The Mechanism of Selenium-induced Skeletal Muscle Dysfunction and Weakness

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February, 2018
Declaration

I, Thomas Wilson, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

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The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.

This thesis does not contain work that I have published, nor work under review for publication.

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Signature: __________________________
Thomas Wilson
February 2018
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Abstract

Background: Selenium toxicity (selenosis) induces skeletal muscle contracture in many animal models, and can result in respiratory failure. A skeletal muscle contracture is a prolonged state of muscle tightness, or tone. Many previous research papers suggest that the main cause of selenite-induced contracture (SIC) is via the oxidation of the ryanodine receptor (RyR), causing uncontrolled Ca\textsuperscript{2+} release. This study investigates the possibility that the mechanism of this contracture can occur by multiple independent mechanisms. These mechanisms involve changes to cellular redox state and Ca\textsuperscript{2+} homeostasis affecting active force production, and possibly contribute to the baseline force increase. The other hypothesis presented in this study is the possibility that the SIC could be caused by mitochondrial-mediated cell death resulting in a rigor contracture.

Methods & Results: In vitro whole-muscle experiments using extensor digitorum longus (EDL) muscle from 6 week old male ARC mice showed selenite (10 mM) initially increased active force by 11.2 ± 1.63 % (n = 6), before progressively decreasing active force (TT50: 26.8 ± 0.85 mins) and concurrently increasing baseline force by a factor of 8.54 ± 0.88. Glutathione (GSH; 10 mM) pre-treatment enhanced the initial force increase up to 17.7 ± 1.51 % (n = 5) upon addition of selenite (10 mM), and prolonged this increase (TT50: 32.7 ± 0.68 mins), whereas pre-treatment with 4-hydroxy tempol (tempol; 20 mM), a superoxide dismutase (SOD) mimic, showed no initial force increase upon the addition of selenite (10 mM) but accelerated force decay (TT50: 14.8 ± 0.49 mins; n = 6). Twitch half relaxation times (1/2RT) were significantly elevated after 6 mins of selenite exposure (control: 3.2 ± 0.54 ms/g; selenite: 6.0 ± 1.18 ms/g; n = 6), with no change in time to peak twitch force (TTP). Both 1/2RT and TTP were elevated once force production had fallen by 50 % (1/2RT: 9.5 ± 1.9 ms/g; TTP: 6.8 ± 1.2 ms/g).

Tetracaine (500 μM; n = 5) was used to block the ryanodine receptor (RyR) preventing the release of Ca\textsuperscript{2+}; however, this had no effect on the SIC, even when extracellular Ca\textsuperscript{2+} was omitted from the bathing solution (n = 3). The possibility that the SIC could be caused by mitochondrial dysfunction was then investigated using flow cytometry. The
results of this experiment showed that selenite causes mitochondrial depolarisation that precedes the loss of plasma membrane integrity and results in cell death.

Single muscle fibres were dissected from whole muscle that had been exposed to selenite and had lost either 20% (Group-A; 18.2 ± 0.3 mins of selenite exposure, $n = 6$) or 50% (Group-B; 26.6 ± 0.9 mins of selenite exposure, $n = 7$) of initial force production. Force recordings from these single fibres showed that Group-A had lost 62 % and Group-B had lost 67 % of maximal $\text{Ca}^{2+}$ activated force. Due to the oxidative nature of selenite exposure, these fibres were exposed to dithiothreitol (DTT, 20 mM), a thiol-specific reducing agent, which was only able to recover a small amount of force (Group-A: 11.9 % recovery; Group-B: 18.9 % recovery). The small amount of force recovery due to DTT exposure suggests that only part of the damage observed in whole muscle is due to reversible thiol oxidation. When skinned fibres were dissected from untreated whole muscle were subsequently exposed to selenite as skinned fibres in isolation, both force production and myofilament $\text{Ca}^{2+}$ sensitivity were markedly reduced ($n = 7$). DTT exposure recovered this reduction in force, but was not able to recover myofilament $\text{Ca}^{2+}$ sensitivity. Repeated exposure of isolated fibres to selenite and DTT solutions showed a reduction in the amount of force able to be recovered upon a second cycle of exposures to selenite and DTT.

**Conclusion:** The prolonged 1/2RT suggests that the initial increase in force production is likely caused by impairment to SERCA activity, reducing fusion frequency with no change in RyR $\text{Ca}^{2+}$ release. The prolonged reduction in SERCA activity could cause less $\text{Ca}^{2+}$ to be released upon stimulation, due to decreased SR $\text{Ca}^{2+}$ accumulation, resulting in decreased active force. Skinned fibre experiments showed selenite exposure reduces maximal force and $\text{Ca}^{2+}$ sensitivity, via direct thiol oxidation. Flow cytometry studies provide evidence that the end result of the SIC could be mitochondrial-mediated cell death. It is likely that this process of cell death is triggered by reaching a threshold of mitochondrial oxidative stress, which results in a rigor contracture driven by a lack of ATP.
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<th>Full Form</th>
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<tbody>
<tr>
<td>1/2RT</td>
<td>Half relaxation time</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>DD</td>
<td>Double distilled</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ECC</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor digitorum longus</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathion peroxidase</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>H⁺</td>
<td>Hyrdogen ion (proton)</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>Lₒ</td>
<td>Optimum length</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition channel</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
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</table>
**Na$_2$Se$_3^{2-}$**  Sodium selenite
**Na$_2$SeO$_4^{2-}$**  Sodium selenate
**NADPH**  Nicotinamide adenine dinucleotide phosphate
**O$_2$**  Oxygen
**O$_2^•$**  Superoxide
**OH•**  Hydroxyl radical
**P$_i$**  Inorganic phosphate
**R**  Correlation coefficient
**R$^2$**  Coefficient of determination
**RNS**  Reactive nitrogen species
**ROH**  Alcohol
**ROS**  Reactive oxygen species
**RSSe$^-$**  Selenopersulphide anion
**RSSeSR**  Selenotrisulphide
**RyR**  Ryanodine receptor
**S1**  Subfragment one
**S2**  Subfragment two
**Se**  Selenium
**Se$^{2-}$**  Selenide
**SeO$_2^{3-}$**  Selenite
**SeO$_4^{2-}$**  Selenate
**SERCA**  Sarco/endoplasmic reticulum Ca$^{2+}$-ATPase
**SIC**  Selenite-induced contracture
**SOD**  Superoxide dismutase
**SR**  Sarcoplasmic reticulum
**T3**  Triiodothyronine
**T4**  Thyroxin
**Tempol**  4-hydroxy tempol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Tm</td>
<td>Tropomyosin</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin</td>
</tr>
<tr>
<td>Tn-C</td>
<td>Troponin-C</td>
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<td>Tn-I</td>
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<td>Tn-T</td>
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</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>TT50</td>
<td>Time to 50 % force rundown</td>
</tr>
<tr>
<td>TTP</td>
<td>Time to peak tension</td>
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1 Introduction

Selenium (Se) is an essential mineral for animals, including humans (Yang et al. 1983), as it is a functional cofactor for many proteins (termed selenoproteins) that play important roles in the regulation of cellular metabolism. Crucial selenoproteins include GSH peroxidase, which helps increase cellular capacity to defend against oxidative stress, and thioredoxin reductase and iodothyronine deiodinase, which are both essential enzymes in regulating metabolism (Arner 2009; Arner 2010; Lu, Berndt & Holmgren 2009).

Se homeostasis is critical for normal cell function. Deficiency in Se causes white muscle disease and Keshan disease, which are mediated by oxidative damage to skeletal muscle (Ishihara et al. 1999; Yang et al. 1983). Conversely, high levels of Se can lead to a disease known as blind staggers disease in livestock, or selenosis in humans (Painter 1941). Cases of selenosis have resulted from changes in dietary intake due to drought, industrial accidents, over-supplementation, and poisonings (Williams & Ansford 2007; Aldosary et al. 2012; Spiller & Pfeifer 2007; MacFarquhar et al. 2010; Schellmann, Raithel & Schaller 1986; Wilson 1962; Alderman & Bergin 1986). Blind staggers disease is not usually diagnosed until the disease has progressed considerably, and symptoms are the same as those described in human selenosis. The symptoms that relate to skeletal muscle dysfunction include ataxia, paralysis, and death from respiratory failure (Wilber 1980; Lin-Shiau, Liu & Fu 1990).

Previous in vitro studies on rodent skeletal muscle have shown that toxic levels of selenite (SeO$_2^{3-}$), a highly oxidative form of selenium, causes skeletal muscle contracture. Selenite-induced contractions in vivo also lead to respiratory failure in broiler chickens (Lin-Shiau, Liu & Fu 1990; Lin-Shiau, Liu & Fu 1989; Koller & Exon 1986). The hypothesised mechanism behind the selenite-induced contracture (SIC) in both skeletal and cardiac muscle is a redox-mediated breakdown in intracellular Ca$^{2+}$ handling, mainly affecting the sarcoplasmic reticulum (SR) (Lin-Shiau, Liu & Fu 1989; Ugur & Turan 2001). Whether this occurs because selenite increases endogenous ROS production, which then oxidises cellular components, or whether selenite directly oxidises cellular components itself, could not be determined from these experiments.
Xia et al. (2004) showed that selenite increases \(\text{Ca}^{2+}\) flux in isolated SR vesicles *via* direct ryanodine receptor (RyR) thiol oxidation and subsequent channel opening. This effect was blocked by GSH, suggestive of a reactive oxygen species (ROS) mediated mechanism (Xia et al. 2004). In combination, the conclusions of these studies provide direct evidence that selenite can directly oxidise thiol groups, and outlines the possibility of indirect oxidative effects by increasing endogenous ROS species – both of which could contribute to the SIC by oxidation of key SR \(\text{Ca}^{2+}\) handling proteins.

Mitochondrial dysfunction is an alternative mechanism that could explain how altered \(\text{Ca}^{2+}\) handling could cause SIC. Previous research has shown that selenite can cause mitochondrial dysfunction *via* a number of different mechanisms as explained in detail in section 1.12. All of the documented selenite-mediated mechanisms capable of inducing mitochondrial dysfunction can result in the irreversible opening of the mitochondrial permeability transition channel (MPT), a high-conductance non-specific cation channel. Opening of MPT causes rapid efflux of cations from the mitochondrial matrix, subsequent loss of mitochondrial membrane potential, respiratory uncoupling, and the release of cytochrome-C leading to cell death (Lemasters et al. 2009; Brookes et al. 2004; Gyorgy et al. 2006; Shilo et al. 2003; Zhu, Xu & Huang 2002; Chung et al. 2006). Therefore, the effect of selenite on mitochondria function cannot be discounted when considering the SIC in muscle tissues.

In this thesis, new data are added to the hypothesis that SIC in skeletal muscle is a result of multiple contributory selenite oxidative mechanisms. These mechanisms are most likely mediated by an increase in endogenous ROS production, as well as possible nonspecific direct effects of selenite-thiol oxidation. Elevated cellular oxidation can cause a transient increase in myofilament \(\text{Ca}^{2+}\) sensitivity, increasing active force production, where further elevation in oxidation level becomes detrimental to myofilament \(\text{Ca}^{2+}\) sensitivity, contributing to a decrease in active force. I hypothesise that the SIC is not dependent on any one specific \(\text{Ca}^{2+}\) source, and that the SIC can occur in the absence of extracellular and SR \(\text{Ca}^{2+}\). Selenite may alter SR \(\text{Ca}^{2+}\) handling processes, which could also account for changes in submaximal active force production. I hypothesise that selenite could also cause mitochondrial dysfunction,
which could account for the increase in baseline force production associated with the SIC, as well as contributing to changes in active force production.

1.1 Dietary intake of selenium

Selenium is a naturally occurring mineral found in soil in many forms, including elemental Se, selenide (Se\(^2\)), selenate (SeO\(_4\)^2\(^-\)), or selenite (SeO\(_3\)^2\(^-\)). Selenate is the dominant form of Se found in the soil, as it is more soluble in soil than other selenium species (Banuelos & Schrale 1989). Se tolerant plants are able to take up Se from the soil and store it in their tissues. Leafy green vegetables (collards), mustard seeds, cabbage, corn, broccoli, cereals, and even grass, are all capable of storing high concentrations of Se (30-1200 mg/kg dry weight) (Banuelos & Schrale 1989). However, such high concentrations of Se in food are only achieved in areas of very high Se content in the soil, such as Enshi, China (Fordyce et al. 2000b).

Dietary Se toxicity is a rare condition, which occurs mainly due to ingestion of vegetables, cereals, and maize grown in areas of high carbonaceous strata (Yang et al. 1983; Fordyce et al. 2000a). These areas have high concentrations of underground carbon rock formation, and have been shown to increase crop content and bioavailability of Se (Fordyce et al. 2000b). One simple solution that can prevent excessive crop Se uptake is to farm in areas away from the carbonaceous strata. However, population pressure and drought do not always permit this (Yang et al. 1983). Available rice crops diminish under drought conditions, as they require a high amount of water, forcing people to eat increased amounts of high Se content leafy green vegetables, corn, and cabbage, all of which are more drought tolerant than rice (Fordyce et al. 2000b; Yang et al. 1983).

Se is mainly excreted from the body via the kidneys. Other mechanisms, usually related to chronic exposure, include a small amount of Se being excreted in the bile and faeces, often as diarrhoea. Se can also be converted to di-methylselenide, which is excreted by the lungs, giving off a garlic odour on the breath (Spiller & Pfiefer 2007). When ingestion exceeds the body’s capacity to excrete Se, acute/chronic symptoms of
selenosis (detailed below) develop (Spiller & Pfiefer 2007; McConnell & Portman 1952).

1.2 Selenosis

Disease associated with high levels of ingested Se is referred to as selenosis in humans, and blind staggers disease in livestock. These two diseases are essentially the same, as damage is caused by the same oxidative mechanism, although the observed onset of disease differs in timing, as humans are able to report symptoms earlier in disease progression than animals. The symptoms of selenosis that relate to skeletal muscle include ataxia, paralysis, and respiratory failure (Lin-Shiau, Liu & Fu 1989; Wilber 1980).

The recommended daily intake of Se is 55 µg/day for male and female adults, and 70 µg/day for pregnant woman (Mistry et al. 2012). The minimum level of dietary Se needed to avoid selenium deficiency has been suggested to be 30 µg/day (Yang et al. 1983). The tolerable upper limit of Se ingestion (the level with no observed adverse effects) is reportedly 400 µg/day (NRC 2000). This limit is a corrected value based on reported cases of people exposed to high Se intake. Observations made of the communities in the Enshi district of south China showed that the highest Se intake per day of a person suffering no adverse effects from selenosis was 913 µg/day (Yang & Zhou 1994). Measurements of blood Se concentration from studies by Yang et al. (1983) showed that the normal mean blood Se level was 1 µM in unaffected individuals, and at the earliest onset of selenosis the mean blood Se level was 40 µM, with a maximum blood Se level of 90 µM in one individual. This led to the conclusion that 800 µg/day would show no adverse effects (NRC 2000). In a study of 142 free-living ranch workers of South Dakota, USA, the highest recorded Se dietary intake was 724 µg/day (Longnecker et al. 1991). This individual showed no adverse effects (Longnecker et al. 1991), consistent with Yang & Zhou’s (1994) findings. However, to account for any degree of uncertainty, this number was then halved to 400 µg/day as a recommended upper limit to daily intake (NRC 2000).
Individuals who are suffering from severe Se related disease present with symptoms such as weakness, fatigue, ataxia, dyspnoea, convulsions, and paralysis (Yang et al. 1983). Although these symptoms are severe, the mechanism behind the disease remains poorly understood. There are few investigations into the specific tissues that are affected by selenosis. Yang et al. (1983) concluded that symptoms such as convulsions and paralysis were nervous system abnormalities, although this study never investigated the mechanism by which selenosis affects the nervous system. However, evidence from more recent studies on isolated organs suggest that skeletal muscle function can be greatly affected by toxic doses of Se (Xia et al. 2004; Lin-Shiau, Liu & Fu 1989). As studies conducted by Lin-Shiau, Liu & Fu (1989) used 100 μM Se concentration directly on the diaphragm muscle, which caused contracture, this suggests that the observed symptoms in studies by Yang et al. (1983) were partly due to effects on skeletal muscle. However, no researchers have investigated the effects of selenite on muscle function any further than the studies of Lin-Shiau, Liu & Fu (1989) and Xia et al. (2004).

