereby the production of hsps by one stressor protects against a subsequent, but different form of assault (Marber et al., 1993; Locke et al., 1995b). In particular, elevated levels of the inducible hsp72, either expressed constitutively or invoked by heat shock, are associated with improved myocardial contractile recovery following ischemia and reperfusion (Karmazyn et al., 1990; Currie et al., 1988; Plumier et al., 1995). It has been suggested that hsp72 acts to salvage denatured proteins produced by the stress of ischemia/reperfusion by facilitating their refolding, chaperoning them to a degradative system, or simply binding them to prevent inappropriate aggregation between these damaged proteins and otherwise viable ones (Hightower, 1991; Marber et al., 1993). Unlike is found for cardiac tissue, a protective role for skeletal muscle has not been consistently shown. Garramore and collaborators (1994) observed that prior heat shock reduced ultrastructural indices of ischemic injury in skeletal muscle. Although these investigators showed that prior heat shock resulted in significantly greater recovery of

creatine phosphate (CP) levels after ischemia, the CP content was still substantially less than that noted under basal conditions (Garra more et al., 1994). Ianuzzo and colleagues (1995) found that heat shock pretreatment did not protect latissimus dorsi muscle against damage from surgery and subsequent ischemia/reperfusion. No investigations, as yet, have examined further the protective role of the heat shock proteins on skeletal muscle contractile function.

Therefore the role of hsp72 in maintaining cardiac versus skeletal muscle function under stressful conditions is unclear and requires further study.

1.2. Muscle Contraction

1.2.1. Mechanics of Contraction

The contractile proteins of striated or skeletal muscle are organized into functional units, called sarcomeres, that contain a repeating striation pattern. The striations observed are a consequence of the overlapping design of the thick filaments, composed primarily of the protein myosin, and the thin filaments, containing mainly actin (Squire et al., 1990). Huxley and Niedergerke (1954) in an early paper proposed that force is generated at individual points of interaction between the filaments. These points of interaction are referred to as crossbridges. The “heads” of the myosin molecules have been thought to individually act in causing force by a rowing-like action against actin. Brenner (1990) proposed that during an isometric contraction, when the muscle is not allowed to shorten, there is an elastic deformation of the crossbridge producing strain or force. Lieber and Bodine-Fowler (1993) demonstrated that the highest isometric force occurs at so-called optimal sarcomere length (L_o) or rest length when the highest overlap between the filaments, and thus, the greatest number of crossbridges occurs.

Based on ultrastructural evidence of the myosin head, it is known that myosin acts as the “motor” for contraction, containing both an actin binding site for crossbridge attachment and an ATPase (adenosine triphosphatase) enzyme for the utilization of ATP energy in the generation of force (Rayment et al., 1993). The ATP hydrolysis cycle has been proposed to link the transition between a weak-binding state (non-force generating) and a strong-binding state (force generating) of the crossbridge (Brenner, 1990).

1.2.2. The Activation of Muscle Contraction

The activation of muscle contraction is the result of motoneurone innervation of muscle fibres. The signal is transmitted as a propagating action potential along the muscle fibre membrane, the sarcolemma. At regular intervals the sarcolemma is reflected into the interior of the fibre forming transverse tubules (T-tubules) to allow the signal to reach the deepest regions of the fibre (Franzini-Armstrong and Porter, 1964). The sarcoplasmic reticulum (SR), a large calcium storage network, forms cisternae on either side of the T-tubules at junctions called the triad (Franzini-Armstrong, 1980). At these junctions, on the T-tubule membrane, there is a dihydropyridine (DHP) receptor, believed to be the voltage sensor in the detection of the propagating action potential (Adams et al., 1990). Cytoplasmic regions of the DHP receptor have been shown to be important in the transmission of the action potential signal to induce calcium release by the SR (Tanabe et al., 1990). The calcium release channel of the SR is a pore-type channel called the ryanodine receptor (Imagawa et al., 1987) and when opened, allows large amounts of calcium to enter the sarcoplasm.

Calcium release is critical for muscle contraction due to its ability to regulate actin for myosin binding. Associated with actin are two sets of regulatory proteins, troponin

and tropomyosin. Calcium binding to troponin leads to conformational changes which cause movement of the troponin and tropomyosin away from their inhibitory positions on actin (Lehman et al., 1994; Tao et al., 1990). This movement is thought to expose the myosin-binding site on actin so that the myosin head can attach in a strong-binding formation and produce force.

Muscle relaxation occurs by removal of calcium from its troponin-binding site and is trans-located primarily into the sarcoplasmic reticulum catalyzed by the sarcoplasmic reticulum ATPase enzyme (MacLennan et al., 1985).

1.2.3. Excitation-contraction coupling

The functional connection between voltage sensing and calcium regulation of muscle contraction is referred to as excitation-contraction coupling (Westerblad et al., 1993). The calcium released in response to a single action potential produces force, called a twitch, with the peak twitch force referred to as P_t . A tetanus is the contractile response to a train of action potentials. The high frequency train does not allow for complete muscle relaxation between the stimuli, so that a “fusion” of contractions takes place, producing a summation of force. As the frequency of stimulation is raised, the force increases until a frequency is reached beyond which there is no further increase in tension [maximal tetanic tension (P_o)].

1.3. Muscle Fatigue

1.3.1. Definition And Description Of Muscle Fatigue

Muscle fatigue can be defined as the failure to maintain a given force output during sustained or repeated contractions (for review see Gibson and Edwards, 1985;

Fitts, 1994). Peak isometric twitch force (P_t) and tetanic force both decline in response to fatigue (Thompson et al., 1992b). Despite its extensive study, the mechanisms underlying muscular fatigue have yet to be clearly defined. The physiological changes which accompany fatigue are many and attempting to determine which of these are causal and how they interact *in vivo* in the decrement of contractile function is a complex task.

The decline in force with fatigue has been attributed to impairment at any of the sites involved in force production from the central nervous system (CNS) to actomyosin interaction. These include CNS fatigue (Davis and Bailey, 1997), reduced motoneurone firing rates (Bigland-Ritchie et al., 1986), neuromuscular transmission failure and reduced sarcolemmal action potential frequency (Krnjevic and Miledi, 1958), defects in excitation-contraction coupling (Gyorke, 1993), and metabolite effects on the contractile apparatus (Godt and Nosek, 1989).

1.3.2. Description of Low frequency fatigue

Low frequency fatigue (LFF) is characterized as a greater loss of muscle force at low frequencies than at high frequencies of stimulation, following fatiguing muscle contractions. The characteristic reduced force at low frequencies, in contrast to nearly complete recovery for high frequencies, has a very slow recovery of hours or sometimes days (Jones, 1996).

Defects in excitation-contraction coupling, specifically relating to calcium handling by the sarcoplasmic reticulum, have been implicated as a primary factor in the observed contractile dysfunction seen with low frequency fatigue. During fatigue, there is a decreased tetanic calcium transient, decreased rate of decay of the calcium transient, and

an increase in resting calcium (Gyorke, 1993). The characteristic reduced recovery of force at the low frequencies of stimulation is thought to be due to the reduced calcium release observed at all frequencies of stimulation in fatigued fibres (Westerblad et al., 1993). The sigmoidal relationship between intracellular calcium and developed force can explain the preferential effects of reduced levels of calcium on force. The sigmoidal relationship indicates that in response to reduced calcium, high frequency forces lying on the upper horizontal plateau portion of the curve would experience small changes in tension, whereas low frequencies lying on the steep portion of the curve would undergo relatively large changes in tension (Westerblad et al., 1993). Therefore, decreases in calcium release could account for the conditions associated with low frequency fatigue.

1.3.3. Structural injury as a causal factor in LFF

Jones (1996) suggested that the slow recovery in low frequency fatigue can be seen as a process of repair and regeneration of protein structure in the muscle fibre and excitation-contraction coupling mechanisms possibly damaged during fatigue.

Ultrastructural changes such as mitochondrial disruption (Nimmo and Snow, 1982) and disrupted sarcomere structure (Belcastro et al., 1988) have been found in response to whole-body exercise.

Structural changes to proteins involved in excitation-contraction coupling (i.e. sarcoplasmic reticulum channels, pumps, etc.) could result in the observed decrement in calcium release and resulting contractile impairment at low frequencies of stimulation (Westerblad et al., 1993). The failure in force production could then persist throughout recovery governed by the time required to return proteins to their native conformations

and normal function. As hsp's may be able to stabilize and promote the proper refolding of damaged proteins (Li et al., 1995), their role in recovering normal protein conformation and function in response to low frequency fatigue may be of use.

1.3.4. Metabolic Effects

Alterations in protein conformation could be due to metabolic changes produced during fatigue. Intense muscular activity results in cellular acidosis (Thompson et al., 1992a). Metzger and Moss (1987) demonstrated that decreasing pH leads to a reduction in maximum force production implicating acidosis as a causal factor in the reduction of force in fatigued fibres. Changes in the ionic environment can affect protein-protein interactions which may be critical in the production of force, such as the actin-myosin interaction. Changes in pH can also affect protein conformation by altering the charge of amino acid side groups and thereby disrupting electrostatic interactions that contribute to the conformational stability of the protein (Pace, 1990). Therefore, exercise-induced acidosis may affect both protein conformation and function of cellular structures involved in the activation of force such as SR channels, membrane-bound pumps, and the myofilaments. As the heat shock proteins may stabilize protein form and function secondary to acidosis (Tomasovic, 1989), they may play a role in maintaining force production in fatigued muscle fibres.

The calcium pool of the sarcoplasmic reticulum, which is the main source of calcium for skeletal muscle contraction, is sufficient to produce muscle damage, if allowed to leak into the cytoplasm (Byrd, 1992). Therefore, it is of interest whether the impairment in calcium handling is related to the muscle injury associated with low

frequency fatigue. Byrd (1992) suggested that the high resting level of cytoplasmic calcium, resulting from the fatigue-induced impairment in excitation-contraction coupling, could initiate degradation of skeletal muscle proteins by calcium-activated proteases. Belcastro (1993) confirmed that calcium-activated neutral protease (calpain) activity is indeed increased in skeletal muscle in response to exercise. Calpain has been shown to induce extensive proteolysis of the calcium release channel of skeletal muscle (Gilchrist et al., 1992). As well, Goodman (1987) found that agents leading to elevated calcium levels led to non-myofibrillar protein breakdown. And Chin and Allen (1996) provided evidence that non-fatiguing stimulation protocols in conjunction with increased intracellular calcium, actually led to reductions in low frequency force. Protease activation by high resting cytoplasmic calcium from fatiguing contractions may lead to microinjury of the contractile apparatus or to damage to sarcoplasmic reticulum channels. It has been suggested by Parkhouse (1988) that proteolysis is a highly selective process, initiated by “marking” of proteins for degradation. If the heat shock proteins can recognize and stabilize these marked proteins, they may protect them from protease action and thereby lessen some of the damage to force generating or calcium handling apparatuses.

During fatigue, damage to the calcium handling mechanisms could be caused by reactive oxygen species. Davies and colleagues (1982) reported increased free radical concentrations following exhaustive exercise. Oxyradicals can attack cellular membranes by lipid peroxidation thereby affecting permeability characteristics and ionic gradients, and can also induce cellular injury by modifying protein conformation (for review see Rice-Evans and Burdon, 1993; Davies and Goldberg, 1987). Specifically, it was noted that reactive oxygen species can damage sarcoplasmic reticulum structure by disrupting

SR calcium ATPase activities (Ishibashi et al., 1996) and calcium release (Brotto and Nosek, 1996). The result of such damage would be that decreased levels of calcium would be available for muscle contraction and forces at low frequencies would be primarily affected. Since high levels of antioxidants can inhibit low frequency fatigue (Reid et al., 1992), the role of oxyradicals in the development of fatigue is strongly implicated. Since the oxyradicals act by specific interactions with susceptible amino acids, affecting secondary and tertiary structure of the proteins (Rice-Evans and Burdon, 1993), hsps may play a role in stabilizing and/or facilitating the refolding of any proteins under oxyradical attack. Protection against such attack is a primary explanation for the efficacy of heat shock preconditioning against damage from ischemia/reperfusion in cardiac muscle (Karmazyn et al., 1990).

1.4. Purpose and Rationale

The purpose of this investigation was to examine whether elevated levels of hsp72 can protect skeletal muscle from the contractile dysfunction associated with low frequency fatigue, with contractility assessed prior to, during, and in recovery from a fatiguing protocol.

Given the protective role hsp70 may play in the cell, particularly with its potential ability to salvage and promote refolding of denatured proteins in response to stress (Hightower, 1991), it seems likely that hsp70 may also perform these functions in a fatigued muscle cell where structural damage to proteins may have taken place. Heat shock has been proposed to protect the myocardium against ischemia/reperfusion by countering the damaging effects of oxyradicals produced during reperfusion (Karmazyn et al., 1990). Exercise also results in the production of oxyradicals in skeletal muscle

(Davies et al., 1982) which may damage functional components involved in the production of force. Nosek (1996) is currently investigating the possible protective effects of hsp70 against oxyradical- and muscle fatigue-induced disruptions of calcium release. Therefore, an investigation of the possible protective role of hsp70 in skeletal muscle fatigue is of interest.

Since low frequency fatigue is long-lasting and, thus, most likely related to alterations in protein conformation, it was of interest in this study whether elevated levels of hsp72 can protect against such damage and the persistent loss of contractile force. If elevated levels of hsps are found to be associated with improved skeletal muscle performance, a possible role as an ergogenic aid may be indicated.

As well, the current investigation may serve to further identify the function(s) of the hsp72 protein. It has been determined recently that hsp72 levels are increased as a result of exercise training (Kelly et al., 1996). It has also been found that a training regimen can enhance resistance to fatigue (Duchateau and Hainaut, 1984; Fitts and Holloszy, 1977). By isolating the effects of hsps on contractility, this study may indicate whether hsps are one of the many variables that lead to the improved muscle performance seen with training.

1.5. Hypotheses

Hypothesis 1:

Since eight weeks of treadmill training in rats leads to both increased hsp72 levels and improved resistance to fatigue, it was of interest whether hsp72 was one of a number of variables leading to improved performance. Therefore, it was hypothesized that

elevation of hsp72 by heat shock 24 hours prior to fatiguing stimulation would provide improved resistance to fatigue.

Hypothesis 2:

Since the presence of increased amounts of hsp72 has been associated with improved recovery of force in cardiac muscle after ischemia/reperfusion, it was hypothesized that high levels of hsp72, induced by heat shock 24 hours prior to stimulation, would be associated with greater and more rapid recovery of skeletal muscle force following fatiguing contractions.

Hypothesis 3:

Damaging effects from heat treatment may still be present 24 hours after heat shock, such as slow recovery from anaesthesia, poor eating habits and weight loss. Since other investigators have found elevated levels of hsp72 remaining four days after heating, this study also investigated the effects of increased hsp72 on plantaris muscle contractility four days after heating. Therefore, it was hypothesized that animals stimulated four days after heat shock, having the benefits of high hsp72 levels and minimized effects of heating, would be most protected against the damaging effects of fatiguing stimulation.

2. Methods

Ethics approval was obtained by the Animal Care Committee (ref. no.: 95253-11).

2.1. Animal Care

Thirty male Sprague-Dawley rats (Charles River) aged four to seven months and weighing between 203 and 480 grams were employed. The animals were maintained on a 12:12 hour dark/light cycle, housed individually at 20 ± 1 degrees C, 50% relative

humidity, and fed and watered ad libitum. Animal weights were recorded before heat treatment and before muscle stimulation protocols.

2.2. Experimental Design

Six subjects were assigned to each of five experimental groups.

Group 1 ***Control*** subjects received no heat shock, *in situ* muscle preparation, or muscle stimulation treatment.

Group 2 ***Surgical Control*** subjects did not receive heat shock or stimulation treatments, but underwent the full surgical procedure and *in situ* muscle preparation. These subjects were in the *in situ* preparation apparatus until body and bath temperatures reached 37 degrees C, and remained there for two hours.

Subjects in group 3 ***Stimulation*** (“*stim*”) did not receive heat shock, but underwent both the surgery and muscle stimulation protocol.

Group 4 ***Heat Shock with Stimulation at 24 hours*** (“*heat 24h*”). Subjects received the heat shock, and 24 hours later were submitted to the surgery and muscle stimulation protocols.

The final set of subjects in Group 5 ***Heat Shock with Stimulation at 4 days*** (“*heat 4d*”) were given heat shock treatment, and underwent surgery and muscle stimulation four days later.

2.3. Heat Shock Protocol

Animals were anaesthetized intraperitoneally with pentobarbital sodium (35 mg/kg) . Subjects underwent whole-body heating using a Sunbeam heating pad. Body temperature was maintained at 41.5 degrees Celsius for 15 minutes, as monitored by

rectal probes. After heat treatment, 10-15 ml of water were administered orally ad libitum. Twenty-four hours or four days of recovery were allowed after heat shock.

2.4. Surgical Methods (*In Situ* Muscle Preparation)

Subjects were anaesthetized with pentobarbital sodium (65mg/kg; supplemented as needed). The left plantaris muscle was isolated by removing the soleus and most of the gastrocnemius, while retaining blood supply and sciatic innervation.

The plantaris muscle was chosen for analysis in this study because of its fast fatiguing characteristics. Fast-twitch glycolytic fibres comprise 41 ± 3 % of the population and 50 ± 3 % of the population consists of fast-twitch oxidative fibres, while only 9 ± 1 % of plantaris fibres are classified as slow-twitch oxidative (Armstrong and Phelps, 1984). The plantaris muscle was also chosen because it expresses only low amounts of hsp72 under basal conditions, but hsp72 production can be elevated significantly 24 hours following a single heat shock treatment (Locke and Tanguay, 1996).

The distal end of the plantaris tendon was ligated with surgical silk and separated from its insertion at the calcaneus. The animal was placed in a prone position with the left hindlimb stabilized, by drilling a pin through the femur at the knee joint. A mineral bath was prepared using the skin as a barrier, so that the hindlimb, plantaris, and exposed sciatic nerve were submerged. Mineral bath contents and body temperature were raised to and maintained at 37 degrees C by a recirculating pump.

2.5. Muscle Stimulation

The distal ligature of the plantaris muscle was attached to a Gould Model UCT2 pressure transducer, with mechanical activity transmitted by a Grass Model 7 Series Polygraph and monitored by a Tektronix 5110 oscilloscope. A Grass S11 stimulator was used to elicit muscle contraction via platinum electrodes in contact with the sciatic nerve. Recordings of contractile force were performed by the Interactive Laboratory System (ILS PC1) software. Calibration of the force transducer was performed with each experiment and remained constant throughout. Calibration was performed by attaching a 25 gram weight to the end of the transducer and recording the force produced.

Rest Measurements: Muscle stimulation began when body and bath temperatures reached 37 degrees C. Stimulus strength was set well above (2-3 volts above) the minimum voltage required to elicit peak isometric twitch force. Resting length was ascertained by introducing small incremental changes in muscle length until maximum isometric force was achieved. Three minutes after the determination of resting muscle length, peak isometric twitch tension (P_i) was elicited at 1 Hz and 0.1 ms twitch duration. Using 1 second train durations, tetani were elicited at frequencies of 10 Hz, 25 Hz, 40 Hz, 60 Hz, and 200 Hz (P_o). Two minutes of rest were allowed between each measure to eliminate any possible force potentiation. Tension was expressed in Newtons per gram of muscle weight (N/g). The twitch to tetanus ratio ($P_i:P_o$) was calculated.