1.3 Metabolic selenoproteins

Se may be incorporated into two amino acids, cysteine (forming selenocysteine), or methionine (forming selenomethionine), and both of these selenium-containing amino acids are incorporated into many proteins (termed selenoproteins) (Jacob et al. 2003). There are five Se containing protein groups and associated isoforms in humans, three of which play major roles in cellular function and development. The remaining two groups of selenoproteins are responsible for Se uptake, storage, and transportation to body tissues (Kryukov et al. 2003).

Important selenoproteins include thioredoxin reductase (TrxR), which is the only known enzyme in humans capable of reducing thioredoxin (Trx) (Moore, Reichard & Thelander 1964). Trx is reduced in the presence of TrxR and nicotinamide adenine dinucleotide phosphate (NADPH), and this reduced form is then able to catalyse the reduction of disulphide bonds on proteins, protecting against oxidative stress (Masutani et al. 1996; Meyer et al. 2009). Alteration in the expression of TrxR has been
linked to many diseases, including cancer, diabetes, cardiovascular and neurodegenerative diseases, and rheumatoid arthritis (Nakamura et al. 1992; Lillig & Holmgren 2007; Chernatynskaya et al. 2011). Se is also an important regulator of cellular metabolism. It is a co-factor of the selenoprotein iodothyronine deiodinase, which converts thyroxin (T4) into triiodothyronine (T3) (Schneider et al. 2006). T3 is a stimulator of cellular metabolism, a regulator of cell proliferation and differentiation, and is essential in foetal development (Pop et al. 1999).

1.4 Glutathione (GSH)

The glutathione (GSH) enzymes, in particular glutathione peroxidase (GSH-Px), are the group of selenoproteins that are vital for normal skeletal muscle function. GSH-Px catalyses the reduction of hydrogen peroxide (H₂O₂) to water in the presence of GSH, leaving GSH in an oxidised state (GSSG), as shown in Equations 1 and 2. Oxidised glutathione is formed by the oxidation of thiol groups on the GSH molecule, which creates disulphide bonds (Rotruck et al. 1973). These disulphide bonds change the structure of the tri-peptide, inhibiting any further action of GSH as an anti-oxidant (Spallholz 1997) (Figure 1). These oxidisable bonds of reduced GSH are protective against oxidative stress. GSH is also able to reduce organic hydroperoxides (ROOH) to H₂O and alcohol (ROH) (Lotscher et al. 1979). These qualities of GSH help prevent lipid peroxidation and further dismutation of H₂O₂ into the more damaging hydroxyl radicals (Letavayova, Vlckova & Brozmanova 2006) (Figure 1).

The disulphide bonds formed during the oxidation of GSH shown in Equation 1 and 2 can be reduced back to their previous state by glutathione reductase (GSR), effectively restoring the anti-oxidant capacity of the GSH protein (Kalpakcioglu & Senel 2008). This GSH redox cycle is essential in maintaining cellular ROS homeostasis. However, H₂O₂, the radical responsible for lipid peroxidation, can be reduced to H₂O and O₂ by the action of the enzyme catalase, shown by Equation 3.

Equation 1

\[ 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow 3\text{GSSG} + 2\text{H}_2\text{O} \]
Equation 2
2GSH + ROOH → 3GSSG + ROH + H₂O

Equation 3
(In the presence of catalase)
2 H₂O₂ → 2H₂O + O₂

Interestingly, both high and low concentrations of dietary Se can result in increased levels of cellular oxidative stress. As Se is a cofactor of GSH, too little Se leads to a net increase in the effects of ROS by reducing the cellular GSH concentration, and thus cellular anti-oxidant handling capacity. Therefore, it is essential to maintain an adequate level of Se in the diet to maintain GSH levels.

![Figure 1: The selenite reduction pathway](image)

Selenite (SeO₃²⁻) can be reduced to elemental selenium (Se⁰), producing superoxide (O₂•⁻) in the presence of glutathione (GSH) in a cellular environment. This O₂•⁻ is then able to be dismutated into H₂O₂ via the action of O₂•⁻ dismutase, which can then be broken down to the hydroxyl radical (OH•) via Fenton reactions with other metal ions (adapted from Spallholz 1997).

Increased oxidative stress caused by low dietary Se intake drastically increases the chance of Keshan disease (a cardiomyopathy) in humans, and white muscle disease in livestock (Andrews, Hartley & Grant 1968; Salonen et al. 1982). White muscle disease is not evident in livestock until observable weakness in standing, curvature of the back, and muscle stiffness occur (Beytut, Karatas & Beytut 2002). Patients with Keshan disease, however, present with cardiac symptoms before obvious skeletal muscle weakness (Collipp & Chen 1981; Kien & Ganther 1983). The mechanism of muscle damage, both cardiac and skeletal, for white muscle disease and Keshan disease is attributed to low GSH-Px activity (Lei et al. 2011; Andrés et al. 1997). Evidence for low GSH-Px activity being attributed to reduced Se intake is shown by the positive effect Se
supplementation has on increasing GSH-Px activity in affected humans and livestock (Andrés et al. 1997; Yang et al. 1983).

Excessive Se intake can lead to the oxidation of GSH, and the production of the oxygen radical, $O_2^{•−}$, and elemental selenium (Spallholz 1997). The oxidation of GSH occurs with GSSeSG as an intermediate, where the ratio of GSH : GSSG (oxidised GSH) determines the level of available GSH to be used as an anti-oxidant (Meyer et al. 1998). Once the ratio of GSH : GSSG favours GSSG, excessive ROS species can accumulate (Kitahara, Seko & Imura 1993). The free radical, $O_2^{•−}$, is a critical cell-signalling molecule; however, excessive increases in the concentration of $O_2^{•−}$ negatively affects cell function (Lamb & Westerblad 2011). Superoxide is able to dismutate to form secondary oxidative products such as $H_2O_2$, which are highly reactive. $H_2O_2$ can be further oxidised via Fenton-like reactions, by reacting with partially reduced metal ions such as iron ($Fe^{2+}$), or copper ($Cu^{+}$) to form a hydroxide radical ($OH^{•}$), both of which can cause oxidative damage (Terada et al. 1999; Spallholz 1997).

1.5 Skeletal muscle structure

To understand the following sections, a firm understanding of normal muscle structure and function is required. Skeletal muscle consists of bundles of muscle fascicles, made up of bundles of muscle cells, known as muscle fibres. These fibres contain specialised organelles, channels, and membrane substructures that couple motor nerve excitation with muscle contraction. The bulk of the muscle fibre is made up of contractile proteins, arranged into sarcomeres, which are responsible for the generation of force and muscle shortening. Sarcomeres are organised in such a way that the contractile proteins are aligned parallel to the longitudinal axis of the muscle fibre. The three dimensional shape of the sarcomere is cylindrical, with adjacent sarcomeres joined at Z discs via the structural protein desmin. Sarcomeres contain both contractile and non-contractile proteins. The two main contractile proteins of the sarcomere are myosin and actin. These two contractile proteins are arranged into thick and thin filaments, where six thick filaments surround each thin filament, contributing to the cylindrical organisation of the sarcomere (Figure 2). The thick filaments are composed of myosin, and the thin filament is mostly composed of polymerised F-actin protein and the regulatory proteins, troponin (Tn) and tropomyosin (Tm). The myosin and actin
proteins arranged in this manner are the components that contribute to generating active force (Wang 1996).

Actin is a spherical globular monomer, with one binding site for myosin. Tropomyosin and troponin provide structural support to the thin filament by binding to actin to form filamentous actin. Tm binds to seven actin molecules, as well as a subunit of Tn, increasing actin’s structural stability (Lorenz, Popp & Holmes 1993). The troponin protein is composed of three subunits, troponin-C (TnC), troponin-T (TnT), and troponin-I (TnI). The TnC subunit binds Ca\(^{2+}\) ions, whereas the TnT subunit attaches to tropomyosin, and the TnI subunit has an inhibitory function (Greaser & Gergely 1973; Mak & Smillie 1981). The overall length of the thin filament is determined by the non-contractile protein, nebulin, which winds around the entire length of the thin filament (Wang 1996).

Myosin is a contractile protein consisting of a straight tail region, which terminates in a globular head portion, termed subfragment 1 (S1). It is this region that enables myosin to interact with actin during cross-bridge cycling, as it contains the actin binding site, and an adenosine tri-phosphate (ATP) binding site, which supplies energy for cross-bridge cycling (Rayment et al. 1993). The S1 region can bind to actin due to the presence of subfragment 2 (S2), which is a flexible link region in the myosin tail. The thick myosin filaments are held in the middle of the sarcomere by the non-contractile protein titin, which links the thick filament to the Z disc, ensuring that myosin remains centred (Figure 2) (Tregear et al. 1998).
Figure 2: Structure of skeletal muscle and the sarcomere

A) The structure of skeletal muscle organelles. A muscle is made up of fascicles, which are bundles of muscle fibres. The muscle fibre contains thick and thin filaments, which are surrounded by the sarcoplasmic reticulum, and adjoining t-tubular system. The myofibrils themselves are organised into a sarcomere (B.), the functional contractile unit of skeletal muscle. B) The general layout of the sarcomere, including its thin (actin) and thick (myosin) contractile filaments. C) The individual components that make up the thick and thin filaments. (Saladin 2010).

1.6 Cross-bridge cycling

Cross-bridge cycling is initiated by the release of Ca\(^{2+}\) from the SR via the RyR Ca\(^{2+}\) release channels. The release of Ca\(^{2+}\) enables the interaction between the proteins of the thin filament (Ebashi & Endo 1968; Hellam & Podolsky 1969). Ca\(^{2+}\) binding to TnC enhances the interaction between TnC and TnI. This weakens the bond between TnI and actin, which allows Tm to change configuration and expose the myosin-binding site on the actin filament (Moore, Huxley & DeRosier 1970). These Ca\(^{2+}\) dependent factors in combination influence the probability of the open myosin binding state of actin, thus increasing the probability of myosin binding and cross-bridge formation (Hancock, Huntsman & Gordon 1997).
Cross-bridge cycling first involves the rapid binding of ATP to its binding site on the myosin S1 region while myosin is bound to actin, forming an actomyosin-ATP (AM•ATP) complex. This is followed by a rapid dissociation of myosin from actin, leaving ATP bound to myosin (M•ATP). The ATP is then hydrolysed into adenosine diphosphate (ADP), and inorganic phosphate (P_i), which remain attached to the myosin head, forming M•ADP•P_i. This form of myosin is able to reassociate with actin to form a weakly bound A-M•ADP•P_i complex, which can then be isomerised into a more strongly bound state, AM•ADP•P_i. From this strong binding state, a subsequent isomerisation occurs to form AM**•ADP•P_i. This process may be stabilised by the release of P_i, leaving AM**•ADP (Cooke 1997).

Force is generated during cross-bridge cycling by the ‘power-stroke’, a process in which the S1 region of myosin is flexed towards its tail while attached to actin. This propels the thin filaments on each side of the sarcomere towards each other. The exact mechanism behind the power-stroke is unknown; however, the transition between the weak binding state (A-M•ADP•P_i) and the strong binding state (AM**•ADP) involves a large change in free energy, and it is during this process that force is produced (White & Taylor 1976; Pate & Cooke 1989).

1.7 Excitation-contraction coupling (ECC)

The excitation of the muscle cell membrane (the sarcolemma) is initiated via the release of acetylcholine (ACh) from the synaptic terminal of a motor nerve at the motor end plate region of the muscle. The released ACh binds ligand-gated receptors (nicotinic ACh receptors) in the synaptic cleft, causing an influx of sodium ions and efflux of potassium ions. This initiates an action potential in the muscle cell, as there is a far greater permeability for sodium ions moving into the cell, due to the electrochemical gradient, than there is for potassium ions moving out of the cell, causing depolarisation of the sarcolemma (for review: (Greig & Jones 2016)). This action potential propagates along the sarcolemma and deep within the muscle fibre via the t-tubule system, which are invaginations of the sarcolemma that descend into the cell (Figure 3).
Dihydropyridine receptors (DHPR) are voltage sensitive L-type calcium channels found along the T-tubules (for review: (Frontera & Ochala 2015)). Depolarisation of the sarcolemma causes a change in configuration of the DHPR, which are located adjacent to the terminal cisternae region of the SR, resulting in a mechanical link forming between the II-III loop of DHPR \( \alpha \) subunit and specialised \( \text{Ca}^{2+} \) release channels, RyR of the SR, which is the main \( \text{Ca}^{2+} \) store in skeletal muscle. The RyR are located in the lateral sac region of the SR, known as the terminal cisternae, where the SR meets the T-tubule system. The SR is a specialised endoplasmic reticulum, which acts as the internal \( \text{Ca}^{2+} \) store. The activation of the RyRs induces calcium release from the SR into the intracellular space, which increases the cytosolic calcium concentration by approximately 1000-fold within 1 millisecond, initiating cross-bridge cycling by the contractile apparatus. SR \( \text{Ca}^{2+} \) ATPase (SERCA) pumps, found in abundance in the SR membrane, are responsible for restoring SR \( \text{Ca}^{2+} \) concentration after \( \text{Ca}^{2+} \) release has ceased. The SERCA pumps reduce cytosolic \( \text{Ca}^{2+} \) concentration, and causes relaxation of the muscle cell by dissociation of \( \text{Ca}^{2+} \) from TnC (for review: (Rebbeck et al. 2014)).

Figure 3: The process of excitation-contraction coupling

The components of excitation-contraction coupling. An action potential (AP) propagates along the sarcolemma, and down the t-tubule system. The AP can then depolarise the voltage sensitive dihydropyridine receptors (DHPR). \( \text{Ca}^{2+} \) release channels in the sarcoplasmic reticulum (SR) membrane then open, allowing \( \text{Ca}^{2+} \) to move into the cytoplasm and initiating contraction. The sarcoplasmic reticulum \( \text{Ca}^{2+} \) ATPase (SERCA) pump, pumps \( \text{Ca}^{2+} \) back into the SR, causing relaxation. The orange arrows demonstrate the path of \( \text{Ca}^{2+} \) during contraction and relaxation. (Image created by Thomas Wilson, 2017).
1.8 Reactive oxygen species and oxidative stress

Endogenously derived ROS can increase the likelihood of skeletal muscle fatigue during exercise (Jackson, Edwards & Symons 1985). Skeletal muscle contraction also promotes the oxidation and overall depletion of GSH, temporarily decreasing the amount of available antioxidant (Sen et al. 1992; Lew, Pyke & Quintanilha 1985). These factors contribute to the development of oxidative stress and muscle damage. Several types of oxidative changes occur during muscle contraction, including reversible protein modification and irreversible damage. Reversible oxidative modifications refer to the oxidation of thiol (sulphur-hydroxide) groups on proteins and lipid peroxidation, which is the oxidation of lipids by hydroxide radicals (Sumida et al. 1989). Irreversible changes refer to protein carbonylation, in which the metal catalysed oxidation of amino acid side chains of proteins results in the formation of carbonyl groups (Cattaruzza & Hecker 2008). This changes the amino acid structure and thus, the protein’s function (Nystrom 2005). During exercise, markers of lipid peroxidation increase (Duthie et al. 1990) as does the degree of protein carbonylation (Barreiro et al. 2005; Guidi et al. 2011). It has also been demonstrated via mass spectrometry that prolonged exposure to oxidative stress damages both contractile and structural proteins of the sarcomere (Fedorova, Kuleva & Hoffmann 2010). Fast glycolytic (type 2) muscles are much more susceptible to irreversible oxidative carbonylation than are slow oxidative (type 1) muscle fibres (Feng et al. 2008).

1.9 Cellular redox state and force development

The effects of oxidative stress in cells are opposed by antioxidant compounds, which act by reducing ROS and/or oxidised components (Hirai et al. 2011; Branco et al. 2012; Murphy, Dutka & Lamb 2008). These compounds exist in delicate balance with ROS in order to optimise cell signalling. While at rest, skeletal muscle fibres are in a mostly reduced state (Lamb & Westerblad 2011; Reid 2001). ROS production increases as muscle fibres perform work (Ji 2015). An initial increase in ROS production can have a positive effect on muscle force production, but if the level of ROS produced exceeds the muscle’s antioxidant buffering capacity, force production begins to fall (Figure 4) (Lamb & Westerblad 2011).
Lamb & Westerblad (2011) showed that exposure of intact mouse fast twitch muscle fibres to H$_2$O$_2$ transiently increased force production after 4 mins, but force then decreased below control values after 8 mins. The force loss due to H$_2$O$_2$ exposure was fully reversible with the application of the thiol-specific reducing agent dithiothreitol (DTT). In another experiment by Lamb & Westerblad (2011), DTT was applied first resulting in a reduction in force, which was then recovered by the addition of H$_2$O$_2$ to the bathing solution. This finding demonstrates how a muscle fibre’s basal redox state sits towards the reduced side of the optimal point for force production, and can be manipulated in either direction in accordance with overall redox state. Reactive nitrogen species (RNS), such as nitric oxide, are also able to influence force production, as they have a negative effect on myofilament Ca$^{2+}$ sensitivity (Dutka et al. 2017). The build up of both ROS and RNS can lead to excessive oxidative damage and can irreversibly alter the function of proteins; therefore, selenite-induced oxidative stress generated by ingestion of excess levels of Se could be causal in selenium toxicity.

![Figure 4: The effect of redox balance on skeletal muscle force production](image)

The red line indicates a figurative change in force production as a function of cellular redox state. The purple traces show the effect of H$_2$O$_2$, a ROS, on force over time. An initial increase in cellular oxidation increases force production from a reduced basal state, but increased oxidation has a negative effect on force production. The green traces to the right indicate a severe loss of force due to oxidation by H$_2$O$_2$ followed by an increase in force production after exposing the same fibre to DTT, a thiol reducing antioxidant. (Adapted from (Lamb & Westerblad 2011)).
1.10 Selenite-induced contracture

Skeletal muscle contracture is the abnormal shortening of a muscle, preventing it from lengthening/relaxing (Mayer & Esquenazi 2009). This is not the same as normal skeletal muscle contraction, which is the process of cortical activation leading to nerve stimulus, causing electro-chemical coupling and cross-bridge cycling. Normal muscle contraction entails the capacity for a muscle to relax and lengthen, which is not possible during contracture. Many different pathologies and trauma can cause contracture, which is often the result of muscle damage leading to fibrosis of muscle cells (Mayer & Esquenazi 2009; Favrot et al. 2009; Canapp & Saunders 2014).

Cellular depletion of ATP can lead to a rigor contracture, which is where the myosin head will not detach from the actin molecule, causing muscle rigidity (White 1970). This would result in the inability to actively produce force, and would also increase baseline force production. Thus, this pathway is a logical mechanism for the SIC. Another intrinsically derived contracture is observed in malignant hyperthermia (MH) (Walker, Yip & Pirmohamed 2014). When muscles are stimulated, voltage-gated activation of DHPR causes the RyR to release Ca²⁺ (Franzini-Armstrong 2004). In cases of MH, the DHPR linkage to the RyR can become fixed when exposed to particular anaesthetics, causing uncontrollable calcium-induced calcium release (CICR) from the RyR that leads to potentially fatal hyperthermia due to contracture (for review; (Rosenberg et al. 2015)). As SIC is hypothesised to arise from RyR dysfunction (Lin-Shiau, Liu & Fu 1989; Xia et al. 2004), muscle stimulation and the linkage of DHPR to RyR could play a role in the SIC. As the aforementioned studies show, SIC occurs within two hours of exposure, factors affecting an intrinsically derived loss of active force production and/or an increase in baseline force in the presence of selenium, form the basis of this thesis.

Lin-Shiau et al (1989, 1990) showed that high levels of Se cause contracture of the diaphragm muscle and therefore, may contribute to the respiratory failure observed in chickens effected by selenium toxicity (Lin-Shiau, Liu & Fu 1990). These experiments were carried out on isolated diaphragm muscle fibres using 0.1 mM sodium selenite at 37 °C. Diaphragm muscle was stimulated to produce a series of twitches and the
authors found that sodium selenite causes skeletal muscle contracture (Figure 5), where active force is diminished and baseline force is markedly increased. The mechanism responsible for this observation is poorly understood. One commonly cited explanation is that Se can alter the function of key proteins involved with cellular Ca\(^{2+}\) homeostasis, resulting in elevated cytosolic Ca\(^{2+}\) concentration and hence an increase in baseline force production (Lin-Shiau, Liu & Fu 1989; Lin-Shiau, Liu & Fu 1990; Xia et al. 2004). Mitochondrial dysfunction is another explanation for this observation, and is a commonly cited mechanism responsible for how selenium can induce apoptosis in cancerous cells (Shen et al. 2001; Kim et al. 2007; Kim & Choi 2008; Guan et al. 2009; Shilo et al. 2003). Both of these hypotheses will be explained further below.

**1.11 Effect of selenite on Ca\(^{2+}\) homeostasis**

The hypothesised mechanism behind the SIC in both skeletal and cardiac muscle is a redox-mediated breakdown in intracellular Ca\(^{2+}\) handling, mainly due to Ca\(^{2+}\) release from the SR. An intracellular Ca\(^{2+}\) source is implicated in SIC, as the removal of Ca\(^{2+}\) from the bathing solution did not prevent the SIC (Lin-Shiau, Liu & Fu 1989; Ugur & Turan 2001). The SIC was inhibited when GSH was added to the bathing solution prior to selenite exposure. However, this inhibiting effect was weaker when GSH was added 20 mins after selenite exposure, and GSH was not able to antagonise the effects of selenite when added 65 mins after selenite exposure. This suggests that Se produces bi-phasic oxidative stress, by initially oxidising thiol groups, which can be reversibly reduced by anti-oxidants such as GSH. The second phase is observed later and produces oxidative damage via an irreversible mechanism (Lin-Shiau, Liu & Fu 1989).
The hypothesis that Se causes dysfunction of internal Ca\(^{2+}\) homeostasis processes was further supported by Xia et al. (2004), who showed that selenite directly increases Ca\(^{2+}\) flux in SR vesicles via RyR thiol oxidation and subsequent channel opening in a dose-dependent manner (Figure 6). This effect was also blocked by GSH, suggesting a ROS mediated mechanism (Xia et al. 2004)(Figure 6). It is possible that the hypothesised changes in Ca\(^{2+}\) handling could lead to the activation of calpains, which are Ca\(^{2+}\) activated protease enzymes (Belcastro 1993). Calpains can be activated by increased ROS, which can then result in mitochondrial-derived cell death (Yokoyama et al. 2014). Selenite activation of caplains by altering Ca\(^{2+}\) handling processes could result in irreversible cell damage, or death, which is consistent with the observations made in the aforementioned studies. The conclusions of these studies outline the possibility that both direct and indirect oxidative mechanisms could contribute to the SIC by oxidation of key SR Ca\(^{2+}\) handling proteins, mostly likely the RyR that regulates Ca\(^{2+}\) release.
Figure 6: The effect of selenium on RyR Ca\textsuperscript{2+} release rate in SR Vesicles

Mean (±SEM) Ca\textsuperscript{2+} rate of loaded SR vesicles is plotted as a function of ebselen concentration ([μM]: solid circles), showing a positive sigmoidal relationship between ebselen concentration and Ca\textsuperscript{2+} release rate. Mg\textsuperscript{2+} 2.2 mM (solid triangle), a RyR antagonist, ruthenium red 5 μM (open circle), and GSH 100 mM (solid square), were all able to reduce the Ca\textsuperscript{2+} release rate from the RyR in the presence of 40 μM ebselen. (Xia et al. 2004).

1.12 Effect of selenite on mitochondrial function

Mitochondrial dysfunction presents an alternative hypothesis to dysfunction of SR Ca\textsuperscript{2+} handling causing SIC. Previous research using various cell types has shown that selenite can cause mitochondrial dysfunction via a number of mechanisms including: 1) direct oxidation of thiols on the mitochondrial membranes (Kim et al. 2002; Chung et al. 2006); 2) over-production of O\textsubscript{2}•⁻ within mitochondria (Kim et al. 2007; Kim, Yun & Kim 2003; Shen et al. 2001; Guan et al. 2009); 3) oxidation of thioredoxin-reductase and GSH-reductase, causing oxidation of NADH (Rigobello et al. 2011; Vlessis & Mela-Riker 1987; Shilo et al. 2003); and 4) direct oxidation of proteins of the mitochondrial permeability transporter (MPT) channel itself (Kim et al. 2002; Kim, Yun & Kim 2003; Shilo et al. 2003). All of these mechanisms can result in the irreversible opening of the MPT channel, a high-conductance non-specific cation channel. Opening of the MPT causes rapid efflux of cations and protons from the mitochondrial matrix, subsequent loss of mitochondrial membrane potential, respiratory uncoupling, and the release of cytochrome-c leading to cell death (Lemasters et al. 2009; Brookes et al. 2004; Gyorgy et al. 2006; Shilo et al. 2003; Zhu, Xu & Huang 2002; Chung et al. 2006). These mechanisms of selenite-induced mitochondrial dysfunction are responsible for a large
component of the chemo-protective effects of selenium against cancer, as selenium uptake is increased in cancerous cells (Shilo et al. 2003; Guan et al. 2009; Kim & Choi 2008; Kim et al. 2007; Shen et al. 2001).

As mentioned above, mitochondrial dysfunction can result in the release of Ca$^{2+}$ and protons into the cytosol. Decreases in extracellular pH to 6.6 in healthy EDL muscle bundles, or cytosolic pH to 6 in chemically skinned single fibres, can significantly decrease myofilament Ca$^{2+}$ sensitivity (Rassier & Herzog 2002; Martyn & Gordon 1988). Studies of intracellular pH changes due to exercise in humans have shown that intracellular pH can fall from 7.14 to between 6.5 – 6.87 at muscle exhaustion (Juel et al. 1990). This observation suggests that even in healthy human subjects, exercise can temporarily overcome the capacity of outward proton homeostasis transport systems, such as the Na$^+$/H$^+$ co-transporter, Na$^+$/Cl$^-$/bicarbonate system, and even the lactate/H$^+$ co-transport system. The mitochondria contain high concentrations of both Ca$^{2+}$ and H$^+$, which can be uncontrollably released through the high conductance MPT channel. In an in vitro whole muscle environment, which lacks a blood supply to aid in H$^+$ buffering, it is plausible to hypothesise that the release of H$^+$ could cause a decrease in myofilament Ca$^{2+}$ sensitivity, and that the release of Ca$^{2+}$ could contribute to the increased baseline force.

Mitochondrial dysfunction also decreases ATP production, which can instigate a rigor contracture (Fink, Stephenson & Williams 1986; White 1970; Han et al. 2003; Yamaguchi 1998). A rigor contracture may only account for the increase in baseline force and lack of active force once muscle fibres begin to die, which would unlikely occur in all muscle fibres of a whole muscle simultaneously. Therefore, peripheral muscle fibres that are exposed to selenite first may begin to die and enter rigor before muscle fibres deeper in the muscle belly. This would decrease the active force and increase the baseline force in a gradual manner. As rigor is possible in the absence of Ca$^{2+}$ (Yamaguchi 1998; Fink, Stephenson & Williams 1986), the SIC may not require an increase in $[\text{Ca}^{2+}]_{\text{cytosol}}$, but could arise from a combination of Ca$^{2+}$ handling dysfunction, decreased myofilament Ca$^{2+}$ sensitivity, a loss of ATP, and possible cell death caused by mitochondrial dysfunction.
2 Aims & Hypotheses

The overall aim of this project was to determine the mechanism of selenium-induced skeletal muscle dysfunction and weakness. The three research foci of this thesis were the effects of selenium on the whole muscle force response, the effects of selenium on mitochondrial function, and the specific effects of selenium on the contractile apparatus. Experiments were designed in order to address the following specific aims:

2.1 Aim 1

To identify the characteristics of selenite-induced skeletal muscle dysfunction and contracture, and whether this contracture is dependent on changes to Ca\(^{2+}\) handling. I aimed to assess the effect of selenite on twitch characteristics, myofilament Ca\(^{2+}\) sensitivity, and RyR SR Ca\(^{2+}\) release.

Aim 1 hypotheses

1. High concentration of selenite acting via a nonspecific redox mechanism causes skeletal muscle dysfunction via multiple mechanisms.
2. Selenite will significantly alter twitch characteristics observed as changes to the rate of force development and relaxation.
3. Selenite will cause an initial increase, followed by a subsequent decline, in myofilament Ca\(^{2+}\) sensitivity.
4. The SIC is independent of external [Ca\(^{2+}\)] and SR Ca\(^{2+}\) release.

2.2 Aim 2

To determine if mitochondrial dysfunction contributes to selenite-induced muscle dysfunction.
Aim 2 hypotheses

1. High concentrations of selenite will depolarise mitochondrial membranes.
2. This depolarisation will precede cell death, and thus implicate the involvement of mitochondrial dysfunction in SIC.

2.3 Aim 3

To investigate the effect of selenite on the contractile apparatus of rodent skeletal muscle, and to determine if these effects contribute to the loss of force observed in whole muscle.

Aim 3 hypotheses

1. The loss of force seen in whole muscle experiments is partly due to direct effects on the contractile apparatus.
2. Selenite will reduce the sensitivity of the contractile apparatus, and thereby reduce maximum force production.
3. The decrease in sensitivity and force production due to selenite exposure is caused by reversible thiol oxidation.
3 Materials & Methods:

3.1 Animals

The Animal Ethics Committee of the University of Western Australia approved the use of animals for this study (RA/100/1324). All animals used in this study were acquired from the Animal Resource Centre (Murdoch, Western Australia). All experiments were conducted using adult (6 week old) male ARC Swiss mice. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013).

3.2 The effect of selenite on intact whole muscle

Experimental animals were anaesthetised using intraperitoneal injection of sodium pentobarbitone (Lethabarb, 40 mg/kg), and were unresponsive to the foot-pinch and corneal reflexes. Animals were monitored constantly during the procedure, and supplemental anaesthesia was administered as required. Once under anaesthesia, animals were placed on a 37 °C heating pad to maintain their core body temperature. The extensor digitorum longus (EDL) muscle was surgically excised, and both tendons of the muscle tied firmly with non-absorbable, black braided surgical silk (size 3.5, Dysilk, Dynek Pty Ltd, Adelaide, South Australia). The excised EDL muscle was mounted in an in vitro muscle test system (1205A, Aurora Scientific Inc., Aurora, Canada). The muscle was positioned in an organ bath super-fused with mammalian Ringer solution (in mM; NaCl 121, KCl 5.4, MgSO$_4$·7H$_2$O 1.2, NaHCO$_3$ 25, HEPES 5, glucose 11.5 and CaCl$_2$ 2, maintained at pH 7.3) and bubbled with carbogen (5 % carbon dioxide, in oxygen, Air Liquide, Western Australia). The organ bath temperature was maintained at 22 or 25 °C (depending on individual experiments), both of which are within the optimum temperature range for in vitro force production (Segal, Faulkner & White 1986; Arbogast & Reid 2004). The organ bath temperature was adjusted accordingly for whole muscle experiments performed at 37 °C. Muscles were stimulated via two
platinum electrodes, which ran parallel to the suspended muscle, connected to a 701B pulse stimulator (Aurora Scientific Inc. Canada). The force output data was recorded and displayed using Dynamic Muscle Control software (Aurora Scientific Inc. Canada). Experimental animals were euthanized following the dissection of the EDL muscle by lethal dose of sodium pentobarbitone (Lethabarb, > 120 mg/kg).

3.2.1 Whole muscle experimental protocol

Optimal muscle length ($L_o$) was determined by adjusting muscle length to produce peak twitch force ($P_t$). This muscle length was then used throughout whole muscle experiment protocols. The force-frequency relationship for the EDL muscle was determined at both 25 °C (in Hz; 10, 20, 30, 40, 60, 80, 100, 120, 150), and 37 °C (in Hz; 20, 40, 60, 80, 100, 120, 150, 200), with a 2 min interval between each stimulation. Submaximal stimulations were used for all whole muscle experiment protocols, given as the frequency that produced ~50 % of maximum force.