Fatigue: The plantaris muscle was stimulated at 40 Hz for 333 milliseconds every second for a total of 5 minutes and 20 seconds as described by Burke and colleagues (1971). The resistance to fatigue was determined by calculating the 'fatigue index'. The fatigue index was calculated as the inverse of the ratio of the maximally potentiated raw peak force to the raw peak force generated 2 minutes later.

Recovery: Immediately post fatigue, active recovery for 5 minutes was performed by stimulating the muscle at 40 Hz for 333 ms duration at 10 second intervals. Active recovery was performed to facilitate the removal of lactate and its potential inhibitory effects on force production. The extent of recovery was then determined by repeating the rest parameters at set time intervals post fatigue: (1) T1, immediately following the active recovery period (5 minutes post fatigue); (2) T2, 25 minutes post fatigue; and (3) T3, 65 minutes post fatigue. The degree of recovery was calculated using the post fatigue tension as a percentage of the initial prefatigue tension for each frequency of stimulation measured. The subjects remained in the surgical preparation after the end of stimulation until 2 ½ hours post fatigue had elapsed.

2.6. Hsp72 Protein Analysis

The plantaris muscles from both left and right hindlimbs were removed, weighed, and immediately frozen in liquid nitrogen. Samples were stored at -70 degrees C.

2.6.1. Gel Electrophoresis

Frozen muscle portions (stored at -70 degrees Celsius) were homogenized in 19 vol of 600 mM NaCl and 15 mM Tris(hydroxymethyl)amino-methane (Tris), pH 7.5. Muscle protein concentrations were determined using the technique described by Lowry and colleagues (1951) using bovine serum albumin (BSA) as the standard. Two hundred micrograms of protein from plantaris muscle homogenates were loaded onto one-dimensional sodium dodecyl sulphate (SDS) 12% polyacrylamide gels. Soleus muscle

homogenate (60 µg) was loaded in one lane as a standard. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).

2.6.2. Protein Transfer, Immunoblotting, And Detection

Proteins were transferred from the gels to nitrocellulose membranes (0.2 µm thickness; Bio-Rad Laboratories) according to the method described by Towbin and colleagues (1979), using the Bio-Rad mini-protean II gel transfer system. After transfer, nitrocellulose membranes were blocked with 5% blotto: blotting grade blocker non-fat dry milk (Bio-Rad) in Tris-buffered saline (TBS: 20mM Tris-HCl, pH 7.5 and 500 mM NaCl) for 6 hours. Blots were washed twice for 5 minutes each in TTBS (TBS plus 0.05% Tween-20). Blots were incubated overnight with a monoclonal hsp72 primary antibody (Stress-Gen) in a 1:500 dilution in TTBS with 2% blotto. After two washes of 5 minutes each in TTBS, blots were incubated with a goat anti-mouse immunoglobulin G secondary antibody conjugated to alkaline phosphatase (Bio-Rad) in a 1:1000 dilution in TTBS with 2% blotto for 3 hours. Blots were washed twice in TTBS and once in TBS, and developed by immersion in bicarbonate buffer (100mM NaCO₃, 1mM MgCl₂, pH9.8) containing 3% (w/v) p-Nitro-blue-tetrazolium chloride p-toluidine salt in 70% N,N-dimethyl-formamide (DMF) and 15% w/v 5-Bromo-4-Chloro-3-Indolyl phosphate in 100% DMF. After development, the blots were washed in ddH₂O and dried. Quantification of bands was performed using an LKB Ultrosan XL Laser densitometer (Model 2202) equipped with an LKB 2220 recording integrator. Soleus muscle protein (60 µg) was used as a standard to allow comparison between different blots.

2.7. Statistical Analyses

Prefatigue contractile data for the three groups that underwent stimulation (stim, heat 24h, heat 4d) were compared by a two-way repeated measures analysis of variance (ANOVA) with one repeated factor (frequency of stimulation). Fatigue indices were analyzed for significant differences between the three groups by a one-way ANOVA. The percent recovery of force post fatigue was compared among the stimulated groups using a three-way repeated measures ANOVA, with the repeated variables being frequency of stimulation and time post fatigue. Hsp72 protein concentrations from all five treatment groups were analyzed for significant differences among the groups by a Kruskal-Wallis one-way analysis of variance on ranks. Following analyses of variance, Tukey's post hoc tests were performed, with the significance level set at $p < 0.05$.

3. Results

3.1. Subject Characteristics

Male Sprague-Dawley rats (203-480 grams) were used in this investigation, with six subjects assigned to each of five groups. Animals in the control and surgical control groups weighed 421 ± 18 grams (mean $\pm$ SEM) and 417 ± 23 grams respectively. Subject characteristics from the three stimulated groups were tabulated in order to compare these subjects in terms of body and muscle weight characteristics (Table 1). As well, these data give an indication of the effect of heat shock preconditioning on these parameters.

Table 1. Subject characteristics for non heat-shocked and heat-shocked rats from the stimulated experimental groups.

	Body Weight - Day of heat shock	Body Weight - Day of Stimulation	Plantaris Muscle Weight	Muscle to Body Weight Ratio (mg/g)
<i>Stim</i> (n=6)	-----	270 $\pm$ 20	0.224 $\pm$ 0.017	0.83 $\pm$ 0.03
<i>Heat 24h</i> (n=6)	260 $\pm$ 15	257 $\pm$ 14	0.211 $\pm$ 0.016	0.82 $\pm$ 0.04
<i>Heat 4d</i> (n=6)	267 $\pm$ 14	286 $\pm$ 11	0.240 $\pm$ 0.012	0.84 $\pm$ 0.02

Values represent means $\pm$ SEM.

The male Sprague-Dawley rats used in the three stimulated groups weighed between 203 and 357 grams. The mean body weights on the day of stimulation were 270 $\pm$ 20 g, 257 $\pm$ 14 g, and 286 $\pm$ 11 g for the stim, heat 24h, and heat 4d groups respectively. Although animals did not show growth by 24 hours after heat shock, by four days of recovery from heat shock continuing growth was noted.

Muscle to body weight ratios were calculated to ensure that heat pretreatment did not induce any gross changes in muscle weight expressed with respect to animal weight. There were no significant differences among the stimulated groups for muscle to body weight ratios ($p > 0.05$). Therefore, it can be concluded that subjects from the three stimulated groups were well matched in terms of subject characteristics prior to stimulation and that heat shock preconditioning did not affect any significant changes in muscle weight.

3.2. Hsp72 Protein Data

Hsp72 levels were measured for plantaris muscles from animals in all experimental groups. The three stimulated groups (stim, heat 24h and heat 4d) had both left (stimulated) and right (non-stimulated) plantaris muscles tested for the presence of

heat shock proteins. Figure 1 is a representative Western blot depicting hsp72 content in rat plantaris muscle from all groups.

Figure 1. Hsp72 content in rat plantaris muscle.

Lane 1: control plantaris; lane 2: surgical control plantaris; lane 3: stimulation control (stim) group, stimulated limb; lane 4: stim group, non-stimulated limb; lane 5: heat-shocked with 24 hours of recovery prior to stimulation (heat 24h), stimulated limb; lane 6: heat 24h, non-stimulated limb; lane 7: heat-shocked with 4 days of recovery prior to stimulation (heat 4d), stimulated limb; lane 8: heat 4d, non-stimulated limb.

A Kruskal-Wallis one-way analysis of variance on ranks was used to compare the heat shock response among the groups. Unstressed, control plantaris showed very low amounts of the hsp72 protein. Compared to control muscles, the surgical preparation alone did not induce further production of hsp72 ($p > 0.05$). Plantaris muscles from the non heat-shocked stimulated group (stim) did not demonstrate significantly higher hsp72 levels compared to control muscles ($p > 0.05$). However, a single 15 minute heat shock at 41.5 degrees C resulted in elevated levels of hsp72 after 24 hours or four days ($p < 0.05$). Whether heat shock was induced 24 hours or four days earlier resulted in no significant difference in the hsp72 detected ($p > 0.05$).

An analysis of the non-stimulated limbs provided assurance that hsp72 levels had been elevated prior to stimulation for those animals that were heat-shocked. Since the non-stimulated limbs had significantly higher hsp72 levels than control muscles ($p < 0.05$) and there was no difference in hsp72 levels between stimulated and non-stimulated limbs

in the heat-shocked animals ($p>0.05$), it can be inferred that hsp72 levels were elevated prior to stimulation. As well, it can be concluded that fatiguing stimulation did not result in further induction of hsp72.

3.3. Contractile Properties

Raw contractile data obtained from the stimulated subject groups (stim, heat 24h, and heat 4d) throughout the experiment are presented in Appendix A.

3.3.1. Prefatigue Parameters

The prefatigue contractile parameters for the three stimulated groups of subjects are presented in Table 2. A two way repeated measures ANOVA, with the repeated variable being frequency of stimulation, was used to compare the contractile responses of the stimulated groups.

Table 2. Isometric contractile properties of plantaris muscle from heat-shocked and non heat-shocked rats prior to fatigue.

	<i>Stim</i>	<i>heat 24h</i>	<i>heat 4d</i>
twitch (P_t), N/g	2.3 ± 0.2	2.4 ± 0.2	2.0 ± 0.2
10 Hz, N/g	2.7 ± 0.2	2.7 ± 0.2	2.3 ± 0.2
25 Hz, N/g	3.5 ± 0.2	4.1 ± 0.6	3.0 ± 0.2
40 Hz, N/g	6.6 ± 0.5	6.6 ± 0.5	5.7 ± 0.5
60 Hz, N/g	10.6 ± 0.3	10.7 ± 0.7	9.6 ± 0.7
200 Hz (P_o), N/g	14.3 ± 0.4	13.8 ± 0.9	13.1 ± 1.0
$P_t:P_o$	0.16 ± 0.01	0.18 ± 0.01	0.16 ± 0.01

Values are means $\pm$ SE; N/g, tension divided by muscle weight; P_t and P_o are the maximum isometric twitch and tetanic tensions, respectively; 10 Hz to 200 Hz represent increasing frequencies of stimulation; $P_t:P_o$ is the twitch to tetanus ratio.