Maximum (stimulation parameters: 120 Hz, 500 ms duration) and submaximal tetanic responses (22 °C & 25 °C: 40 Hz; 500 ms duration; 37 °C: 80 Hz, 500 ms duration), single muscle twitches, and baseline force levels were recorded before and after the addition of sodium selenite to the bathing solution (22 °C & 25 °C: 10 mM; 37 °C: 0.1 mM; Sigma-Aldrich Inc.). Recordings were taken every 2 mins over a 70 min period, with sodium selenite (10 mM) dissolved in Krebs solution added after 10 mins of stable recordings. The justification for the use of such high concentrations of sodium selenite in this thesis is described as part of section 4.1.1, and section 4.1.5. Peak active force amplitude and baseline force were recorded at each time point. It must be noted that the stimulation frequency used for each condition was different (25 °C = 40 Hz, 37 °C = 80 Hz); however, both of these frequencies correspond to ~50 % of maximum force production at each respective temperature. If a stimulation frequency of 40 Hz is used at 37 °C, the increased energetics of the system prevents any tetanic fusion, resulting in a series of single twitches, thus a higher frequency must be used. All recordings were sampled at a frequency of 2000 Hz. Data points of interest between treatment groups were analysed using unpaired 2-tailed multiple Student’s t-tests, with $\alpha = 0.05$. All data analysis, unless otherwise stated, was performed using GraphPad Prism.
software (Graphpad Prism 6, GraphPad Software Inc.). Data for all whole muscle experiments are presented as mean values ± SEM. Comparisons conducted within groups in order to analyse changes in relative force production were completed using paired 2-tailed Student’s t-tests. Changes to the relative time to peak tension (TTP) and relative half-relaxation (1/2RT) times, were analysed using repeated measures one-way ANOVA with post hoc Dunnett analysis.

At normal physiological temperature (37 °C), a plasma selenite concentration of 0.1 mM is a toxic dose (Koller & Exon 1986). The results of the work done by Lin-Shiau (1989) showed contracture induced by 0.1 mM at 37 °C did not occur in rat diaphragm preparations until ~100 mins of exposure. Intact muscle in a 37 °C in vitro environment will decay at a much faster rate due to the effects of temperature alone (Segal, Faulkner & White 1986; Moopanar & Allen 2005; Arbogast & Reid 2004); therefore, these intact whole muscles studies were performed at room temperature (22 - 25 °C). Due to the decrease in temperature, the selenite concentration was increased to 10 mM. In order to demonstrate that 10 mM selenite at 25 °C (n = 6) is comparable to a physiologically relevant model, a 37 °C temperature control group were exposed to 0.1 mM selenite. We also compared the effects of 10 mM selenite at 25 °C (n = 6) and 22 °C (n = 5), in order to relate the results of other experiments that had to be performed at 22 °C to this data set. Linear regressions were fitted to these data, in order to compare Hill slope values of these data sets. The Hill slope measured in the 22 and 25 °C data sets were used to calculate a $Q_{10}$ value for the SIC using Equation 4.

**Equation 4**

\[ Q_{10} = \left( \frac{R2}{R1} \right) ^ {\frac{10}{T2 - T1}} \]

\[
R1 = \text{Hill slope at 22 °C}; R2 = \text{Hill slope at 25 °C}; T1 = 22 °C; T2 = 25 °C
\]

The $Q_{10}$ value is a calculated rate of change of a reaction if the temperature was increased by 10 °C. In *in vivo* conditions, values for $Q_{10}$ range from -1.5 - 3 (Bennett 1990), where negative values reflect a decrease in performance, 1 - 1.5 reflect neutral temperature dependence, 2 reflects moderate dependence, and 3 or greater reflects high temperature dependence. The $Q_{10}$ value reflects the altered reaction rate; hence, a $Q_{10}$ of 3 means the reaction rate would be expected to increase by a factor of 3.
3.2.2 Effect of tetracaine and antioxidants on SIC

Tetracaine is a potent blocker of the RyR channels of the sarcoplasmic reticulum (Laver & van Helden 2011; Csernoch et al. 1999), which are the main Ca^{2+} release channels responsible for initiating cross-bridge cycling events. We applied tetracaine to our stimulation protocol to investigate the role of RyR-in selenium-induced muscle dysfunction. Tetracaine (Sigma-Aldrich) was dissolved in dimethyl-sulphide (DMSO; Univar Chemicals) and added to the organ bath after 10 mins of stable recordings, at a final concentration of 500 μM and a DMSO : Krebs ratio of 1 : 2000. Selenite (10 mM) was added to the bath 10 mins after the addition of tetracaine. For studies concerning the effect of the anti-oxidants glutathione (GSH) (Sigma-Aldrich) and 4-hydroxy tempo (tempol) (Sigma-Aldrich), the anti-oxidants were added to the bathing solution before commencing the whole muscle protocol in 10 mM and 20 mM concentrations, respectively.

3.3 UV - spectrophotometry

During experiments explained in section 4.1.3, the addition of selenite to an organ bath containing GSH created a red solution of colloid consistency. Further research into this observation showed the existence of an abiotic reaction between selenite and GSH, in which GSH is able to reduce selenite into less toxic selenium species. Cui et al (2008) used mass spectrometry to propose a three-step reaction equation for an abiotic solution containing selenite and GSH, shown by equation 5.

Equation 5

UV – spectrophotometry was performed to quantify the observations made in section 4.1.3 between the abiotic reaction of GSH and sodium selenite. To do this, maximum
peak absorbance above 200 nm wavelength was recorded using a Cary 60 UV – Vis
spectrophotometer, with a 1 cm optical glass cuvette (Agilent Technologies). Samples
were made by the addition of GSH, sodium selenite, or both, into DD water. The
concentrations used for control samples containing either GSH, or sodium selenite,
were prepared at 50 mM concentrations, in order to show how little either compound
affects absorbance individually, even at extremely high concentrations. The
concentration used for samples containing both GSH and sodium selenite were made
to molar ratios of 1 : 1 (Sodium selenite : GSH) and 1 : 25, in order to reflect the molar
ratios used as part of this thesis (1 : 1) and those used as part of previous research (1 : 25) (Lin-Shiau, Liu & Fu 1989). The concentrations of GSH and sodium selenite used in
these combined solutions (1 : 1 ratio; sodium selenite 0.5 mM : GSH 0.5 mM; 1 : 25
ratio; sodium selenite 0.05 mM : GSH 1.25 mM) were far less than what was used
elsewhere in this thesis, as higher concentrations of these two substances creates too
many precipitated particles to use UV - spectrophotometry. All solutions were made
into 6 samples to be used as repeated measures, and the mean (±SEM) light
absorbance of each solution was compared between groups using a one-way ANOVA.

3.4 Flow cytometry for mitochondrial membrane potential

Low passage (< 10) murine C2C12 myoblast cells (ATCC) were used to assess
mitochondrial function via flow cytometry. Cells were grown in Dulbecco’s modified
Eagle’s medium with 1x GlutaMAX (DMEM) (Gibco) containing 10 % v/v foetal bovine
serum (FBS) (Serana) until 80 % confluent. Cells were then exposed to selenite (10 mM
in phosphate buffered saline) at room temperature (22 °C) for 0, 10, 20, or 30 mins
and then harvested (Accutase; Gibco) for flow cytometry to determine mitochondrial
depolarisation and cell viability.

Cellular ratios of viability (a loss of plasma membrane integrity visualised by
fluorescence of the membrane-impermeable dye: 7-AAD+) and mitochondrial
depolarization (visualised by fluorescence of a proprietary MitoPotential dye: MPd+;
Merck Millipore) were measured using flow cytometry. Triplicate samples were
individually analysed on a Muse flow cytometer (Merck Millipore) using Muse
MitoPotential Assay kits (Merck Millipore), following the manufacturer’s protocols,
and gating was set using control cells (0 mins selenite exposure) and maintained for each treatment. Gated cell counts of > 1000 events (cells) were collected, and percentage counts per gate generated using Muse 1.1.2 Analysis software (Merck Millipore) and imported into Excel (Microsoft). Data are presented as mean ± SEM of 3 independent cell culture replicates, and paired sample 2-tailed Student’s t-tests were performed to determine statistical significance of treatments.

3.5 Single skinned fibre preparation

Single muscle fibres were isolated from the EDL muscle of 6-week old male ARC mice euthanized via overdose of intraperitoneal pentobarbital (Lethabarb, 120 mg/kg). The EDL muscle was used for these experiments as it is composed of 96% fast twitch muscle fibre, which helps reduce potential variability in results due to fibre type selection (Agbulut et al. 2003). The muscle fibres were dissected from the EDL muscle while it was bathed in paraffin oil. An isolated muscle fibre was clamped at one end using a pair of fine tipped forceps fixed to the experimental apparatus, and the other was secured to a force transducer (SI Heidelberg; KG4A; Germany) using very fine surgical thread (Bakker & Berg 2002). The fibres were maintained at room temperature (22 °C) in a 2 ml perspex bath containing highly buffered physiological solution (Sol. A; in mM; K⁺ 117, Na⁺ 36, ATP (total) 8, free Mg²⁺ 1; creatine phosphate 10, EGTA²⁻ (total) 50, HEPES 60, NaN₃ 1, at pH = 7.10). NaN₃ was added to destroy mitochondria, preventing mitochondrial Ca²⁺ flux. The muscle fibres were then stretched by 20% of their slack length to bring muscle fibre towards optimal length (Lamb & Stephenson 1990). Cell membranes were permeabilised by a 12 min exposure to the polyethylene detergent Triton X-100 before commencing any experiments. For experiments pertaining to section 4.3.1, muscle fibre width was measured over three sites along the length of each fibre. The cross sectional area (CSA) of fibres was calculated by Equation 6, where A represents the largest measure of diameter and B represents the smallest measure of diameter. Force responses were recorded (400 Hz) using a Powerlab data acquisition unit (ADInstruments) attached to a PC.

Equation 6

\[ \text{CSA} = \frac{\pi AB}{4} \]
The effect of Se on Ca\(^{2+}\) sensitivity and maximum force production of the contractile apparatus in EDL muscle fibres was tested by exposing muscle fibres to solutions of different free [Ca\(^{2+}\)] before and after exposure to Se. These solutions were highly Ca\(^{2+}\)-buffered, and contained different free [Ca\(^{2+}\)], prepared by combining different proportions of Sol. A and Sol. B, where Sol. B had a difference in [EGTA\(^{2+}\)] and [Ca-EGTA] (Stephenson & Williams 1981). The [EGTA\(^{2+}\)] of Sol. A was 0.77 mM, whereas the [Ca-EGTA] of Sol. B was 49.33 mM, where the free [Ca\(^{2+}\)] in solution was calculated using a \(K_{app}\) for EGTA of 4.78 x 10\(^{6}\) M\(^{-1}\) (Fink, Stephenson & Williams 1986). Sodium selenite (10 mM, Sigma-Aldrich) and DTT (20 mM, Sigma-Aldrich) were added directly to individual baths containing Sol. A only. Muscle fibres were bathed in these solutions for set periods of time, with no change observed in resting tension. It is important to note that fibres from the experiments of sections 4.3.2-4.3.4 were never exposed to selenite in solution containing [Ca\(^{2+}\)] high enough to initiate any contraction. This is because preliminary experiments showed osmotic interference from sodium selenite (10 mM), reducing muscle fibre contraction. The fibres were placed in a washout solution before being exposed to any [Ca\(^{2+}\)] large enough to cause contraction.

Fibres were briefly washed by exposure to Sol. A between force recordings to return force to baseline values. Force recordings after exposure to any treatment were made relative to force recordings of pre-treatment recordings to account for any loss of force over time. Force responses to solutions of increasing free [Ca\(^{2+}\)] were expressed as a percentage of maximum Ca\(^{2+}\) activated force, which were then plotted as a function of pCa. Sigmoidal curves were fitted to these data, with Hill-slope coefficient and pCa\(_{50}\) values (pCa value producing 50 % of maximum force) calculated accordingly. Differences in myofilament sensitivity to [Ca\(^{2+}\)] and maximal force production before and after exposure to treatment were compared using 2-tailed Student’s t-tests. To determine statistical significance in experiments comparing the difference between two groups only, or before and after treatment in a single group, paired or unpaired sample 2-tailed Student’s t-tests were used.
3.5.1 The effect of whole muscle selenite exposure on single skinned fibre force production

For experiments conducted in section 4.3.1, a whole EDL muscle was exposed to selenite (10 mM; 25 °C) as described in section 3.2.1. Submaximal force in these muscles was allowed to decay by 20 % (group-A) or 50 % (Group-B), before removing the muscle from the organ bath and preparing it for single skinned fibre dissection. Fibres dissected from these muscles were tested without directly exposing the fibre itself to selenite. Therefore, any differences in force production, compared to time matched control muscles, are due to the effects of selenite on the myofilament proteins that occurred in intact whole muscles.

3.5.2 The direct effect of selenite on single skinned fibre force production

For experiments conducted in sections 4.3.2 onwards, healthy EDL muscle was dissected from an animal and immediately prepared for single skinned fibre dissection. The muscles these fibres were dissected from were not exposed to selenite at any point; the single fibres dissected from these healthy muscles were only exposed to selenite once they had been chemically skinned. As the chemical skinning process permeabilises any lipid membrane, the cytosolic contents diffuse out into the bathing solution, so the effects of selenite on the contractile apparatus can be studied without the interference of other cellular organelles, or cytosolic enzymes. Therefore, any effects of selenite exposure in the experiments of section 4.3.2 onwards are due to direct effects of selenite on the myofilament proteins, and not because of any other cellular mechanisms.
3.6 How these methods address the aims

The contribution of each method and how it pertains to the aims of this thesis are outlined below, in figure 7.

Each square represents a possible causal influence in selenium-induced muscle dysfunction and contracture. Calcium handling: The main variables of the whole muscle study that infer changes in calcium handling are the muscle twitch characteristics, where changes in the speed of contraction/relaxation can indirectly infer changes in Ca\textsuperscript{2+} release and reuptake events that effect active force production. The use of tetracaine and Ca\textsuperscript{2+} free bathing solution in whole muscle studies was tested the requirement of SR Ca\textsuperscript{2+} release via the RyR, and the requirement of extracellular Ca\textsuperscript{2+}. Mitochondria: Whether or not mitochondrial dysfunction could play a role in the SIC observed in whole muscle was investigated using flow cytometry of C2C12 muscle cells. This method assesses the integrity and the polarisation of both the cell and mitochondrial membranes. By using this method it was possible to ascertain whether or not selenite was able to cause mitochondrial membrane depolarisation, and whether or not this depolarisation occurs before or after cell death. Contractile apparatus: The whole muscle method was used to assess the effect of selenite on frequency dependent force production at different key stages of the characterised selenite-induced active force response. If force production differs between twitch, submaximal, and maximal tetanic force in response to selenite exposure, it is possible that this could be due to effects on myofilament Ca\textsuperscript{2+} sensitivity. This was directly examined by using the single skinned fibre technique. The data collected from all of these methods combined will provide the framework for this masters thesis.
4 Results

4.1 Whole muscle studies

4.1.1 The effect of selenite on submaximal force generation at 37 °C and 25 °C

As mentioned in section 3.2.1, in vitro rodent whole-muscle studies are best performed at 25 °C. The most relevant of the previous studies examining Se-induced muscle dysfunction (Lin-Shiau 1989) was performed using diaphragm strips at 37 °C using 0.1 mM selenite. Therefore, a comparable model for testing the effects of selenite at 25 °C was established. The decrease in force, in the absence of selenite, after 70 mins at 25 °C (7.6 ± 1.4 %; n = 5) was significantly less than that at a 37 °C (16.6 ± 2.7 %; n = 5; p < 0.001) (Figure 8 A). It is possible that part of the 8.9 % difference observed in this experiment was due to fatigue arising from the higher stimulation frequency at 37 °C (80 Hz) compared to 25 °C (40 Hz). Higher stimulation frequency had to be used at 37 °C, as fusion frequency increases with temperature. However, this effect was considered to be minimal as the relative intensity of stimulation is constant between both conditions.