Although there was a trend of reduced force values for the heat 4d animals, a comparison of the force (N/g) generated at various frequencies of stimulation indicated that heat shock had no statistically significant effect on the prefatigue tension ($p>0.05$) (Fig 2). No statistically significant differences were found between any of the three treatment groups. As expected, force increased in response to increasing frequencies of stimulation ($p<0.01$).

Figure 2. Prefatigue force-frequency curves for plantaris muscle from non heat-shocked and heat-shocked rats. Each individual point represents the group mean and the bars represent the SEM.

Expressing the force at each frequency as a percentage of the maximum force at P_0 (200 Hz) for each group (Fig. 3) demonstrates that heating had no effect on the relative force-frequency relationship, as the curves from all three groups can be superimposed.

Figure 3. Prefatigue relative force-frequency curves for plantaris muscle from non heat-shocked and heat-shocked rats. Each point represents the group mean and bars indicate the SEM.

Twitch to tetanus ratios ($P_t:P_o$) obtained prior to fatigue are presented in Table 2.

The ratios for the heat 24h (0.18 ± 0.01) or heat 4d animals (0.16 ± 0.01) were not different from rats in the stim group (0.16 ± 0.01) ($p > 0.05$), indicating that heat preconditioning did not affect initial twitch to tetanus ratios.

3.4. Fatigue

During the fatiguing stimulation protocol initially described by Burke and collaborators (1971), a typical fatigue profile for the rat plantaris muscle consisted of an initial potentiation of tetanic force, followed by a rapid decline in force and by slow decrements thereafter. The peak potentiated tetanic tensions for the stim, heat 24h, and heat 4d animals occurred at 13 seconds, 11.7 seconds, and 14.5 seconds, respectively.

Most muscles completed the rapid decline in force approximately one minute from the beginning of the fatiguing contractions.

The fatigue index can be used as an indicator of resistance to fatigue. The fatigue index is determined by calculating the inverse of the ratio of the potentiated peak force to the peak force generated two minutes later. Table 3 shows a comparison of the fatigue indices from the three stimulated groups. The fatigue indices for animals heat-shocked twenty-four hours or four days before stimulation (0.16 ± 0.03 and 0.30 ± 0.05 , respectively) were not statistically different from each other or from a fatigue index of 0.18 ± 0.05 obtained by control (stim) animals ($p > 0.05$). Therefore, heat shock did not significantly improve skeletal muscle resistance to fatigue.

Table 3. Fatigue indices for plantaris muscles from non heat-shocked and heat-shocked rats.

Group	Fatigue Index
stim	0.18 ± 0.05 (6)
heat 24h	0.16 ± 0.03 (6)
heat 4d	0.30 ± 0.05 (6)

Values are mean $\pm$ SEM (*n*). Fatigue Index: inverse of the ratio of the maximally potentiated peak force to the peak force developed 2 minutes later.

3.5. Recovery

Recovery of force was determined by expressing the post fatigue force as a percentage of the prefatigue force for each frequency of stimulation. Force recovery was measured immediately following the active recovery period (five minutes post fatigue; T1), 25 minutes post fatigue (T2), and 65 minutes post fatigue (T3). The results of a three-way ANOVA with two repeated measures (time post fatigue and frequency of stimulation), used to compare the force recovery of non heat-shocked and heat-shocked

subjects, is presented in Table 4. Figure 4 graphically represents the percent of force recovery obtained by subjects in each of the stimulated groups at increasing time intervals post fatigue.

Table 4. A comparison of the recovery of force among heat-shocked and non heat-shocked rats: Summary table of a three-way ANOVA with two repeated measures.

Effect	df	F	p-level
Group (BS)	2	2.4992	.115667
Time (WS)	2*	199.2243*	.000000*
Frequency (WS)	5*	285.5010*	0.000000*
Group X Time	4*	4.3689*	.006670*
Group X Frequency	10	.7909	.637413
Time X Frequency	10*	42.9451*	0.000000*
Group X Time X Frequency	20	.9065	.579521

BS - between subject variable, WS - within subject variable. * indicates statistical significance at $p < 0.05$. Group - experimental group (stim, heat 24h, heat 4d). Time - time post fatigue. Frequency - frequency of stimulation.

During the recovery from fatigue, main effects were found for both time of recovery and frequency of stimulation ($p < 0.01$). However, an interaction between time post fatigue and frequency was also statistically significant ($p < 0.01$). Appendix B contains the report for the Tukey post hoc test, for the interaction between the variables time post fatigue and frequency of stimulation, and lists the p values for the multiple comparisons tested. The curves depicted in Fig. 4 (2A, B, C) are a good representation of the conditions of low frequency fatigue. Maximum tetanic force (200 Hz tension) gained nearly full recovery immediately after fatigue (T1) and remained above 90% of prefatigue force for the entire experiment (at T2 and T3). The recovery of force for 200 Hz stimulation was not significantly different among the three time periods post fatigue ($p > 0.05$).

Although the lower frequency forces (twitch, 10 Hz, 25 Hz, 40 Hz, and 60 Hz) demonstrated statistically improved force recoveries over time post fatigue ($p < 0.01$),

2A

B

C

Figure 4. Percent of force recovery for plantaris muscle from fatiguing stimulation in non heat-shocked (stim) and heat-shocked (heat 24h, heat 4d) rats. Percent of force recovery was obtained by expressing the post fatigue tension as a percentage of the prefatigue tension for each frequency of stimulation. Each point indicates the group mean and bars show the SEM.

Panel 1: Interaction of the variables group and time post fatigue expressed over all frequencies of stimulation; * represents significant differences from stim group across all frequencies of stimulation ($p < 0.05$). **Panel 2:** Expansion of Panel 1 indicating force recovery response to different frequencies of stimulation. **A:** T1 (5 minutes post fatigue); **B:** T2 (25 minutes post fatigue); **C:** T3 (65 minutes post fatigue).

these lower frequencies of stimulation had significantly lower force recovery values compared to P_o for all time periods measured ($p < 0.01$). Most notably, the force recovery for 40 Hz was dramatically depressed.

P_i immediately post fatigue (T1) retained significantly higher percentages of initial force than every other frequency measured, except for 200 Hz ($p < 0.01$). However, by T2 and T3, twitch force recovery was not statistically different from 10 Hz and 25 Hz frequencies ($p > 0.05$), and all were significantly higher than the depressed recovery observed at 40 Hz ($p < 0.01$). In contrast, by T3, 60 Hz force had recovered 76 % of prefatigue force averaged across all groups. This uniform depression of the three lowest frequencies (1 Hz, 10 Hz, 25 Hz) and the obvious delay in recovery for 40 Hz at 65 minutes post fatigue signifies the persistence of force loss for low frequencies of stimulation. This selective impairment at the low frequencies is consistent with the phenomenon of low frequency fatigue.

The interaction of the effects of group (stim, heat 24h, and heat 4d) and time post fatigue (T1, T2, T3) was also significant ($p < 0.01$) (Fig. 4, Panel 1). The Tukey post hoc test results for the interaction of the variables 'group' and 'time post fatigue' are given in Appendix C. Differences in force recovery found between the groups were significant across all frequencies of stimulation, since the three-way interaction between group, time post fatigue, and frequency of stimulation was not significant ($p > 0.05$).

As depicted in Figure 4 (Panel 1), the non heat-shocked (stim) group displayed higher percentages of force recovery than the heat-shocked animals that had four days of recovery (heat 4d) for all three time periods post fatigue (T1, T2, T3) ($p < 0.05$). At 65 minutes post fatigue (T3), both groups of heated animals (heat 24h and heat 4d) significantly lagged behind the group of non-heated animals (stim group) in the recovery

of prefatigue force ($p < 0.01$). The heated groups were not statistically different from one another at any time post fatigue ($p > 0.05$).

Although subjects in each of the groups showed improved recovery of prefatigue force over time, non-heated animals in the stim group had the benefit of a faster degree of recovery. Heat pretreatment did not protect against the force decrements seen with low frequency fatigue and actually delayed the recovery of force

4. Discussion

The purpose of this study was to investigate the potential protective effects of hsp72 against the skeletal muscle dysfunction associated with low frequency fatigue.

4.1. Subject Characteristics

Animals did not experience growth 24 hours after heat shock treatment. However, four days following heat shock animals demonstrated continuing growth, indicating their improved recovery from heat shock in terms of body weight.

Animals from the three stimulated groups exhibited no significant differences in muscle to body weight ratios. Therefore, heat shock did not induce any gross changes in muscle weight such as might be indicated in response to water movement. Thus, any differences in contractile performance among the groups could be said to be due to other effects of heat shock rather than changes to muscle weight.

4.2. Hsp72 Protein Data

As revealed by Western blotting (Fig. 1), plantaris muscles from control rats showed very little or undetectable amounts of hsp72. Locke and Tanguay (1996) have also reported very low amounts of hsp72 in rat plantaris muscle under basal conditions.

The findings of this study confirm that the cellular content of the inducible hsp70 protein can be increased significantly in rat skeletal muscle by a 15 minute period of whole-body heat shock at 41.5 degrees C. Ianuzzo and colleagues (1995) also found that a single period of whole-body hyperthermia was sufficient to induce production of hsp72 in skeletal muscle. In agreement with other investigators (Karmazyn et al., 1990; Kiang et al., 1994), hsp72 content was significantly elevated above control conditions 24 hours and four days after heat shock. The increased levels of hsp72 detected were due solely to the hyperthermic treatment as neither the surgery (surgical control) nor the fatiguing stimulation protocols (stim) alone could induce production. The non-stimulated limbs of the heat-shocked subjects showed elevated levels of hsp72 compared to control muscles. Therefore, it could be inferred that hsp72 protein levels were elevated prior to stimulation and if hsp72 should affect contractile function it was present.