After characterising the independent effects of temperature, the effect of Se on contractile function was then investigated at 25 °C (10 mM; n = 6) and 37 °C (0.1 mM; n = 5; Figure 8 C & D). In both cases, an initial transient increase in active force is observed, followed by a progressive decline in active force for the remainder of the experiment. The initial transient increase in active force was larger at 25 °C (10 mM) (11.2 ± 1.6 %) compared to 37 °C (0.1 mM) (3.4 ± 0.5 %), P = 0.045), when compared to respective pre-exposure force production. After 70 mins of submaximal stimulation in the presence of selenite at 25 °C (10 mM), active force was virtually abolished, having declined to only 1.1 ± 0.4 % of pre-exposure force. At 37 °C (0.1 mM), the decline in active force was markedly less than at 25 °C (10 mM), having only declined to 31.6 ± 4.9 % of pre-exposure force.
The average time to 50% active force (TT50) at 25 °C (10 mM: 26.8 ± 0.9 mins) was significantly shorter than the TT50 time observed in the 37 °C (0.1 mM: 55.2 ± 2.5 mins, P < 0.0001; Figure 8 C). Linear regressions were fitted to these data to characterise the rate of active force decay. The force decay in the 25 °C (10 mM) group was bi-phasic and therefore fitted with two linear regressions. There was a significant increase in the rate of force decay after 20 mins of selenite exposure (from -2.3 ± 0.03 %/min (R = 0.99) to -4.3 ± 0.2 %/min (R = 0.99; P < 0.001). This change in slope corresponds directly to a significant increase in baseline force in the 25 °C (10 mM) group after 20 mins of selenite exposure, which peaked at 8.5 ± 0.9 times the original baseline after 70 mins (Figure 8 D). These results suggest a significant interaction between the increase in baseline force and the muscle’s ability to actively produce force (Figure 8 C & D). These characteristics were not observed in the 37 °C (0.1 mM) group, as baseline force had not increased significantly compared to control after 70 mins of selenite exposure (Se 0.1 mM: 1.6 ± 0.3; control: 0.5 ± 0.1; P = 0.3). For this reason, the 37 °C (0.1 mM) group had only one linear regression fitted to characterise the loss of active force (-1.1 ± 0.03 %/min; R = 0.99).
4.1.2 The effect of selenite on twitch, submaximal and maximal force production

These experiments were designed to characterise the effect of selenite on frequency-dependent force production and twitch characteristics of skeletal muscle at different points of the selenite-induced force response as described in 4.1.1. To do this, a single muscle twitch, a submaximal (40 Hz), and a maximal tetanic stimulation (120 Hz) were applied to intact EDL muscles (n = 6) at different time points following the addition of selenite (10 mM), at 25 °C. Figure 8 illustrates that submaximal force production increases to its peak 6 mins following the addition of selenite; therefore, a single
twitch and a maximal tetanus were also stimulated at this time point. Once submaximal force production had decreased by ~50 % (T = 25 mins), a single twitch and maximal tetanus were again stimulated and compared to submaximal force production.

There was no significant difference in maximum twitch force at T = 6 mins, compared to its pre-exposure control (99.9 ± 2.0 %; P = 0.97; Figure 9). However, submaximal (40 Hz) and maximal tetanic (120 Hz) force production both showed transient increases at T = 6 mins, of 12.2 ± 1.5 % (P < 0.001) and 5.9 ± 1.0 % (P = 0.002), respectively. The increase observed in submaximal force was significantly greater than the increase in maximal force (P = 0.006). After 25 mins of selenite exposure, twitch and submaximal force had both fallen by approximately ~50 %, 45.7 ± 6.7 % and 48.4 ± 3.6 %, respectively (P = 0.27). However, tetanic force had fallen by 65.2 ± 5.2 % of original control levels, a significantly greater reduction than twitch and submaximal force (P = 0.008; Figure 9).

The twitch recordings were also used to assess the effects of selenite on the time-course of contraction and relaxation, thus providing information relating to the effect of selenite on SR Ca\textsuperscript{2+} handling events. Time to peak twitch force (TTP) and half relaxation time (1/2RT) are used as measures of the rate of SR Ca\textsuperscript{2+} release and reuptake. The TTP and 1/2RT values were analysed as absolute values and as values normalised to respective twitch force amplitude, to attempt to account for changes in total twitch force amplitude, giving a measure of ms/g of force. There were no statistically significant changes to TTP at either time point, nor normalised TTP at T = 6 mins when compared to respective pre-exposure TTP (Table 1; P > 0.05); however, normalised TTP at T = 25 mins was significantly slower (9.5 ± 1.9 ms/g) compared to pre-exposure values (3.2 ± 0.5 ms/g) (Table 1; P = 0.03). There was a significant increase in 1/2RT and normalised 1/RT at T = 6 mins and T = 25 mins, when compared to pre-exposure values (Table 1).
Figure 9: The effect of selenite on force production at different stimulation frequencies.

Mean (±SEM) (D) % Normalised active force production at different stimulation frequencies - twitch (1 Hz), submaximal (40 Hz) and maximum tetanic (120 Hz) - where values are made relative to their corresponding force production pre-selenite exposure (10 mM) at 25 °C. T = 6 mins corresponds to the peak in submaximal force increase following selenite exposure observed in previous experiments. Raw traces for pre-exposure, T = 6 mins, and T = 25 mins, of twitch (A), submaximal (B), and maximal tetanic (C) raw responses are shown. (n = 6). (** = P < 0.01, *** = P < 0.001).

Table 1: Effect of sodium selenite on single muscle twitch speed characteristics.

Mean (±SEM) time to peak (TTP) and half-relaxation times (1/2RT) for mouse EDL muscles exposed to sodium selenite (10 mM). TTP and 1/2RT values are also shown as normalised values relative to the magnitude of contracture (ms/g). (**Indicates significant difference to pre-exposure value, calculated via RM one way ANOVA (n = 6, F (1,3,6) = 12.7), * = P <0.05, ** = P < 0.01).

<table>
<thead>
<tr>
<th></th>
<th>Pre-exposure</th>
<th>T = 6 mins</th>
<th>T = 25 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTP (ms)</td>
<td>22 ±2</td>
<td>28 ±4</td>
<td>24 ±4.5</td>
</tr>
<tr>
<td>1/2RT (ms)</td>
<td>20.8 ±2.1</td>
<td>38.5 ±5.0**</td>
<td>42.7 ±6.5 *</td>
</tr>
<tr>
<td>TTP (ms/g)</td>
<td>3.2 ±0.3</td>
<td>3.8 ±0.3</td>
<td>6.8 ±1.2 *</td>
</tr>
<tr>
<td>1/2RT (ms/g)</td>
<td>3.2 ±0.5</td>
<td>6.0 ±1.2 *</td>
<td>9.5 ±1.9 *</td>
</tr>
</tbody>
</table>
4.1.3 The effect of tempol and GSH on selenite-induced muscle dysfunction

As all hypotheses concerning the effect of high concentrations of selenite on cellular function revolve around redox mechanisms (2.1-2.3), I investigated the effects of altering the cellular redox environment using either pro-oxidant or antioxidant pre-treatment.

Tempol is a SOD mimetic and accelerates the dismutation of $\text{O}_2^-\text{ into H}_2\text{O}_2$, which can act as a pro-oxidant in higher concentrations. Muscle exposed to tempol alone, at a pro-oxidant concentration (20 mM), showed an average active force rundown of 19.6 ± 2.6 % ($n = 5$), with no change in baseline force after 70 mins (1.2 ± 0.1; $P = 0.9$; Figure 10 A). This loss of active force was significantly greater than in no-tempol controls (7.8 ± 1.4 %; $n = 5$; $P < 0.0001$), consistent with its pro-oxidant nature at high concentrations. Pre-treatment with tempol (20 mM) and exposure to selenite (10 mM) (94.8 ± 2.5 %; $n = 5$), produced significantly less relative force than selenite (10 mM) alone (111.2 ± 1.6 %; $n = 6$; $P < 0.0001$; Figure 10 C) at $T = 6$ mins. Pre-treatment with tempol significantly reduced TT50 time (14.8 ± 0.5 mins; $P < 0.0001$) and significantly increased the rate of active force decay (-3.8 ± 0.3 %/min; $P = 0.016$), compared to selenite alone (26.8 ± 0.9 mins, -2.9 ± 0.1 %/min).

Exposure to the antioxidant GSH (10 mM) alone showed an average active force rundown of 11.6 ± 2.7 % ($P = 0.83$; Figure 10 A) with no increase in baseline force production after 70 mins, which is not different to that observed in controls without GSH (Figure 10 B; $P = 0.23$). However, when selenite was added to the bathing solution containing GSH (10 mM), the solution turned a bold red/orange colour with colloid consistency. Further inquiry into this reaction indicated that GSH can abiotically reduce selenite into elemental selenium, which appears red with a colloid consistency in solution (For details: section 3.3). This observation is further examined in section 4.1.4.
Relative active force production was significantly higher in muscle exposed to selenite (10 mM) and GSH (10 mM) (117.7 ± 1.5 %; n = 5) compared to selenite alone (10 mM) (111.2 ± 1.6 %; n = 6; P < 0.0001; Figure 10 C), after 6 mins of exposure. GSH pre-treatment prolonged this transient active force increase and delayed the subsequent rapid force decay, resulting in a significant rightward shift in TT50 time (32.7 ± 0.7 mins), compared to selenite alone (26.8 ± 0.9 mins; P < 0.001). No difference in the rate of active force decay was observed between GSH pre-treated muscles (-2.6 ± 0.1 %/min) and selenite alone (-2.8 ± 0.1 %/min; P = 0.076). These data are consistent with the hypothesis that the mechanism affecting active force production, both the increase and decrease, is highly redox sensitive.

Normalised baseline force production was significantly elevated in all treatment groups after 70 mins of exposure to selenite (10 mM; P < 0.0001; Figure 10 D). The peak normalised baseline force recorded for both GSH and tempol pre-treatment was not significantly different to muscles exposed to selenite alone (selenite: 8.6 ± 0.9; GSH and selenite: 8.5 ± 1.9; P = 0.99; tempol and selenite: 8.2 ± 0.6; P = 0.99).
Figure 10: Effect of tempol and GSH on the selenite-induced force response.
Mean (±SEM) normalised active force (A & C) and normalised baseline force (B & D) for all groups. Force recordings were taken at 25 °C, with 40 Hz stimulation, applied to mouse EDL muscle. For groups pretreated with GSH (10 mM, diamonds) or tempol (20 mM, triangles), respective anti-oxidants were added and force stabilised before commencing experiments. Forces were normalised to the force at T = 0 mins. (n = 5 for all groups).

4.1.4 The abiotic reduction of selenite by GSH

Following observations made of the interaction between GSH (10 mM) and selenite (10 mM) during the whole muscle experiments of section 4.1.3, UV-spectrophotometry was performed to quantify the existence of different chemical species in solution. To test this, high concentrations of selenite and GSH were individually examined, followed by the effects of combining the two compounds in the same solution at a ratio of either 1 : 1, or 1 : 25 (selenite : GSH). The peak absorbance
values recorded for selenite (50 mM; n = 6) alone in DD water and GSH (50 mM; n = 6) alone in DD water were far less than either ratio of selenite and GSH combined (Figure 11). These data show that selenite and GSH alone have little effect on absorbance. Absorbance recorded for solution containing a 1 : 1 ratio of selenite (0.5 mM) : GSH (0.5 mM) (n = 6), as used in this study (10 mM : 10 mM), was much greater than absorbance recorded for solution containing a 1 : 25 ratio of selenite (0.05 mM) : GSH (1.25 mM) (n = 6), as used in Lin-Shiau (1989) (0.1 mM : 2.5 mM) (P < 0.0001). These data demonstrate a shift in chemical composition of abiotic solutions containing sodium selenite and GSH. This change indicates that the effective concentration of each compound has likely changed, which could have a strong influence on their redox potential.

Figure 11: The abiotic reduction of selenite by GSH.  
Mean (±SEM) peak light absorbance recorded by UV-spectrophotometry, for wavelengths greater than 200 nm. The table lists the composition of each solution and the molar ratio of compounds (where applicable). The appearance of the 1:1 ratio solution clearly appears orange/red to the eye of the observer, whereas the 1 : 25 ratio solution appears normal with small amounts of grey precipitate at the bottom of the vessel. (n = 6 for all solutions).
4.1.5 Effect of temperature and calculating $Q_{10}$

As some experimental methods following this section could only be performed at 22 °C, a comparative study was performed to determine the effect of temperature on selenite-induced muscle dysfunction using data recorded at 25 °C (10 mM; $n = 6$) and 22 °C (10 mM; $n = 5$). There were no significant differences observed in the average active force rundown, or baseline force, after 70 mins between control muscles at 25 °C and 22 °C in the absence of selenite (Figure 12 A; $P = 0.99$).

At 22 °C, transient increase in active force production of 11.3 ± 1.5 % occurred at $T = 6$ mins, which was not different at 25 °C (11.2 ± 1.6 %; $P = 0.99$; Figure 12 A). The average TT50 time at 22 °C (52.1 ± 0.8 mins) was significantly slower than at 25 °C (26.8 ± 0.9 mins; $P < 0.0001$). The rate of active force decay, measured as the %/min loss from peak active force to when force had decayed by 50 %, was greater at 25 °C (-2.8 ± 0.1 %/min) when compared to 22 °C (-1.3 ± 0.02 %/min). A $Q_{10}$ value of 12.2 was calculated using these rate values, demonstrating an extremely high temperature dependence of the selenite-induced force response. Baseline force was significantly elevated at both temperatures after 70 mins, but slower to rise at 22 °C (25 °C: $P < 0.0001$; 22 °C: $P < 0.0001$; 22 °C Vs. 25 °C: $P = 0.61$; Figure 12 B).

These data show that temperature affects the time to onset of contracture, observed as a faster rate of active force decrease and an earlier increase in baseline force, but does not affect the magnitude of contracture. This experiment also showed far less variability in the increase in baseline force between the 20 min and 60 min time points, showing that 22 °C would be a better model for studying changes in baseline force.
4.1.6 The effect of selenite on baseline force

One hypothesis of the cause of SIC is that selenite oxidises key thiol groups of the RyR, which causes Ca\(^{2+}\) to be released from the SR, causing an increase in baseline force production. To test this hypothesis, I investigated the effect that blocking the RyR using tetracaine (500μM), has on the selenite-induced baseline force increase. After 10 mins of exposure to tetracaine (500 μM), active force was reduced to 2.4 ± 0.2 % (T = 20 mins: n = 4; Figure 13 A), with active force remaining inhibited after another 80 mins after exposure to tetracaine, indicating the RyR was effectively blocked for the entire experimental protocol.

For muscle exposed to tetracaine and selenite, tetracaine was first added to abolish active force, with selenite added to this bathing solution 10 mins later. The increase in maximum baseline force in the presence of tetracaine (500 μM) and selenite (10 mM) after 70 mins was not significantly different to selenite (10 mM) alone (tetracaine and selenite: 6.6 ± 0.6; n = 5; selenite alone: 6.4 ± 0.9; n = 5; P = 0.99; Figure 13 B). Figure 13 B shows that at the 55 mins time point, the tetracaine and selenite group appear to produce greater baseline force than selenite alone (selenite alone: 3.5 ± 0.8; selenite
and tetracaine: 6.6 ± 0.4); however, this was not significantly different (p = 0.12). This experiment was repeated in Ca²⁺-free Krebs solution, and no difference in baseline force was observed after 70 mins compared to the aforementioned groups (6.5 ± 0.8; n = 3; P = 0.99; Figure 13 B). These results indicate that the selenite-induced rise in baseline force is not due to an increase in cytosolic Ca²⁺ from the SR, or from an extracellular Ca²⁺ source.

The effect of muscle stimulation on the selenite-induced rise in baseline force was also investigated. This experiment compared the difference in baseline force production in muscles that were stimulated at 40 Hz every 2 mins, to unstimulated muscles (0 Hz), in the presence of selenite (10 mM). These experiments were performed at 22 °C, as results of section 4.1.5 demonstrated a lower degree of variability in baseline force in the presence of selenite (10 mM) at 22 °C compared to 25 °C. There was no significant difference in baseline force between stimulated and unstimulated muscles at 70 mins (40 Hz: 6.4 ± 0.9; n = 5; 0 Hz: 6.4 ± 1.8; n = 5; P = 0.99; Figure 13 B). This result indicates that the amplitude of baseline force increase is not dependent on the amount of muscle contraction, as no active force was produced in the unstimulated group.

Figure 13: The effect of selenite on baseline force production.

A) Mean (±SEM) normalised submaximal (40 Hz) active force production for muscle exposed to tetracaine (500 μM) alone. 10 mins of healthy force recording were taken before tetracaine (500 μM) was added to the bathing solution (indicated by the first vertical dotted line), abolishing active force production by T = 20 mins. The second dotted vertical line represents the time point at which selenite was added for muscles seen in B (T = 0 mins).

B) Mean (±SEM) normalised baseline force for muscles exposed to selenite (10 mM) at 22 °C, in mouse EDL muscle. Groups: no stimulation (0 Hz, squares), control (circles), tetracaine (500 μM, triangles), or tetracaine (500 μM) in Ca²⁺ free solution (diamonds). (Control: n = 5, 0 Hz selenite only: n = 5, tetracaine/selenite: n = 5, tetracaine/selenite/Ca²⁺ free: n = 3).
4.2 Mitochondrial depolarisation studies

The overall aim of these mitochondrial studies was to determine the effect of selenite on mitochondrial depolarisation, which is a precursor event resulting in severe mitochondrial dysfunction, which leads to cell death. The aim of this experiment was to determine if selenite exposure causes mitochondrial depolarisation (visualised by fluorescence of a proprietary MitoPotential dye: MPd+; Merck Millipore) prior to loss of viability (a loss of plasma membrane integrity visualised by fluorescence of the membrane-impermeable dye 7-AAD+). Whether or not selenite is able to cause mitochondrial depolarisation before or after death, will provide evidence to suggest that mitochondrial dysfunction is a causal influence in the SIC, or just an end result.