4.3. Baseline Contractile Parameters

The contractile characteristics of non-heated and heated subjects were determined under control conditions prior to fatigue to ensure that heat shock preconditioning had no effect upon the initial ability to generate force.

The maximum tetanic tensions (P_o) generated by plantaris muscles from rats in the non-heated, control (stim) group gave a mean of 14.3 ± 0.4 N/g (mean $\pm$ SEM), which is very similar to a P_o of 15.3 N/g reported by Kandarian and White (1990), but is

approximately 30-35% lower than the maximum tetanic forces given by Olha and colleagues (1988) and Gardiner and Olha (1987). The discrepancies between the forces reported in this study and others could merely be due to differences in equipment. The mean twitch to tetanus ratio determined for non-heated animals in this study (0.16 ± 0.01) is similar to a mean of 0.15 obtained by Roy and colleagues (1982) and 0.14 noted by other investigators (Olha et al., 1988; Gardiner and Olha, 1987). The similarity of the twitch to tetanus ratios indicates that the calcium active state in response to a single stimulus relative to the tetanus is comparable. Therefore, this evidence provides assurance that the ability to activate normal excitation-contraction coupling mechanisms has been achieved in this investigation.

None of the prefatigue contractile parameters differed significantly for animals that were heat-shocked 24 hours or four days prior to stimulation compared to control but stimulated (stim) subjects. However, it was noted that plantaris muscles from animals heated four days earlier had a trend toward a reduction in these parameters prior to fatigue, as demonstrated in Fig. 2. When the force (N/g) was normalized to a percentage of the force generated at 200 Hz, the force-frequency relationships from all three stimulated groups could be superimposed (Fig. 3). It can be inferred from the similar curves in Fig. 3 that heat pretreatment did not alter the ability to activate/inactivate normal calcium transients. The maintenance of normal excitation-contraction coupling after heat shock can also be inferred by the similar twitch to tetanus ratios among the groups. Ratios for the heat 4d (0.16 ± 0.01) or heat 24h animals (0.18 ± 0.01) were not different from subjects in the stim group (0.16 ± 0.01).

4.4. Resistance To Fatigue

The fatigue protocol used for rat plantaris muscle was one initially described by Burke and colleagues (1971), which, in this study, resulted in an initial potentiation of tetanic tension, followed by a rapid force decline, then by smaller changes in force. All muscles achieved maximum potentiation by 19 seconds, suggesting that the process of potentiation is not influenced by heat shock. The rapid decline in force typically ended approximately one minute from the beginning of the fatigue protocol. This pattern of initial potentiation followed by a rapid decline of tetanic force has also been reported for rat plantaris muscle by Gardiner and Olha (1987).

The fatigue index can be used as a measure of resistance to fatigue or endurance capacity. A mean fatigue index of 0.18 ± 0.05 was recorded for plantaris muscle from non-heated (stim) animals. The mean fatigue index noted here is similar, but slightly lower than a mean of 0.22 for whole plantaris muscle reported by Gardiner and Olha (1987) who used the fatigue protocol of Burke and collaborators (1971) and the same calculation for the fatigue index.

It was hypothesized that increased levels of hsp72 would be associated with improved resistance to fatigue. Since exercise training leads to both increased hsp72 levels and endurance capacity (Kelly et al., 1996; Duchateau & Hainaut, 1984), this study was designed to determine whether hsps were one of the many variables leading to improved endurance. Unexpectedly, there was no statistically significant difference between non-heated and heat-shocked animals in the resistance to fatigue ($p = 0.077$). However, it should be noted that the heat 4d animals having a mean fatigue index of 0.30 ± 0.05 , showed a tendency to improved endurance compared to heat 24h (0.16 ± 0.03) and stim (0.18 ± 0.05) subjects. As indicated in Appendix A, subject #6 in the stim group

attained a fatigue index of 0.41, which provided an outlying response compared to the other stim subjects. If this outlying data point were removed, a statistically significant difference would have been found between the stim and heat 4d groups ($p < 0.05$).

However, this data point was retained for two reasons. First, all other prefatigue and post fatigue data for this subject were well within the range of the other subjects in the stim group. As well Gardiner and Olha (1987) reported that rats in their study attained a maximum fatigue index of 0.37, not very different from an index of 0.41 noted in this study. Therefore, it was felt to be inappropriate to remove the entire data set for this subject because only one measure was questionable.

4.5. Recovery Of Prefatigue Force

Recovery of force was determined by calculating the percent recovery of the prefatigue force for each frequency of stimulation. Recovery was measured immediately following the active recovery period (five minutes post fatigue; T1), 25 minutes post fatigue (T2), and 65 minutes post fatigue (T3).

There were main effects found for both time of recovery and frequency of stimulation on the recovered force. As time post fatigue progressed, the general trend was for an improved recovery of force. Ionic and metabolic changes would have been reversed fairly quickly following fatigue (Jones, 1996), particularly in response to an active recovery. Therefore, a slow trend toward recovery may suggest that reparative processes against damage to protein structure incurred by fatigue are progressively leading to greater recovery of force. Force was generally depressed for the low frequencies but improved for higher frequencies of stimulation.

These main effects should be qualified, however, in that there was also a significant interaction between these two variables. Although the lower frequencies of stimulation (1 Hz, 10 Hz, 25 Hz, 40 Hz, 60 Hz) demonstrated greater recovery of prefatigue force over time post fatigue, at all times periods 200 Hz force recovery was significantly greater than other frequencies. Maximum tetanic force achieved over 90% of prefatigue force five minutes post fatigue (Fig. 4, Panel 2), and showed slight, but non-significant improvements over the rest of the experiment. In contrast, the lower frequencies of stimulation listed above remained depressed for the entire experiment, showing much slower recovery of force.

Edwards and colleagues (1977) demonstrated this discrepancy between the recovery of low and high frequency forces in an early study, reporting a “long-lasting element of fatigue” for the low frequencies of stimulation which lasted several hours of recovery time. In the current study, at 65 minutes post fatigue, the depression in force recovery at the low frequencies of stimulation compared to the greatly improved recovery for 60 and 200 Hz frequencies, emphasizes the incredible persistence of the decrement in low frequency force. This phenomenon is referred to as low frequency fatigue and has been reported by many other investigators. In accordance with the current study, Westerblad and colleagues (1993) found nearly complete recovery of maximum tetanic force immediately post fatigue, while the low frequency recoveries were substantially reduced. Low frequency fatigue has been associated with the reduced tetanic $[Ca^{2+}]_i$ noted in fatigued fibres, and is due primarily to a reduced release of calcium or slowed rate of release of calcium from the sarcoplasmic reticulum (Westerblad et al., 1993). Due to the sigmoidal function of the force - $[Ca^{2+}]_i$ relationship, low frequencies of stimulation lying on the steep portion of the curve would experience relatively large decreases in force for

small changes in $[Ca^{2+}]_i$; maximum tetanic frequency, falling on the upper horizontal part of the curve would only experience minor deficits in force for moderate falls in $[Ca^{2+}]_i$ (Westerblad et al., 1993). The incredible depression in force recovery for 40 Hz frequency of stimulation could result if it lay on the steepest portion of the force - calcium curve. This rationale could explain the extraordinary depressant effect on force for 40 Hz frequency in response to reduced calcium availability. Long lasting low frequency fatigue has been proposed to be due to structural damage to calcium channels which has been incurred during the intense fatiguing contractions, since any metabolites normally associated with decreased force would have been removed (Chin and Allen, 1996). The idea of ultrastructural damage being the basis for the long-lasting element of low frequency fatigue has also been supported by Jones (1996).

At T1, it is clear that twitch recovery of force was significantly higher than the other low frequencies of stimulation, including 60 Hz recovery (Fig. 4, 2A). By T2 and T3, twitch force recovery was not significantly different than 10 Hz and 25 Hz. Twitch potentiation, that is elevation of twitch force output in fatigued muscle, is probably a function of "post tetanic potentiation" (PTP). PTP has been purported to be caused by phosphorylation of a myosin light chain kinase, which has been shown to increase maximum isometric force at submaximal levels of calcium (Metzger et al., 1989). Since calcium release is reduced in fatigued fibres (Westerblad et al., 1993), myosin light chain phosphorylation could be particularly efficient in improving the sensitivity of force to calcium levels. Vandenboom and Houston (1996) have shown that conditioning stimuli given to muscles exhibiting low frequency fatigue result in increased myosin light chain phosphorylation and twitch force. Since the first measurement of twitch recovery at T1 was taken 10 seconds after the last tetanus of the active recovery period, the active

recovery may have acted as conditioning stimuli to increase the phosphate content of the myosin light chain and twitch force. If two minutes of rest was given after the active recovery period, as per that given between all contractile parameters measured thereafter, twitch force recovery would probably have been closer to that found for 10 Hz and 25 Hz frequencies. The twitch potentiation may have also been due to more calcium remaining in the cytoplasm following the last tetanus of the active recovery period, since there is a decreased rate of decay of the calcium transient in fatigued muscle fibres (Gyorke, 1993). The result would be a greater calcium activation of twitch force.