To this end, experimental conditions resulting in submaximal lethality were determined by optimising exposure levels (10 mM for 60 mins) at room temperature (22 °C). These conditions provide experimental variables that were amenable to discern the effects of selenite on mitochondrial depolarisation. The empirically determined experimental conditions were such that maximum selenite exposure duration (60 mins) killed approximately 20 % of cells (20.0 % ±0.5), showing a significant increase when compared to time-matched PBS-only controls (1.2 % ±0.2; P < 0.0001).

I then conducted a time course experiment over a 60 min time period, to investigate whether mitochondria become depolarised before or after they become unviable. To determine whether mitochondrial membranes depolarised before plasma membrane integrity failed, I compared the proportion of cells with viable plasma membranes and depolarised mitochondria at each time point to the proportion at 0 mins. As expected, selenite induced mitochondrial depolarisation in cells with viable plasma membranes in a time-dependent manner (Figure 14). The number of cells with unviable cell membranes and depolarised mitochondria increased in equal magnitude to those that remained viable with depolarised mitochondria at each time point (Figure 14).
These data show that a high number of cells are likely undergoing mitochondrial depolarisation while their plasma membranes are still viable, before the integrity of the plasma membrane subsequently fails. This would indicate that the effects of selenite toxicity may first observed in mitochondria, before the plasma membrane is overtly affected, and that these effects are a potential precursor to later cell death. This supports the hypothesis that mitochondrial dysfunction leading to a rigor contracture is a possible mechanism involved in the SIC.

![Graph](image.png)

**Figure 14:** The effect of selenite on mitochondrial membrane depolarisation and cell viability.

Mean (±SEM) percentage of cells counted with depolarised mitochondrial membranes, using flow cytometry of C2C12 cells, exposed to sodium selenite (10 mM) 0, 10, 20, 30, or 60 mins. Values are expressed as; total percentage of cells with depolarised mitochondria (square), live cells with depolarised mitochondria (solid circle), and dead cells with depolarised mitochondria (open circle).
4.3 Isolated single skinned fibre studies

4.3.1 Effect of whole muscle selenite exposure on skinned fibre force production

Based on the experiments outlined above (section 4.1), the contractile apparatus is a possible site of selenite-mediated contractile dysfunction. To study the effects of selenite on the contractile apparatus, intact EDL whole-muscles were exposed to selenite (10 mM) as described previously. These muscles were removed from the organ bath after normalised active force production had decreased by 20 % (Group-A; n = 6), or 50 % (Group-B; n = 7). A control group (n = 4), not exposed to selenite, was stimulated for equal time as Group-B to control for time and procedural effects. Single muscle fibres were then dissected from these muscles, chemically skinned, and the contractile properties examined. Skinned muscle fibres from Group-A produced 8.0 ± 0.8 N/cm², which is not different to skinned muscle fibres from Group-B (7.0 ± 1.2 N/cm²; P = 0.9; Figure 15 A). The degree of force loss in Group-B was ~67 % lower than the normalised force produced by time matched control muscle fibres (21.1 ± 0.04 N/cm²; P < 0.0001; Figure 15 B).

To determine if the decreased force found in muscle fibres from selenite-exposed whole muscle was due to oxidation of protein thiol groups, fibres were then exposed to the thiol reducing agent, DTT (20 mM). Group-A muscle fibres showed a ~12 % increase in specific-force production, following a 5 mins exposure to DTT (8.9 ± 0.9 N/cm²; p = 0.0016; Figure 15 A). Group-B muscle fibres showed a ~19 % increase in specific-force production, following a 5 min exposure to DTT (8.4 ± 1.4 N/cm²; p = 0.0017; Figure 15 B). When the relative degree of force recovery was compared, no difference was found (P = 0.72), indicating the degree of reversible thiol oxidation was already maximal in Group-A.
Figure 15: Force production in single skinned muscle fibres dissected from whole-muscles exposed to selenite.

Mean (±SEM) specific force (N/cm$^2$) for the control group (black) ($n = 4$), the selenite (10 mM) exposed fibres; before (white) and after DTT (20 mM) exposure (striped) ($n = 7$) is shown. Muscle fibres in selenite groups were dissected from intact muscles that had been exposed to selenite (10 mM), which had lost; A) 20% of normalised force (Group-A), and B) 50% of normalised force (Group-B). (control; $n = 4$, Group-A: $n = 6$; Group-B: $n = 7$)

### 4.3.2 Time-dependent effect of selenite on force production of skinned fibres

In these experiments, the direct effect of acute selenite exposure on the contractile apparatus was examined. A time-course study was conducted to assess the relative change in force production of skinned muscle fibres over time. Force production was measured before exposing fibres to selenite for 2, 10, 20, and 30 mins. After this exposure, the fibres were placed in a wash solution, before recording maximum force production following selenite exposure. The presence of reversible thiol oxidation was assessed by subsequent exposure to DTT (20 mM) for 10 mins, after which point the fibres was again washed out and maximal force recorded, thus allowing any force recovery to be analysed.

There was a significant decrease in normalised force production after 2 mins of selenite exposure ($p = 0.02$: Figure 16). Force loss had become maximal from the 10 min time point, significantly less than what was observed after 2 mins ($P = 0.014$), but
not different to the 30 min time point \((P = 0.99)\). Exposure to DTT for 10 mins increased force production to pre-exposure control values in all groups \((P > 0.05)\), which represents a significant recovery of selenite-induced force loss in all groups \((2 \text{ mins}: 99.1 \pm 0.8 \% ; n = 5; p < 0.001; 10 \text{ mins}: 99.3 \pm 3.6 \% ; n = 6; P = 0.008; 20 \text{ mins}: 98.3 \pm 0.7 \% ; n = 5; P = 0.002; 30 \text{ mins}: 96.5 \pm 2.2 \% ; n = 5; P < 0.0001)\).

Figure 16: Effect of selenite exposure time on maximum force production in single skinned fibres.

A) Example traces are shown for pre-exposure maximal force, maximal force following a 10 min selenite exposure, and maximal force following a 10 min DTT exposure, all in the same fibre (scale bar provided). B) Mean \((\pm \text{SEM})\) normalised maximum force production of single skinned muscle fibres after different lengths of selenite \((10 \text{ mM})\) exposure (white bars), and subsequent maximum force production after 10 mins of DTT \((20 \text{ mM})\) exposure (striped bars). Force values were normalised to maximum pre-exposure force production. Letters depict between group differences. \((\text{Effect of DTT on recovery: } ^* = P < 0.01, ^{**} = P < 0.001, ^{***} = P < 0.0001)\). \((2 \text{ mins}: n = 5, 10 \text{ mins}: n = 6, 20 \text{ mins}: n = 5, 30 \text{ mins}: n = 5)\).
4.3.3 Effect of a repeated cycle of selenite and DTT exposure on force production

These experiments were designed firstly, to assess the effect of a repeated second series of exposures to selenite (10 mM) and DTT (20 mM) on force production, and secondly, to determine if force production recovers without DTT exposure. Muscle fibres were divided into a repeated exposures group (Group-1; \( n = 6 \)) and a self-recovery group (Group-2; \( n = 5 \)), based on the series of solutions they were exposed to (Figure 17). Maximum force production was recorded before initial exposure to selenite, and in between each consecutive solution change for both groups. Muscle fibres were exposed to selenite for a maximum of 10 mins per exposure, as this is a sufficient amount of time for selenite to decrease force production maximally (Figure 16). Muscle fibres were also exposed to DTT for 10 mins per exposure, as this was shown to be sufficient for full force recovery.

For Group-1, the initial exposure to selenite decreased force production by \( 24.7 \pm 3.2 \% \) compared to pre-exposure force, equal to that of Group-2 (\( P = 0.42 \)). The first exposure to DTT was able to fully recover this loss of force, showing no significant difference to pre-exposure values (\( 99.3 \pm 3.6 \% \); \( P = 0.85 \)). The second exposure to selenite decreased force production to the same extent as after the first exposure (Group-1: \( T = 10 \) mins vs \( T = 30 \) mins; Figure 17; \( P = 0.09 \)). The second exposure to DTT, however, did not fully recover force to pre-exposure values (\( 85.5 \pm 4.4 \% \); \( P = 0.02 \)), but still recovered \( \sim 20.9 \% \) (\( P = 0.008 \)).

The series of solutions used for Group-2 were designed to assess whether or not force production would recover without DTT exposure. To do this, muscle fibres were exposed to selenite for 10 mins, followed by two rounds of 10 min incubations in selenite/DTT free solution, with force production recorded between each round. The final 10 mins incubation with DTT was included in this experiment to ensure that force was still recoverable, and to account for any force rundown between groups. The initial 10 min selenite exposure decreased muscle fibre force production by \( 21.4 \pm 2.9 \)
% compared to pre-exposure force. The two subsequent 10 min incubations in selenite/DTT free solution showed no recovery in force production (T = 20 mins & T = 30 mins; P = 0.18; Figure 17). No difference in force production was observed between Groups 1 and 2 at T = 30 mins (P = 0.11; Figure 17). The final exposure to DTT for 10 mins, in Group-2, showed full force recovery to pre-exposure values (P < 0.001; Figure 17).

Figure 17: Effect of a repeated cycle of selenite and DTT exposure on force production.

Mean (±SEM) relative maximum force production of single skinned muscle fibres are shown, where force values recorded after each solution exposure were made relevant to pre-exposure values. The series of solutions each group was exposed to is shown on the figure, where selenite is 10mM and DTT is 20mM. Letters depict within group differences, where no difference is found between data points of the same letter and each letter represents a difference to the other letters. (Group-A: n = 6; Group-B: n = 5). (Effect of DTT between groups: * = P < 0.05, *** = P < 0.001).
4.3.4 Effect of selenite on the sensitivity of the contractile apparatus

The previous three sections (4.3.1 - 4.3.3) demonstrate that selenite can cause dysfunction of the contractile apparatus affecting maximal force production; however, it remains unclear whether direct selenite oxidation is capable of affecting myofilament Ca\(^{2+}\) sensitivity. To address this, fibres were exposed to a series of solutions of increasing free [Ca\(^{2+}\)]. Force recordings for each Ca\(^{2+}\) solution were made before and after a 10 min selenite (10 mM) exposure. The same fibres were then exposed to DTT (20 mM) for 10 mins, and then exposed to the Ca\(^{2+}\) solutions to determine if any changes in myofilament Ca\(^{2+}\) sensitivity were due to reversible thiol oxidation. Fibres were placed in washout solutions before and after being placed in the increasing free [Ca\(^{2+}\)] solutions.

When sigmoidal regression curves were fitted to these data, analysis of EC\(_{50}\) values showed a rightward shift following exposure to selenite (-6.2 \pm 0.04 pCa; \(n = 7; R^2 = 0.95\)) when compared to pre-exposure controls (-6.3 \pm 0.03 pCa; \(n = 7; R^2 = 0.91; P = 0.007\); Figure 18). The Hill slope coefficient was significantly lower following selenite exposure (2.5 \pm 0.1), when compared to pre-exposure controls (3.4 \pm 0.3; \(P = 0.02\)). The decrease in Hill slope could account for the rightward shift in EC\(_{50}\), as the higher [Ca\(^{2+}\)] seem to be more affected than the lower [Ca\(^{2+}\)]. Subsequent DTT exposure increased the Hill slope coefficient (2.9 \pm 0.2; \(P = 0.039\); Figure 18 D) back to pre-exposure control values, but shifted the EC\(_{50}\) values further to the right (-6.1 \pm 0.03; \(R^2 = 0.94; P = 0.012\)).

The effect of DTT alone on myofilament sensitivity was also investigated. A control recording of [Ca\(^{2+}\)] dependent force in the absence of selenite was taken, before bathing the fibres in selenite/DTT free solution for 20 mins. Fibres were then exposed to DTT (20 mM) for 10 mins, and the [Ca\(^{2+}\)] dependent force was then retested (Figure 18 D). The EC\(_{50}\) values recorded following the exposure to DTT (20 mM) alone showed a significant rightward shift in EC\(_{50}\) value (-6.2 \pm 0.01; \(n = 5; R^2 = 0.98; P = 0.044\)), but were significantly less than EC\(_{50}\) values of fibres exposed to selenite and DTT (\(P =\) 0.007).
This indicates that the rightward shift in EC50 following selenite, and subsequent DTT, exposure is not due to time or DTT exposure.

Figure 18: Effect of selenite on myofilament Ca^{2+} sensitivity.

A) Representative pCa force response traces are shown for pre-exposure pCa, post-selenite exposure, and post-DTT exposure, recorded in one fibre (scale bar given). Mean (±SEM) pCa curves for single skinned muscle fibres before selenite exposure (circles), after 10 mins of selenite (10 mM) exposure (solid squares), and after subsequent 10 mins of DTT (20 mM) exposure (triangles). A DTT alone group (open square) that was not exposed to selenite is also shown. This group was exposed to the pCa solution twice, 10 mins apart, to control for time and procedure. Nonlinear trend lines were fitted to these data to determine the EC50 value and Hill slope coefficient after each exposure. (Selenite and DTT exposed fibres: n = 7, DTT only: n = 5).
5 Discussion

The overall aim of this thesis was to determine the mechanism underlying selenite-induced skeletal muscle dysfunction and contracture. By studying the effects of selenite using a whole muscle approach, flow cytometry, and the single skinned fibre method, it was possible to determine the effect of selenite on different aspects of muscle function and how these contribute to selenite-induced muscle dysfunction.

Sodium selenite is a highly oxidative inorganic salt and can oxidise many cellular targets in many different cell types. Previous studies have established that selenite acts via changes in the levels of ROS in muscle, possibly via both direct interactions and by increasing endogenous ROS. Using an intact whole skeletal muscle model, I have shown that selenite can enter muscle cells and cause significant dysfunction. The effects of selenite are observed on active force production, where force transiently rises upon initial exposure, before falling acutely. The effects of selenite are also observed in the six-fold increase in baseline force. The combination of the loss of active force and the increase in baseline force are the two main aspects of the SIC. These findings suggest that the effects of selenite could be caused by oxidation to the SERCA pump proteins, possibly via both an increase in endogenous ROS levels in the cell, as well as direct oxidative effects of selenite. Results of this thesis also support the hypothesis that selenite exposure causes dysfunction of the contractile apparatus, and the mitochondria. Each of the three target areas, the SERCA pump, the contractile apparatus, and the mitochondria, can explain multiple components of selenite-induced dysfunction in whole-muscle.

5.1 Optimising the whole muscle method

These experiments were designed to find a treatment condition that would best reflect the physiological effects of selenite, whilst minimising the detrimental effects of higher temperature on skeletal muscle viability, to allow the effects of selenite to be examined in isolation. The active force rundown after 70 mins in the absence of selenite was significantly greater at 37 °C (16.6 ± 2.7 %) than at 25 °C (7.6 ± 1.4 %). The
initial transient increase in submaximal force, induced by exposure to selenite, was significantly lower at 37 °C (0.1 mM) compared to 25 °C (10 mM). This is consistent with previous research that shows \textit{in vitro} experiments performed at 37 °C display increased basal ROS production (Andrade et al. 1998; Andrade, Reid & Westerblad 2001; Mollica et al. 2012; Lamb & Westerblad 2011). The force/redox function suggests that an increase in basal ROS production will result in an increase in force, but prolonged oxidation will cause a decrease in force (for review: (Reid 2001; Cheng et al. 2016)).

At 37 °C (0.1 mM) there was no increase in baseline force, even after 70 mins, and no change in the rate of active force loss was observed. At 25 °C the onset of the rise in baseline force \((T = 20 \text{ mins})\) coincided with a significant increase in the rate of active force loss. It is therefore possible that the mechanism responsible for the increase in baseline force is linked to the mechanism affecting active force. As there was no appreciable increase in baseline force at 37 °C (0.1 mM), which is a major characteristic of SIC, and the amount of active force decay due to temperature alone was significant, the 25 °C (10 mM) model was chosen as a more appropriate condition to study the effects of selenite on \textit{in vitro} whole muscle.

Some of the experiments conducted in this thesis, such as the skinned fibre method and flow cytometry, could only be performed at 22 °C; therefore, a series of experiments were conducted to compare the selenite-induced muscle dysfunction at 22 °C and 25 °C in response to the same selenite concentration (10 mM). The rate of active force loss was significantly slower at 22 °C compared to 25 °C, resulting in a \(Q_{10}\) value of 12.2, which reflects extreme temperature dependence in this model. The high \(Q_{10}\) value supports the requirement of such a large difference in selenite concentration used at 37 °C (0.1 mM) and 25 °C (10 mM). As the effect of temperature is so large, dropping the temperature by 12 °C, from 37 to 25 °C, would reduce the effective toxicity of 0.1 mM selenite at 25 °C. Future studies could improve on this model, by studying the dose-response of incrementally increasing selenite concentration from 0.1 mM at 25 °C.
The baseline force increased to approximately the same level at both 22 °C and 25 °C by the end of the experiment. However, the rate of increase in baseline force was slower at the lower temperature; consequently, the rise in baseline force had plateaued prior to the cessation of the experiment at 25 °C, but not for the 22 °C. It is possible that the baseline force at 22 °C would have continued to rise, but these data still allow the study of the characteristics of SIC at 22 °C. It was also found that the increase in baseline force at 22 °C is far less variable throughout most time points than what is observed at 25 °C, and for that reason, experiments investigating the effects of selenite on the increase in baseline force (section 4.1.6) were performed at 22 °C.

These temperature results also provide a reasonable basis for the possible clinical use of monitored hypothermia as a treatment option for acute selenosis patients. It is conceivable that due to the extremely high $Q_{10}$ value, decreasing the core body temperature could slow the progression of disease and allow clinicians more time to make further treatment decisions.

5.2 The effect of cellular redox state on selenite-induced dysfunction

It is likely that the increase in active force production is, at least in part, caused by an increase in endogenous ROS production, as it complies with the known understanding of how force reacts to changes in cellular redox state. As the force/ROS function is a positive parabolic curve with basal levels of ROS resting on the reduced ascending limb of the curve, the addition of selenite was expected to cause an initial increase in force, followed by a subsequent decrease. Tempol, being a SOD mimetic, accelerates the dismutation of $\text{O}_2^*$ to $\text{H}_2\text{O}_2$, which can be further dismutated to the hydroxyl radical (Spallholz 1997). Tempol was used at a high concentration (20 mM) to serve as a positive oxidative control. This was confirmed in the tempol only control data, which showed significantly more run down compared to no tempol control data. These results showed tempol pre-treated muscles exposed to selenite produced no initial increase in force and a greater rate of force loss. This result is consistent with tempol
(20 mM) increasing the amount of basal oxidation in the cell, thus shifting the cellular redox state to the descending limb of the force-ROS function, prior to the addition of selenite. It is possible that the disproportionate dismutation of $O_2•^-$ into its more damaging metabolites, $H_2O_2$ and the hydroxyl radical, caused by tempol, could account for this increase in the rate of force loss. Prolonged exposure to $H_2O_2$ has a dose-dependent negative effect on myofilament $Ca^{2+}$ sensitivity (Lamb & Westerblad 2011), which could also explain why tempol pre-treated muscles show no initial transient increase in force. The effects of tempol (20 mM) pre-treatment on the active force response, induced by selenite, are consistent with selenite-induced muscle weakness being redox sensitive.

Similarly, it was hypothesised that pre-treatment with the antioxidant GSH would bias the basal ROS state of the cell further down the ascending limb of the force-ROS function, towards being more reduced than normal. This hypothesis is consistent with the greater relative magnitude and prolonged initial increase in active force observed in GSH pre-treated muscles, after just 2 mins of selenite exposure. Past studies have shown that exposure to ROS, in the presence of elevated GSH, can further enhance myofilament $Ca^{2+}$ sensitivity, via the process of glutathionylation, which could also help explain this observed increase in active force (Murphy, Dutka & Lamb 2008; Spencer & Posterino 2009). Furthermore, if pre-treatment with GSH did create a more reduced cellular environment, it would presumably increase the amount of oxidation, and time, required to decrease active force production. This hypothesis is consistent with the delayed decrease in active force when selenite was added after pre-treatment with GSH. GSH can also decompose $H_2O_2$ and the hydroxyl radical (Kalpakcioglu & Senel 2008; Lamb & Westerblad 2011), which may have helped delay the onset of excessive oxidative stress.

However, a red colloid solution was observed upon the addition of selenite to solution containing GSH, which suggests the presence of elemental selenium (Staicu, Van Hullebusch & Lens 2015; Staicu et al. 2015). Upon further investigation it was discovered that GSH could directly reduce cytotoxic selenite ions to relatively less toxic selenium-GSH compounds and elemental selenium in the absence of enzymes (Tetteh et al. 2014; Lampis et al. 2014; Staicu, Van Hullebusch & Lens 2015; Staicu et al. 2015).
The red appearance of the solution observed is given empirical gravitas by the results in section 4.1.4, which demonstrate that the two compounds in the same solution at a 1 : 1 molar ratio created a very large increase in light absorbance. Past studies show that a ratio of selenite to GSH of 1 : 2 results in a 40 % reduction of selenite in 5-20 mins, and with a ratio of 1 : 4 this increases to an 80 % reduction of selenite after 10 mins (Kessi & Hanselmann 2004). The 1 : 25 ratio used by Lin-Shiau (1989) would likely have removed the majority of selenite from solution in less than 10 mins, which is most probably why their use of GSH showed no contracture. It is possible that the 1 : 1 ratio used as part of this thesis would, therefore, result in less than 40 % of extracellular selenite being reduced by GSH, where this process would likely take more than 10 mins. This provided ample time for selenite to enter muscle fibres before being reduced by GSH. It is most likely that this simple abiotic reaction occurs concurrently with the mechanistically sound explanations for the observed differences in force production, which themselves are consistent with the antioxidant effects of GSH. However, it is impossible to separate the different contributions of the reduction of selenite concentration by GSH, and thereby toxicity, and the possible antioxidant effects of GSH.

When examining the effect of selenite on the baseline force production of whole muscle, the magnitude of increase does not appear to be as redox sensitive as with the decrease in active force. While pre-treatment with GSH or tempol have pronounced effects on the rate of active force decline, no significant differences were observed in baseline force increase when compared with selenite alone (T = 70 mins). GSH pre-treated muscles exposed to selenite appear to have a slightly different slope to the increase in baseline force observed in the selenite only group. As baseline force had not reached a plateau in GSH pre-treated muscles, it is possible that it could have continued to rise. What is more important to consider is that the selenite concentration was, presumably, decreased significantly via abiotic reactions with GSH in these muscles. The possible reduction in selenite concentration supports the idea that the magnitude of baseline force increase does not appear as sensitive to redox changes as the active force does. From these experiments, it is clear that a ROS sensitive process mediates selenite-induced loss of active force and triggers the
increase in baseline force, but it is still not clear what components of skeletal muscle are being affected.

5.3 The effect of Ca^{2+} handling impairment on selenite-induced dysfunction

Previous literature hypothesised that selenite primarily affects the Ca^{2+} handling system of skeletal muscle, via oxidation of the RyR (Xia et al. 2004; Lin-Shiau, Liu & Fu 1989). Analysis of twitch characteristics recorded after 6 mins of selenite exposure showed no change in TTP values, suggesting that selenite does not alter SR Ca^{2+} release through oxidation of the RyR. This is supported by the observation that blocking the RyR with tetracaine had no effect on the increase in baseline force involved in the SIC. Both of these results suggest that selenite exposure affects muscle function via mechanisms that are independent of the RyR.

Analysis of twitch characteristics did, however, show a significant increase in the 1/2RT, which suggests that selenite significantly decreases the function of the SERCA pump. Previous studies have shown that the function of SERCA is redox sensitive, where cysteine residues found on the SERCA protein can become oxidised, inhibiting their function (Qin et al. 2014; Li et al. 2015). It is therefore hypothesised that oxidation of cysteine residues on SERCA could be responsible for the selenite-induced changes observed in active force and baseline force.

The increase in 1/2RT could explain the increase in submaximal force production, and why there was no increase in twitch force, at the 6 min time point. If SERCA function is impaired by selenite, this would mean that more Ca^{2+} remains in the cytosol between each concurrent electrical stimulus, increasing force summation for a given submaximal stimulation frequency. As a twitch recording is the result of a single electrical stimulus, and therefore independent of summation, it would not increase in the same manner as submaximal force. This can be observed in Figure 9 B, where it is clear that the first initial force response of the 40 Hz stimulus before force summation
is not different between pre-exposure and following 6 mins of selenite exposure. Once force begins to summate, it is clear that force increases more so with every concurrent electrical pulse after 6 mins of selenite exposure, compared to pre-exposure.

The increase observed in submaximal force was proportionally greater than what was observed with maximal force production. The slower removal of Ca\(^{2+}\) from the cytosol, due to impaired SERCA function, could increase the amount of force summation, thereby increasing submaximal force. As Ca\(^{2+}\) release is maximal during tetanic stimulation, it is possible that the increase in maximal tetanic force is due to an increase in myofilament Ca\(^{2+}\) sensitivity. Previous studies have shown that different ROS and NRS can have different effects on myofilament Ca\(^{2+}\) sensitivity, where increases in H\(_2\)O\(_2\) can lead to an initial increase in sensitivity, which subsequently decreases with prolonged exposure (Lamb & Westerblad 2011), whereas NO has a negative effect on myofilament Ca\(^{2+}\) sensitivity (Dutka et al. 2017). This mechanism could explain the biphasic nature of active force response induced by exposure to selenite, with an increase in H\(_2\)O\(_2\) first increasing myofilament sensitivity, while prolonged H\(_2\)O\(_2\) exposure would decrease it, aided by the presence of NOS.

However, unlike the results of previous studies showing an increase in ROS leading to an increase in myofilament Ca\(^{2+}\) sensitivity (Lamb & Westerblad 2011), the chemically skinned fibre model used in this thesis showed no increase in maximal force, or Ca\(^{2+}\) sensitivity, at any point. This difference to the literature could be because previous researchers reporting an initial increase in myofilament Ca\(^{2+}\) sensitivity in the presence of ROS used intact muscle bundles, rather than chemically skinned fibres that lack cytosolic contents and functional organelles. This difference would suggest that if a component of the increase in force production observed in our whole-muscle studies is caused by an increase in myofilament Ca\(^{2+}\) sensitivity, the mechanism responsible for this must require an intact cellular environment.

Submaximal force had decreased by ~50 % after 25 mins of selenite exposure, corresponding with a significant increase in both 1/2RT and TTP twitch characteristics. This is consistent with the hypothesis that selenite may be oxidising components of the SERCA pump, reducing SR Ca\(^{2+}\) accumulation. This would result in less Ca\(^{2+}\) being
released from the SR upon electrical stimulation, accounting for the increased TTP, and ultimately resulting in less force being produced. Maximal force production was affected to a greater extent than twitch and submaximal force at this time point, consistent with the fact that contractions requiring maximal amounts of Ca\textsuperscript{2+} release from the SR are more affected than those requiring less Ca\textsuperscript{2+}.

It is possible that the increase in cytosolic Ca\textsuperscript{2+} could cause an increase in baseline force production, which could explain why tetracaine had no effect on the SIC. If the loss of active force is caused by cytosolic Ca\textsuperscript{2+} accumulation via impairment of the SERCA pumps, or another nonspecific SR Ca\textsuperscript{2+} leak mechanism, it is likely that its influence on the increase in baseline force would be scalar in the same manner observed in active force. However, no scalar qualities were observed in baseline force. Temperature and changes to the cellular redox environment may alter the onset of baseline force increase to a small degree, but they did not alter the maximum amplitude of baseline force increase. This suggests that as much as cytosolic Ca\textsuperscript{2+} accumulation could contribute to the decrease in active force and increase in baseline force to some degree, it is unlikely the main driving force behind the increase in baseline force.

When whole-muscle force production had decreased by 20 % due to selenite exposure, the myofilament force production of single skinned muscle fibres had dropped by 62 % compared to normalised control force. Similarly, a 50 % reduction in whole-muscle force production resulted in skinned fibre force decreasing by 68 % compared to time matched controls. This suggests that the effect of whole muscle selenite exposure on the contractile apparatus is maximal after 20 mins of selenite exposure. At least part of the difference observed in force production between whole muscle exposed to selenite and skinned fibres from those muscles, could be that selenite remained in the muscle after it was removed from the organ bath. Once the selenite-exposed whole muscle was removed from the organ bath it was thoroughly washed in normal Krebs solution; however, it is possible that some selenite remained inside the muscle fibres and continued to damage the contractile apparatus. A small, but significant, amount of force was recovered in these fibres when they were exposed to DTT, a specific thiol reducing agent (Adamczyk, Bal & Krezel 2015), showing that
part of the decrease in force production is caused by reversible thiol oxidation. These experiments provide strong evidence that selenite exposure, as whole-muscle, affects the loss of active force by mechanisms that damage the contractile apparatus; however, they cannot explain if this damage is caused by direct or indirect mechanisms.

The irreversible effect of selenite on the contractile apparatus could be caused by selenite-induced endogenous mechanisms, such as the activation of calpains, or protein carbonylation. If selenite exposure does impair SERCA function, leading to an accumulation of Ca^{2+} in the cytosol, calpains could become activated damaging the muscle and impacting its ability to produce force. Calpains can also be activated by increased ROS (Yokoyama et al. 2014), suggesting calpains could be contributing to the irreversible selenite-induced damage observed irrespective of Ca^{2+} handling dysfunction. An increase in cellular ROS production could also increase protein carbonylation, which could contribute to the irreversible damage and loss of active force. Fast glycolytic (type 2) muscles are more susceptible to irreversible oxidative carbonylation than are slow oxidative (type 1) muscle fibres (Feng et al. 2008), making this a likely outcome given the use of EDL muscle (type 2) in this model. Thiols can become irreversibly oxidised when exposed to excessive ROS (Adachi et al. 2004; Ying et al. 2007), which could be a third possible contributing influence to the irreversible damage and loss of active force.

5.4 The direct effect of selenite on the contractile apparatus

Experiments conducted by chemically skinning healthy muscle fibres before exposing them to selenite provide additional evidence that selenite can directly affect the contractile apparatus in the absence of all other cytosolic contents and cellular influences. These experiments showed a decrease in maximal Ca^{2+} induced force production, corresponding with a decrease in the Hill slope of the force-pCa curve. The decreased Hill slope shows that selenite has a greater effect on Ca^{2+} sensitivity at higher Ca^{2+} concentrations. It is possible that the reduction in the Hill slope was, in part, responsible for the disproportionate loss of maximal force, compared to twitch and submaximal force observed in whole muscle.
It is likely that the reduced maximal Ca$^{2+}$ activated force and myofilament Ca$^{2+}$ sensitivity caused by selenite exposure in skinned fibres are due to direct selenite-induced oxidation, as there are no cytosolic components or functional organelles in this model. Selenite is most likely oxidising thiol groups of the contractile apparatus, as subsequent exposure to DTT was able to recover all force production and restore the Hill slope. The amount of force loss caused by exposure to selenite plateaued by 10 mins, suggesting a discrete number of thiols become oxidised upon selenite exposure in this model. The ability of selenite to directly oxidise thiols is consistent with thiols being crucial to cell signalling during muscle fatigue, as they have the ability to become reversibly oxidised as a protective mechanism against excessive oxidative stress (Dutka et al. 2017). The direct oxidation of thiols of the contractile apparatus by selenite exposure causes maximal Ca$^{2+}$ induced force production to be more affected than submaximal, as demonstrated by the reduced Hill slope. This effect of selenite-induced reduction in myofilament Ca$^{2+}$ sensitivity is consistent with observations of the whole muscle force response at T = 25 mins, described above.