The long time of recovery required for low frequency forces again implicates the need for time to repair damage incurred during fatigue. If structural damage to proteins has taken place, the possible chaperoning and refolding functions of the hsp72 may be of use in promoting recovery (Tomasovic, 1989). It was hypothesized that subjects receiving prior heat shock, with a resulting elevation in the levels of the protective hsp72 protein, would have improved recovery of force compared to non-heated animals. The results, however, indicate that heat pretreatment actually delayed the recovery of isometric force following fatigue. From five minutes to 65 minutes post fatigue, animals heat-shocked four days earlier had a significantly reduced recovery of force compared to control (stim) subjects. As well, at the last time period (65 minutes post fatigue), subjects in the stim group had significantly greater force recovery than both heat-shocked groups (24 hour and four day recovery) across all frequencies of stimulation (Fig. 4, Panel 1).

These findings are surprising given that heat shock-induced hsp72 elevation has been shown time and time again to be associated with improved recovery of force following ischemia/reperfusion in cardiac muscle (Locke et al., 1995b; Currie et al., 1988; Karmazyn et al., 1990). The improved myocardial recovery has been proposed to be due

to an association between heat shock preconditioning and protection against oxyradical-induced damage that occurs during reperfusion. The activity of catalase, an endogenous antioxidant, is increased post heat shock, and treatment with 3-amino-1,2,4-triazole, an inactivator of catalase, can inhibit the heat shock-enhanced myocardial protection (Karmazyn et al., 1990). This data by Karmazyn and collaborators (1990) provides evidence that heat shock protects the myocardium against ischemia/reperfusion by an associated increase in catalase to protect against oxyradical-induced damage. However, Plumier and colleagues (1995) have shown that transgenic animals expressing constitutively high levels of the hsp72 protein can still show improved myocardial contractile recovery following ischemia/reperfusion, despite no increases in catalase activity. It seems that the hsp72 protein, itself, is acting directly to repair oxyradical-induced reperfusion injury in cardiac muscle. Since both oxyradicals and cytoplasmic calcium can be elevated in muscle fatigue (Davies et al., 1982; Gyorke, 1993), and both elements have the potential to stimulate proteolysis and structural injury to proteins (Zeman et al., 1985; Davies and Goldberg, 1987), the same protective response of heat shock might be seen in skeletal muscle.

Questions regarding the protective ability of hsp72 for skeletal muscle have been raised previously. Although Garramore and colleagues (1994) found that heat-shocked animals had better recovery of creatine phosphate levels following ischemia, the content was still substantially reduced compared to control values. Ianuzzo and colleagues (1995) determined that heat shock could not provide the protection to skeletal muscle that has been shown in cardiac muscle. The current study was undertaken to examine further the protective role of hsp72 in a different model, low frequency fatigue, which focused primarily on skeletal muscle function. The soleus muscle, as opposed to the plantaris,

may be more related to cardiac muscle in terms of its fatiguing characteristics and thus, may have provided a better comparison to the hsp72 response in cardiac muscle. The plantaris muscle was used because it can be fatigued quite readily and severely, and hsp72 can be elevated significantly by heat shock (Locke and Tanguay, 1996). Therefore, if hsp72 is to have a protective influence, it should be most apparent in the plantaris muscle.

The specific impairment in low frequency force recovery is the hallmark feature of low frequency fatigue which has been proposed to be due to problems in calcium release (Chin and Allen, 1996). Given that the sarcoplasmic reticulum is the primary source of calcium for contraction in skeletal muscle but plays a minor role in cardiac contractility (Berne and Levy, 1993), this site may be the basis for the discrepancy between the ability of hsps to protect cardiac versus skeletal muscle function. The sarcoplasmic reticulum may be particularly sensitive to damage by oxyradicals (Brotto and Nosek, 1996; Ishibashi et al., 1996) which can be produced in response to both exercise (Davies et al., 1982) and ischemia/reperfusion (Karmazyn et al., 1990). If local damage to the SR has taken place in either cardiac and/or skeletal muscle, and the hsps are not able to repair this damage, cardiac muscle would be less affected than skeletal muscle. Thus, in the case of skeletal muscle, the extensive damage to the SR and the resulting impairment of contractile function may overshadow any other protective influence the hsps have on other cellular processes.

Another possible reason for the lack of agreement in the ability of hsps to protect cardiac versus skeletal muscle may lie in the methods of force measurement. The Langendorff heart preparations involved electrically pacing the heart at 320 beats per minute and inducing maximal contractile force by one set pre-load (Locke et al., 1995b).

If gradations in force production were examined in cardiac preparations, observations similar to those in skeletal muscle may be forthcoming.

The inability of hsp72 to protect against the long-term effects of low frequency fatigue could also be the result of an accumulation of stressful assaults that were beyond the protective capacity of the heat shock proteins. Ianuzzo and colleagues (1995) proposed that the inability of hsp72 to protect skeletal muscle against ischemic insult may have been due to the multiple stresses of heat shock, subsequent surgery, and ischemia which were beyond the limited capacity of the heat shock system. In this study, the heat shock, surgery and intense muscle contractions may also have challenged this protective pathway. In particular the fatigue protocol used in this study was fairly intense given that the muscle was stimulated at 40 Hz. However, in order to examine the potential benefits of the hsp72 protein, it was required that the plantaris muscle be significantly fatigued.

A final explanation for the contrasting results of the protective influence of the hsps in cardiac versus skeletal muscle, may be that low frequency fatigue is not caused by protein damage. Heat shock proteins have been proposed to counter denaturing conditions by repairing or removing abnormal proteins (Tomasovic, 1989). However, if the unfolding of proteins does not occur, hsps would not have a role in protecting against the contractile decrement in low frequency fatigue.

4.6. Detrimental Effects Of Heat Shock

It is proposed in this study that the heat shock pretreatment not only did not protect against, but may have caused, cellular injury that resulted in the poor performance of the heat-shocked animals, causing increased susceptibility to damage from fatigue.

Yellon and colleagues (1992) found that whole-body heat stress could not limit

myocardial infarction in an *in situ* heart preparation, and suggested, as this study has, that whole-body hyperthermia may have deleterious results that may negate any beneficial effects of hsp elevation. At four days of recovery from heat shock, continued growth of the animals was noted. Therefore, poor recovery from heat shock in terms of body weight cannot be used to justify the depressed response of the heat 4d animals to fatigue. As well, muscle to body weight ratios from both heat pretreated groups were not significantly different from those of the control (stim) group, suggesting that some other effect of heat shock was responsible for the compromised performance.

Hyperthermia has been shown to cause significant biochemical alterations in the cell. Total cellular protein breakdown has been shown to increase during hyperthermia (Hall-Angeras et al., 1990). As well, intracellular concentrations of both calcium and superoxide anion are elevated in response to increases in temperature (Drummond et al., 1986; Salo et al., 1991). Both calcium ions and reactive oxygen species are able to activate the breakdown of cellular protein (Goodman, 1987; Rice-Evans and Burdon, 1993), leading to thermal injury. Therefore, although elevations in temperature can lead to the protective induction of heat shock proteins, the heat treatment itself may cause subtle structural damage to proteins in the cell.

The long-term effects of the biochemical alterations induced by heat shock are unknown. In this study, one of the initial hypotheses stated that those subjects having a longer recovery time from heat shock (four days) would have the benefit of elevated hsp72 levels and reduced detrimental heating effects. Although animals in the heat 4d group recovered better than rats in the heat 24h group in terms of growth, it appears as if the animals in the heat 4d group actually sustained greater muscle impairment from the initial heat treatment. The heat 4d animals not only showed reduced force recovery

compared to control animals throughout the whole recovery period, but may also have shown a trend toward reduced baseline contractility prefatigue. The similar muscle to body weight ratios among the stimulated groups indicates that the malfunction was probably not due to changes in relative muscle weight secondary to the heat treatment that may have influenced force production. It seems as though the heat treatment, via certain biochemical alterations, has activated a cascade of events leading to greater impairment at four days compared to 24 hours. This concept of a delayed time course of injury following stress has been studied extensively with respect to eccentric muscle contractions. Friden and Lieber (1992) suggested that the degradation of structure that takes place hours to days following the initial injury may be the result of enhanced calcium-activated, or lysosomal proteolytic enzyme activity. In this investigation, the potential increase in calcium levels induced by heat shock could have activated cellular proteases, which may have impaired protein function more at four days than at 24 hours of recovery. The damage incurred even at 24 hours may have rendered these muscles more susceptible to further insult. Heat pretreated muscles may then be more susceptible to the stress associated with low frequency fatigue since reparative mechanisms may be occupied by the first assault (heat shock).

4.7. Implications

Heat shock therapy as a means of introducing protection against a subsequent assault may be contraindicated given the other biochemical alterations that could have deleterious effects on cellular viability. Investigators who currently use heat stress conditioning as a means to limit myocardial infarction and/or damage from ischemia / reperfusion in skeletal or cardiac muscle should be cautioned against this method, unless

it can be shown more clearly that cardiac muscle reacts to heat shock in a significantly different manner. The heat shock response is sensitive and can lead to many other cellular events beside heat shock protein elevation. Although the bulk of the results reported in recent literature tout the protective effects of heat shock, the current study calls into question the benefits of this therapy in improving skeletal muscle performance. This is not to say that heat shock proteins are not protective. However, when using the heat stress treatment, it is difficult to separate the potential benefits of the hsps and the negative effects of heat shock. Clearly, the heat shock response should not be used as an ergogenic aid to improve skeletal muscle performance.

4.8. Future Study

It should be emphasized that this study does not refute that heat shock proteins have a protective effect. Therefore, to further elucidate the role of hsps in skeletal muscle, some other means of elevating the hsps should be utilized that does not have so many confounding variables as does heat shock. Transgenic animals, those which constitutively express high amounts of the hsp72, could be used, without the detrimental effects of the stress condition normally inducing their elevation. Through this procedure, a more direct demonstration of the effects of hsps on the subsequent insult could be observed.

Positive results obtained from cardiac muscle cannot be automatically assumed for skeletal muscle, as the findings obtained from this study and others lead to contradictory answers as to whether or not hsps can protect skeletal muscle at all.