Past studies have shown that changes in myofilament Ca$^{2+}$ sensitivity due to ROS exposure, or induced fatigue, can occur without any change in maximal force production (Moopanar & Allen 2005). Others that have found a ROS-induced decrease in myofilament Ca$^{2+}$ sensitivity have also observed a return of sensitivity, when the muscle is exposed to DTT (Andrade et al. 1998). These past studies, and the studies of this thesis, suggest that a change in myofilament Ca$^{2+}$ sensitivity precedes change in maximal force production. As these experiments are performed with fixed Ca$^{2+}$ concentration, these results support the hypothesis that a portion of the force loss observed in whole-muscle could occur due to a decrease in myofilament Ca$^{2+}$ sensitivity (Lamb & Westerblad 2011), which could be independent of any Ca$^{2+}$ handling dysfunction. However, this experimental model is not able to explain any increase in active force production observed in whole-muscle experiments, suggesting that the increase in active force, and thereby any increase in myofilament Ca$^{2+}$ sensitivity associated with it, requires a healthy, intact muscle fibre system.
It should be noted that exposure to both selenite and then DTT increased the EC50 more than either substance alone. This difference illustrates a mechanism involving cycles of oxidation and reduction that seems to have an irreversible impact on the contractile apparatus. This was also demonstrated when examining maximal force production following repeated cycles of exposure to selenite and DTT, where a single repeated cycle showed a decrease in the ability of DTT to recover maximal force production to pre-exposure values. As protein carbonylation requires a more complex system of Fenton-like reactions with other metal ions to produce H$_2$O$_2$ (Cattaruzza & Hecker 2008), it is improbable that it plays a role in this experimental model. Thiols can become irreversibly oxidised when exposed to excessive oxidation (Adachi et al. 2004; Ying et al. 2007), and this becomes more likely in the absence of GSH (Fahey 2001), which is consistent with the skinned fibre model. The natural antioxidant buffering system present in a healthy whole-muscle could be repetitively reducing thiols that are repetitively oxidised by selenite exposure. These oxidation and reduction cycles could, therefore, be one possible reason why only a small amount of force could be recovered in fibres dissected from whole-muscles that had been exposed to selenite.

The results of this study support both the hypotheses that changes to active force production are caused by changes to Ca$^{2+}$ homeostasis, and by changes to the function of the contractile apparatus. Neither hypothesis is so unlikely that it could not be involved, and yet neither hypothesis is so dominant that it could be the only responsible mechanism. Therefore, it is most likely that both the changes in Ca$^{2+}$ homeostasis and the changes in myofilament Ca$^{2+}$ sensitivity are responsible for the changes in active force production. However, these mechanisms cannot explain the non-scalar characteristic observed in the increase in baseline force.
5.5 The effect of mitochondrial depolarisation on selenite-induced dysfunction

The flow cytometry experiments in this thesis showed that selenite affects the viability of mitochondria, causing mitochondrial membrane depolarisation prior to subsequent cell death. The high number of live cells with depolarised mitochondrial membranes showed that it is possible that the mitochondria could become depolarised in the presence of selenite in our whole-muscle experiments. It must be acknowledged that C2C12 myoblasts are not functionally identical to rodent whole muscle. However, it is clear that selenite is able to penetrate both cell types and cause significant dysfunction, therefore, it is not unlikely that this process could also occur in adult EDL fibres. The results of this experiment suggest that the mitochondria are a major site of selenite-induced dysfunction; thus, it is highly likely that mitochondrial dysfunction is a causal influence in the SIC.

When considering the possible impact of mitochondrial depolarisation on active force, respiratory uncoupling and the subsequent reduction in available ATP must be considered. The SERCA pumps require ATP to pump \( \text{Ca}^{2+} \) back into the SR to cause muscle relaxation; with reduced ATP, this process becomes impaired. Once submaximal force had decreased by 50 %, a contracture had occurred, and there was a significant increase in the 1/2RT. This demonstrates that if the mitochondria are affected by selenite exposure in whole muscle it could be influencing active force via impairment to the SERCA pumps, consistent with the hypotheses presented in section 5.3. Another mechanism that could influence both active and baseline force production is the irreversible opening of the MPT channel. The MPT channel has been observed to irreversibly open during mitochondrial depolarisation, and by direct oxidation of mitochondrial thiols by selenite (Kim et al. 2002; Chung et al. 2006; Kim, Yun & Kim 2003; Shilo et al. 2003). If this occurs, it can release the mitochondrial matrix store of \( \text{Ca}^{2+} \) into the cytosol, which is enough to cause the observed increase in baseline force. The MPT channel also releases an extremely high concentration of \( \text{H}^{+} \) into the cytosol, which has a negative effect on myofilament \( \text{Ca}^{2+} \) sensitivity (Rassier & Herzog 2002; Martyn & Gordon 1988).
Blocking the RyR by using tetracaine, and removing Ca$^{2+}$ from the bathing solution had no impact on the magnitude of the SIC. Attempting to alter the cellular redox state of the cell by using tempol or GSH pre-treatment similarly had no effect. These observations suggest that the increase in baseline force is not influenced by RyR Ca$^{2+}$ release or non-specific cellular redox events. One mechanism that could cause an equally proportioned increase in baseline force, i.e. that is not scalar according to changes in ROS and/or Ca$^{2+}$ homeostasis, is mitochondria-mediated cell death resulting in a rigor contracture. I have provided evidence that selenite can cause cell death via mitochondria-mediated pathways, involving the depolarisation of mitochondria of live cells. If this does indeed occur in whole-muscle, it would result in the cessation of ATP production. No changes in Ca$^{2+}$ handling processes are required during a rigor contracture, as they can occur in the absence of Ca$^{2+}$ (Yamaguchi 1998; Fink, Stephenson & Williams 1986), which would account for the fact that the baseline force increase was unaffected when the RyR was blocked in the absence of extracellular Ca$^{2+}$. However, it has been shown that calpains can become activated by increased ROS, which can result in mitochondrial-derived cell death (Yokoyama et al. 2014). This suggests that if Ca$^{2+}$ does begin to accumulate in the cytosol, affecting active force production as hypothesised in section 5.3, this could also trigger calpain activation and cause mitochondrial-derived cell death. This supports the possibility of a link between the mechanisms affecting baseline force and those influencing active force, as described in section 4.1.1.

The magnitude of a rigor contracture would not be as sensitive to ROS and/or Ca$^{2+}$ homeostasis changes, as its main driving force is the decrease in ATP availability. It is likely that there will be an unequal rate of selenium diffusion between fibres resulting in temporal differences of muscle fibre functional compromise throughout the muscle belly. This hypothesis could account for at least part of the observed gradual decrease in active force and the gradual increase in baseline force. This idea is most likely not the only factor affecting the active and baseline forces, but if cell death is involved in the SIC, this mechanism would likely cause the end result.
6 Conclusion

In conclusion, the factors affecting the changes in active force involved in the SIC are redox sensitive, and are likely the result of both direct selenite thiol oxidation and a selenite-mediated increase in endogenous ROS. These oxidative changes affect the function of the SERCA pump and the myofilaments, both of which could be responsible for causing the initial increase in active force, followed by the acute decrease. The dysfunction of the SERCA pump could cause cytosolic Ca\(^{2+}\) accumulation, which could partly explain the increase in baseline force. However, it is possible that the increase in baseline force involved in the SIC could be caused by a more complex mechanism, as it does not appear to be affected by redox changes, nor RyR Ca\(^{2+}\) release. The results of the flow cytometry experiments of this thesis provide evidence that selenite exposure is capable of causing mitochondrial depolarisation, as a precursor to cell death. If selenite exposure does cause mitochondrial depolarisation in whole muscle, a rigor contracture that gradually spreads throughout the whole muscle could ensue. Whether this mechanism is caused by a direct selenite interaction, or an increase of endogenous ROS, remains unclear. As much as the aforementioned impairment to SERCA and the myofilaments may affect active force production, improvement to these components may not prevent mitochondrial-derived cell death. For this reason, selenite-induced mitochondrial dysfunction in skeletal muscle should form a major focus point for future studies.
7 Future directions

Selenite is able to directly oxidise many cellular thiol targets as well as inducing an increase in endogenous ROS production, which can lead to severe loss of muscle function, cell death, and on an organism level, death by respiratory failure (Lin-Shiau, Liu & Fu 1990). The only observed beneficial treatments both in the literature and in this thesis are either pre-treatment with GSH or reducing temperature (Lin-Shiau, Liu & Fu 1989). This indicates that the main pathways of treatment are through causing a reduction in selenite’s oxidative capacity, by either reducing it to less toxic forms or reducing the body temperature, due to selenite’s extremely high Q_{10} value.

Before moving towards using in vivo animal models, there are some in vitro experiments that should be performed, in order to better understand the mechanism and kinetics of how GSH is affected when exposed to selenite. One such study would be to pre-treat whole muscles in the same manner used in this thesis, before changing the GSH containing solution for normal Krebs solution. This would allow observation of any GSH loading effects that could protect against oxidative damage, when the muscle is subsequently exposed to selenite following the washout. If protective effects similar to the results of this thesis were observed, the implications for an in vivo study aiming to increase cellular GSH content would be very promising.

As exposure of whole muscle to selenite was hypothesised to cause dysfunction to the SERCA pumps, investigating this effect further is critical to the full understanding of selenium toxicity. A positive control for this hypothesis would be to block SERCA via the use of TBQ, a reversibly SERCA channel blocker. This should exacerbate any muscle dysfunction caused by SERCA impairment. Another idea would be to identify the exact location of any oxidation of cysteine residues of the SERCA protein, which could pinpoint the site of selenite-induced SERCA dysfunction. It would also be possible to perform Ca^{2+} imaging experiments on flexor hallicus brevis (FHB) muscles, which are small enough for Ca^{2+} imaging to be performed as whole muscle. This would have the added advantage of leaving the SR Ca^{2+} handling system intact. This would allow the
direct measurement of Ca\(^{2+}\) release and reuptake from the SR, providing evidence of the involvement of RyR Ca\(^{2+}\) and SERCA mediated Ca\(^{2+}\) reuptake in selenite-induced dysfunction. By omitting Ca\(^{2+}\) from the bathing solution in this experiment, and/or by using tetracaine to block the RyR Ca\(^{2+}\) release, the involvement of Ca\(^{2+}\) in the SIC could be further studied. To add to this, blocking the MPT channel of mitochondria could elicit the influence of Ca\(^{2+}\) release from the mitochondria in the SIC.

As one of the major sites of selenite-induced cellular dysfunction is the mitochondrion, further *in vitro* studies should be performed to evaluate pathways affected by selenite exposure. A possible protocol would be to block the MPT channel before exposing C2C12 cells to selenite, in the same manner conducted in this thesis. By blocking the MPT channel, if the cells are able to stay alive for longer, or fewer cells show depolarised mitochondria, then the use of an MPT channel blocker in whole muscle could yield interesting results regarding the attenuation/prolonged onset of SIC. If this did yield positive results, it could be synergistic to combine this treatment with a GSH pre-loading protocol.

Looking forward towards the development of possible *in vivo* prophylactic treatments on an organism level, *in vivo* studies using N-acetyl cysteine (NAC) could provide an answer. NAC is a precursor to the production of endogenous GSH (Martinez et al. 2012), which could help protect animals against toxic doses of selenite. By administering NAC to rats orally through drinking water, assessment of their plasma GSH levels can be made *via* tail vein cannulation. Once their GSH levels have increased significantly, selenite could be introduced through food chum, or drinking water. The animal’s physical state would have to be monitored closely, looking for changes in their coat, skin, and nails, as these are the first areas of physically observable symptoms. Their activity level before and after the introduction of selenite could be measured, as a proxy for general health and well-being. Rats are large enough to take tail vein blood samples, so researchers would be able to assess blood concentrations of selenite (or different selenium species), as well as GSH levels.

There are limitations on experiments related to recovering force production and cell or animal viability following exposure to selenite, as we know that acute ROS damage in
intact cells is not entirely reversible. Therefore, trying to reverse this process using animals or animal material seems unethical, at this time. Based on the results of this thesis and logical inference, the best course of possible treatment for a human or animal exposed to toxic levels of selenite would be a combination of the following: monitored hypothermia, dialysis, blood exchange transfusion, intra-venous GSH, and heavy metal chelation therapy. Dialysis could help lessen the concentration of selenium in the blood, as selenite is excreted via urine. Blood exchange transfusion is removal of a patient’s blood with concurrent transfusion of new blood, in order to help reduce the selenium concentration. Intra-venous GSH could be able to decrease the oxidative capacity of toxic selenium species to less toxic forms, such as elemental selenium. Heavy metal chelation would help reduce selenium concentration in the blood, and would also help reduce further oxidative stress occurring via the Fenton reaction shown in Figure 1 – where $O_2•^-$ is able to dismutate into more damaging radicals in the presence of heavy metals such as iron, magnesium, and copper.

Thus, future work from the results of this thesis lies in determining more specific effects of selenite on SERCA proteins and the mitochondria, and identifying oxidised thiols, as well as determining the effect of pre-loading whole muscle with GSH in vitro, before moving towards any in vivo studies.
References


Aldosary, BM, Sutter, ME, Schwartz, M & Morgan, BW 2012, 'Case series of selenium toxicity from a nutritional supplement', *Clinical toxicology*, vol. 50, no. 1, pp. 57-64.


Arner, ES 2009, 'Focus on mammalian thioredoxin reductases-important selenoproteins with versatile functions', Biochimica et Biophysica Acta, vol. 1790, no. 6, pp. 495-526.


Chernatynskaya, AV, Looney, B, Hu, H, Zhu, X & Xia, CQ 2011, 'Administration of recombinant human thioredoxin-1 significantly delays and prevents autoimmune diabetes in nonobese diabetic mice through modulation of autoimmunity', *Diabetes Metabolism Research and Reviews*, vol. 27, no. 8, pp. 809-812.


Greaser, ML & Gergely, J 1973, 'Purification and properties of the components from troponin', *Journal of Biological Chemistry*, vol. 248, no. 6, pp. 2125-2133.


Kessi, J & Hanselmann, KW 2004, 'Similarities between the abiotic reduction of selenite with glutathione and the dissimilatory reaction mediated by Rhodospirillum rubrum and Escherichia coli', *Journal of Biological Chemistry*, vol. 279, no. 49, pp. 50662-50669.


Kim, TS, Jeong, DW, Yun, BY & Kim, IY 2002, 'Dysfunction of rat liver mitochondria by selenite: induction of mitochondrial permeability transition through thiol-oxidation', *Biochemical and Biophysical Research Communications*, vol. 294, no. 5, pp. 1130-1137.

Kim, TS, Yun, BY & Kim, IY 2003, 'Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide', *Biochemical Pharmacology*, vol. 66, no. 12, pp. 2301-2311.

Kitahara, J, Seko, Y & Imura, N 1993, 'Possible involvement of active oxygen species in selenite toxicity in isolated rat hepatocytes', *Archives of Toxicology*, vol. 67, no. 7, pp. 497-501.

Koller, LD & Exon, JH 1986, 'The two faces of selenium-deficiency and toxicity--are similar in animals and man', *Canadian Journal of Veterinary Research*, vol. 50, no. 3, pp. 297-306.


Laver, DR & van Helden, DF 2011, 'Three independent mechanisms contribute to tetracaine inhibition of cardiac calcium release channels', *Journal of Molecular and Cellular Cardiology*, vol. 51, no. 3, pp. 357-369.

Lei, C, Niu, X, Ma, X & Wei, J 2011, 'Is selenium deficiency really the cause of Keshan disease?', *Environmental Geochemistry and Health*, vol. 33, no. 2, pp. 183-188.


Lu, J, Berndt, C & Holmgren, A 2009, 'Metabolism of selenium compounds catalyzed by the mammalian selenoprotein thioredoxin reductase', Biochimica et biophysica acta, vol. 1790, no. 11, pp. 1513-1519.


Pate, E & Cooke, R 1989, 'Addition of phosphate to active muscle fibers probes actomyosin states within the powerstroke', *Pflugers Arch*, vol. 414, no. 1, pp. 73-81.


Schneider, MJ, Fiering, SN, Thai, B, Wu, SY, St Germain, E, Parlow, AF, St Germain, DL & Galton, VA 2006, 'Targeted disruption of the type 1 selenodeiodinase gene (Dio1) results in marked changes in thyroid hormone economy in mice', *Endocrinology*, vol. 147, no. 1, pp. 580-589.


Sen, CK, Marin, E, Kretzschmar, M & Hanninen, O 1992, 'Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization', *Journal of Applied Physiology*, vol. 73, no. 4, pp. 1265-1272.


Spallholz, JE 1997, 'Free radical generation by selenium compounds and their prooxidant toxicity', *Biomedical Environmental Sciences*, vol. 10, no. 2-3, pp. 260-270.


Yamaguchi, M 1998, 'Modulating factors of calcium-free contraction at low [MgATP]: a physiological study on the steady states of skinned fibres of frog skeletal
Yang, G & Zhou, R 1994, 'Further observations on the human maximum safe dietary selenium intake in a seleniferous area of China', *Journal of Trace Elements and Electrolytes in Health and Disease*, vol. 8, no. 3-4, pp. 159-165.