In terms of the initial question whether hsps were one of the many variables leading to the improved resistance to fatigue following exercise training, this study cannot

give a clear answer. The use of transgenic animals may be the only way of isolating their role.

5. Conclusions

The findings from this study indicate that heat shock induced production of hsp72 does not protect against low frequency fatigue in rat plantaris muscle. Elevation of hsp72 levels did not result in improved resistance to fatigue, and therefore, *hypothesis 1* must be rejected. *Hypothesis 2* must also be rejected given that increased levels of hsp72 did not lead to greater or more rapid recovery from fatigue. In fact, heat shock preconditioned subjects actually demonstrated reduced recovery of prefatigue force compared to non-heated animals. In *hypothesis 3*, it was proposed that subjects having four days of recovery would benefit from the increased hsp72 levels and reduced effects of anaesthesia and heating. However, this last hypothesis must also be rejected, since animals having four days of recovery from heat shock seemed to have more negative effects of the heat pretreatment than those having 24 hours of recovery.

Heat shock therapy should not be used to elevate skeletal muscle hsp72 levels as a protective mechanism against fatigue given its potential to compromise cell viability.

Appendix A. Raw Contractile Data For Rat Plantaris Muscle

Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Group	stim	stim	stim	stim	stim	stim	heat 24h	heat 24h	heat 24h	heat 24h	heat 24h	heat 24h	heat 4d	heat 4d	heat 4d	heat 4d	heat 4d	heat 4d
Pre-fatigue force (N/g)																		
twitch	3.02	2.28	1.88	2.22	2.25	1.96	1.97	2.93	2.46	1.95	2.80	2.27	1.56	1.93	1.96	2.72	2.07	1.84
10 Hz	3.56	2.65	2.14	2.64	2.82	2.30	2.27	3.40	2.79	2.18	3.13	2.65	1.79	2.14	2.24	3.14	2.40	2.18
25 Hz	4.41	3.42	3.24	3.36	3.68	2.97	2.81	4.22	7.01	2.73	4.23	3.49	2.27	2.69	2.76	3.92	3.12	2.94
40 Hz	8.34	6.57	4.90	6.25	6.80	6.97	5.10	6.87	8.45	5.20	6.97	6.99	4.73	4.92	4.93	7.88	6.48	5.46
60 Hz	10.65	9.81	9.90	11.34	11.55	10.30	9.37	11.07	11.85	9.35	13.29	9.20	6.91	9.13	9.65	11.24	9.38	11.44
200 Hz	13.67	13.62	13.93	15.60	15.40	13.54	11.59	14.62	14.24	12.55	17.86	12.03	9.24	11.93	13.14	15.11	12.74	16.43
tw:tetanus	0.22	0.17	0.13	0.14	0.15	0.14	0.17	0.20	0.17	0.16	0.16	0.19	0.17	0.16	0.15	0.18	0.16	0.11
Fatigue																		
fatigue index	0.08	0.16	0.13	0.17	0.15	0.41	0.08	0.10	0.21	0.20	0.23	0.10	0.17	0.43	0.18	0.42	0.21	0.36
Recovery at T1																		
twitch	1.79	1.51	0.90	1.44	0.97	1.03	1.04	1.33	0.96	0.94	1.32	1.22	0.94	0.72	0.95	1.01	1.00	1.05
tw recovery	59.27	66.23	47.87	64.86	43.11	52.55	52.79	45.39	39.02	48.21	47.14	53.74	60.26	37.31	48.47	37.13	48.31	57.07
10 Hz	1.87	1.23	1.03	1.09	0.83	0.84	1.02	0.94	0.80	0.75	0.96	1.13	0.81	0.61	0.78	0.98	0.90	0.88
10 Hz recovery	52.53	46.42	48.13	41.29	29.43	36.52	44.93	27.65	28.67	34.40	30.67	42.64	45.25	28.50	34.82	31.21	37.50	40.37
25 Hz	2.20	1.42	1.36	1.14	1.14	1.07	1.17	1.11	0.84	0.86	1.07	1.44	1.06	0.76	0.80	1.03	0.94	0.98
25 Hz recovery	49.89	41.52	41.98	33.93	30.98	36.03	41.64	26.30	11.98	31.50	25.30	41.26	46.70	28.25	28.99	26.28	30.13	33.33
40 Hz	3.16	2.06	2.26	1.91	1.95	1.92	2.30	1.86	1.38	1.54	1.55	2.53	1.80	1.18	1.19	1.68	1.72	1.47
40 Hz recovery	37.89	31.35	46.12	30.56	28.68	27.55	45.10	27.07	16.33	29.62	22.24	36.19	38.05	23.98	24.14	21.32	26.54	26.92
60 Hz	5.92	4.68	4.76	5.32	3.99	3.90	5.35	4.28	3.06	3.31	3.40	5.55	4.02	2.34	2.78	3.51	4.01	3.28
60 Hz recovery	55.59	47.71	48.08	46.91	34.55	37.86	57.10	38.66	25.82	35.40	25.58	60.33	58.18	25.63	28.81	31.23	42.75	28.67
200 Hz	10.72	12.98	14.42	15.18	15.33	12.36	13.25	13.73	10.80	11.32	16.62	11.90	9.46	9.64	10.58	13.63	12.53	16.03
200 Hz recovery	78.42	95.30	103.52	97.31	99.55	91.29	114.32	93.91	75.84	90.20	93.06	98.92	102.38	80.80	80.52	90.21	98.35	97.57
Recovery at T2																		
twitch	1.84	1.38	1.14	1.29	1.02	0.93	1.31	1.40	1.00	0.90	1.02	1.43	0.97	0.54	0.88	1.14	0.88	0.96
tw recovery	60.93	60.53	60.64	58.11	45.33	47.45	66.50	47.78	40.65	46.15	36.43	63.00	62.18	27.98	44.90	41.91	42.51	52.17

Subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Group	stim	stim	stim	stim	stim	stim	heat	heat	heat	heat	heat	heat	heat	heat	heat	heat	heat	heat
10 Hz	2.10	1.60	1.33	1.50	1.13	1.02	1.49	1.82	1.05	0.99	1.24	1.69	1.07	0.60	0.94	1.29	1.01	1.07
10 Hz recovery	58.99	60.38	62.15	56.82	40.07	44.35	65.64	53.53	37.63	45.41	39.62	63.77	59.78	28.04	41.96	41.08	42.08	49.08
25 Hz	2.65	2.00	1.60	1.92	1.52	1.31	1.85	2.72	1.22	1.28	1.74	2.29	1.38	0.83	1.16	1.75	1.64	1.40
25 Hz recovery	60.09	58.48	49.38	57.14	41.30	44.11	65.84	64.45	17.40	46.89	41.13	65.62	60.79	30.86	42.03	44.64	52.56	47.62
40 Hz	3.85	2.84	2.56	3.01	2.95	2.33	2.93	3.68	1.90	2.22	2.55	3.61	2.11	1.40	1.78	2.71	2.45	2.21
40 Hz recovery	46.16	43.23	52.24	48.16	43.38	33.43	57.45	53.57	22.49	42.69	36.59	51.65	44.61	28.46	36.11	34.39	37.81	40.48
60 Hz	7.47	6.35	6.94	8.02	6.56	5.29	6.60	7.50	4.78	5.49	5.30	7.58	4.78	3.04	4.68	5.89	5.52	5.08
60 Hz recovery	70.14	64.73	70.10	70.72	56.80	51.36	70.44	67.75	40.34	58.72	39.88	82.39	69.18	33.30	48.50	52.40	58.85	44.41
200 Hz	14.02	12.19	14.98	15.67	15.90	13.08	12.65	13.73	11.56	12.12	17.06	12.28	8.14	9.91	12.18	14.57	12.58	16.20
200 Hz recovery	102.56	89.50	107.54	100.45	103.25	96.60	109.15	93.91	81.18	96.57	95.52	102.08	88.10	83.07	92.69	96.43	98.74	98.60
Recovery at T3																		
twitch	2.59	1.80	1.50	1.71	1.60	1.40	1.43	1.92	1.28	1.23	1.48	1.74	1.02	0.75	1.09	1.59	1.06	1.29
tw recovery	85.76	78.95	79.79	77.03	71.11	71.43	72.59	65.53	52.03	63.08	52.86	76.65	65.38	38.86	55.61	58.46	51.21	70.11
10 Hz	3.02	1.96	1.74	1.92	1.83	1.72	1.59	2.13	1.38	1.40	1.74	1.93	1.14	0.86	1.20	1.78	1.19	1.38
10 Hz recovery	84.83	73.96	81.31	72.73	64.89	74.78	70.04	62.65	49.46	64.22	55.59	72.83	63.69	40.19	53.57	56.69	49.58	63.30
25 Hz	3.66	2.48	2.29	2.46	2.28	2.21	1.94	2.86	1.67	1.76	2.12	2.62	1.41	1.22	1.55	2.37	1.87	1.88
25 Hz recovery	82.99	72.51	70.68	73.21	61.96	74.41	69.04	67.77	23.82	64.47	50.12	75.07	62.11	45.35	56.16	60.46	59.94	63.95
40 Hz	4.86	3.87	3.30	3.70	3.48	3.27	3.02	3.97	2.71	2.64	3.13	4.52	2.19	1.82	2.28	3.60	2.78	3.08
40 Hz recovery	58.27	58.90	67.35	59.20	51.18	46.92	59.22	57.79	32.07	50.77	44.91	64.66	46.30	36.99	46.25	45.69	42.90	56.41
60 Hz	9.22	8.10	8.88	10.39	8.93	8.11	7.94	8.30	7.56	7.58	7.93	8.87	4.41	4.55	6.57	8.46	7.41	7.48
60 Hz recovery	86.57	82.57	89.70	91.62	77.32	78.74	84.74	74.98	63.80	81.07	59.67	96.41	63.82	49.84	68.08	75.27	79.00	65.38
200 Hz	14.13	12.91	15.01	15.81	16.83	13.79	13.63	12.03	11.02	12.26	17.13	12.94	6.62	9.36	12.55	14.86	12.74	16.40
200 Hz recovery	103.37	94.79	107.75	101.35	109.29	101.85	117.60	82.28	77.39	97.69	95.91	107.56	71.65	78.46	95.51	98.35	100.00	99.82

Note: Recovery values are given as absolute force (N/g) followed by the percent recovery of prefatigue force.

Appendix B. Tukey HSD Test: Interaction of Time of Recovery and Frequency of Stimulation

Instructions for use.

1. The levels of each of the variables (TIME and FREQUENCY) are represented by numbers in the leftmost column and are defined in the legend given below (e.g. the number 6 under FREQUENCY refers to 200 Hz; the number 2 under TIME refers to T2).
2. Each combination of TIME and FREQUENCY is represented by a bracketed numeral “{}”, and is also shown with its mean value in the headings above the table.
3. How does one compare combinations?

Example 1:

To determine if the 60 Hz frequency force recovery at T2 was significantly higher than the 60 Hz force recovery at T1:

- a. 60 Hz at T2 is represented by the combination of “5” under FREQUENCY and “2” under TIME or “{11}”.
- 60 Hz at T1 is represented by the combination of “5” under FREQUENCY and “1” under TIME or “{5}”.
- b. To determine the p value, find {5} across the top and {11} along the left side and find where they intersect. This p value is significant ($p < 0.05$) as $p = .00036$.

Example 2 (a non significant comparison):

To determine if the twitch force recovery at T2 is significantly different than the 25 Hz recovery at T2.

- a. twitch at T2 is represented by the combination of “1” under FREQUENCY and “2” under TIME or “{7}”.
- 25 Hz at T2 is represented by the combination of “3” under FREQUENCY and “2” under TIME or “{9}”.
- b. The p value reported 1.000000 is non significant ($p > 0.05$).

Results

TIME	FREQ.	(1) 50.48500	(2) 37.82944	(3) 33.66611	(4) 29.98056	(5) 40.49222	(6) 93.41500	(7) 50.28611
1	1 (1)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	1.000000
1	2 (2)	.000036*		.124431	.000036*	.851858	.000036*	.000036*
1	3 (3)	.000036*	.124431		.000036*	.000059*	.000036*	.000036*
1	4 (4)	.000036*	.000036*	.301093	.301093	.000036*	.000036*	.000036*
1	5 (5)	.000036*	.851858	.000059*	.000036*	.000036*	.000036*	.000036*
1	6 (6)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
2	1 (7)	1.000000	.000036*	.000036*	.000036*	.000036*	.000036*	1.000000
2	2 (8)	.999999	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
2	3 (9)	.999999	.000036*	.000036*	.000036*	.000036*	.000036*	1.000000
2	4 (10)	.000036*	.173429	.000036*	.000036*	.999926	.000036*	.000036*
2	5 (11)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
2	6 (12)	.000036*	.000036*	.000036*	.000036*	.000036*	.673248	.000036*
3	1 (13)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
3	2 (14)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
3	3 (15)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
3	4 (16)	1.000000	.000036*	.000036*	.000036*	.000036*	.000036*	.999992
3	5 (17)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
3	6 (18)	.000036*	.000036*	.000036*	.000036*	.000036*	.520319	.000036*

Values under group headings represent means; tabulated are the p values for the multiple comparisons tested
 * indicates significance at $p < 0.05$

TIME								
	1 - T1	2 - T2	3 - T3					
FREQUENCY								
	1 - twitch	2 - 10 Hz	3 - 25 Hz	4 - 40 Hz	5 - 60 Hz	6 - 200 Hz		

TIME	FREQ.		(8) 49.46556	(9) 49.46278	(10) 41.82778	(11) 58.33389	(12) 96.44111	(13) 65.90334	(14) 64.12833
1	1	(1)	.999999	.999999	.000036*	.000036*	.000036*	.000036*	.000036*
1	2	(2)	.000036*	.000036*	.173429	.000036*	.000036*	.000036*	.000036*
1	3	(3)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
1	4	(4)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
1	5	(5)	.000036*	.000036*	.999926	.000036*	.000036*	.000036*	.000036*
1	6	(6)	.000036*	.000036*	.000036*	.000036*	.673248	.000036*	.000036*
2	1	(7)	1.000000	1.000000	.000036*	.000036*	.000036*	.000036*	.000036*
2	2	(8)		1.000000	.000036*	.000036*	.000036*	.000036*	.000036*
2	3	(9)	1.000000		.000036*	.000036*	.000036*	.000036*	.000036*
2	4	(10)	.000036*	.000036*		.000036*	.000036*	.000036*	.000036*
2	5	(11)	.000036*	.000036*	.000036*		.000036*	.000036*	.001305*
2	6	(12)	.000036*	.000036*	.000036*	.000036*		.000036*	.000036*
3	1	(13)	.000036*	.000036*	.000036*	.000036*	.000036*		.997049
3	2	(14)	.000036*	.000036*	.000036*	.001305*	.000036*	.997049	
3	3	(15)	.000036*	.000036*	.000036*	.038424*	.000036*	.741018	.999994
3	4	(16)	.990532	.990386	.000036*	.000052*	.000036*	.000036*	.000036*
3	5	(17)	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*	.000036*
3	6	(18)	.000036*	.000036*	.000036*	.000036*	1.000000	.000036*	.000036*

TIME	FREQ.	(15) 63.00111	(16) 51.43222	(17) 76.03222	(18) 96.70111
1	1	.000036*	1.000000	.000036*	.000036*
1	2	.000036*	.000036*	.000036*	.000036*
1	3	.000036*	.000036*	.000036*	.000036*
1	4	.000036*	.000036*	.000036*	.000036*
1	5	.000036*	.000036*	.000036*	.000036*
1	6	.000036*	.000036*	.000036*	.520319
2	1	.000036*	.999992	.000036*	.000036*
2	2	.000036*	.990532	.000036*	.000036*
2	3	.000036*	.990386	.000036*	.000036*
2	4	.000036*	.000036*	.000036*	.000036*
2	5	.038424*	.000052*	.000036*	.000036*
2	6	.000036*	.000036*	.000036*	1.000000
3	1	.741018	.000036*	.000036*	.000036*
3	2	.999994	.000036*	.000036*	.000036*
3	3	.000036*	.000036*	.000036*	.000036*
3	4	.000036*	.000036*	.000036*	.000036*
3	5	.000036*	.000036*	.000036*	.000036*
3	6	.000036*	.000036*	.000036*	.000036*

Appendix C. Tukey HSD Test: Interaction of Time of Recovery and Group

GROUP TIME		(1)	(2)	(3)	(4)	(5)	(6)	(7)
		51.68833	61.57222	78.30750	46.08111	58.60583	68.22567	45.16472
1	1 (1)	.000521*	.000521*	.000149*	.116855	.024640*	.000149*	.040385*
1	2 (2)	.000149*		.000149*	.000149*	.818036	.034348*	.000149*
1	3 (3)		.000149*		.000149*	.000149*	.000426*	.000149*
2	1 (4)	.116855	.000149*	.000149*	.000154*	.000154*	.000149*	.999901
2	2 (5)	.024640*	.818036	.000149*	.000149*	.000700*	.000700*	.000151*
2	3 (6)	.000149*	.034348*	.000426*	.000149*	.000151*	.000149*	.000149*
3	1 (7)	.040385*	.000149*	.000149*	.999901	.000151*	.000149*	.010569*
3	2 (8)	.999742	.001896*	.000149*	.034562*	.086877	.000149*	
3	3 (9)	.000321*	.999999	.000149*	.000149*	.670010	.062441	.000149*

GROUP TIME		(8)	(9)
		52.73056	62.06500
1	1 (1)	.999742	.000321*
1	2 (2)	.001896*	.999999
1	3 (3)	.000149*	.000149*
2	1 (4)	.034562*	.000149*
2	2 (5)	.086877	.670010
2	3 (6)	.000149*	.062441
3	1 (7)	.010569*	.000149*
3	2 (8)		.000992*
3	3 (9)	.000992*	

Values under group headings represent means; tabulated are the p values for the multiple comparisons tested; * indicates significance at $p < 0.05$

GROUP	1 - slim 2 - heat 24h 3 - heat 4d
TIME	1 - T1 (5 min. post-fatigue) 2 - T2 (25 min. post-fatigue) 3 - T3 (65 min. post-fatigue)

Appendix D. Glossary of Terms

cross-tolerance - prior treatment with one stressor protects against a subsequent, but different form of assault

excitation-contraction coupling - the functional connection between voltage sensing and calcium regulation of muscle contraction

fatigue index - inverse of the ratio of the maximally potentiated peak force to the peak force generated two minutes later

HSE - heat shock element (DNA sequence which is bound by HSF)

HSF - heat shock transcription factor

hsp - heat shock protein

HSP - referring to the heat shock gene or gene products

hsp72 - 72 kilodalton heat shock protein

Hz - frequency of stimulation

isometric contraction - muscle produces force with no change in muscle length

low frequency fatigue (LFF) - characterized as the greater loss of muscle force at low frequencies than at high frequencies of stimulation, following fatiguing contractions

muscle fatigue - the failure to maintain a given force output during sustained or repeated contractions

P_o - the maximum tetanic tension

P_t - the peak twitch force in response to a single action potential

$P_t: P_o$ - twitch to tetanus ratio

potentiation - activity-dependent increase in isometric twitch tension

sarcoplasmic reticulum (SR) - a large intracellular store of calcium

tetanus - the contractile response to a train of action potentials

thermotolerance - resistance to normally lethal temperatures

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IMAGE EVALUATION TEST TARGET (QA-3)

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